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Plasticity in Reproduction and Survival under Dynamic Socio-Sexual Environment: Empirical Evidence from *Ephesia kuehniella* Zeller (Lepidoptera: Pyralidae)

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Plasticity in Reproduction and Survival under Dynamic Socio-Sexual Environment: Empirical Evidence from *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae)

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Abstract

Using an important pest of stored products, *Ephestia kuehniella* Zeller, I tested a number of theoretical predictions regarding strategies taken by males and females for resource allocations in response to dynamic socio-sexual environment. I demonstrate that males only respond to mean sperm competition levels and eupryrene sperm are produced both before and after emergence. Lifetime reproductive fitness in males depends on the number of copulations they can achieve, rather than the number of sperm ejaculated in each copulation. Regardless of whether males are exposed to rivals or not during their early adulthood, copulation duration and sperm allocation are not positively correlated, indicating that copulation duration cannot be used as a correct estimate of sperm allocation. Contrary to the previous prediction that males invest more in courting in the presence of rivals, my experiments demonstrate that males allocate more resource to courtship in the presence of additional females, which reduces their lifetime copulation frequency and fecundity. This finding offers a novel explanation for the success of mating disruption strategy using sex pheromones in pest management. Contradicting the previous prediction that females are more promiscuous under a female-biased condition and choosier in a male-biased sex ratio, my results show that perception of additional males makes females more receptive so that they mate more times and fertilise more eggs. Females call more when no additional mates or females are present than when either additional mates or females are present, suggesting that perception of no additional conspecifics by females may trigger them to allocate more energy for calling for further mating opportunities. Although virgin females lay similar numbers of eggs in all treatments, they start oviposition earlier and live shorter in the presence of conspecific males or females, supporting previous predictions that higher reproductive rate may accelerate senescence. Virgin females produce fewer eggs in male-biased than in female-biased sex ratio, suggesting that they reduce reproductive investment during their early life for mating opportunities under male-biased conditions. My studies provide insight into the plasticity in reproduction and survival under dynamic socio-sexual environment for animals with sexual reproduction in general and for this insect in particular.
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I would like to dedicate my thesis to my beloved family, particularly my parents.
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Chapter 1

General Introduction

1.1 Introduction

Lepidoptera is a large order of insects including moths and butterflies, some of which are serious pests of crops and stored products (Powell 2003). There are about 70 moth species infesting stored products from Tineidae, Heliodinidae, Yponomeutidae, Cosmopterygidae, Gelechiidae, Torticidae, Pieridae, Noctuidae and Pyralidae (Hill 2002). The Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), is one of the most important pests of stored products in storehouses and flour mills, causing economic losses (Hill 2002; Pandir & Sahingoz 2013). The damage caused by larvae to wheat flour is characteristic of webbing and silken galleries that can block machinery (Hansen & Jensen 2002; Hill 2002). The larvae prefer powdered foods but also attack the whole grains or bran (Ferro 1976).

The Mediterranean flour moth is a multivoltine insect which can survive in flours with low moisture content (Munro 1966; Ferro 1976; Jacob & Cox 1977). On white flour, this species can survive at the temperature ranging from 12 to 28°C and the humidity from 0 to 75% (Jacob & Cox 1977). In favorable conditions of 25°C and 75% RH, it takes 50 days to complete its life cycle with a relatively long larval period of about 40 days (Brindley 1930; Jacob & Cox 1977). It may develop continually throughout the year in the warm mills without diapause (Munro 1966; Jacob & Cox 1977). The ease of access and rearing along with short developmental time makes this species useful material in the laboratory rearing of parasitoids and predators for the biological control of other pests (Hansen & Jensen 2002; Mollá et al. 2013; De Puysseleyr et al. 2014) and for the studies of evolutionary biology (Xu & Wang 2009a, 2009b, 2010b).
1.2 Relevance of the research

A study of the reproductive behaviour of an insect provides essential information for our understanding of its life history, sexual selection mechanisms and evolutionary ecology (Hughes & Reynolds 2005; Bonduriansky et al. 2008). Since Darwin (1871) proposed the sexual selection theory, the mechanisms involved in male-male competition and female mate choice have been extensively studied by biologists.

Local sex ratio or socio-sexual environment often highly varies temporally and spatially, which may alter behavioural traits among individuals, and those individuals that can adapt to such variations may be selected as the fittest (Bretman et al. 2011a). Behavioural plasticity is of particular significance to animals because it enables animals to respond to short-term environmental fluctuations and to maximise fitness in the face of uncertainty (Komers 1997; West-Eberhard 2003; Bretman et al. 2011a). The plasticity in response to dynamic socio-sexual environment, particularly in males, has been widely reported in different taxa (Bretman et al. 2011a). However, there is still a substantial scope for theoretical and experimental investigations into the effect of various factors (e.g., sex ratio, mating history, age and social status of two sexes, mating systems, etc.) on behavioural and physiological plasticity of both males and females (Bretman et al. 2011a, b; Esfandi et al. 2015).

When exposed to a selection pressure, both males and females may attempt to increase their reproductive success through intrasexual and intersexual (epigamic) competition (Price 1997; Panhuis et al. 2001). In polyandrous mating systems where females copulate with more than one male, males are induced to compete for mates (Price et al. 2014). Therefore, male-male competition results in the evolution of reproductive strategies regarding energy allocation for reproductive and non-reproductive traits as well as ejaculate allocation among successive matings based on the sperm competition risk/intensity and the possibility of future mating(s) (Parker et al. 1997). Although various studies have addressed mating behavioural strategies in both sexes (e.g., Lize et al. 2014; Ramm & Stockley 2014; Simmons et al. 2014; Xu & Wang 2014), the factors affecting their decisions and mechanisms involved are still poorly understood (Pizzari et al. 2002; Bretman et al. 2011a; Delbarco-Trillo 2011). Moreover, few studies have quantified the cost currency of male pre-copulatory courtship displays.
and its trade-off against other fitness currencies, particularly the number of mates inseminated and sperm transferred by males in their lifespan, in response to dynamic socio-sexual environment (Scharf et al. 2013).

Parker’s (1970) sperm competition (SC) theory suggests that intrasexual competition may occur from pre-copulatory (such as mate choice) to post-copulatory stages (such as sperm competition) (Andersson & Simmons 2006) where males compete for fertilising eggs. The outcomes of sperm competition determine the paternity of competing males and accordingly their reproductive success (Parker 1990). So far, many studies have supported (e.g., Boschetto et al. 2011; Klemme & Firman 2013 Chaudhary et al. 2016) but some contradicted (e.g., Bernasconi et al. 2006; Cramer et al. 2013; Edme et al. 2016) these predictions. The mechanisms behind sperm competition are still largely unknown.

Empirical studies on insects and other animals have demonstrated that males may respond to sperm competition risk/intensity by adjusting the number of sperm transferred, prolonging copulation duration, etc. to increase their short-term and lifetime reproductive success (Wedell et al. 2002a, b; Parker & Pizzari 2010). In lepidopteran insects, males produce two types of sperm, the fertile eupyrene and infertile apyrene sperm (Silberglied et al. 1984; Poiani 2006). Therefore, males’ ejaculate adjustment may include the number and/or ratio of sperm types (Wedell et al. 2002b) in response to socio-sexual environment. Most studies have focused on the male prudence in the allocation of energy and ejaculate budget in response to socio-sexual environment (Bretman et al. 2009; Ingleby et al. 2010; Price et al. 2012; Bretman et al. 2013a; Xu & Wang 2014). However, little is known about how females respond to dynamic socio-sexual environment.

As discussed earlier, many questions still remain to be answered, for instance:
(1) Whether both males and females can detect the presence and sex ratio of conspecifics through clues without physical contact?
(2) Whether and how dynamic socio-sexual environment affects mating behaviour of both sexes?
(3) Whether and how socio-sexual environment affects lifetime reproductive investment of both sexes?

(4) Whether perception of socio-sexual environment affects reproductive behaviour and longevity of virgin individuals of both sexes?

1.3 Aims and objectives

The aims of the present study were to address the above questions using *E. kuehniella*, with two objectives:

1) To test whether and how males adjust their investment in reproduction and survival in response to socio-sexual environment, and

2) To examine whether and how females adjust their investment in reproduction and survival in response to socio-sexual environment.
Chapter 2

General Literature Review

2.1 Introduction

This chapter reviews the current knowledge on reproductive behaviours relevant to my studies on *E. kuehniella*.

2.2 Classification of *Ephestia kuehniella*

This species was first described by Zeller in 1879 and its taxonomic position is:

Order: Lepidoptera

Superfamily: Pyraloidea

Family: Pyralidae

Subfamily: Phycitinae

Genus: *Ephestia*

Species: *kuehniella*

2.3 General biology of *Ephestia kuehniella*

Being a holometabolous species, *E. kuehniella* has four life stages: egg, larva, pupa, and adult (Figure 2.1). The egg is flat/round type (Iossa et al. 2016) and white in colour, and it turns lightly yellow as the embryo develops (Figure 2.1A) (Brindley 1930). The first instar larva is white or pink and sparsely covered with hairs. A late instar male larva can be identified by its testes, which are visible through the skin on the dorsal side of its abdomen (Figure 2.1B) (Brindley 1930). Mature larvae construct silken cocoons in which they pupate (Brindley 1930). The pupa is pale green in color and gradually turns to reddish brown on the dorsal side of the thorax and yellowish brown on the ventral surface (Figure 2.1C). In the pupal stage, the sex can be recognized according to the appearance of the genital area on the ventral side of last abdominal segments (Figures 2.1E and 2.1F). The adult is one of the largest members of the genus *Ephestia* with a wingspan of 20-25 mm (Hill 2002). Forewings are pale-grey, speckled brown and white, and hindwings are white (Figure 2.1D).
Figure 2.1 Morphology of *Ephesia kuehniella*: (A) egg, (B) mature larvae (top, female; bottom, male, arrow indicates the developing testes), (C) pupae (top, male; bottom, female), (D) adult moth, (E) male pupa (arrow indicates two bumps separated by a narrow groove on abdominal segment), (F) female pupa (arrow indicates genital area that appears as a narrow groove on segment), (G) terminal abdomen of male adult (displays a pair of claspers; arrow indicates aedeagus), and (H) terminal abdomen of female adult (arrow indicates the ovipositor).
When reared on wheat flour at 28-30°C with RH of 60-70%, it takes 4-5 days for eggs to hatch, 40-50 days for larvae to complete development through six instars, 8-10 days for pupal stage, and male and female adults live for 7-11 and 6-10 days, respectively (Brindley 1930; Tarlack et al. 2014). Adults do not feed. They usually mate at the night of emergence and oviposit at the next night following mating. Females call by protruding their abdomen to release a sex pheromone and males perform courtship by wing-fanning and approaching toward calling females (Trematerra 1997).

2.4 Reproductive system of Lepidoptera

Lepidopteran insects have relatively similar reproductive systems with a pair of gonads that are connected to a common duct by individual ducts. In both sexes, the reproductive system is coordinated by hormones and transcription factors which are controlled by the physiological and environmental factors (Klowden 2013).

In most lepidopteran families (ditrysians) the female reproductive system (Norris & Richards 1932) includes a pair of ovaries each connected to a lateral oviduct (Figure 2.2). Lateral oviducts join a median oviduct, vestibulum, which enters a saclike structure called bursa copulatrix. Bursa is physically distinct from the common oviduct and open to outside via the vulva (copulatory opening on segment 8). A sperm duct, ductus seminalis, connects the bursa with the median oviduct; sperm move via the sperm duct from the spermatophore to median oviduct and then through the spermathecal duct to spermatheca where sperm are stored, and an accessory gland is attached to the apex of spermatheca (Chapman 1998; Gillott 2005). Ovipore, the second reproductive opening which is used for oviposition, is located on segment 9. However, in some moth families (monotrysians) the only single terminal genital opening serves for both copulation and oviposition (Gillott 2005).
In lepidopteran males, testes are fused and form a single median organ (Gillott 2005; see Figure 2.3). Sperm are released into vas deferens and then move to vesicula seminalis. They quickly leave seminal vesicles and enter to duplex, where they are stored. Duplex is continuous with an elongate accessory gland and posteriorly joins the unpaired ejaculatory duct (or simplex). Like the locomotive activity, sperm migration has a circadian rhythm in *E. kuehniella* (Riemann et al. 1974; Závodská et al. 2012).
2.5 Sperm heteromorphy and spermatogenesis in Lepidoptera

Sperm heteromorphism has been reported in a wide variety of invertebrates including spiders, centipedes, and insects (Swallow & Wilkinson 2002). Butterflies and moths (Lepidoptera) produce anucleated (apyrene) and nucleated (eupyrene) sperm (Figure 2.4) but only eupyrene can fertilise eggs (Meves 1902). Apyrene sperm are smaller and released as individual sperm whereas eupyrene sperm are much larger and
remain in bundles even after copulation (Figure 2.4) (Cook & Wedell 1996; Karr & Walters 2015).

![Image of Eupyrene and Apyrene](image)

**Figure 2.4** Eupyrene (eupy) in a bundle and apyrene (apy) of *Ephestia kuehniella*.

Several hypotheses have been put forward to explain the roles of apyrene sperm in the ejaculate of Lepidoptera (Silberglied et al. 1984; Holman & Snook 2006; Dallai 2014): (1) high motility in apyrene sperm may assist the transfer of eupyrene sperm (Cook & Wedell 1996; Friedländer et al. 2005), (2) they may represent nutrient for female zygote or sperm in the storage (Riemann & Gassner 1973), (3) apyrene sperm may play a role in sperm competition through displacing a former male’s sperm or by interfering with rival sperm (Silberglied et al. 1984), and (4) they may fill the spermatheca (cheap filler hypothesis) and thus delay female remating and decrease its receptivity (Drummond 1984).

Depending on the species, eupyrene spermatogenesis starts during larval instars and stops after pupation, whereas apyrene spermatogenesis begins just before or after
pupation and may continue in imago (Lai-Fook 1982; Lachance & Olstad 1988; Friedlander 1997). Both types of sperm are released from the testis into vas deferens and then into the duplex, where they are stored. Since certain numbers of sperm are constantly released from the testes to the duplex, the number of sperm stored in the duplex increases with age [e.g., in *E. kuehniella* (Thorson & Riemann 1977) and *Heliotis virescens* (Fabricius) (Proshold 1991)]. The timing of release of two types of sperm may also be different. In *E. kuehniella*, in the first day after emergence, the release of apyrene sperm into the duplex starts earlier than that of eupyrene sperm (Riemann et al. 1974). Similarly, in gypsy moth, *Lymantria dispar* (L.), apyrene sperm release into the vas deference about two hours earlier than eupyrene sperm (Giebultowicz et al. 1988). Riemann et al. (1974) suggest that the factors controlling the release of the sperm from testes may not be closely related to those factors which control earlier stages of spermatogenesis, i.e., endocrine system and ecdysone hormone because the differential circadian rhythm of sperm release could still be detected in the adult stages when the ecdysone is absent (Riemann et al. 1974). Giebultowicz et al. (1988) demonstrate that in gypsy moth sperm release from testes into the vas deferens and also from the latter into the seminal vesicles are photoperiod- and/or temperature-dependent.

In sperm heteromorphic systems, not only the sperm number but also the proportion of sperm types might be strategically adjusted in response to sperm competition (SC) (Snook 1998; Wedell & Cook 1999b). In their study on *Pieris rapae* (L.), Cook and Wedell (1996) reported that when paired with virgin females, males transfer higher proportions of eupyrene sperm in the second ejaculate than in the first one. These authors also demonstrate that *P. rapae* males tailor their second ejaculate according to the female mating history, providing a higher proportion of eupyrene sperm to virgin females than previously mated ones (Wedell & Cook 1999b). Such increase in the proportion of eupyrene sperm in the second copulation is attributed to higher probability of encountering non-virgin females in the second copulation, and a strategy which could be potentially advantageous in sperm competition through outnumbering the rival male sperm (Cook & Wedell 1996; Wedell & Cook 1999b). Strategic allocation of sperm type has also been reported in the lesser wax moth, *Achroia grisella* (Fabricius), whose virgin adults transfer a higher proportion of
eupyrene sperm in their first copulation after they have perceived higher competition risk (i.e., presence of a rival in their early adulthood), than those males that are kept singly (Jarrige et al. 2015). They suggest that such adaptation to sperm competition may serve as a strategy for increasing fertilization success and thereby the reproductive fitness.

Strategic allocation of sperm types when males encounter different levels of SC risk may be an indication of a functional association between the sperm types (e.g., Holman & Snook 2008) and evolution of sperm heteromorphism in these species (Swallow & Wilkinson 2002). For example, the apyrene sperm of a male may act as a “cheap filler” (Baker & Bellis 1989), diluting rival males’ sperm and increasing the fertilization success of the male (Silberglied et al. 1984; Swallow & Wilkinson 2002). Under such scenario, male success in SC may be a complex function of the numbers and proportions of the two types of sperm in the competing ejaculates (Parker 1990). Although male adaptive plasticity in ejaculate adjustment has been widely studied across taxa, the mechanisms behind the differential variation in the sperm number and types in Lepidoptera are not yet clear (Jarrige et al. 2015). Furthermore, since most studies have tested just the first few copulations, the lifetime effect of variation in socio-sexual environment and SC levels on the ejaculate composition or sperm ratio is largely unknown.

2.6 Mating system and strategy

The mating system refers to the way in which individuals obtain mates, the number of mates they acquire, and pair bond and its characteristics (Krebs & Davies 2009). In general, different mating systems are classified based on the dispersion of males and/or females, or pattern of promiscuity in either sex. Based on the patterns of promiscuity, there are two main mating systems in animals: (1) monogamy, where an individual has only one sex partner during his/her lifetime or at any one time, and (2) polygamy, where an individual has more than one partner during his/her lifetime or at any one time. In the latter case, it is called polygyny in males, polyandry in females, or polygynandry/polyandrygyny that occurs in both sexes simultaneously (Shuster & Wade 2003).
Male reproductive success usually depends upon the spatial and temporal availability of sexually receptive females, and as a result, males normally search for females (Choe & Crespi 1997; Shuster & Wade 2003). In most mammals and insects males provide little nutrition to females during mating and thus females have to look for resources to achieve higher reproductive potential (Krebs & Davies 2009). Accordingly, female dispersion should be affected by resource availability in space and time while male dispersion should be influenced by female dispersion (Shuster & Wade 2003).

Although females may not experience the same increase in their reproductive success by mating with more mates as males may do, recent studies show that female’s reproductive success may also depend on the number of matings (Kvarnemo & Simmons 2013). Females may mate multiply for nutrition from ejaculate (Abraham et al. 2011a), higher fertility (Abraham et al. 2011b), offspring fitness (Schausberger et al. 2016), and/or genetic diversity within brood (Forsman et al. 2007).

2.7 Mating behaviour

In many animals with internal fertilisation, mating sequence could be divided into eight subsequent steps (Choe & Crespi 1997): (1) formation of pair via mate seeking, gathering in the mating location as a result of extrinsic stimuli, and signaling (calling) of one sex, (2) courtship where one sex attempts to reduce resistance of the opposite sex to copulation, (3) copulation where the genital organs of both sexes are engaged, (4) insemination where sperm enter the female reproductive track, (5) post-copulatory phase where mate-guarding, mating plugs and/or prolonged mating occur, (6) fertilisation where the fusion of gametes occurs, (7) parental care where both or one sex invests in offspring rearing, and (8) bonding which is long-term cooperative parental care.

In lepidopteran insects mating success largely depends on a female’s receptivity, and a female’s receptivity to mate is affected by the male’s courtship display which reflects his quality as a potential mate (Wedell 2005). In some butterfly species males court females prior to mating by releasing pheromones to promote mating acceptance of females (Andersson et al. 2003). However, in many moth species, females usually
release long-range sex pheromones to attract males while males release unstable volatile molecules to stimulate females during courtship (Wedell 2005). In pyralid species the courtship behaviour has been categorised into two patterns (Phelan & Baker 1990): (1) simple courtship - after locating the female by response to her sex pheromone, the male attempts to copulate by lateral abdominal thrusts under the female’s wings without embellished courtship behaviour, and (2) complex interactive courtship - after locating a female, the male engages in head to head position and strikes the female on the head and thorax through which the male scent structures get involved with female antennae (Figure 2.5A-E). Afterward, the male attempts to copulate by a dorsal-lateral thrust of the abdomen towards the female’s genitalia (Figure 2.5F) (Phelan & Baker 1990).

The mating behaviour of *E. kuehniella* is similar to that illustrated by Phelan and Baker (1990) for *E. elutella* (Hübner) (Figure 2.5). Sexually mature females protrude their abdomens between the wings while ovipositors are extended along with abdominal movement, facilitating the release of the sex pheromone (Cotter Jr 1967). Females stay in this receptive position calling (Norris & Richards 1932) for hours. Males start seeking females through rapid running while fluttering wings (Cotter Jr 1967). After locating a female, the male approaches the female with fluttering wings and moves his abdomen dorsolaterally trying to achieve a grasp with claspers (Cotter Jr 1967). Following the successful grasp, the male stops fluttering and aligns his body parallel to that of female and copulation occurs.
Figure 2.5 Mating sequence of *E. elutella*, male on the left: (A) the female calls (releasing sex pheromone) by raising the tip of her abdomen and the male approaches with fanning wings the calling female from the front; (B) the male touches the female in a head-to-head position while the female keeps her abdomen elevated and the male continues wing fanning; (C) the female lowers her abdomen; (D) the male delivers head-thump and the female raises abdomen; (E) male conducts dorsal-lateral thrust, and (F) successful copulation occurs in end-to-end position. From Phelan and Baker (1990).

2.8 Sexual selection

In his attempt to explain male’s exaggerated ornaments, Darwin (1871) proposed another form of selection, “sexual selection”, in which individuals are selected
not for their best survival (as per natural selection; Darwin 1859) but based on their success to compete for mate or mating opportunities and thereby increase their reproductive success (Andersson & Iwasa 1996; Clutton-Brock 2007). Similar to natural selection, which has been suggested to vary in accordance with the temporal and spatial variation in the environmental conditions, sexual selection (and its major behavioural forces) may also be subject to such fluctuations (Gosden & Svensson 2008; Cornwallis & Uller 2010; Miller & Svensson 2014). For example, since population density or local sex ratio may change in time and space, the strength, form, or direction of sexual selection may change accordingly (Miller & Svensson 2014; Evans & Garcia-Gonzalez 2016).

2.8.1 Forces behind sexual selection

According to the sexual selection theory, two main selection forces operate independently: (1) intrasexual selection where individuals of the same sex (typically males) compete with each other to acquire a mate from the opposite sex, and (2) intersexual selection which is the result of preferential mate choice by one sex for particular individual of the opposite sex (usually choice of males by females) (Clutton-Brock 2007; Miller & Svensson 2014).

Current understanding of sexual selection theory is that males achieve higher reproductive potential by reducing their investment in gamete production and parental care, which increases the number of the sexually active males relative to the number of receptive females (i.e., operational sex ratio, OSR, Emlen & Oring 1977) and thus intensifies intrasexual competition among males (Clutton-Brock 2007). Greater intrasexual competition between males causes stronger selection on the secondary sexual characters which demonstrate the male’s quality (Parker 1979; Clutton-Brock 2007).

The major concepts shaping the understanding of the sexual selection theory are Bateman’s (1948) principle and Trivers’ (1972) parental investment theory. Bateman (1948) proposed that compared to females, males have a steeper slope of the gradient of mating frequency and reproductive success (Bateman gradient), the intrasexual
competition would be more intense in males than in females (Byers & Dunn 2012; Collet et al. 2014). Trivers’ (1972) theory, on the other hand, predicted that investment in one offspring (to increase offspring’s chance of surviving) would reduce the parent’s ability to invest in the other. Therefore, lower investment in offspring would provide that sex with the possibility of higher reproduction rate, which in turn, may result in a surplus of that sex with higher intrasexual competition (Parker & Simmons 1996). In other words, sexual selection is expected to be stronger in the sex (conventionally males) that has a trivial investment in offspring production. However, the above predictions are generally based on the assumption of conventional sex role for males and females with an even sex ratio in the population (Rodriguez-Munoz et al. 2010).

Female mate choice also generates selective force on male ornaments (Miller & Svensson 2014). It is a complex component of sexual selection subject to different environmental parameters affecting its selection pressure on male traits (Andersson & Simmons 2006; Miller & Svensson 2014). Although sperm production capability along with the effect of environmental and social circumstances may make males choosy (Edward & Chapman 2011b; Miller & Svensson 2014), so far female mate choice has been better studied than male mate choice (Andersson & Simmons 2006; Miller & Svensson 2014).

Sexual selection includes three different stages (Miller & Svensson 2014): (1) pre-copulatory sexual selection, which covers all aspects of mate acquisition and choice and the factors affecting them; (2) in-copulatory selection, which concerns the effect of environmental factors, more specifically the socio-sexual environment, on mating latency, copulation duration and ejaculate adjustment, and (3) post-copulatory sexual selection, where sperm competition between ejaculates of different males and female manipulation of sperm are generally focused.

### 2.8.2 Pre-copulatory sexual selection

It is generally assumed that females should be choosier than males as the former invest more than the latter in reproduction (Dougherty & Shuker 2014; Sbilocumbo & Martin 2014). When the optimal reproductive fitness strategies of the sexes are
conflicting, sexual conflict between them arises (Parker 1979; Evans & Garcia-Gonzalez 2016). For example, the optimal rate of copulation is an obvious conflict between males and females where males generally copulate more times than females (Arnqvist & Rowe 2005; Evans & Garcia-Gonzalez 2016). The conflict has probably resulted in the evolution of two distinct strategies in males and females regarding the optimal copulation rate: male coercion to copulate and female resistance (Andersson 1994; Rice 1996; Rice & Holland 1997; Holland & Rice 1998, 1999). Evidence for substantial costs of mating to females (e.g., Arnqvist & Nilsson 2000; Simmons 2005; Wong & Candolin 2005) explains the existence of such resistance in females, and coevolution of the male’s traits in order to overcome such resistance (Chapman et al. 2003; Edward & Chapman 2011b).

Although the cost of reproduction has been well documented in females, evidence for that in males is much scarcer (Hoefler 2008; Papadopoulos et al. 2010; Wedell 2010). However, research shows that male sexual activities [e.g., mate attraction, courtship, male-male competition, and copulation] also appear to be costly (Dewsbury 1982; Wedell et al. 2002; Kotiaho & Simmons 2003; Wedell 2010)]. As a result, males should have pre-copulatory mate preference particularly when there is a great variance in female quality or when the cost of mate searching is low (Bonduriansky 2001; Kokko & Monaghan 2001; Tigreros et al. 2014).

Several mechanisms involved in mate choice have been suggested to explain the occurrence of pre-copulatory sexual selection (reviewed in Andersson & Simmons 2006): (1) direct phenotype effect - male ornaments are a reflection of their advantages, for example, higher quality territory, nutrition or protection; (2) sensory bias - male ornaments could initially develop for other reasons, such as foraging, but males with these traits become more favorable; (3) Fisher’s (1930) sexy sons model - choosing an ornamented male will result in sons and daughters carrying alleles of attractiveness; (4) good genes - females choose older males as their age is an indication of their higher survival capability, and (5) genetic compatibility - choosing a mate that might complement the genome of the chooser which is considered as non-additive benefits. Direct phenotype effect and sensory bias mechanisms are related to fitness advantages of directly chosen traits while according to the sexy sons or good genes models, a
preference is indirectly selected as it is genetically correlated to directly selected traits (Andersson & Simmons 2006).

### 2.8.3 In-copulatory sexual selection

Although sperm are vastly smaller than eggs, they are transferred in huge numbers and their production is still limited and costly (Dewsbury 1982). Therefore, males may adjust ejaculate size during copulation based on the female quality (Simmons 2001). Empirical studies have found that males regulate their ejaculate size based on female age, size and reproductive status or perceived risk/intensity of SC (Parker 1970; Pitnick & Brown 2000; Wedell et al. 2002b; Xu & Wang 2009b).

SC theory predicts that risk and/or intensity of male-male competition on fertilizing a set of ova determines the resource allocation in a given copulation (Parker et al. 1996, 1997; Parker 1998) as well as the lifetime investment in reproduction (Wedell et al. 2002b; Parker & Pizzari 2010). It has been suggested that immediate risk [presence of rival(s) in the mating arena] or intensity of SC (the number of males present in the mating arena) affects male investment in a given copulation: an increase in immediate risk of SC always increases the number of sperm allocated in a single copulation while an increase in immediate intensity of SC reduces the number of sperm allocated in a single copulation (Parker et al. 1996, 1997; Engqvist & Reinhold 2005). However, the effect of mean risk (the average number of rivals) or intensity of SC (the average probability of female remating) would be reflected in the lifetime investment in reproduction (Parker et al. 1996, 1997; Engqvist & Reinhold 2005). Generally, males should increase the relative investment in spermatogenesis with the increase of the mean risk/intensity of SC. For example, perceived SC by individuals during their developmental stages (e.g., Gage 1995), or adopted mating strategies based on male condition (e.g., sneaker males), could elevate the mean SC and, as a result, cause irreversible adaptation such as larger testis size in males (Simmons 2001; Engqvist & Reinhold 2005; Bertram et al. 2013). The ultimate fitness benefit of such strategies would be a higher paternity share in the next generation and fertilization success. While some empirical tests of optimal sperm allocation strategies have supported the predictions of SC theory (e.g., Wedell & Cook 1999a, b; Solensky & Oberhauser 2009;
In a rapidly changing socio-sexual environment, the effectiveness by which a male can detect and accordingly respond to the potential risk or intensity of SC is the key to maximizing his fitness (Parker & Pizzari 2010; Bretman et al. 2011a). It has been documented in many species that different characteristics of male ejaculate (e.g., sperm quality, morphology, or seminal fluid compositions) may be subject to modification in response to the level of SC; however, the total number of sperm in the ejaculate seems to be the most reliable, although not the only, determinant of fertilization and paternity success (Parker & Pizzari 2010; Kelly & Jennions 2011).

2.8.4 Post-copulatory sexual selection

In general, three types of post-copulatory sexual selection have been proposed (Miller & Svensson 2014; Eberhard 2015): cryptic female choice (Thornhill 1983; Eberhard 1996), sperm competition (Parker 1970; Simmons 2001), and sexually antagonistic coevolution between males and females (Holland & Rice 1998; Arnqvist & Rowe 2005). Moreover, four major mechanisms are believed to be involved in post-copulatory sexual selection through sperm competition or cryptic female choice (Danielsson 1998; Parker & Pizzari 2010): (1) sperm storage space (fair raffle) — the simplest mechanism of sperm competition in which all sperm ejaculated have equal chance for fertilization (Parker 1990); (2) sperm inequalities (loaded raffle) — raffle becomes biased in favour of one or other male due to intrinsic or extrinsic factors; (3) degree of sperm mixing — non-random mixing of sperm from different ejaculates before fertilization, and (4) physical manipulation of ejaculate by either sex and sperm selection by females.

Under such post-copulatory selective pressure, males may attempt to ultimately promote their paternity for which they may undergo morphological adaptations (e.g., male complex genital morphology, Arnqvist 1998) to guard their mates after copulation (Simmons 2001), manipulate ejaculate components (e.g., Avila et al. 2011), and/or adjust sperm expenditure based on the sperm competition risk/intensity (Parker 1982,
In some species [e.g., *Callosobruchus maculatus* (Fabricius) (Edvardsson & Canal 2006), *Nysius huttoni* White (Wang et al. 2008), or *Menochilus sexmaculatus* (Fabricius) (Chaudhary et al. 2016)] prolonged copulation is used by males as a mechanism of mate guarding in response to the high competition risk. Furthermore, perception of rivalry has been reported to increase sperm number [e.g., *A. grisella* (Jarrige et al. 2015), *E. kuehniella* (Xu and Wang 2014)], sperm motility [e.g., *D. melanogaster* (Lupold et al. 2012)] or proportion of fertile sperm in male ejaculate [e.g., *A. grisella* (Jarrige et al. 2015)].

Females, on the other hand, may bias the fertilization through some proximate mechanisms in pre-storage and storage of sperm (Firman et al. 2017): (1) control over the timing and order of mating (Xu & Wang 2010a; Chaudhary et al. 2016); (2) differential sperm ejection, digestion, incapacitation, and uptake (Holman & Snook 2008; Lupold et al. 2012; Friesen et al. 2016); (3) differential sperm storage (Ward 2000; Miller & Pitnick 2002); (4) selective fertilization by female as a result of the interaction of secretions of the female’s reproductive tract and sperm (Oliver & Evans 2014; Alonzo et al. 2016; Rosengrave et al. 2016), and (5) sperm-egg signaling (Ghaderi et al. 2011; Stapper et al. 2015). For example, *E. kuehniella* females influence the fertilisation by re-mating sooner and thereby biasing it towards the second male ejaculate (Xu & Wang 2010b). In *D. melanogaster*, females terminate the process of storage and displacement of second male sperm by ejecting all the sperm located in the bursa (Lupold et al. 2013). It has been reported in this study that the timing of sperm ejection had a particularly strong effect on the absolute and relative numbers of each male’s sperm remaining in storage, thereby determining the fertilisation.
Chapter 3

General Methodology

3.1 Introduction

In this chapter, the general methodology applied throughout the research is described, including the materials, procedures, and definitions.

3.2 Materials

**Breeding colony:** A colony of *E. kuehniella* was established from the larvae in infested flour collected at Turks Poultry, Foxton, New Zealand, in 2013. All these larvae were allowed to pupate in the original food. Newly emerged adults (10 males + 10 females) were then maintained in an oviposition cylinder (Figure 3.1).

**Oviposition cylinders:** Transparent plastic cylinders (6 cm diameter × 8 cm height, LabServ, Auckland) with the interior wall lined with porous plastic sheets (aperture diameter = 0.15 mm) were used for egg laying (Figure 3.1). Twenty oviposition cylinders were set up. Eggs laid on the sheet (Figure 3.2) between the second and fifth oviposition days were collected daily from each cylinder and transferred into Petri dishes for hatching.
**Figure 3.1** Oviposition cylinder with 20 adult moths.

**Figure 3.2** Plastic sheet for oviposition.

**Glass tubes:** Glass tubes (2 cm diameter × 7.5 cm height) with a plastic lid with a hole (1 cm diameter) in the middle covered with two layers of cloth mesh (70 apertures per inch) were used for keeping individual pupae and adults (Figure 3.3).
**General Methodology**

*Figure 3.3* Glass tubes used to maintain pupae and newly emerged adults before experiments.

**Petri dishes:** Petri dishes (8.5 cm diameter × 1.5 cm height) were used for egg incubation.

**Larval rearing cylinders and standard larval diet:** Transparent plastic cylinders (8 cm diameter × 10 cm height, LabServ, Auckland) were used for larval mass rearing (Figure 3.4A), each with 50 g standard diet and 200 larvae. Twenty larval rearing cylinders were set up for colony maintenance and experiments (Figure 3.4B). A piece of wrapped paper towel was placed in each cylinder for pupation. The diet consisted of 43.5% wholemeal wheat flour, 43.5% maize meal, 3.0% yeast, and 10% glycerine. The ingredients were simply mixed up and then kept in a freezer before using.
Figure 3.4 Breeding colony of *E. kuehniella*: (A) larval rearing cylinder, and (B) humidity containers, each with 10 larval rearing cylinders.

**Dissecting microscopes:** Leica MZ12 (Switzerland) (Figure 3.5) and Olympus SZ III (Japan) dissecting microscopes were used for dissecting adults and counting larvae. Olympus SZ III was also used for recording other biological parameters.

Figure 3.5 Insect dissection using a dissecting microscope.
Phase-contrast microscope: A phase-contrast microscope (Olympus BX51, Japan) with a micrometer eyepiece was used for sperm counting (Figure 3.6).

Figure 3.6 Sperm counting using a phase-contrast microscope.

Electronic scale: An electronic balance (Mettler Toledo AG135, Switzerland) with a readability of 0.00001g was used for weighing pupae (Figure 3.7).
**Experimental device:** A device consisting of 15 experimental containers and an air divider was constructed for experiments (Figure 3.8). The experimental container was made of two identical transparent plastic cylinders (8 cm diameter × 10 cm length) connected to each other with a Parafilm on external walls. The two cylinders were separated with a metal mesh (2.8 apparatus per mm) in between, allowing free air movement. One cylinder was used as the mating chamber where a male and a female were held and the other for accommodating rival males or additional females. The mating chamber had a lid at the end with a hole (3 cm diameter) in the middle that was covered with a fabric (cloth) mesh (2.8 apparatus per mm).
Figure 3.8 Experimental device.

The air from a compressed air tap was filtered through activated charcoal, measured via an airflow meter, humidified by passing through distilled water, and then blown into the air divider, a bigger transparent plastic cylinder (15 cm diameter × 20 cm height), from which the air was equally divided into 15 silicone pipes (0.5 cm in diameter), each of which was connected to a neighboring chamber. The air was blown through the neighboring chamber to the mating chamber and then out through the hole at the end of the mating chamber. The air speed was set to allow the air in all 15 experimental containers to be replaced once per minute. All containers were placed horizontally on an experimental bench. All experiments were carried out during the scotophase and a red light (Sylvania, F36W/Red, Holland) was used for illumination under the above environmental conditions.

3.3 Environmental conditions

The breeding colony was maintained and all experiments carried out at 25 ± 1°C and 60 ± 10% RH with a photoperiod of 14:10 h (L:D) in a bioassay room.
3.4 Procedures

**Adult weight assessment:** As the adult weight is positively correlated with the pupal weight (Xu & Wang 2009b), pupal weight was considered as an index of adult body weight. In this colony mean pupal weight (mean ± SD) was 23.1 ± 2.1 mg for males and 24.4 ± 2.4 mg for females. I categorized pupal weight as light (< mean -1 SD), average (mean ± 1 SD), or heavy (> mean +1 SD).

**Sperm extraction and count:** After termination of copulation, the female was immediately separated and dissected for sperm count according to Koudelova and Cook (2001). Under the dissection microscope (× 40 magnification) (Figure 3.5), the spermatophore (Figure 3.9B) was separated from the bursa copulatrix (Figure 3.9A) on a glass slide and then ruptured using a fine needle to release sperm in a droplet of distilled water (Figure 3.9C). Afterwards, the sample was washed off into a glass vial and diluted with distilled water up to 30 ml. Eight 10-μl subsamples were taken using a Gilson autopipette and then placed on slides and allowed to dry under a dust cover. Each slide was examined under the phase-contrast microscope (× 100 magnification) (Figure 3.6) and the number of eupyrene sperm bundles and apyrene sperm (Figure 3.9D) were counted. The total number of eupyrene sperm was calculated by multiplying the number of bundles by 256 (the number of eupyrene sperm per bundle). The total number of apyrene sperm was calculated by multiplying the mean number of apyrene per slide by the dilution factor.
Figure 3.9 Sperm extraction procedure: (A) bursa copulatrix containing two spermatophores, (B) spermatophore separated from bursa, (C) sperm cloud inside a spermatophore, and (D) eupyrene (eupy) sperm bundles surrounded by apyrene (apy) sperm.

To count the number of sperm produced by virgin males, I dissected males after death as well as newly emerged ones and removed the testes (Figure 3.10), seminal vesicle, and vas deferens under the dissecting microscope. The number of eupyrene and apyrene sperm in those organs was estimated as described above.
Egg extraction and count: Females were removed immediately after mating and transferred into the oviposition cylinders. To determine the fertility, I collected and counted eggs daily and then incubated them in Petri dishes. Three-d-old eggs were observed under the dissecting microscope (Figure 3.5), and eggs with black dots (larval heads) were recorded as fertile (Xu et al. 2007).

For virgin females, the number of mature eggs in the ovaries was counted by dissecting females after death in a drop of 1% saline solution on a glass slide under the dissecting microscope. The ovaries were separated out and immersed in 1% acetocarmine for 10 s to stain the eggs before being transferred to clean saline solution. The chorion of mature eggs prevents the stain but immature eggs absorb the stain (Edwards 1954). A similar procedure was used for the mated females after their death.
3.5 Behaviour assessment

Eight TECHview cameras (QV-3034, Singapore) were set up 20 cm above the mating chambers to record the behaviour of the focal insects. Video recording started one hour before the onset of the scotophase and continued until one hour into the scotophase. Mating behaviours in terms of male pre-fanning time and wing fanning duration, female calling duration, and mating latency and duration (see Section 3.6) were measured by reviewing the videos. The frequency of male wing fanning was also recorded.

3.6 Definitions

Pre-fanning time: Duration from introduction of both sexes into the experimental cylinders to the start of first wing fanning by the focal male.

Wing fanning (courtship) duration: Total duration in which a male fans its wings.

Calling duration: Total duration in which a female protrudes her abdomen tip.

Mating latency: Although the focal male and female were introduced into the mating chamber one hour before the scotophase, they remained inactive until the start of the scotophase. As a result, mating latency is defined as the duration between the start of the scotophase and initiation of copulation.

Mating duration: Duration from initiation of mating (male and female are engaged by the tip of their abdomen) until the end of copulation (both insects separate completely).

Pre-oviposition period: The period from adult emergence to the onset of oviposition.

Oviposition period: The period from the first to the last oviposition.

Fecundity: The total number of sperm ejaculated by a male in its lifetime was recorded as male fecundity, and total number of eggs produced by a female in her lifetime as female fecundity.
**Fertility:** The number of the eggs with black dots after three days incubation was recorded as female fertility.

### 3.7 Statistical analysis and reported values

All analyses were done using SAS software (SAS 9.4, SAS Institute Inc., NC, USA). Rejection level was set at $\alpha < 0.05$. Reported values were means $\pm$ SE.
Chapter 4

Male Reproductive Outputs and Survival in Response to Socio-sexual Environment

4.1 Introduction

Sperm competition (SC) theory (Parker 1970) seeks evolutionary stable strategy (ESS) for ejaculate allocation (e.g., Parker et al. 1997; Wedell et al. 2002a; Parker & Pizzari 2010; Bretman et al. 2011a; delBarco-Trillo 2011; Parker et al. 2013), predicting that with increasing risk of sperm competition (SCR) males gain fitness by increasing ejaculation and that with increasing intensity of sperm competition (SCI) the male ESS is to reduce investment. In these models, female promiscuity is proposed to determine SCR and SCI levels and precopulatory energy costs in males. So far, most empirical studies (e.g., Wedell & Cook 1999a, b; Bretman et al. 2009; Ingleby et al. 2010; Price et al. 2012; Bretman et al. 2013a; Xu & Wang 2014) have supported while some (e.g., Cook & Gage 1995; Ramm & Stockley 2007; Worthington et al. 2013; Zizzari et al. 2013) contradicted these predictions.

Most evolutionary biologists have investigated the female mating frequency and male ejaculate allocation separately assuming fixed levels of the opposite sex’s strategies (Alonzo & Pizzari 2010; Abe & Kamimura 2015). However, these strategies of sexes are likely to coevolve (Abe & Kamimura 2015). Recently, by incorporating dynamic reproductive strategies of both sexes Requena and Alonzo (2014) predict that male ejaculate allocation and female promiscuity are negatively correlated, and Abe and Kamimura (2015) foresee that female-biased conditions make males parsimonious and females promiscuous. These two most recent models support some components and contradict others of the previous SC models. The differences in predictions between models may result from different conceptual frameworks-based (e.g., Requena & Alonzo 2014), experimental designs (e.g., Engqvist & Reinhold 2005), and/or life history strategies of study species. Parameters for life history strategies may include...
whether adults feed, how often both sexes mate, whether ejaculation and sperm storage sites are different, how females manipulate the use and storage of sperm from different males, and whether males produce a spermatophore during mating, as examples.

Engqvist and Reinhold (2005) point out that many empirical experiments used to test theoretic predictions about SCR and SCI may be inappropriately designed. They illustrate four common pitfalls: (1) ignoring the difference between immediate and mean SCR and SCI levels — the accurate test for the first set of predictions concerning immediate SCR and SCI should be to test for differences in allocation of the males’ present sperm reserves at a given mating and for the second set of predictions concerning mean SCR and SCI to test for differences in sperm production before mating; (2) incorrect manipulation of immediate SCI — the number of males nearby should be different from the number of competing ejaculates, and thus, introducing more males will always increase immediate SCR, which has a positive rather than negative effect on ejaculate size; (3) ignoring past and future risk in studies of immediate SCR and SCI — males should take the females’ past mating history and future mating probability into account when estimating immediate SCR and SCI; and (4) ignoring the impact of sex ratio — similar to pitfall (1) above, a relatively long-term manipulation of sex-ratio to study immediate SC is inappropriate. Several additional points are also worth mentioning. First, although females may play important roles in male reproductive plasticity (Bretman et al. 2011a; Requena & Alonzo 2014; Abe & Kamimura 2015), they are often conditioned (e.g., virgin females reared in groups) before mating while the impact of such treatment on SC predictions is not considered (e.g., Price et al. 2012; Bretman et al. 2013b; Moatt et al. 2013; Worthington et al. 2013). Second, the effect of different life history strategies of study species on research outcomes (and thus predictions) is rarely considered in SC studies (Ingleby et al. 2010). Third, in many SC studies, authors measure various parameters for only one mating event rather than lifetime reproductive activities (e.g., Bretman et al. 2009, 2010, 2013a, 2013b; Lize et al. 2012a; Price et al. 2012; Worthington et al. 2013). Fourth, copulation duration is usually considered an accurate measure of sperm allocation (e.g., Bretman et al. 2011a; Price et al. 2012; Moatt et al. 2013) but these two parameters are not positively correlated in some species (e.g., Gilchrist & Partridge 2000; the present study). Fifth, sperm production mediated by mean SCR and SCI levels may increase in
both male-biased and female-biased conditions because males may gain reproductive fitness by increasing sperm production after exposure to rivals (Wedell et al. 2002a; Parker & Pizzari 2010) and additional mates (Abe & Kamimura 2015).

One of the earlier examples of costs of sexual activity to males is made by Partridge and Farquhar (1981). It is now an accepted notion that reproduction is costly for males (Scharf et al. 2013). The ultimate cost of reproduction for males is investment in current reproduction at the expense of future reproduction and longevity (Scharf et al. 2013). The first trade-off lies at the foundation of sperm allocation models assuming a limited amount of sperm and time, which should be optimally distributed (e.g., Wedell et al. 2002a; Parker & Pizzari 2010; Lize et al. 2012a; Price et al. 2012; Zizzari et al. 2013; Xu & Wang 2014). The second trade-off predicts that sexual activities shorten male longevity (e.g., Kotiaho & Simmons 2003; Simmons & Kotiaho 2007; Hoefler 2008; Jordan & Brooks 2010; Papadopoulos et al. 2010; Wedell 2010; Bretman et al. 2013b; Xu & Wang 2014) although male longevity and reproduction may not always be traded off against each other (Janowitz & Fischer 2010). Precopulatory courtship displays by males are considered costly across taxa including humans (e.g., Cordts & Partridge 1996; Clutton-Brock & Langley 1997; Hoback & Wagner 1997; Kotiaho & Simmons 2003; Hunt et al. 2004; Simmons & Kotiaho 2007; Hoefler 2008; Papadopoulos et al. 2010; Wedell 2010; Gersick & Kurzban 2014). It is generally accepted that females are choosier in the male-biased sex ratio, leading to higher male precopulatory expenditure than in the female-biased sex ratio, resulting in lower male precopulatory expenditure (Emlen & Oring 1977). So far, few studies have quantified the cost currency of male courtship displays and its trade-off against other fitness currencies, particularly the number of mates inseminated and sperm transferred by males in their lifespan, in response to dynamic socio-sexual environment (Scharf et al. 2013).

Unlike many other study models (e.g., Drosophila) whose adults continue to feed throughout their life and may adjust food ingestion rate in response to the socio-sexual environment, E. kuehniella adults do not feed and thus males have “fixed” resources, obtained during the larval stage, for their lifetime reproductive and survival fitness. Therefore, this is an ideal species for studies on male strategic investment in
precopulatory activities, ejaculate production and allocation, copulation frequency, and longevity, under dynamic socio-sexual context. Female *E. kuehniella* produce a sex pheromone to attract males for mating (Calvert & Corbet 1973). Although there is no direct evidence for the existence of a male sex pheromone in *E. kuehniella*, Corbet and Lai-Fook (1977) suggest that *E. kuehniella* males might produce a courtship pheromone based on morphological features between the seventh and eighth abdominal segments. Adult *E. kuehniella* of both sexes have well-developed hearing organs (Pérez & Zhantiev 1976), and ultrasonic pulses emitted by wing-fanning males during courtship may play a significant role in mating behaviour (Trematerra & Pavan 1995). These features allow both sexes of *E. kuehniella* to perceive the presence of nearby conspecific adults without physical contact. The male produces and transfers a spermatophore into the female’s bursa during copulation (Xu & Wang 2010b). Adults become sexually mature a few hours after emergence; female calling, male courtship and copulation peak in the last few hours of the scotophase, and copulation can continue into the first hour of the photophase (Xu et al. 2008). Both sexes copulate multiply (Xu & Wang 2009a) and in each copulation, the male ejaculates more sperm than necessary for fertilization of the full egg load of a female (Xu & Wang 2009b). The last male that copulates with a copulated female has sperm precedence (Xu & Wang 2010b).

In the present study, the term “SC levels” was used rather than “SCR levels” and “SCI levels” for two reasons. First, Engqvist and Reinhold (2005) suggest that the number of males nearby may be different from the number of competing ejaculates (SCI) in a female and Bretman et al. (2010) demonstrate that there is no detectable effect of increasing the number of rivals above one in *D. melanogaster*. Second, *E. kuehniella* males transfer similar number of sperm to virgin and once copulated females (Xu & Wang 2010a), suggesting that males may not adjust ejaculate allocation based on SCI levels in this species. Therefore, SC levels tested in this study refer to SCR group.

Based on the knowledge outlined above, I carried out a series of experiments using *E. kuehniella* to examine whether and how males adjusted their investment in various reproductive activities and survival in response to immediate and mean SC levels. Newly emerged focal males were either exposed (+E) or unexposed (-E) to rivals/mates prior to experiments. In his lifespan I offered a virgin female to a focal
male once a day in the presence of (1) no other individuals, (2) virgin males, or (3) virgin females, which could be perceived by him without physical contact, and recorded the copulation duration and number of sperm ejaculated in each copulation, lifetime number of copulations, and longevity of the male. Male courtship (wing fanning) duration and mating latency under the above socio-sexual contexts were measured. Because -E focal males were individually kept prior to experiments, they had not been exposed to sperm competition pressure before their first copulation. As a result, only immediate SC levels were in action in the first copulation — allocation of sperm reserves in response to SC levels. However, both mean and immediate SC levels might play roles in subsequent copulations because the focal males had experienced different socio-sexual conditions during the previous mating episode(s) — both production and allocation of sperm in response to SC levels. Because +E focal males were exposed to rivals or mates in their early adulthood, both mean and immediate SC levels were in action throughout the experiments with these males.

In this chapter, I tested three hypotheses: (1) the focal male increases sperm allocation to his mate and prolongs the copulation in the presence of rivals, and the opposite is the case in the presence of additional females; (2) as a result, in the presence of rivals the focal male lives shorter and in his lifetime has fewer number of copulations, faster depletion of sperm supply and faster decrease of copulation duration over successive copulations, and the opposite is the case in the presence of additional females, and (3) in the presence of additional males the focal female is choosier, mating latency is longer and the focal male invests more in courting, and the opposite is the case in the presence of additional females.

4.2 Materials and Methods

4.2.1 Focal Males without Exposure to Other Individuals prior to Pairing (-E)

All insects used for this experiment were 1-d-old and virgin except the focal males that were 1-d-old and virgin only at the start of the experiments (see below). The focal males were kept individually prior to pairing.
4.2.1.1 Reproductive performance and longevity

To determine whether and how perception of the presence of rivals and additional mates by the focal male in the mating chamber affected his longevity and lifetime reproductive performance, I set up three treatments using the experimental device (see Section 3.2) where the focal male perceived: (1) rivals (+M-E) — one male and one female in the mating chamber and five males in the neighbouring chamber, (2) additional females (+F-E) — one male and one female in the mating chamber and five females in the neighbouring chamber, and (3) neither rivals nor additional females (CONT) — one male and one female in the mating chamber and no insect in the neighbouring chamber. This experimental design allowed the focal male in the mating chamber to perceive rivals or additional mates in the neighbouring chamber via chemical and/or acoustic cues but did not allow him to come into contact with the latter. Insects were introduced into their chambers one hour before the onset of the scotophase. Insects quickly settled and remained still until the start of the scotophase. Fifteen replicates were performed for each treatment (only 14 replicates for +M due to the accidental death of a focal male). To avoid the effect of chemical residues left on experimental containers, three sets of 15 containers were used, each for one treatment.

Observation commenced immediately after the scotophase started. For each mating chamber, the female was immediately removed after the termination of copulation and dissected for spermatophore extraction under the dissecting microscope (see Section 3.2). The number of sperm transferred by the male was counted under the phase-contrast microscope (see Section 3.2) according to Koudelova and Cook (2001) (see Section 3.4). The total number of sperm ejaculated by the male in his lifetime was recorded as male fecundity. Insects in the neighbouring chambers were removed after copulations were complete in all containers about one hour into the photophase. One hour before the onset of the next scotophase a female was offered to the focal male and five or no insects introduced into the neighbouring chamber according to the treatment. The procedure was repeated until the death of the focal male. As a result, the focal male was exposed to +M or +F treatment for 12 hours a day (one hour before the scotophase + 10 hours in the scotophase + one hour in the photophase). Copulation duration of each mating, lifetime number of copulations achieved and sperm ejaculated by the focal male and the longevity of the focal male were recorded.
4.2.1.2 Mating latency and courtship display

Males perform courtship display by fanning their wings when they encounter or perceive calling females (Trematerra & Pavan 1995; Xu et al. 2008; Xu & Wang 2009b). Therefore, wing fanning duration was used as an index of courtship duration by males. To determine mating latency and courtship duration under different socio-sexual context, I performed three treatments as in the previous experiment and recorded the behaviour of the focal males using the cameras (see Section 3.4). The recording was made between the start of the scotophase and commencement of copulation for the first day of pairing. Videos were reviewed, and the mating latency and wing fanning duration were recorded with a stopwatch. Twelve replicates were carried out for each treatment.

4.2.2 Focal Males with Exposure to Other Individuals prior to Pairing (+E)

The design was the same as the previous experiments (-E) except the focal male’s pre-pairing exposure to other individuals (+E).

4.2.2.1 Reproductive performance and longevity

To determine whether and how exposure to other individuals during the focal male’s early adulthood and then the perception of the presence of rivals and additional mates by the focal male affected his longevity and lifetime reproductive performance, I set up three treatments using the experimental device (see Section 3.2) where the focal male perceived: (1) rivals (+M+E) — one male and one female in one chamber and five males in the neighbouring chamber, with the focal male being exposed to five males in the neighbouring chamber for 24 hours before paired with the focal female; (2) additional mates (+F+E) — one male and one female in one chamber and five females in the neighbouring chamber, with the focal male being exposed to five females in the neighbouring chamber for 24 hours before paired with the focal female, and (3) control (CONT) - one male and one female in one chamber and no insect in the neighbouring chamber, with both focal male and focal female being kept individually before paired. Fifteen replicates were performed for each treatment.
Parameters recorded included mating latency for the first mating, lifetime number of copulations achieved, copulation duration, sperm ejaculated and longevity.

### 4.2.2.2 Courtship display

Male wing fanning duration was recorded between the start of the scotophase and commencement of copulation for the first day of pairing using the cameras (see Section 3.4) as in the previous experiment. Videos were reviewed and wings recorded using a stopwatch. Twelve replicates were carried out for each treatment.

### 4.2.3 Statistical Analysis

A goodness-of-fit test (Shapiro–Wilk test, SWT; UNIVARIATE procedure) was used to test the distribution of data. Data on the mean number of sperm transferred by –E males in a given mating (Figure 4.1, Tables 4.2 and 4.3) and mean total number of sperm (eupyrenerene and apyrenerene) transferred by both –E and +E males in lifetime (Figures 4.3 and 4.9) and mating frequency of +E males (Figure 4.8), were normally distributed and thus analysed using an analysis of variance (ANOVA, GLM procedure) followed by Tukey’s Studentized multiple range test.

Data became normally distributed after square-root transformation for the mean number of copulations of –E males (Figure 4.2) and mean eupyrenerene and apyrenerene sperm transferred by +E males in the first copulation (Figure 4.7), and after ‘ln(x)’ transformation for the mean copulation duration of both –E and +E males (Table 4.1 for –E males; Table 4.4 for +E males) and mean wing fanning duration of both –E and +E males (Figures 4.6B and 4.12B). These transformed data were then analysed using ANOVA followed by Tukey’s Studentized multiple range test.

Data on mean eupyrenerene and apyrenerene sperm transferred by +E males in the successive matings (Tables 4.5 and 4.6), and mating latency of –E males (Figure 4.6A) and +E males (Figure 4.12A) were not normally distributed even after transformation and thus analysed non-parametrical ANOVA (GLM procedure) followed by Bonferroni (Dunn) t Tests for multiple comparisons. Male survival was analysed using a Life test (LIFETEST procedure) (Figures 4.4 and 4.10).
Data on the relationships between copulation duration/sperm ejaculated and successive copulations of –E males (Figure 4.5) and +E males (Figure 4.11) were analysed using a generalized linear model (GLM, GENMOD procedure) because they were not normally distributed. The slopes of linear lines were compared using the contrast likelihood rate test (LR) with treatments as covariates in the GLM model.

4.3 Results

4.3.1 Focal Males without Exposure to Other Individuals prior to Pairing (-E)

4.3.1.1 Sperm allocation and copulation duration for the first copulation

Figure 4.1 shows sperm allocations by –E focal males in their first copulation under different SC levels. The number of eupyrene and apyrene sperm transferred by –E focal males was not significantly different between treatments although they allocated slightly more eupyrene sperm in +M treatment (ANOVA: $F_{2,41} = 1.30, P = 0.2826$ for eupyrene; $F_{2,41} = 0.45, P = 0.6387$ for apyrene) (Figure 4.1). Copulation duration in the first copulation was also similar for all treatments (ANOVA: $F_{2,41} = 1.32, P = 0.2786$) (Table 4.1).

![Figure 4.1](image.png)

**Figure 4.1** Mean (± SE) number of eupyrene ($\times 256$) and apyrene ($\times 3000$) sperm ejaculated by –E focal males in their first copulation under different SC levels. For each category, bars with the same letters are not significantly different ($P > 0.05$).
Table 4.1 Mean copulation duration (hour) associated with −E focal males in successive copulations under different SC levels.

<table>
<thead>
<tr>
<th>Copulation</th>
<th>CONT</th>
<th>+M</th>
<th>+F</th>
<th>F (df)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
<td>2.49 ± 0.14 a</td>
<td>2.55 ± 0.10 a</td>
<td>2.27 ± 0.14 a</td>
<td>1.32 (2,41)</td>
<td>0.2782</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>2.68 ± 0.16 a</td>
<td>2.78 ± 0.20 a</td>
<td>2.48 ± 0.28 a</td>
<td>0.50 (2,38)</td>
<td>0.6105</td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt;</td>
<td>2.94 ± 0.21 a</td>
<td>3.06 ± 0.26 a</td>
<td>2.80 ± 0.24 a</td>
<td>0.23 (2,32)</td>
<td>0.7958</td>
</tr>
<tr>
<td>4&lt;sup&gt;th&lt;/sup&gt;</td>
<td>3.44 ± 0.22 a</td>
<td>3.20 ± 0.29 a</td>
<td>3.07 ± 0.31 a</td>
<td>0.40 (2,23)</td>
<td>0.6749</td>
</tr>
<tr>
<td>5&lt;sup&gt;th&lt;/sup&gt;</td>
<td>3.52 ± 0.32 b</td>
<td>3.28 ± 0.11 b</td>
<td>4.83 ± 0.66 a</td>
<td>4.53 (2,15)</td>
<td>0.0289</td>
</tr>
<tr>
<td>6&lt;sup&gt;th&lt;/sup&gt;</td>
<td>4.21 ± 0.66 a</td>
<td>4.27 ± 0.21 a</td>
<td>0.01 (1,6)</td>
<td>0.9236</td>
<td></td>
</tr>
<tr>
<td>7&lt;sup&gt;th&lt;/sup&gt;</td>
<td>4.11 ± 1.16 a</td>
<td>3.21 ± 0.71 a</td>
<td>0.33 (1,3)</td>
<td>0.6059</td>
<td></td>
</tr>
</tbody>
</table>

Means (± SE) with the same letters in rows are not significantly different (P > 0.05).

4.3.1.2 Lifetime mating frequency, copulation duration, sperm allocation, and longevity

−E focal males in +F copulated significantly fewer times in their lifetime (ANOVA: F<sub>2,41</sub> = 6.81, P = 0.0028; Figure 4.2).

![Figure 4.2](image-url) Mean (± SE) lifetime number of copulations of −E focal males under different SC levels. Bars with the same letters are not significantly different (P > 0.05).
The mean total number of eupyrene and apyrene sperm transferred by −E focal males in their lifetime was significantly lower in +F than in +M and CONT (ANOVA: $F_{2,41} = 7.40$, $P = 0.0018$ for eupyrene; $F_{2,41} = 4.83$, $P = 0.0131$ for apyrene; Figure 4.3). However, the longevity of the −E focal males was similar in all three treatments (Lifetest: $x^2 = 2.43$, $P = 0.2961$; Figure 4.4).

Figure 4.3 Mean (± SE) total number of eupyrene ($\times 256$) and apyrene ($\times 3000$) sperm ejaculated by −E focal males during their lifetime under different SC levels. For each category, bars with the same letters are not significantly different ($P > 0.05$).

Figure 4.4 Survival of −E focal males under different SC levels. Lines with the same letters are not significantly different ($P > 0.05$).
Table 4.2 Mean number of eupyrene (× 256) sperm ejaculated by –E focal males in successive copulations under different SC levels.

<table>
<thead>
<tr>
<th>Copulation</th>
<th>CONT</th>
<th>+M</th>
<th>+F</th>
<th>F (df)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
<td>33.67 ± 3.20 a</td>
<td>41.00 ± 4.10 a</td>
<td>35.93 ± 2.07 a</td>
<td>1.30 (2,41)</td>
<td>0.2835</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>27.00 ± 1.76 b</td>
<td>33.31 ± 1.60 a</td>
<td>28.23 ± 1.67 b</td>
<td>4.56 (2,38)</td>
<td>0.0168</td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt;</td>
<td>31.33 ± 2.26 a</td>
<td>34.00 ± 3.74 a</td>
<td>30.00 ± 1.91 a</td>
<td>0.18 (2,32)</td>
<td>0.8361</td>
</tr>
<tr>
<td>4&lt;sup&gt;th&lt;/sup&gt;</td>
<td>29.00 ± 2.29 a</td>
<td>31.27 ± 2.71 a</td>
<td>35.80 ± 5.09 a</td>
<td>0.76 (2,23)</td>
<td>0.4791</td>
</tr>
<tr>
<td>5&lt;sup&gt;th&lt;/sup&gt;</td>
<td>22.50 ± 2.19 a</td>
<td>28.86 ± 3.16 a</td>
<td>21.00 ± 4.40 a</td>
<td>1.52 (2,15)</td>
<td>0.2506</td>
</tr>
<tr>
<td>6&lt;sup&gt;th&lt;/sup&gt;</td>
<td>25.75 ± 2.95 a</td>
<td>23.75 ± 3.47 a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7&lt;sup&gt;th&lt;/sup&gt;</td>
<td>23.00 ± 2.00 a</td>
<td>16.50 ± 2.50 a</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Means (± SE) with the same letters in rows are not significantly different (P > 0.05).

Table 4.3 Mean number of apyrene (× 3000) sperm ejaculated by –E focal males in successive copulations under different SC levels.

<table>
<thead>
<tr>
<th>Copulation</th>
<th>CONT</th>
<th>+M</th>
<th>+F</th>
<th>F (df)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
<td>19.54 ± 1.76 a</td>
<td>18.71 ± 1.98 a</td>
<td>17.50 ± 2.48 a</td>
<td>0.45 (2,41)</td>
<td>0.6407</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>14.00 ± 1.74 a</td>
<td>20.01 ± 3.35 a</td>
<td>15.27 ± 2.64 a</td>
<td>1.56 (2,38)</td>
<td>0.2233</td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt;</td>
<td>14.72 ± 0.83 a</td>
<td>15.76 ± 2.39 a</td>
<td>17.64 ± 4.61 a</td>
<td>0.02 (2,32)</td>
<td>0.9802</td>
</tr>
<tr>
<td>4&lt;sup&gt;th&lt;/sup&gt;</td>
<td>11.75 ± 2.07 a</td>
<td>15.14 ± 2.12 a</td>
<td>13.87 ± 2.60 a</td>
<td>0.73 (2,23)</td>
<td>0.4927</td>
</tr>
<tr>
<td>5&lt;sup&gt;th&lt;/sup&gt;</td>
<td>12.85 ± 2.12 a</td>
<td>11.65 ± 2.60 a</td>
<td>4.50 ± 0.70 a</td>
<td>2.92 (2,15)</td>
<td>0.0849</td>
</tr>
<tr>
<td>6&lt;sup&gt;th&lt;/sup&gt;</td>
<td>9.63 ± 3.18 a</td>
<td>4.53 ± 0.65 a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7&lt;sup&gt;th&lt;/sup&gt;</td>
<td>7.71 ± 1.46 a</td>
<td>4.32 ± 0.32 a</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Means (± SE) with the same letters in rows are not significantly different (P > 0.05).
–E focal males in +M transferred significantly more eupryrene sperm in their second copulation than in +F or CONT (Table 4.2). However, there was no significant difference between treatments in the number of apyrene sperm transferred by –E focal males in their successive matings (Table 4.3). For a given successive mating, no significant difference was found in copulation duration between treatments, except that in the fifth mating it was significantly longer for –E focal males in +F treatment (Table 4.1).

Sperm transferred declined (Figure 4.5A-B) and copulation duration increased (Figure 4.5C) with successive copulations. However, there was no significant difference in slopes between treatments (LR: $\chi^2 = 4.79, 2.36$ and 1.14 for eupryrene, apyrene and copulation duration, respectively; $P > 0.05$) (Figure 4.5).
Figure 4.5 Relationship between the number of eupyrene (×256)/apyrene (×3000) sperm ejaculated/copulation duration (hour, CD) and the order of matings (MN) in –E focal males under different SC levels. (A) Eupyrene sperm: CONT, eupyrene = exp (3.5357 – 0.0576 MN) (R^2 = 0.098, F1,68 = 7.44, P = 0.0081); +M, eupyrene = exp (3.7899 – 0.0983 MN) (R^2 = 0.2080, F1,62 = 16.29, P = 0.0002); +F, eupyrene = exp (3.5935 – 0.0638 MN) (R^2 = 0.1069, F1,41 = 4.91, P = 0.0323). (B) Apyrene sperm: CONT, apyrene = exp (3.0273 – 0.1249 MN) (R^2 = 0.1898, F1,68 = 15.93, P = 0.0002); +M, apyrene = exp (3.1652 – 0.1683 MN) (R^2 = 0.2209, F1,62 = 17.58, P = 0.0001); +F,
apyrene = \exp (3.0348 - 0.1358 \text{MN}) (R^2 = 0.1054, F_{1,41} = 4.83, P = 0.0337). (C) Copulation duration: CONT, CD = \exp (0.8115 + 0.0945\text{MN}) (R^2 = 0.3109, F_{1,68} = 30.68, P < 0.0001); +M, CD = \exp (0.8838 + 0.0703 \text{MN}) (R^2 = 0.2083, F_{1,62} = 16.31, P < 0.0001); +F, CD = \exp (0.6116 + 0.1584 \text{MN}) (R^2 = 0.3532, F_{1,41} = 22.39, P < 0.0001). All original data were used for analysis but only mean (± SE) values were presented.

4.3.1.3 Mating latency and courtship duration

My data show that socio-sexual environment had no effect on mating latency (ANOVA: F_{2,23} = 1.56, P = 0.2253) (Figure 4.6A). In all treatments –E focal males fanned their wings before copulation occurred. Most wing fanning took place in the last few hours of the scotophase. The focal males in +F performed wing fanning five to eight times longer than those in CONT and +M (ANOVA: F_{2,33} = 34.35, P < 0.0001) (Figure 4.6B). The wing fanning duration was not significantly different between CONT and +M (Figure 4.6B).
Figure 4.6 Mean (± SE) mating latency (A) and wing fanning duration (B) in –E focal males under different SC levels. Columns with the same letters are not significantly different (P > 0.05).
4.3.2 Focal Males with Exposure to Other Individuals prior to Pairing (+E)

4.3.2.1 Sperm allocation and copulation duration for the first copulation

+E focal males allocated significantly more eupyrene sperm in their first copulation in +M than in +F and CONT, however, they transferred similar number of apyrene sperm in all treatments (ANOVA: $F_{2,41} = 7.59$, $P = 0.0016$ for eupyrene, and $F_{2,41} = 2.26$, $P = 0.1167$ for apyrene) (Figure 4.7). Copulation duration in the first copulation was similar for all treatments (ANOVA: $F_{2,41} = 2.30$, $P = 0.1135$) (Table 4.4).

![Figure 4.7](image)

Figure 4.7 Mean (± SE) number of eupyrene ($\times 256$) and apyrene ($\times 3000$) sperm ejaculated by +E focal males in their first copulation under different SC levels. For each category, bars with the same letters are not significantly different ($P > 0.05$).

4.3.2.2 Lifetime mating frequency, copulation duration, sperm allocation, and longevity

+E focal males in +M copulated significantly more times than in +F and CONT (ANOVA: $F_{2,42} = 15.01$, $P < 0.0001$) (Figure 4.8). However, for any given copulation no significant difference in copulation duration was detected between treatments (Table 4.4).
Figure 4.8 Mean (± SE) lifetime number of copulations of +E focal males under different SC levels. Bars with the same letters are not significantly different (P > 0.05).

Table 4.4 Mean copulation duration (hour) associated with +E focal males in successive copulations under different SC levels.

<table>
<thead>
<tr>
<th>Copulation</th>
<th>CONT</th>
<th>+M</th>
<th>+F</th>
<th>F (df)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>1.76 ± 0.15 a</td>
<td>2.03 ± 0.11 a</td>
<td>1.78 ± 0.13 a</td>
<td>2.30 (2,41)</td>
<td>0.1131</td>
</tr>
<tr>
<td>2nd</td>
<td>3.19 ± 1.22 a</td>
<td>2.32 ± 0.13 a</td>
<td>2.22 ± 0.13 a</td>
<td>1.28 (2,41)</td>
<td>0.2889</td>
</tr>
<tr>
<td>3rd</td>
<td>2.30 ± 0.14 a</td>
<td>2.98 ± 0.29 a</td>
<td>2.51 ± 0.15 a</td>
<td>1.76 (2,39)</td>
<td>0.1854</td>
</tr>
<tr>
<td>4th</td>
<td>4.86 ± 2.02 a</td>
<td>3.31 ± 0.17 a</td>
<td>3.28 ± 0.24 a</td>
<td>0.79 (2,35)</td>
<td>0.4618</td>
</tr>
<tr>
<td>5th</td>
<td>3.21 ± 0.27 a</td>
<td>3.41 ± 0.36 a</td>
<td>2.70 ± 0.25 a</td>
<td>1.24 (2,28)</td>
<td>0.3048</td>
</tr>
<tr>
<td>6th</td>
<td>3.50 ± 0.60 a</td>
<td>3.79 ± 0.28 a</td>
<td>4.37 ± 0.42 a</td>
<td>0.66 (2,16)</td>
<td>0.5304</td>
</tr>
<tr>
<td>7th</td>
<td>2.05 ± 1.00 a</td>
<td>4.03 ± 0.52 a</td>
<td>4.38 ± 0.54 a</td>
<td>0.87 (2,10)</td>
<td>0.4484</td>
</tr>
<tr>
<td>8th</td>
<td>5.11 ± 0.50 a</td>
<td>5.59 ± 0.00 a</td>
<td>0.04 (1,6)</td>
<td>0.8481</td>
<td></td>
</tr>
<tr>
<td>9th</td>
<td>5.19 ± 1.00 a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10th</td>
<td></td>
<td></td>
<td>4.38 ± 0.29 a</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Means (± SE) with the same letters in rows are not significantly different (P > 0.05).
The mean total number of eupyrene and apyrene sperm transferred by +E focal males in their lifetime was significantly higher in +M than in CONT and +F (ANOVA: \( F_{2,42} = 33.61, P < 0.0001 \) for eupyrene, and \( F_{2,42} = 4.5, P = 0.0169 \) for apyrene) (Figure 4.9). The longevity of +E focal males was significantly shorter in +F than in CONT (Lifetest: \( x^2 = 9.49, P = 0.0087 \)) (Figure 4.10).

**Figure 4.9** Mean (± SE) number of eupyrene (× 256) and apyrene (× 3000) sperm ejaculated by +E focal males during their lifetime under different SC levels. For each category, bars with the same letters are not significantly different (\( P > 0.05 \)).

**Figure 4.10** Survival of +E focal males under different SC levels. Lines with the same letters are not significantly different (\( P > 0.05 \)).
In the first four copulations +E focal males in +M transferred significantly more eupyrene sperm than in CONT and +F, however, the number of eupyrene sperm ejaculated in the subsequent copulations was not significantly different between treatments (Table 4.5). The number of apyrene sperm transferred was not significantly different between treatments, except for the second mating where it was significantly higher in +M and +F than in CONT (Table 4.6).

**Table 4.5** Mean number of eupyrene ($\times 256$) sperm ejaculated by +E focal males in successive copulations under different SC levels.

<table>
<thead>
<tr>
<th>Copulation</th>
<th>CONT</th>
<th>+M</th>
<th>+F</th>
<th>$F_{(df)}$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
<td>38.43 ± 2.59 b</td>
<td>47.00 ± 2.08 a</td>
<td>34.73 ± 2.26 b</td>
<td>7.59 (2,41)</td>
<td>0.0016</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>26.60 ± 1.52 b</td>
<td>37.67 ± 1.39 a</td>
<td>25.93 ± 1.11 b</td>
<td>21.35 (2,41)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt;</td>
<td>26.17 ± 2.21 b</td>
<td>33.60 ± 1.61 a</td>
<td>25.53 ± 1.76 b</td>
<td>6.35 (2,39)</td>
<td>0.0041</td>
</tr>
<tr>
<td>4&lt;sup&gt;th&lt;/sup&gt;</td>
<td>18.44 ± 2.03 b</td>
<td>27.93 ± 1.23 a</td>
<td>19.18 ± 2.02 b</td>
<td>10.54 (2,35)</td>
<td>0.0003</td>
</tr>
<tr>
<td>5&lt;sup&gt;th&lt;/sup&gt;</td>
<td>21.22 ± 2.96 a</td>
<td>23.29 ± 1.71 a</td>
<td>19.43 ± 1.19 a</td>
<td>1.23 (2,27)</td>
<td>0.3082</td>
</tr>
<tr>
<td>6&lt;sup&gt;th&lt;/sup&gt;</td>
<td>13.50 ± 2.25 a</td>
<td>20.62 ± 1.59 a</td>
<td>19.33 ± 3.18 a</td>
<td>3.26 (2,17)</td>
<td>0.0634</td>
</tr>
<tr>
<td>7&lt;sup&gt;th&lt;/sup&gt;</td>
<td>20.00 ± 0.00 a</td>
<td>18.78 ± 1.04 a</td>
<td>12.50 ± 2.50 a</td>
<td>2.77 (2,9)</td>
<td>0.1157</td>
</tr>
<tr>
<td>8&lt;sup&gt;th&lt;/sup&gt;</td>
<td>15.57 ± 1.62 a</td>
<td>10.00 ± 0.00 a</td>
<td>12.50 ± 2.50 a</td>
<td>1.25 (1,6)</td>
<td>0.3068</td>
</tr>
<tr>
<td>9&lt;sup&gt;th&lt;/sup&gt;</td>
<td>11.50 ± 0.50</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Means (± SE) with the same letters in rows are not significantly different (P > 0.05).
Table 4.6 Mean number of apyrene (× 3000) sperm ejaculated by +E focal males in successive copulations under different SC levels.

<table>
<thead>
<tr>
<th>Copulation</th>
<th>CONT</th>
<th>+M</th>
<th>+F</th>
<th>F(df)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>11.89 ± 1.76 a</td>
<td>14.89 ± 1.05 a</td>
<td>12.45 ± 1.37 a</td>
<td>2.26 (2,41)</td>
<td>0.1167</td>
</tr>
<tr>
<td>2nd</td>
<td>7.89 ± 0.74 b</td>
<td>11.58 ± 0.83 a</td>
<td>10.91 ± 1.55 a</td>
<td>6.37 (2,41)</td>
<td>0.0039</td>
</tr>
<tr>
<td>3rd</td>
<td>12.14 ± 2.83 a</td>
<td>9.45 ± 0.73 a</td>
<td>11.70 ± 1.84 a</td>
<td>0.32 (2,39)</td>
<td>0.7295</td>
</tr>
<tr>
<td>4th</td>
<td>7.96 ± 0.07 a</td>
<td>9.80 ± 0.99 a</td>
<td>11.03 ± 1.92 a</td>
<td>1.28 (2,35)</td>
<td>0.2904</td>
</tr>
<tr>
<td>5th</td>
<td>10.69 ± 2.12 a</td>
<td>7.10 ± 0.65 a</td>
<td>9.33 ± 1.51 a</td>
<td>0.93 (2,27)</td>
<td>0.4057</td>
</tr>
<tr>
<td>6th</td>
<td>7.00 ± 1.18 a</td>
<td>5.31 ± 0.81 a</td>
<td>8.13 ± 1.41 a</td>
<td>0.52 (2,18)</td>
<td>0.6014</td>
</tr>
<tr>
<td>7th</td>
<td>8.13 ± 0.00 a</td>
<td>3.18 ± 0.60 a</td>
<td>4.94 ± 0.19 a</td>
<td>2.51 (2,10)</td>
<td>0.1311</td>
</tr>
<tr>
<td>8th</td>
<td>1.46 ± 0.45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9th</td>
<td>1.25 ± 0.50</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Means (± SE) with the same letters in rows are not significantly different (P > 0.05).

Similar to the -E experiments above (see Section 4.3.1) the number of sperm transferred +E focal males declined (Figure 4.11A and B) while copulation duration increased (Figure 4.11C) with successive copulations. However, the number of eupyrene sperm ejaculated declined significantly slower in +F than in CONT and +M (LR: $\chi^2_2 = 6.25$, $P = 0.0439$) (Figure 4.11A). The number of apyrene sperm declined faster in +M than in CONT and +F (LR: $\chi^2_2 = 52.62$, $P < 0.0001$) (Figure 4.11B). There was no significant difference in slopes between treatments in copulation duration over successive copulations (LR: $\chi^2_2 = 0.97$, $P = 0.6162$) (Figure 4.11C).
Figure 4.11 Relationships between the number of eupyrene ($\times 256$)/apyrene ($\times 3000$) sperm ejaculated/copulation duration (hour, CD) and the order of matings (MN) in +E focal males under different SC levels. (A) Eupyrene sperm: CONT, eupyrene = exp $(3.8018 - 0.1943 \times MN)$ ($R^2 = 0.4459$, $F_{1,68} = 54.73$, $P < 0.0001$); +M, eupyrene = exp $(4.0330 - 0.1813 \times MN)$ ($R^2 = 0.6516$, $F_{1,108} = 201.97$, $P < 0.0001$); +F, eupyrene = exp $(3.6461 - 0.1494 \times MN)$ ($R^2 = 0.4720$, $F_{1,67} = 59.90$, $P < 0.0001$). (B) Apyrene sperm: CONT, apyrene = exp $(2.4453 - 0.0540 \times MN)$ ($R^2 = 0.0719$, $F_{1,68} = 5.27$, $P = 0.0248$); +M, apyrene = exp $(3.0127 - 0.2466 \times MN)$ ($R^2 = 0.6173$, $F_{1,108} = 174.20$, $P < 0.0001$);
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\[ F, \text{ apyrene} = \exp(2.6796 - 0.1077 MN) \quad (R^2 = 0.0930, F_{1,67} = 6.87, P = 0.0108). \]  
\[ \text{(C)} \]

Copulation duration: \( \text{CONT, CD} = \exp(0.4905 + 0.1286 MN) \quad (R^2 = 0.3489, F_{1,65} = 34.84, P < 0.0001); \) \( \text{+M, CD} = \exp(0.6969 + 0.1058 MN) \quad (R^2 = 0.3925, F_{1,108} = 69.77, P < 0.0001); \) \( \text{+F, CD} = \exp(0.4712 + 0.1496 MN) \quad (R^2 = 0.5562, F_{1,67} = 83.96, P = 0.0004). \) All original data were used for analysis but only mean (± SE) values were presented.

\section*{4.3.2.3 Mating latency and courtship duration}

\( +E \) focal males in \( +M \) started copulating significantly earlier than in \( \text{CONT} \) and \( +F \) in their first mating (ANOVA: \( F_{2,41} = 28.35, P < 0.0001 \)) (Figure 4.12A).

Similar to the results of the \(-E\) experiments above (see Section 4.3.1), \( +E \) focal males fanned their wings before copulation occurred, and the males in \( +F \) performed significantly longer wing fanning than those in \( \text{CONT} \) and \( +M \) (ANOVA: \( F_{2,33} = 6.47, P = 0.0167 \)) (Figure 4.12B).
Figure 4.12 Mean (± SE) mating latency (A) and wing fanning duration (B) in +E focal males under different SC levels. Columns with the same letters are not significantly different (P > 0.05).

4.4 Discussion

SC theory (Parker 1970; Wedell et al. 2002a; Parker & Pizzari 2010) predicts that SC levels would determine the copulation duration and ejaculate allocation of the focal male where the male increases sperm allocation to his mate and prolongs the copulation in the presence of rivals, and the opposite is the case in the presence of additional females. To determine whether immediate SC levels affect sperm allocation and copulation duration, I compare the treatments for the first copulation before which time all insects were kept individually (–E experiments). My results show that the –E focal male allocated similar number of eupyrene and apyrene sperm from his reserves (Figure 4.1) to the mate and had similar copulation duration regardless of presence or absence of immediate SC effect (Table 4.1). These findings phenotypically contradict
the predictions of the SC model (Wedell et al. 2002a; Parker & Pizzari 2010), sperm use and storage model (Requena & Alonzo 2014) and sperm economy model (Abe & Kamimura 2015). Although not tested directly in the –E experiments, it is possible that the lack of phenotypic response to immediate socio-sexual context could result from female–male interactions (or conflicts). For example, with elevated SC levels males may attempt to increase sperm allocation (Wedell et al. 2002a; Parker & Pizzari 2010) but females may intend to mate again for genetic diversity via the last sperm precedence mechanism (Xu & Wang 2010a) and thus accept fewer sperm; or with reduced SC levels males may attempt to save sperm to inseminate more mates (Wedell et al. 2002a; Parker & Pizzari 2010) but females may attempt to collect more sperm (Requena & Alonzo 2014).

To assess the effects of both immediate and mean SC levels on sperm allocation and copulation duration, I took into consideration the results from both –E and +E experiments. First, I examined the first copulation of +E focal males which were exposed to males or females for 24 h before pairing. My results show that the focal male allocated significantly more eupryrene sperm to the mate in +M+E than in +F+E and CONT with similar number of apyrene sperm transferred across treatments (Figure 4.7) but copulation duration remained the same regardless of presence or absence of a SC effect (Table 4.4). These findings strongly support the predictions of the SC model in terms of sperm allocation (Wedell et al. 2002a; Parker & Pizzari 2010), suggesting that pre-pairing exposure to rivals is essential to detection of any SC effect and males only respond to mean SC levels in *E. kuehniella*. It is also suggested that copulation duration and eupryrene number are not positively correlated in this species. Furthermore, my findings challenge the common view on lepidopteran sperm production that eupryrene spermatogenesis stops after pupation (Lai-Fook 1982; Lachance & Olstad 1988; Friedlander 1997; Friedländer et al. 2005) because +E focal males are treated after their emergence. Second, I evaluated all copulation events during the focal males’ lifetime where -E focal males had perceived different socio-sexual environments for about 12 hours a day since the first mating event and +E focal males for 24 hours before pairing and 12 hours a day since the first mating event. In –E experiments I have not found any
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difference in sperm allocation (Tables 4.2 and 4.3) and copulation duration (Table 4.1) in any given copulation between treatments except for the second copulation, where the focal male transferred significantly more eupyrene sperm in +M-E than in CONT and +F-E (Table 4.2), and fifth copulation, where the copulation duration was significantly longer in +F-E than in CONT and +M-E (Table 4.1). Although the discrete patterns in these two copulations cannot be explained using any model at the present, these results phenotypically contradict the SC prediction. In a test of the effect of mean SC levels on sperm allocation in a cricket, Worthington et al. (2013) also indicate that males do not prudently adjust the number of sperm they transfer to mates based on mean SC levels, agreeing with my findings. However, Bretman et al. (2010) and Parker (2015, per. communication) point out that the length of exposure to rivals may be critical in determining male responses to the mean SC levels. My results suggest that without the 24-hour pre-pairing exposure to rivals, daily 12-hour exposure is not sufficient to trigger a measurable response of the male to mean SC levels. Similar to the findings in –E experiments, there was no difference in copulation duration (Table 4.4) in any given copulation between treatments in +E experiments. However, focal males in +M+E transferred significantly more eupyrene sperm to their mates than in +F+E and CONT during their first four copulations (Table 4.5) while the apyrene sperm transferred were similar in any given copulation between treatments except the second copulation (Table 4.6). These findings have three implications: (1) the predictions of the SC model in terms of sperm allocation are supported but the impact of the pre-pairing exposure to rivals only lasts for the first four copulations; (2) copulation duration and sperm allocation in any given copulation are not positively correlated regardless of whether focal males are exposed to rivals or not before pairing, and (3) focal males usually only adjust allocation of fertile eupyrene sperm in response to mean SC levels detected during the early adulthood before pairing. Although the extent may vary, male adjustment of sperm allocation in response to pre-pairing exposure to rivals occurs in other species including moths (Gage & Baker 1991; Cook & Wedell 1996; Wedell & Cook 1999a, b; Bretman et al. 2009; Price et al. 2012; Garbaczewska et al. 2013; Jarrige et al. 2015; Rouse & Bretman 2016). The increased eupyrene sperm allocation in response to higher mean SC levels may help males achieve greater paternity share in the SC battle (Parker et al. 1996, 1997; Wedell et al. 2002a).
When I examined my findings longitudinally, I found that in all treatments of both –E and +E experiments the ejaculate size decreased over successive copulations (Figures 4.5A-B and 4.11A-B), fitting the model on reproductive output declines with age of adults having fixed resources obtained during the immature stages (Begon & Parker 1986). In addition, copulation duration increased in successive copulations in all treatments regardless of whether focal males were exposed to rivals prior to pairing or not (Figures 4.5C and 4.11C). These results demonstrate a negative correlation between sperm allocation and copulation duration over males’ lifetime, contradicting findings in many other studies (e.g., Schofl & Taborsky 2002; Garcia-Gonzalez & Gomendio 2004; Prokop & Vaclav 2005; Bretman et al. 2009; Price et al. 2012; Lize et al. 2012b; Moatt et al. 2013). In *E. kuehniella*, a 24 hour recovery period between copulations is necessary for a male to be able to deliver a spermatophore filled with sperm to a female; if the intermating period is shorter, he can still transfer a spermatophore with no or few sperm (Xu & Wang 2011). This feature may explain why copulation duration and sperm number are not positively correlated over successive copulations in this species.

Furthermore, in both –E and +E experiments there was no significant difference in slopes between treatments in copulation duration over successive copulations (Figures 4.5C and 4.11C), suggesting that copulation duration cannot be used as a measure to detect males’ response to SC levels in *E. kuehniella*. If the mean SC levels played a role in both sperm production and allocation, then it would be expected that the decrease in ejaculate size over successive copulations would be faster in +M than in +F. In –E experiments (Figure 4.5A-B), the likelihood rate test did not show any significant difference in the slopes of regression lines for sperm depletion between treatments, indicating that the mean SC levels set in -E experiments (about 12 hours a day since the first mating event) are not sufficient to affect sperm production and allocation. However, in +E experiments, the number of eupyrene sperm ejaculated declined significantly slower in +F than in CONT and +M (Figure 4.11A) and the number of apyrene sperm declined significantly faster in +M than in CONT and +F (Figure 4.11B), strongly suggesting that pre-pairing exposure to rivals during the early adulthood is necessary to trigger males’ response to mean SC levels in *E. kuehniella*.

The lifetime reproductive fitness of focal males also differs between –E and +E experiments. For example, the focal male in –E+F had significantly fewer copulations
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(Figure 4.2) and transferred significantly lower number of sperm (Figure 4.3) than in +M-E and CONT, the latter two of which were not significantly different. However, the focal male in +M+E had significantly more copulations (Figure 4.8) and transferred significantly greater number of sperm (Figure 4.9) than in +F+E and CONT, the latter two of which were not significantly different. The findings from -E experiments contradict Xu and Wang (2014) and cannot be explained by the SC prediction while those from +E experiments support Xu and Wang (2014) and SC theory (Parker 1970; Wedell et al. 2002a; Parker & Pizzari 2010), further suggesting that pre-pairing exposure to rivals during the early adulthood increases sperm production and allocation in *E. kuehniella*. Results of the present study indicate that the lifetime number of sperm transferred by the focal male was, in fact, the function of his lifetime number of copulations, contradicting the prediction by the sperm economy model: female-biased conditions make males parsimonious and females promiscuous, that is both sexes mate more times under female-biased conditions (Abe & Kamimura 2015). In a previous study on *E. kuehniella*, Xu and Wang (2009b) demonstrate that (1) a male can inseminate up to nine females in his lifetime, ejaculating between 3,400 and 11,000 eupyrene sperm per copulation, and (2) once-mated females produce the same number of offspring (fertility) regardless of the number of eupyrene sperm received within the above range. This study shows that the number of females a male can mate during his life determines his lifetime reproductive fitness. In the present study, females received an average of 4,100–10,500 (Table 4.2) or 3,900-12,000 eupyrene sperm (except the ninth copulation with ca. 2,900 eupyrene sperm; Table 4.5) in a given copulation, falling into the range reported by Xu and Wang (2009b). It is thus strongly suggested that the reduced number of copulations by the focal male in +F-E (Figure 4.2) leads to a decline in his lifetime reproductive fitness and increased number of copulations by the focal male in +M+E (Figure 4.9) results in an increase in his lifetime reproductive fitness. Furthermore, in their lifetime –E+M focal males ejaculated an average of >38,000 eupyrene sperm (Figure 4.2) while +M+E focal males transferred an average of >56,000 eupyrene sperm (Figure 4.9), indicating that pre-pairing exposure to rivals during the early adulthood increases sperm production.

According to Emlen and Oring (1977), females should be choosier in the male-biased sex ratio, leading to longer mating latency. However, my results from neither –E
nor +E experiments support this notion. In –E experiments, mating latency was between 7 and 8 hours with no significant difference between treatments (Figure 4.6A) whereas in +E experiments, this was significantly shorter in +M+E than in +F+E and CONT (Figure 4.12A). My findings have at least two implications: (1) mean SC levels experienced by males in early adulthood play a key role in mating latency and (2) males are in the control of mating latency. Furthermore, several recent studies have tested sperm production rate and mating success in response to SC levels. For example, *D. melanogaster* males increase sperm production rate after exposed to rivals for a lengthy period of time (Moatt et al. 2014). The simultaneously hermaphroditic flatworm *Macrostomum lignano* Ladurner, Schärer, Salvenmoser and Rieger raised under higher SC levels produce sperm faster (Giannakara et al. 2016). In a study on the butterfly *Bicyclus anynana* (Butler), Kehl et al. (2015) show that young males with higher number of eupyrene sperm are more likely to succeed in mating than those with lower eupyrene sperm number. Therefore, the shorter mating latency (Figure 4.12A) and higher lifetime fecundity (Figure 4.9) found in +M+E focal males suggest that their pre-pairing exposure to rivals not only increases but also accelerates sperm production in *E. kuehniella*.

Increasing empirical evidence shows that precopulatory courtship displays by males are costly across taxa including humans (e.g., Cordts & Partridge 1996; Clutton-Brock & Langley 1997; Hoback & Wagner 1997; Kotiaho & Simmons 2003; Hunt et al. 2004; Simmons & Kotiaho 2007; Hoefler 2008; Papadopoulos et al. 2010; Wedell 2010; Gersick & Kurzban 2014). For example, in a moth *Ostrinia furnacalis* Guenée of the Pyraloidea, to which *E. kuehniella* belongs, the wing beat rate in courtship is almost twice as high as that in flight (Nakano et al. 2008), demonstrating at least one set of costs of wing fanning during courtship: energy. To determine the insight into why the focal male had lower lifetime number of mates inseminated (and lower number of sperm transferred) in the presence of additional mates, I tested the precopulatory courtship behaviour by the focal male for variation in response to dynamic socio-sexual environments. A previous study on mating behaviour of *E. kuehniella* (Trematerra & Pavan 1995) indicates that wing fanning by males is the major component of courtship behaviour and functions to attract females or make females receptive for mating. In both –E and +E experiments, focal males in +F fanned their wings for significantly longer
period before copulation than those in CONT and +M and the wing fanning duration was not significantly different between the latter two treatments (Figures 4.6B and 4.12B). This result strongly suggests that it is the increased courtship displays that reduce the lifetime copulation frequency and fecundity of the focal males in +F-E in *E. kuehniella*, contradicting the prediction where, in the presence of rivals, the focal male invests more in courting (Emlen & Oring 1977). In a more recent meta-analysis Weir et al. (2011) have also shown that courtship rate decreases rather than increases as the sex ratio becomes more male-biased because courtship is costly, supporting, in part, the findings of the present study that as compared to CONT, focal males did not increase courtship rate regardless of whether focal males were exposed to rivals before pairing.

Similar to findings in Janowitz and Fischer (2010) results of –E experiments show that male longevity was the same in all treatments (Figure 4.4) although –E+F focal males copulated significantly fewer times (Figure 4.2) and ejaculated significantly fewer sperm in their lifetime (Figure 4.3). Therefore, my findings from –E experiments do not demonstrate a clear trade-off between longevity and reproductive outputs in *E. kuehniella* in response to socio-sexual contexts, contradicting findings and predictions in many other studies (e.g., Kotiaho & Simmons 2003; Simmons & Kotiaho 2007; Hoefler 2008; Jordan & Brooks 2010; Papadopoulos et al. 2010; Wedell 2010; Bretman et al. 2013b; Scharf et al. 2013). However, as compared to CONT, although higher courtship cost in +F+E reduced focal males’ longevity (Figure 4.10), it did not lower their lifetime copulation frequency and fecundity (Figures 4.8 and 4.9). These findings suggest that pre-pairing exposure to mates also plays a role in adjustment of resource allocation by focal males, i.e. males allocate more resource for sperm production at the cost of longevity in response to perception of higher mating opportunity during their early adulthood, partially supporting the model by Abe and Kamimura (2015).

One could argue that the extremely female-biased sex ratio in +F treatments is not frequently encountered in nature and thus males are not adapted to it. Therefore, the male perception of calling signals produced by multiple females in +F may overstimulate the focal male so that he performs more courtship displays, costing his future reproductive outputs in –E+F treatment and longevity in +F+E treatment. These findings may have important implications for management of insect pests that use sex
pheromones. For example, synthetic female sex pheromones have now been used to disrupt matings for the control of *E. kuehniella*, achieving promising outcomes (e.g., Trematerra & Spina 2013; Trematerra et al. 2013). The rationale of the mating disruption approach using sex pheromone dispensers is to release a large amount of synthetic pheromones to the environment so that it is difficult for males to locate their mates, reducing the pest population size in the next generation. Here, my findings offer a novel explanation for the success of mating disruption approach: the tactic can also lower male reproductive outputs or longevity by increasing male courtship costs.

In conclusion, I demonstrate that males only respond to mean SC levels and pre-pairing exposure to rivals during the early adulthood is necessary to trigger males’ responses in *E. kuehniella*. My findings contradict many previous studies because copulation duration and sperm allocation are not positively correlated regardless of whether focal males are exposed to rivals or not before pairing. After pre-pairing exposure to rivals, males not only increase but also accelerate sperm production in *E. kuehniella*, challenging the common view on lepidopteran sperm production that eupyrene spermatogenesis stops after pupation. Without pre-pairing exposure to rivals or mates, males do not show a clear trade-off between longevity and reproductive outputs in response to socio-sexual contexts. However, following pre-pairing exposure to mates, males allocate more resource for sperm production on the cost of longevity. My study contradicts the common prediction that males invest more in the courting in presence of rivals because *E. kuehniella* males perform more courtship display in the presence of additional mates which costs either reproductive outputs in -E experiments or longevity in +E experiments. Finally, my findings offer a novel explanation for the success of mating disruption approach: the tactic can also lower male reproductive outputs or longevity by increasing male courtship costs.
Chapter 5

Female Reproductive Outputs and Survival in Response to Socio-sexual Environment

5.1 Introduction

Bateman’s (1948) principle predicts that males’ reproductive success increases linearly with the increase in the number of the matings they have, and selective pressure favours those prudent males possessing the ability to adjust their resource between and within mating bouts to achieve higher reproductive success (Wedell et al. 2002b; Parker & Pizzari 2010). However, females’ reproductive fitness depends on their own offspring production (Bateman 1948), which is affected by the number of sperm they receive per mating (Parker & Birkhead 2013; Abe & Kamimura 2015). Females have developed mechanisms to facilitate full fertilization of the eggs, which may make the sperm competition (SC) and pre-mating competition more costly for males (Birkhead et al. 1993; Westneat 2010). As a result, a conflict of fitness interest between males and females over the mating rate may occur: males attempt to increase the number of matings by which they achieve higher paternity share and decrease the possibility of female re-mating, whereas females endeavour to achieve an optimal mating frequency to ensure higher fertility and fecundity and minimize deleterious effects from re-mating (Parker 1979; Gavrilets et al. 2001; Wedell 2001; Torres-Vila 2013). However, reproductive strategies and mating behaviour of females in response to socio-sexual environment are far more understudied as compared to those in males (Kasumovic et al. 2008; Bretman et al. 2011a; Alonzo & Pizzari 2013).

The optimal mating rate for females (which guarantees the full fertilization of their egg load and compensates the costs associated with re-mating) and the effect of socio-sexual environment or operational sex ratio (OSR) on female re-mating behaviour are central to studies investigating evolutionary consequences of polyandry (Arnqvist & Nilsson 2000; Taylor et al. 2014). Different reactions of males and females to the biased
OSR determine the optimal mating rate (Weir et al. 2011). For example, in a male-biased sexual environment, females are more selective in accepting mating (Tinghitella et al. 2013; Atwell & Wagner 2014; Judge et al. 2014). When the local sex ratio is female-biased, however, females are expected to be more receptive (Stoffer & Uetz 2015). Moreover, a female-biased population is also predicted to increase intrasexual competition amongst females (Wedell et al. 2002b).

Requena & Alonzo (2014) predict that there should be a negative correlation between male ejaculate allocation and female promiscuity. They consider these two factors ‘independent’ which contradicts the traditional assumption of the SC model: female mating is the main determinant of risk and intensity of SC (Parker 1998; Parker & Pizzari 2010). Abe and Kamimura (2015) argue that male and female mating strategies are likely to coevolve and thus the optimal mating rate will depend on the adopted strategies of the other sex. These models along with all empirical studies clearly emphasize the importance of the role females play in the evolution and coevolution of reproductive strategies and the fitness consequences for both sexes (Pizzari & Wedell 2013). However, females’ responses to the variations in their sociosexual environment and the associated costs/benefits have received much less attention (den Hollander & Gwynne 2009; Fan et al. 2015).

The costs of mating could be the result of higher predation risk, sexually transmitted disease, time and energy consumption, harassment or male-male competition-derived costs, which affect female reproductive fitness and/or longevity (Arnqvist & Nilsson 2000; Wedell 2001; Parker & Birkhead 2013). Mating rate beyond the optimal level may thus be considered “deleterious” as it may decrease females’ fertility (Arnqvist & Nilsson 2000; Pizzari & Wedell 2013). However, despite the potential harms of re-mating, empirical studies (e.g., Tregenza & Wedell 2002; Arnqvist et al. 2005; Wilson & Tomkins 2015; Egan et al. 2016) suggest that females tend to increase the number of matings to maximize their reproductive fitness (Arnqvist & Nilsson 2000; South & Lewis 2011). For example, multiple mating increases the reproductive outputs of females (e.g., Wiklund et al. 1993, 1998; Billeter et al. 2012; Okada et al. 2013, 2015; Lee et al. 2014; Fan et al. 2015) through nutrient supply (e.g., ejaculates or nuptial gifts), sperm replenishment, and genetic benefits (Arnqvist &
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Nilsson 2000; Wedell 2001; Parker & Birkhead 2013). Mating in females may also affect their longevity. For example, mating and re-mating may have negative impacts on female longevity in some species (e.g., Partridge et al. 1986, 1987; Partridge & Fowler 1990; Chapman et al. 1995; Pletcher 1996; Mangan 1997; Edward et al. 2011; Wedell et al. 2002a; Fan et al. 2015) but multiply-mated females may live longer in others (e.g., Wiklund et al. 1993, 1998; Wigby and Chapman 2004; Lee et al. 2014).

Although one mating is sufficient for fertilization of the full egg load in *E. kuehniella*, females tend to re-mate (Xu & Wang 2009b). It has also been argued that females prefer different males for re-mating to gain genetic diversity in offspring (Xu & Wang 2009a). Before the occurrence of mating, females call with protruding abdominal tip and release sex pheromones and males approach calling females (Xu et al. 2008; Xu & Wang 2009a, b). Males’ responses to dynamic socio-sexual environment have been reported in this species (e.g., Xu & Wang 2014; Esfandi et al. 2015; Chapter 4). However, prior to the current study, nothing was known about how females behaved in response to dynamic socio-sexual environment.

On the basis of the above literature review, I carried out a series of experiments to evaluate how socio-sexual environment affected female mating behaviour, reproductive outputs, and longevity in *E. kuehniella*. During her lifetime I offered one virgin male to the focal female in the mating chamber every day in the presence of five virgin females, five virgin males, or no other individuals in the neighbouring chamber. Because pre-pairing exposure to males or females remarkably changed males’ response to the socio-sexual environment (see Chapter 4), I also compared treatments with and without pre-exposure in this chapter. The parameters I recorded included mating latency, number of matings, fecundity and fertility, longevity, and calling duration.

In this chapter I tested three hypotheses: (1) the focal female is choosier and has lower mating rate in the presence of additional males whereas the opposite is the case in the presence of rival females; (2) as a result, in the presence of additional males the focal female has lower fecundity and fertility and lives longer, and the opposite is the case in the presence of rival females, and (3) in the presence of additional males, mating
latency is longer and time spent by females on calling is shorter, and the opposite is the case in the presence of rival females.

5.2 Materials and methods

5.2.1 Focal females without exposure to other individuals prior to pairing (-E)

All insects used for this experiment were 1 d old and virgin except the focal females that were 1 d old and virgin only at the start of the experiments (see below). The focal females were kept individually prior to pairing.

5.2.1.1 Reproductive performance and longevity

To determine whether and how perception of the presence of rivals and additional males by the focal female in the mating chamber affected her longevity and lifetime reproductive performance, three treatments were set up using the experimental device (see Section 3.2) where the focal female perceived: (1) additional males (+M-E) — one male and one female in the mating chamber and five males in the neighbouring chamber, (2) rival females (+F-E) — one male and one female in the mating chamber and five females in the neighbouring chamber, and (3) neither additional males nor rival females (CONT) — one male and one female in the mating chamber and no insect in the neighbouring chamber. This experimental design allowed the focal female in the mating chamber to perceive rival females or additional mates in the neighbouring chamber via chemical and/or acoustic cues but did not allow her to be in physical contact with the latter. Insects were introduced into their chambers one hour before the onset of the scotophase. Insects quickly settled and remained still until the start of the scotophase. Fifteen replicates were performed for each treatment except in CONT since one of the females did not copulate. To avoid the effect of chemical residues left on experimental containers, three sets of 15 containers, each for one treatment were used.

Observation commenced immediately after the scotophase started. For each mating chamber, the focal male was immediately removed after the termination of copulation. All other insects were removed until one hour into the photophase. The focal female was kept singly in an oviposition cylinder until the next scotophase, and
other insects were discarded or moved to the colony. One hour before the onset of the next scotophase the focal female was offered a male and five or no insects were introduced into the neighbouring chamber according to treatment. As a result, the focal female was exposed to +M or +F treatments for 12 hours on each day (one hour before the scotophase + 10 hours in the scotophase + one hour in the photophase). The procedure was repeated until the death of the focal female. Mating latency, copulation duration, lifetime number of copulations accepted by the focal female, and longevity of the focal female were recorded.

5.2.1.2 Oviposition performance

To determine oviposition performance of each focal female, eggs laid in the oviposition cylinder and mating chamber were collected and counted daily and then incubated in Petri dishes. To determine the fertility (hatching rate), 3-d-old eggs were observed under the dissecting microscope (see Section 3.2), and eggs with black dots (larval heads) were recorded as fertile (Xu et al. 2007). To count unlaid eggs, the number of mature eggs in the ovaries of the focal female after death, I dissected the dead female in a drop of 1% saline solution on a glass slide under the dissecting microscope. The ovaries were separated out and immersed in 1% acetocarmine for 10 s to stain the eggs before being transferred to clean saline solution. The chorion of mature eggs prevents the stain but immature eggs absorb the stain (Edwards 1954). The total number of eggs produced was the sum of eggs laid and mature eggs remaining in the ovaries. Pre-oviposition and oviposition period were recorded for each female.

5.2.2 Focal females with exposure to other individuals prior to pairing (+E)

The design was similar to the previous experiments (-E) except the focal female’s pre-pairing exposure to other individuals (+E).

5.2.2.1 Reproductive performance and longevity

To determine whether and how exposure to other individuals during the focal female’s early adulthood and then the perception of the presence of rival females and
additional males by the focal female affected her longevity and lifetime reproductive performance, three treatments were set up using the experimental device (see Section 3.2) where the focal female perceived: (1) additional males with pre-pairing exposure (+M+E) — one male and one female in one chamber and five males in the neighbouring chamber, with the focal female being exposed to five males in the neighbouring chamber for 24 hours before paired with the focal male, (2) rival females with pre-pairing exposure (+F+E) — one male and one female in one chamber and five females in the neighbouring chamber, with the focal female being exposed to five females in the neighbouring chamber for 24 hours before paired with the focal male, and (3) Control (CONT) — one male and one female in one chamber and no insect in the neighbouring chamber, with both focal female and focal male being kept individually before paired. Fifteen replicates were performed for each treatment.

Parameters recorded included mating latency, lifetime number of copulations achieved, copulation duration, pre-oviposition and oviposition period, daily number of eggs laid, number of eggs unlaid, lifetime number of eggs produced, fertility, and longevity.

5.2.2.2 Calling behaviour

Females display calling behaviour which elicits males’ attempt to mate. They flex their abdomen upwards with abdominal segments 8-10 extruded and release the sex pheromone from their abdominal tip (Dickins 1936; Traynier 1970). This behaviour was used as an index of the female calling behaviour which was recorded under different socio-sexual contexts. To determine female calling behaviour, three treatments were set up as in the previous experiment and the focal female’s behaviour was recorded using cameras (see Section 3.4). The recording was made between the start of the scotophase and commencement of copulation. Videos were reviewed, and calling display duration was recorded with a stopwatch. Four replicates were carried out for each treatment for 10 successive days from the onset of the experiment. All insects used for this experiment were 1 d old and virgin, except the focal females whose age and mating experience varied since the first pairing.
5.2.3 Statistical analysis

A goodness-of-fit test (Shapiro-Wilk test) was used to test the distribution of data. Data on the mean copulation latency and copulation duration in a given mating (Tables 5.4 and 5.5), pre-oviposition and oviposition period of +E females (Table 5.6) were normally distributed and analysed using an analysis of variance (ANOVA, GLM procedure) followed by Tukey’s Studentized multiple range test.

Data on mean mating duration (Figure 5.1C), mean total number of produced eggs (Figure 5.5A), mean daily number of eggs laid, and oviposition period of –E females (Table 5.3) as well as the mean number of eggs unlaid by +E females (Table 5.6) were ln(\(x\)) transformed before ANOVA. Data became normally distributed after square-root transformation for the mean total number of laid eggs (Figure 5.10A), and mean calling duration of +E females (Figure 5.7D).

Data on mating frequency, mating latency, and fertility in both –E females (Figures 5.1A-B and 5.5B) and +E females (Figures 5.7A-B and 5.10B), as well as the mean number of unlaid eggs in –E females (Table 5.3), mean copulation duration (Figure 5.7C), pre-oviposition and oviposition period, and mean daily number of laid eggs of +E females (Table 5.6) were not normally distributed even after transformation and thus were subject to a non-parametric ANOVA followed by the multiple Bonferroni (Dunn) range test. Female survival in both –E females and +E females were analysed using a Life test (LIFETEST procedure) (Figures 5.3 and 5.9).

The oviposition pattern over females’ lifespan of –E females (Figure 5.6) and +E females (Figure 5.11) were analysed using a generalized linear model (GLM, GENMOD procedure) because they were not normally distributed. The slopes of linear lines were compared using contrast likelihood rate test with social-sexual environments as a covariate in the generalized linear model. The possibility of mating and oviposition for both –E females (Figures 5.2 and 5.4) and +E females (Figures 5.8 and 5.12) were analysed using logistic linear model (LOGISTIC procedure) and the difference between combination treatments (i.e., social-sexual environments \(\times\) ages) was compared by a multiple logistic likelihood rate (LR) test. Because more than 80% of eggs were laid within the first five days after pairing and few matings occurred five days after pairing, I
only analysed the data on the possibility of mating and oviposition for the first five days after pairing.

5.3 Results

5.3.1 Focal females without exposure to other individuals prior to pairing (-E)

5.3.1.1 Copulation frequency, duration, mating latency and longevity

Females in +M accepted significantly more copulations than in CONT (ANOVA: F_{2,41} = 7.02, P = 0.0023) (Figure 5.1A). The logistic likelihood rate test indicates that the possibility of female mating was usually significantly higher in +M than in +F and CONT during the first three days ($\chi^2_{14} = 105.33, P < 0.0001$) (Figure 5.2).

There was no significant difference in the mating latency or copulation duration between treatments (ANOVA: F_{2,83} = 1.41, P = 0.2499 for mating latency; F_{2,83} = 0.06, P = 0.9418 for copulation duration) (Figure 5.1B-C). Similar results were obtained when the mating latency or copulation duration was compared between treatments in the subsequent copulations (Tables 5.1 and 5.2). Furthermore, female longevity was not affected by the social-sexual environment (Life test: $\chi^2_{2} = 0.63, P = 0.7298$) (Figure 5.3).
Figure 5.1 Mean (± SE) copulation frequency (A), mating latency (B) and copulation duration (C) of –E focal females under different socio-sexual environments. For each category, bars with the same letters are not significantly different (P > 0.05).
Table 5.1 Mean mating latency (hour) associated with –E focal females in successive copulations under different socio-sexual environments.

<table>
<thead>
<tr>
<th>Copulation</th>
<th>CONT</th>
<th>+M</th>
<th>+F</th>
<th>F (df)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>7.39 ± 0.22 a</td>
<td>8.07 ± 0.39 a</td>
<td>7.72 ± 0.33 a</td>
<td>0.95 (2,41)</td>
<td>0.394</td>
</tr>
<tr>
<td>2nd</td>
<td>7.74 ± 0.67 a</td>
<td>7.88 ± 0.51 a</td>
<td>8.15 ± 0.61 a</td>
<td>0.10 (2,23)</td>
<td>0.9013</td>
</tr>
<tr>
<td>3rd</td>
<td>8.10 ± 0.87 a</td>
<td>7.69 ± 0.57 a</td>
<td>1.16 (1,8)</td>
<td>0.7036</td>
<td></td>
</tr>
<tr>
<td>4th</td>
<td>8.04 ± 0.54 a</td>
<td>5.88 ± 1.73 a</td>
<td>0.68 (1,4)</td>
<td>0.4563</td>
<td></td>
</tr>
</tbody>
</table>

Means (± SE) with the same letters in rows are not significantly different (P > 0.05).

Table 5.2 Mean copulation duration (hour) associated with –E focal females in successive copulations under different socio-sexual environments.

<table>
<thead>
<tr>
<th>Copulation</th>
<th>CONT</th>
<th>+M</th>
<th>+F</th>
<th>F (df)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>1.95 ± 0.10 a</td>
<td>1.94 ± 0.09 a</td>
<td>1.87 ± 0.14 a</td>
<td>0.15 (2,41)</td>
<td>0.8649</td>
</tr>
<tr>
<td>2nd</td>
<td>1.65 ± 0.10 a</td>
<td>1.82 ± 0.08 a</td>
<td>1.85 ± 0.13 a</td>
<td>0.72 (2,23)</td>
<td>0.4961</td>
</tr>
<tr>
<td>3rd</td>
<td>1.92 ± 0.15 a</td>
<td>2.29 ± 0.25 a</td>
<td>1.69 (1,8)</td>
<td>0.2302</td>
<td></td>
</tr>
<tr>
<td>4th</td>
<td>1.88 ± 0.13 a</td>
<td>2.09 ± 0.34 a</td>
<td>0.57 (1,4)</td>
<td>0.4922</td>
<td></td>
</tr>
</tbody>
</table>

Means (± SE) with the same letters in rows are not significantly different (P > 0.05).
**Figure 5.2** Probability of mating of -E focal females in the first five days since first pairing under different socio-sexual environments. Columns with the same letters are not significantly different (P > 0.05).

**Figure 5.3** Survival of –E focal females under different socio-sexual environments. Lines with the same letters are not significantly different (P > 0.05).
5.3.1.2 Oviposition performance

There was no significant difference in pre-oviposition period, oviposition period, daily number of eggs laid and number of eggs remained in females’ ovaries after death between treatments (Table 5.3). However, mating significantly delayed female oviposition in +M ($\chi^2 = 32.82$, P = 0.0135) (Figure 5.4).

Table 5.3 Reproductive outputs of -E focal females under different socio-sexual environments.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CONT</th>
<th>+M</th>
<th>+F</th>
<th>F (df)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-oviposition period (days)</td>
<td>1.71±0.24 a</td>
<td>2.13±0.35 a</td>
<td>1.93±0.41 a</td>
<td>0.24 (2,41)</td>
<td>0.7883</td>
</tr>
<tr>
<td>Oviposition period (days)</td>
<td>4.14±0.23 a</td>
<td>4.93±0.34 a</td>
<td>3.93±0.27 a</td>
<td>3.20 (2,41)</td>
<td>0.0511</td>
</tr>
<tr>
<td>Daily no. of eggs laid</td>
<td>19.04±2.99 a</td>
<td>21.42±3.61 a</td>
<td>17.89±3.04 a</td>
<td>0.23 (2,189)</td>
<td>0.7975</td>
</tr>
<tr>
<td>No. of eggs unlaid</td>
<td>1.93±0.60 a</td>
<td>6.20±2.33 a</td>
<td>2.07±0.50 a</td>
<td>1.31 (2,41)</td>
<td>0.2816</td>
</tr>
</tbody>
</table>

Means (± SE) with the same letters in each row are not significantly different (P > 0.05).

Figure 5.4 Probability of oviposition of –E focal females in the first five days since first
pairing under different socio-sexual environments. Columns with the same letters are not significantly different (P > 0.05).

The total number of eggs laid was not significantly different between treatments, although females laid more eggs in +M than in +F and CONT (ANOVA: F_{2,41} = 2.89, P = 0.0967) (Figure 5.5A). However, fertility was significantly higher in +M than in +F (ANOVA: F_{2,41} = 8.21, P = 0.0065) (Figure 5.5B).

![Figure 5.5](image_url)

**Figure 5.5** Mean (± SE) lifetime number of eggs laid (A) and fertility rate (B) of −E focal females under different socio-sexual environments. For each category, bars with the same letters are not significantly different (P > 0.05).

As shown in Figure 5.6, with increasing female age the daily number of eggs laid significantly increased (x^2 = 690.99, 891.58 and 241.95 for CONT, +M and +F, respectively; P < 0.0001) and then significantly decreased (x^2 = 830.00, 1045.63 and 403.62 for CONT, +M and +F, respectively; P < 0.0001) in all social-sexual environments. The likelihood rate test indicates that with increasing female age, the
daily number of eggs laid increased significantly faster in CONT than in +M and +F, and increased significantly faster in +M than in +F ($\chi^2 = 244.45$, $P < 0.0001$); however, it decreased significantly faster in CONT than in +M, and decreased significantly faster in +M than in +F ($\chi^2 = 297.15$, $P < 0.0001$) (Figure 5.6).

**Figure 5.6** Daily number of eggs laid (DNE) by −E focal females under different socio-sexual environments. CONT, DNE = exp ($1.1005 + 1.4960$ Age $– 0.2049$ Age$^2$) ($R^2 = 0.4105$, $F_{2,139} = 48.41$, $P < 0.0001$); +M, DNE = exp ($0.9793 + 1.2537$ Age $– 0.1399$ Age$^2$) ($R^2 = 0.2763$, $F_{2,156} = 29.78$, $P < 0.0001$); +F, DNE = exp ($2.3197 + 0.5705$ Age $– 0.0773$ Age$^2$) ($R^2 = 0.1654$, $F_{2,151} = 14.96$, $P < 0.0001$).
5.3.2  Focal females with exposure to other individuals prior to pairing (+E)

5.3.2.1 Copulation frequency, duration, mating latency, calling duration, and longevity

In their lifetime, females in +M copulated significantly more times than in +F (ANOVA: $F_{2,42} = 3.46, P = 0.0421$) (Figure 5.7A). However, there was no significant difference between treatments in mating latency (ANOVA: $F_{2,65} = 1.43, P = 0.2467$) (Figure 5.7B) or copulation duration (ANOVA: $F_{2,65} = 2.03, P = 0.1396$) (Figure 5.7C). The calling duration was significantly longer in CONT than in other treatments (ANOVA: $F_{2,33} = 8.62, P = 0.0010$) (Figure 5.7D).

For each treatment, the possibility of copulation decreased significantly with increasing female age ($x^2_{1,4} = 120.13, P < 0.0001$) (Figure 5.8). For each mating, there was no significant difference in the mating latency or copulation duration between treatments (Tables 5.4 and 5.5), except the mating latency in the first copulation where it was significantly longer in +F than in +M. Female longevity was similar in all treatments (Life test: $x^2_{2} = 0.21, P = 0.8992$) (Figure 5.9).
Figure 5.7 Mean (± SE) copulation frequency (A), mating latency (B), copulation duration (C) and total calling duration (D) associated with +E focal females under different socio-sexual environments. For each category, bars with the same letters are not significantly different (P > 0.05).
**Table 5.4** Mean mating latency (hour) associated with +E focal females in successive copulations under different socio-sexual environments.

<table>
<thead>
<tr>
<th>Copulation</th>
<th>CONT</th>
<th>+M</th>
<th>+F</th>
<th>(F_{(df)})</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1\textsuperscript{st}</td>
<td>7.58 ± 0.62 a</td>
<td>5.96 ± 0.79 b</td>
<td>9.16 ± 0.24 a</td>
<td>7.81 ( (2,44) )</td>
<td>0.0012</td>
</tr>
<tr>
<td>2\textsuperscript{nd}</td>
<td>7.78 ± 1.58 a</td>
<td>8.99 ± 0.50 a</td>
<td>2.60 ± 0.00 a</td>
<td>2.64 ( (2,13) )</td>
<td>0.1088</td>
</tr>
<tr>
<td>3\textsuperscript{rd}</td>
<td>9.46 ± 0.18 a</td>
<td>8.67 ± 0.00 a</td>
<td>3.95 ( (1,3) )</td>
<td>0.1409</td>
<td></td>
</tr>
</tbody>
</table>

Means (± SE) with the same letters in rows are not significantly different (P > 0.05).

**Table 5.5** Mean copulation duration (hour) associated with +E focal females in successive copulations under different socio-sexual environments.

<table>
<thead>
<tr>
<th>Copulation</th>
<th>CONT</th>
<th>+M</th>
<th>+F</th>
<th>(F_{(df)})</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1\textsuperscript{st}</td>
<td>1.92 ± 0.13 a</td>
<td>2.22 ± 0.11 a</td>
<td>1.92 ± 0.12 a</td>
<td>1.97 ( (2,44) )</td>
<td>0.1520</td>
</tr>
<tr>
<td>2\textsuperscript{nd}</td>
<td>2.56 ± 0.60 a</td>
<td>2.18 ± 0.19 a</td>
<td>2.57 ± 0.00 a</td>
<td>0.28 ( (2,13) )</td>
<td>0.7582</td>
</tr>
<tr>
<td>3\textsuperscript{rd}</td>
<td>2.24 ± 0.12 a</td>
<td>1.83 ± 0.00 a</td>
<td>2.35 ( (1,3) )</td>
<td>0.2230</td>
<td></td>
</tr>
</tbody>
</table>

Means (± SE) with the same letters in rows are not significantly different (P > 0.05).
Figure 5.8 Probability of mating of +E focal females in the first five days since first pairing under different socio-sexual environments. Columns with the same letters are not significantly different (P > 0.05).

Figure 5.9 Survival of +E focal females under different socio-sexual environments. Lines with the same letters are not significantly different (P > 0.05).
5.3.2.2 Oviposition performance

The pre-oviposition period was not significantly different between treatments (Table 5.4). However, the oviposition period was significantly longer in +F than in +M (Table 5.6). The daily number of eggs laid or the number of eggs remaining in females’ ovaries after death was not significantly different between treatments (Table 5.6).

The pattern of oviposition possibility was similar for all treatments with no significant difference ($\chi^2_{11} = 13.23, P = 0.2786$) (Figure 5.10). Females in +M laid significantly more eggs in their lifetime than in CONT (ANOVA: $F_{2,42} = 5.18, P = 0.0098$) (Figure 5.11A) and had significantly higher fertility rate than in +F (ANOVA: $F_{2,42} = 5.52, P = 0.0074$) (Figure 5.11B).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CONT</th>
<th>+M</th>
<th>+F</th>
<th>$F_{(df)}$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-oviposition period (days)</td>
<td>3.20±0.14 a</td>
<td>3.27±0.20 a</td>
<td>3.13±0.09 a</td>
<td>0.00(2,42)</td>
<td>0.9966</td>
</tr>
<tr>
<td>Oviposition period (days)</td>
<td>5.07±0.47 ab</td>
<td>4.40±0.16 b</td>
<td>5.67±0.23 a</td>
<td>6.58(2,42)</td>
<td>0.0033</td>
</tr>
<tr>
<td>Daily no. of eggs laid</td>
<td>15.29±2.46 a</td>
<td>22.11±3.41 a</td>
<td>18.94±2.99 a</td>
<td>0.16(2,424)</td>
<td>0.8538</td>
</tr>
<tr>
<td>No. of eggs unlaid</td>
<td>3.60±1.29 a</td>
<td>0.47±0.32 a</td>
<td>1.40±0.50 a</td>
<td>2.24(2,42)</td>
<td>0.1189</td>
</tr>
</tbody>
</table>

Means (± SE) with the same letters in each row are not significantly different ($P > 0.05$).
**Figure 5.10** Probability of oviposition of +E focal females in the first five days since first pairing under different socio-sexual environments. Columns with the same letters are not significantly different (P > 0.05).

**Figure 5.11** Mean (± SE) lifetime number of eggs laid (A), and fertility rate (B) associated with +E focal females under different socio-sexual environments. For each category, bars with the same letters are not significantly different (P > 0.05).
The daily number eggs laid by +E females significantly increased ($x^2_1 = 976.19, 1490.94$ and $1309.16$ for CONT, +M and +F, respectively; $P < 0.0001$) and then significantly decreased ($x^2_1 = 1019.09, 1548.53$ and $1349.87$ for CONT, +M and +F, respectively; $P < 0.0001$) with the increase of age (Figure 5.12). The likelihood rate test indicates that with increasing age, the daily number of eggs laid increased significantly faster ($x^2_2 = 53.28, P < 0.0001$) and decreased significantly faster in +F and +M than in CONT ($x^2_2 = 78.25, P < 0.0001$) (Figure 5.12).

![Daily number of eggs laid (DNE) by +E focal females under different socio-sexual environments.](image)

**Figure 5.12** Daily number of eggs laid (DNE) by +E focal females under different socio-sexual environments. CONT, DNE = exp ($-2.6612 + 2.5983 \text{Age} - 0.2646 \text{Age}^2$) ($R^2 = 0.4782, F_{2,166} = 76.07, P < 0.0001$); +M, DNE = exp ($-2.8697 + 2.6007 \text{Age} - 0.2433 \text{Age}^2$) ($R^2 = 0.4855, F_{2,171} = 80.68, P < 0.0001$); +F, DNE = exp ($-4.2282 + 3.3500 \text{Age} - 0.3374 \text{Age}^2$) ($R^2 = 0.5865, F_{2,171} = 121.27, P < 0.0001$).

### 5.4 Discussion

Theories predict that a female-biased condition makes males parsimonious and females promiscuous (Abe & Kamimura 2015) but females should be choosier in the male-biased sex ratio, leading to longer mating latency (Emlen & Oring 1977; Kvarnemo & Ahnesjo 1996). However, my results do not support these predictions. In both –E and +E experiments, the mean mating latency was between 7 and 8 hours with no significant difference detected between treatments (Figures 5.1B and 5.7B). Similarly, there was no significant difference in mating latency in any given copulation
Female Reproductive Outputs and Survival

between treatments except the first mating in +E experiment where mating latency was significantly shorter in +M than in +F, but that in neither +M nor +F was significantly different from that in CONT (Tables 5.1 and 5.4). These results indicate that *E. kuehniella* females are less likely to respond to the social-sexual environments.

If females are choosier in a male-biased environment as predicted by theories (e.g., Kvarnemo & Ahnesjo 1996; Tinghitella et al. 2013; Atwell & Wagner 2014; Judge et al. 2014; Abe & Kamimura 2015; Stoffer & Uetz 2015), then they should have fewer matings in such context. My results show that in both –E and +E experiments, females mated significantly more times in +M than in +F or in CONT (Figures 5.1A and 5.7A), contradicting the above theoretical predictions. When I looked into the probability of mating in relation to socio-sexual environment, I found that the focal females in +M were more receptive to the courting males compared to those in +F and CONT, especially in the first two days of pairing (Figures 5.2 and 5.8). My findings strongly suggest that *E. kuehniella* females are more receptive rather than choosier under male-biased sex ratio. Furthermore, higher mating frequency in the focal females in +M should not be attributed to the greater male effort in courtship because males made significantly less courtship display in the male-biased socio-sexual environment (Figures 4.6B and 4.12B in Chapter 4). In males, copulation duration increased in successive copulations in all treatments (Figures 4.5C and 4.11C in Chapter 4) due to aging in males but in females this remained the same in all copulations (Figures 5.1C and 5.7C, Tables 5.2 and 5.5), suggesting that *E. kuehniella* females have no response to socio-sexual environment in copulation duration.

In lepidopteran moths, females call and release sex pheromones to attract males for mating. Females are expected to call less under a male-biased sex ratio (Sadek, 2012; Rehermann et al. 2016) but more in a female-biased sex ratio (Stoffer & Uetz 2015). However, this notion is not supported by my findings where *E. kuehniella* females called significantly more when no additional individuals were present (CONT) than when they were exposed to either males (+M) or females (+F), with no difference between the two treatments (Figure 5.7D). The present study indicates that *E. kuehniella* females respond to socio-sexual environment very differently from their male counterparts which have significantly longer courtship display when additional females
Female Reproductive Outputs and Survival

are present (Figures 4.6B and 4.12B in Chapter 4). It is likely that females reduce calling duration in response to increased local population density regardless of sex ratio. In other moth species *Grapholita molesta* (Busck) (Stelinski et al. 2006) and *Lobesia botrana* (Denis & Schiffermüller) (Harari et al. 2015), calling is shorter in female-biased sex ratio. These findings suggest that female calling in response to socio-sexual environment could be species specific.

Despite the potential harms of multiple mating, females tend to increase the number of matings to maximize their reproductive fitness (Arnqvist & Nilsson 2000; South & Lewis 2011) through nutrient supply (e.g., ejaculates or nuptial gifts) (e.g., Wiklund et al. 1993, 1998; Billeter et al. 2012; Okada et al. 2013, 2015; Lee et al. 2014; Fan et al. 2015), sperm replenishment, genetic benefits (Arnqvist & Nilsson 2000; Wedell 2001; Parker & Birkhead 2013), or longevity (e.g., Wiklund et al. 1998; Wedell et al. 2002b; Wigby & Chapman 2004; Okada et al. 2013, 2015; Lee et al. 2014). However, under even sex ratio, re-mating does not increase fertility, fecundity or longevity in *E. kuehniella* females (Xu & Wang 2009a). In the present study, although +M and +F treatments had no effect on female longevity (Figures 5.3 and 5.9) and little impact on fecundity (Figures 5.5A and 5.11A), focal females had significantly higher fertility rate in +M than in +F regardless of whether pre-pairing exposure occurred (Figures 5.5B and 5.11B). This finding indicates that perception of additional males makes females more receptive so that they mate more times (Figures 5.1A and 5.7A) and fertilise more eggs (Figures 5.5B and 5.11B).

Regardless whether focal females were exposed to other individuals in their early adulthood, with the increase of age their daily fecundity significantly increased and then significantly decreased in all treatments and CONT (Figures 5.6 and 5.12). However, the daily fecundity patterns were different depending on whether pre-pairing exposure occurred. In –E experiment, +M females increased their daily fecundity significantly faster during their first half of life and decreased significantly faster compared to +F females (Figures 5.6). The opposite was the case in +E experiments (Figure 5.12). My results suggest that pre-pairing exposure alters females’ lifetime resource allocation patterns for oviposition. Although the underlying mechanisms are not clear, my results on probability of copulation (Figures 5.2 and 5.8) and oviposition
(Figures 5.4 and 5.10) under different socio-sexual environment may provide a clue for future exploration.

Overall, my study demonstrates that *E. kuehniella* females do respond to variations of socio-sexual environment, providing new information on females’ reproductive behaviour for future exploration of insight into females’ reproductive strategies under different socio-sexual environment. I show that females (current chapter) and males (Chapter 4) use different strategies to gain reproductive fitness under given socio-sexual contexts. Finally, none of the hypotheses proposed in the Introduction is supported by my findings.
Chapter 6

Reproductive Outputs and Survival of Virgin Moths under Different Socio-sexual Environment

6.1 Introduction

In the previous chapters, I investigated the effects of socio-sexual environment on reproductive and somatic fitness, where focal males and females were offered partners of the opposite sex and allowed mating to occur. Darwin’s (1871) theory predicts two main selection forces behind sexual selection that operate independently: (1) intrasexual selection where individuals of the same sex (typically males) compete with each other to acquire a mate from the opposite sex, and (2) intersexual selection which is the result of preferential mate choice by one sex for particular individual of the opposite sex (usually choice of males by females) (Clutton-Brock 2007; Miller & Svensson 2014). A consequence of sexual selection is that for a given sex, the winners mate and losers may remain virgin temporarily or even for lifetime (Gowaty & Hubbell 2013).

Mating failures due to sexual selection may be more common in males than in females because sexual selection is not expected to act on females in the same way as it does on males (Clutton-Brock 2007, 2009). However, mating failures in females do exist in nature in both haplodiploid [e.g., fig wasps species (West et al. 1998)] and diploid species [e.g., butterflies *Parnassius clodius* Menetries and *P. smintheus* Doubleday (Calabrese & Fagan 2004; Calabrese et al. 2008)]. In haplodiploid species, mated females produce both fertilised and unfertilised eggs that give rise to diploid female and haploid male offspring, respectively, while virgin females produce only unfertilised eggs that develop into haploid males (Quicke 1997). In diploid species such as *E. kuehniella*, however, only fertilised eggs can develop to diploid male and female offspring and unfertilised eggs fail to hatch. Therefore, virgin females of a diploid species have no reproductive rewards from oviposition.
Sexual selection causing variation of mating success between sexes or within a sex may have shaped the evolution of the life-history strategy of a species. The life-history theories predict that organisms have a limited amount of resources which must be competitively allocated to growth, reproduction, survival, and maintenance (Cody 1966; Stearns 1992; Cichoń 2001). Therefore, resources invested in one function cannot be used in another, leading to trade-offs (Stearns 1992; Roff & Fairbairn 2007). Based on these assumptions, two traditional life history trade-offs are proposed (Stearns 1992; Edward & Chapman 2011a): (1) current reproduction versus future reproduction, and (2) current reproduction versus future survival. The trade-offs between current reproduction and future reproduction/survival have long been recognised as a prominent feature of life history trajectories in many species (Traverse et al. 2015). Because socio-sexual environment may change in time and space (Miller & Svensson 2014; Evans & Garcia-Gonzalez 2016), organisms with sexual reproduction are supposed to adjust investment in reproduction and survival in response to such variations; parameters measured include sperm ejaculation (e.g., Parker 1970; Kotiaho & Simmons 2003; Simmons & Kotiaho 2007; Hoefler 2008; Jordan & Brooks 2010; Papadopoulos et al. 2010; Wedell 2010; Parker & Pizzari 2010; Bretman et al. 2011a; Bretman et al. 2013b; Scharf et al. 2013), oviposition (e.g., Arqvist & Nilsson 2000; den Hollander & Gwynne 2009; Xu & Wang 2009a; Requena & Alonzo 2014; Taylor et al. 2014; Fan et al. 2015) and longevity (e.g., den Hollander & Gwynne 2009; Xu & Wang 2009a; Lize et al. 2014; Abe & Kamimura 2015; Fan et al. 2015).

Previous reports show that *E. kuehniella* males respond to increased mean SC levels by increasing sperm production and allocation (see Chapter 4) and *D. melanogaster* males raised under male-biased sex ratio increase sperm competitive ability (Nandy et al. 2013). However, the sizes of testis and accessory gland do not differ in virgin *D. melanogaster* males raised under male- and female-biased sex ratio, suggesting that sperm competitive ability are not due to the evolution of testis/gland size or strategic ejaculate investment (Chechi et al. 2017). Various studies suggest that virgin females of various species may buy time for future opportunities of mating and reproduction by reducing reproduction during their early life (e.g., de Clercq & Degheele 1997; Torres et al. 1997; Fauvergue et al. 2008; Steiner & Ruther 2009;
Soares et al. 2011; Xu & Wang 2011). So far, strategies adopted by virgin males and females under different socio-sexual environment are still largely unknown.

In this chapter, I investigated how virgin males and females of *E. kuehniella* adjusted their investment in reproduction and somatic maintenance in response to the socio-sexual environment. I carried out a series of experiments to test how the perception of the presence of rivals or potential mates affected their reproduction and longevity. During his/her lifetime the focal virgin male/female was maintained in a chamber in the presence of five virgin females, five virgin males, or no other individuals in a neighbouring chamber, and not allowed to mate. The number of eggs laid by the focal virgin females was counted daily, and immediately after death, the insects were dissected to count the number of sperm produced by focal virgin males and the number of eggs that remained in the ovaries of focal virgin females.

Here I tested four hypotheses: (1) the focal virgin male invests more in sperm production in the presence of rivals but allocates more energy to courting in the presence of additional females; (2) as a result, the focal virgin male may have similar longevity regardless of whether rivals or additional mates are present; (3) the focal virgin female has shorter pre-oviposition period and higher fecundity in the presence of males and the opposite is the case in the presence of females, and (4) as a result, the focal virgin female lives shorter in the presence of males, and the opposite is the case in the presence of females.

6.2 Materials and Methods

6.2.1 Reproduction, survival and courtship of virgin males

This experiment was to determine whether and how the perception of the presence of rivals and potential mates by the focal virgin male affected his lifetime sperm produced, courtship performance and longevity. Three treatments were set up using the experimental device (see Section 3.2) where the focal male perceived: (1) presence of rivals (+M), – a virgin male in an experimental chamber and five virgin males in a neighbouring chamber, (2) presence of potential mates (+F) – a virgin male in an experimental chamber and five virgin females in a neighbouring chamber, and (3)
neither rivals nor potential mates (CONT) – a virgin male in an experimental chamber and no insect in a neighbouring chamber. When they were 1 d old, the focal virgin males were individually introduced into the experimental chambers along with the neighbouring insects according to the treatment, one hour before the onset of the scotophase. The focal males were exposed to the neighbouring insects throughout their life and their longevity was recorded. Neighbouring insects were replaced with 1-d-old virgin ones daily one hour before the scotophase until the death of the focal males.

The dead focal males were dissected to count the number of sperm (eupyrene and apyrene) in their testes, seminal vesicle and vas deferens. Furthermore, I also dissected newly emerged (0-d-old) and 1-d-old virgin males without any exposure to other insects to count the number of sperm in the above reproductive organs. All insects used for experiments were of average weight (Section 3.4). For each treatment, fifteen replicates were performed. To avoid the effect of chemical residues left on experimental chambers, three sets of 15 chambers, each for one treatment, were used.

To determine the effect of socio-sexual environment on the frequency and duration of male wing fanning, I recorded the behaviours of four focal males in each treatment for 10 days during the scotophase using cameras (see Section 3.4) from the first day when they were introduced into the experimental chambers.

### 6.2.2 Reproduction and survival of virgin females

This experiment was to determine whether and how the perception of the presence of rivals and additional mates by a virgin female affected their lifetime egg production and longevity. Three treatments were set up using the experimental device (see Section 3.2), where the focal females perceived: (1) presence of potential mates (+M) – a virgin female in an experimental chamber and five virgin males in a neighbouring chamber, (2) presence of rivals (+F) – a virgin female in an experimental chamber and five virgin females in a neighbouring chamber, and (3) neither potential mates nor rivals (CONT) – a virgin female in an experimental chamber and no insect in the neighbouring chamber. All insects used were 1-d-old, virgin and of average weight (Section 3.4). Insects in all treatments were introduced into their chambers one hour before the onset of the scotophase. In all treatments, insects in the neighbouring
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chambers were replaced daily with 1-d-old virgin ones one hour before the scotophase according to the treatments until the death of the focal females. The focal females were exposed to the neighbours during the photophase and scotophase for their entire life. Fifteen replicates were performed for each treatment. To avoid the effect of chemical residues left on experimental chambers, three sets of 15 chambers, each for one treatment were used.

To determine oviposition performance of focal females, eggs laid were collected daily and counted. In all treatments, the number of unlaid eggs was also counted using the method described in Section 5.2.1.2. The total number of eggs produced was the sum of eggs laid and mature eggs remaining in the ovaries. Pre-oviposition and oviposition period and longevity were recorded for each female.

6.2.3 Statistical analysis

A goodness-of-fit test (Shapiro-Wilk test) was used to test the distribution of data. Data on number of eupyrene sperm (Figure 6.1A), pre-oviposition period (Figure 6.4) and total number of eggs produced (Figure 6.5) were normally distributed and thus analysed using an analysis of variance (ANOVA, GLM procedure) followed by Tukey’s Studentized multiple range test.

Data on the relationship between the daily number of eggs laid and age (Figure 6.7) were analysed using a generalized logistic linear model (GLLM, GENMOD procedure) because they were not normally distributed. The slopes of linear lines were compared using an analysis of covariance (ANCOVA) with treatments as the covariate in the GLMM model followed by the contrast likelihood rate test (LR) for multiple comparisons.

Data on apyrene sperm (Figure 6.1B) and total fanning duration (Figure 6.2B) became normally distributed after ln(x) transformation and then analysed using ANOVA followed by Tukey’s Studentized multiple range test. Data on the pre-wing-fanning period of daily exposure (Figure 6.2A) and daily number of eggs laid (Figure 6.6) were not normally distributed even after transformation and thus subject to a non-
parametric ANOVA followed by a multiple Bonferroni (Dunn) range test. Female and male survival were analysed using a Life test (LIFETEST procedure) (Figures 6.3 and 6.8).

6.3 Results

6.3.1 Reproduction, survival and courtship of virgin males

Virgin males produced significantly greater numbers of eupyrene sperm in +M than in CONT and +F with the lowest amount detected for newly emerged males (ANOVA: $F_{4,76} = 33.88$, $P < 0.0001$) (Figure 6.1A). The number of apyrene sperm produced was significantly higher in +M and CONT than in other treatments (ANOVA: $F_{4,76} = 28.20$, $P < 0.0001$) (Figure 6.1B).

**Figure 6.1** Mean (± SE) number of eupyrene sperm ($\times 256$) (A) and apyrene sperm ($\times 3000$) (B) in the reproductive tracts of males under different socio-sexual environments. For each category, bars with the same letters are not significantly different ($P > 0.05$).
Virgin males in +F started their wing fanning significantly earlier than in other treatments (ANOVA: $F_{2.25} = 35.96$, $P < 0.0001$) (Figure 6.2A). During the first ten days of exposure, +F males fanned their wings significantly longer than other treatments (ANOVA: $F_{2.9} = 29.70$, $P < 0.0001$) (Figure 6.2B).

Virgin males in +F treatment lived significantly shorter than those in +M and CONT (Lifetest: $x^2 = 21.97$, $P < 0.0001$) which had similar longevity ($P > 0.05$) (Figure 6.3).

**Figure 6.2** Mean (± SE) pre-wing fanning duration (A) and mean total wing fanning duration (B) of males during the first 10 days under different socio-sexual environments. For each category, bars with the same letters are not significantly different ($P > 0.05$).
**Figure 6.3** Male survival under different socio-sexual environments. Lines with the same letters are not significantly different (P > 0.05).

### 6.3.2 Reproduction and survival of virgin females

Virgin females in CONT had significantly longer pre-oviposition period than in +M and +F (ANOVA: $F_{2,42} = 18.10$, $P < 0.0001$; Figure 6.4A). However, there was no significant difference in the oviposition period between treatments (ANOVA: $F_{2,42} = 0.60$, $P = 0.5532$; Figure 6.4B).
Virgin females in all treatments laid similar number of eggs during their lifetime (ANOVA: $F_{2,42} = 0.68, P > 0.05$) (Figure 6.5A). Although not significant (ANOVA: $F_{2,42} = 0.20, P > 0.05$), +M females had substantially fewer mature eggs remaining in their ovaries when dead as compared to other females (Figure 6.5B). However, when taking the total number of mature eggs produced into consideration, virgin females in +F produced significantly more eggs during their lifespan than did in +M (ANOVA: $F_{2,42} = 3.28, P = 0.0477$) (Figure 6.5C).

**Figure 6.4** Mean (± SE) pre-oviposition period (A), and oviposition period (B) of virgin females under different socio-sexual environments. For each category, bars with the same letters are not significantly different ($P > 0.05$).
Figure 6.5 Mean (± SE) total number of eggs laid (A), eggs unlaid (B), and eggs produced by virgin females (C) under different socio-sexual environments. For each category, bars with the same letters are not significantly different (P > 0.05).
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Figure 6.6 Mean (± SE) daily number of eggs laid by virgin females under different socio-sexual environments. For each category, bars with the same letters are not significantly different (P > 0.05).

The daily number of eggs laid by females during their oviposition period was not significantly different between treatments ($F_{2,404} = 1.86, P = 0.1565$) (Figure 6.6). When looking into the daily oviposition pattern, I found that the number of eggs laid significantly increased with females’ age during most of their oviposition period ($\chi^2_1 = 256.13, 340.38$ and $168.81$ for CONT, +M and +F, respectively; $P < 0.0001$) and then significantly decreased nearing the end their lifespan ($\chi^2_1 = 184.19, 257.37$ and $103.45$ for CONT, +M and +F, respectively; $P < 0.0001$) (Figure 6.7). However, it increased significantly faster (LR: $\chi^2_2 = 49.92$, $P < 0.0001$) and decreased significantly faster in CONT and +M than in +F (LR: $\chi^2_2 = 24.35$, $P < 0.0001$) (Figure 6.7).
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Figure 6.7 Number of eggs laid per day by virgin females after emergence in relation to age, under different socio-sexual environments. CONT: Eggs = \( \exp (-4.330 + 0.8109 \text{Age} - 0.0251 \text{Age}^2) \) (\( R^2 = 0.3504, F_{2,254} = 68.50, P < 0.0001 \)); +F: Eggs = \( \exp (-0.517 + 0.4657 \text{Age} - 0.0194 \text{Age}^2) \) (\( R^2 = 0.1513, F_{2,190} = 17.18, P < 0.0001 \)); +M: Eggs = \( \exp (-2.550 + 0.8212 \text{Age} - 0.0331 \text{Age}^2) \) (\( R^2 = 0.3147, F_{2,199} = 45.69, P < 0.0001 \)).

Virgin females in CONT lived significantly longer than in +M or +F (Lifetest: \( x^2 = 10.82, P = 0.0045 \)), longevity of which was not significantly different (\( P > 0.05 \)) (Figure 6.8).

Figure 6.8 Survival of virgin females under different socio-sexual environments. Lines with the same letters are not significantly different (\( P > 0.05 \)).
6.4 Discussion

To determine whether and how virgin males adjusted their reproductive strategy in response to socio-sexual environment, I compared the reproductive behaviour, sperm production and survival of virgin males under different socio-sexual contexts. The sperm competition (SC) theory (Parker 1970) predicts that with increasing SC levels males gain fitness by increasing ejaculation (e.g., Parker et al. 1997; Wedell et al. 2002b; Parker & Pizzari 2010; Bretman et al. 2011a; delBarco-Trillo 2011; Parker et al. 2013). My results indicate that virgin males produced significantly more eupyrene and apyrene sperm in +M than in +F (Figure 6.1), consistent with findings for mated males (Figures 4.3 and 4.7) and supporting the prediction of SC theory (Parker 1970). These findings again challenge the prediction that in lepidopterans eupyrene spermatogenesis stops after pupation (Lai-Fook 1982; Lachance & Olstad 1988; Friedlander 1997; Friedländer et al. 2005). My current findings also suggest that sperm production of E. kuehniella males in response to the socio-sexual environment is independent of their mating state.

When looking at the total number of sperm produced by virgin males during their early life, I found that males carried substantial number of eupyrene and apyrene sperm at emergence (Figure 6.1), indicating that these sperm are produced before emergence and partially supporting the prediction that eupyrene spermatogenesis starts during or before pupation (Lai-Fook 1982; Lachance & Olstad 1988; Friedlander 1997; Friedländer et al. 2005). However, although newly emerged and 1-d-old virgin males produced similar number of apyrene sperm (Figure 6.1B), 1-d-old virgin males almost doubled the number of eupyrene sperm produced in 24 hours (Figure 6.1A), contradicting the above prediction. These findings demonstrate that eupyrene sperm are produced both before and after emergence in E. kuehniella.

My results indicate that the increase of eupyrene sperm production in response to higher SC levels did not compromise the longevity of the virgin males because those in +M and CONT had similar survival probability (Figure 6.3). However, +F virgin males started courting significantly earlier (Figure 6.2A), performed significantly longer courtship (Figure 6.2B) and had significantly shorter longevity (Figure 6.3) than +M and CONT ones. It is thus strongly suggested that courtship is costlier to males than
sperm production in this species. The substantial cost of courtship displays has been demonstrated across taxa (e.g., Cordts & Partridge 1996; Clutton-Brock & Langley 1997; Hoback & Wagner 1997; Kotiaho & Simmons 2003; Hunt et al. 2004; Simmons & Kotiaho 2007; Hoefler 2008; Nakano et al. 2008; Papadopoulos et al. 2010; Wedell 2010; Gersick & Kurzban 2014).

To evaluate whether and how virgin females adjusted their reproductive strategy in response to socio-sexual environment, I compared the pre-oviposition period, number of eggs laid and produced, daily oviposition patterns and survival of virgin females under different socio-sexual contexts. My results indicate that both +M and +F virgin females started laying eggs significantly earlier than CONT ones (Figure 6.4A), suggesting the presence of conspecifics triggers oviposition in virgin females. Furthermore, I did not find any difference in daily and lifetime oviposition between treatments (Figures 6.5A and 6.6). These findings do not appear to support the prediction that virgin females reduce reproductive investment during their early life for future opportunities of mating (e.g., de Clercq & Degheele 1997; Torres et al. 1997; Fauvergue et al. 2008; Steiner & Ruther 2009; Soares et al. 2011; Xu & Wang 2011). However, virgin females produced significantly fewer eggs (total number of eggs produced: eggs laid + eggs unlaid) in +M than in +F (Figure 6.5C), partially supporting the above prediction, i.e., virgin females slow egg production for future mating opportunities under male-biased sex ratio.

My results show that although daily oviposition followed a similar trend among all virgin females [the number of eggs laid significantly increased with females’ age during most of their oviposition period and then significantly decreased nearing the end of their lifespan (Figure 6.7)], both increase and decrease were significantly slower in +F than in CONT and +M. However, the mechanisms behind need further investigation.

Theoretical and empirical studies suggest that higher reproductive rate may result in greater damage to the soma and thus accelerate senescence (McNamara et al. 2009; Kirkwood & Austad 2000; Salmon et al. 2001; Wang et al. 2001; Edward & Chapman 2011; Travers et al. 2015). Compared to virgin females in CONT, those in +M and +F lived significantly shorter (Figure 6.8). This may be attributed to the fact
that virgin females in +M and +F started laying eggs significantly earlier than CONT ones (Figure 6.4A). The lower reproductive rate during the early life of CONT virgin females may allow them to invest more resource in somatic maintenance and repair (Kirkwood & Austad 2000; Travers et al. 2015) and thus delay the senescence due to aging (Kirkwood & Austad 2000; Bonduriansky et al. 2008).

In conclusion, eupyrene spermatogenesis takes place both before and after emergence in E. kuehniella. Virgin males produce more sperm in +M than in +F, supporting the prediction of SC theory. The increase of sperm production in response to higher SC levels does not reduce virgin males’ longevity but +F virgin males start courting earlier, perform longer courtship and have shorter longevity than +M and CONT ones. These findings strongly suggest that courtship is costlier to males than sperm production in this species. Both +M and +F virgin females start laying eggs earlier and live shorter than CONT ones, suggesting that higher reproductive rate early in life contributes to lower survival. However, virgin females produce fewer eggs in +M than in +F, partially supporting the prediction that virgin females reduce reproductive investment for future mating opportunities under male-biased sex ratio.
Chapter 7

General Discussion

7.1 Introduction

During my PhD studies, I carried out a large number of experiments to investigate how males and females *E. kuehniella* adjusted their resource allocations to reproduction and somatic maintenance in response to dynamic socio-sexual environment. I statistically analysed all data and attempted to interpret my findings. In this chapter, I summarise and discuss my main findings.

7.2 Male response to immediate and mean sperm competition levels

Sperm competition (SC) models (Parker et al. 1996, 1997) make two sets of specific predictions over male’s response to socio-sexual environment: (1) immediate SC – concerning how much of his current sperm reserves a male should allocate to a specific copulation, i.e. the male should allocate a larger amount of his present sperm reserves to the copulation when the SC level is high, and (2) mean SC – concerning how much of his energy budget a male should allocate to sperm production, i.e. the male should invest a larger proportion of his reproductive effort in sperm production when the SC level is high. In the present study, I have tested both predictions.

To test the first prediction, I compared the treatments for the first copulation before which time all insects were kept individually (–E experiment). I show that the –E focal male allocated similar number of sperm from his reserves to the mate regardless of presence or absence of immediate SC effect (Table 4.1). These findings suggest that males do not adjust sperm allocation in response to immediate sperm competition levels, phenotypically contradicting the prediction of the SC model (Wedell et al. 2002b; Parker & Pizzari 2010). To test the second prediction, I examined the first copulation of +E focal males which were exposed to males or females for 24 h before pairing. My results indicate that the focal male allocated significantly more eupyrene
sperm to the mate in +M+E than in +F+E and CONT with similar number of apyrene transferred across treatments (Figure 4.7), supporting the prediction of the SC model. My findings strongly suggest that pre-pairing exposure to rivals is essential to detection of any SC effect and that males only respond to mean SC levels in *E. kuehniella*.

I then evaluated all copulation events during the focal males’ lifetime in both -E and +E experiments. My study demonstrates that focal males in +M+E transferred significantly more eupyrene sperm to their mates than in +F+E and CONT during their first four copulations (Table 4.5). This finding further suggests that focal males adjust allocation of eupyrene sperm in response to mean SC levels detected during the early adulthood before pairing. The increased eupyrene sperm allocation in response to higher mean SC levels may help males achieve greater paternity share in the SC battle (Parker et al. 1996, 1997; Wedell et al. 2002b). In –E experiments, however, I have not found any difference in sperm allocation (Tables 4.2 and 4.3) in any given copulation between treatments except for the second copulation. It is possible that without the 24-hour pre-pairing exposure to rivals, daily 12-hour exposure is not sufficient to trigger a measurable response of the male to mean SC levels. Bretman et al. (2010) and Parker (2015, per. communication) also point out that the length of exposure to rivals may be critical in determining male responses to the mean SC levels.

When I examined my findings longitudinally, I found that in all treatments of both –E and +E experiments the ejaculate size decreased over successive copulations (Figures 4.5A-B and 4.11A-B), fitting the model on reproductive output declines with age of adults having fixed resources obtained during the immature stages (Begon & Parker 1986). If the mean SC levels play a role in both sperm production and allocation, then it would be expected that the decrease in ejaculate size over successive copulations would be faster in +M than in +F. In –E experiments (Figure 4.5A-B), the likelihood rate test did not show any significant difference in the slopes of regression lines for sperm depletion between treatments, further indicating that the mean SC levels set in -E experiments (about 12 hours a day since the first mating event) are not sufficient to affect sperm production and allocation. However, in +E experiments, the number of eupyrene sperm ejaculated declined significantly slower in +F than in CONT and +M.
(Figure 4.11A) and the number of apyrene sperm declined significantly faster in +M than in CONT and +F (Figure 4.11B), strongly suggesting that pre-pairing exposure to rivals during the early adulthood is necessary to trigger males’ response to mean SC levels in *E. kuehniella*.

### 7.3 Relationship between copulation duration and sperm allocation

SC theory (Parker 1970; Wedell et al. 2002b; Parker & Pizzari 2010) predicts that SC levels would determine the copulation duration and ejaculate allocation of the focal male where in the presence of rivals the male increases sperm allocation to his mate by prolonging the copulation. Probably, as a result, copulation duration is often considered an accurate measure of sperm allocation in many studies (e.g., Bretman et al. 2011a; Price et al. 2012; Moatt et al. 2013). However, copulation duration and sperm allocation may not be positively correlated (e.g., Gilchrist & Partridge 2000).

First, I examined the first copulation for both –E and +E experiments. In –E experiment both sperm allocation and copulation duration did not change across treatments (Figure 4.1, Table 4.1). However, the focal male allocated significantly more eupryrene sperm to the mate in +M+E than in +F+E and CONT (Figure 4.7) while copulation duration remained the same regardless of presence or absence of SC effect (Table 4.4). These findings suggest that copulation duration and eupryrene number are not positively correlated in this species.

I then evaluated all copulation events during the focal males’ lifetime in -E and +E experiments. In –E experiment I did not find any difference in sperm allocation (Tables 4.2 and 4.3) and copulation duration (Table 4.1) in most copulations between treatments. Although focal males in +M+E transferred significantly more eupryrene sperm to their mates than in +F+E and CONT during their first four copulations (Table 4.5), there was no difference in copulation duration (Table 4.4) in any given copulation between treatments in +E experiments. These findings further reveal that copulation duration and sperm allocation are not positively correlated regardless of whether focal males are exposed to rivals or not before pairing.
Finally, I examined all copulations longitudinally in both –E and + experiments. I found that in all treatments the ejaculate size decreased (Figures 4.5A-B and 4.11A-B) and copulation duration increased (Figures 4.5C and 4.11C) over successive copulations regardless of whether focal males were exposed to rivals prior to pairing or not. These results demonstrate a negative correlation between sperm allocation and copulation duration over males’ lifetime, contradicting findings in many other studies (e.g., Schofl & Taborsky 2002; Garcia-Gonzalez & Gomendio 2004; Prokop & Václav 2005; Bretman et al. 2009; Price et al. 2012; Lize et al. 2012b; Price et al. 2012; Moatt et al. 2013). Furthermore, in both –E and +E experiments there was no significant difference in slopes between treatments in copulation duration over successive copulations (Figures 4.5C and 4.11C). These findings confirm that copulation duration cannot be used as a measure to detect males’ response to SC levels and as an estimate of sperm allocation in *E. kuehniella*.

### 7.4 Sperm production during adulthood

Previous models envisage that sperm production may increase in both male-biased and female-biased conditions because males may gain reproductive fitness by increasing sperm production after exposure to rivals (Wedell et al. 2002b; Parker & Pizzari 2010) and additional mates (Abe & Kamimura 2015). In Lepidoptera, however, it is generally believed that eupyrene spermatogenesis stops after pupation (Lai-Fook 1982; Lachance & Olstad 1988; Friedländer 1997; Friedländer et al. 2005).

To investigate whether and how my results fit with the above predictions, I looked into the data from both –E and +E experiments. I found that in their lifetime – E+M focal males ejaculated an average of > 38,000 eupyrene sperm (Figure 4.2) while +M+E focal males transferred an average of > 56,000 eupyrene sperm (Figure 4.9). These findings indicate that *E. kuehniella* males increase sperm production if they are exposed to rivals during the early adulthood, supporting the prediction by Wedell et al. (2002b) and Parker and Pizzari (2010). However, my results do not support that by Abe & Kamimura (2015). Furthermore, my findings challenge the prediction that eupyrene spermatogenesis stops after pupation (Lai-Fook 1982; Lachance & Olstad 1988;
In a recent study on butterfly *Bicyclus anynana* (Butler), Kehl et al. (2015) reveal that young males with higher numbers of eupyrene sperm are more likely to succeed in mating than those with lower eupyrene sperm numbers. My present study demonstrates that +M+E focal males had shorter mating latency (Figure 4.12A) and higher lifetime fecundity (Figure 4.9) than +F+E and CONT ones. It is thus suggested that their pre-pairing exposure to rivals not only increases but also accelerates sperm production in *E. kuehniella* males. Furthermore, several recent studies have tested sperm production rate and mating success in response to SC levels, generating similar results. For example, *D. melanogaster* males increase sperm production rate after exposed to rivals for a lengthy period of time (Moatt et al. 2014), and the simultaneously hermaphroditic flatworm *Macrostomum lignano* Ladurner, Schärer, Salvenmoser & Rieger raised under higher SC levels produce sperm faster (Giannakara et al. 2016).

### 7.5 Courtship cost in males

In their study on *E. kuehniella*, Xu and Wang (2009b) report that: (1) a male can inseminate up to nine females in his lifetime, ejaculating between 3,400 and 11,000 eupyrene sperm per copulation, and (2) once-mated females produce the same number of offspring (fertility) regardless of the number of eupyrene sperm received within the above range. My results indicate that –E females received an average of 4,100–10,500 eupyrene sperm (Table 4.2) and +E females obtained 3,900-12,000 eupyrene sperm (Table 4.5) in a given copulation, falling into the range reported by Xu and Wang (2009b). Furthermore, the reduced number of copulations by the focal male in +F-E (Figure 4.2) led to a decline in his lifetime reproductive fitness and increased number of copulations by the focal male in +M+E (Figure 4.9) resulted in an increase in his lifetime reproductive fitness. Therefore, the lifetime number of sperm transferred by the focal male is, in fact, the function of his lifetime number of copulations.

Precopulatory courtship displays by males are costly across taxa (e.g., Cordts & Partridge 1996; Clutton-Brock & Langley 1997; Hoback & Wagner 1997; Kotiaho &
Simmons 2003; Hunt et al. 2004; Simmons & Kotiaho 2007; Hoefler 2008; Papadopoulos et al. 2010; Wedell 2010; Gersick & Kurzban 2014). To determine why the focal male had lower lifetime number of mates inseminated (and lower number of sperm transferred) in the presence of additional mates, I examined the precopulatory courtship behaviour by the focal male in response to dynamic socio-sexual environments. In both –E and +E experiments, focal males in +F fanned their wings for significantly longer period before copulation than those in CONT and +M (Figures 4.6B and 4.12B). This result strongly suggests that it is the increased courtship displays that reduce the lifetime copulation frequency and fecundity of the focal males in +F-E in *E. kuehniella*, contradicting the prediction where, in the presence of rivals, the focal male invests more in courting (Emlen & Oring 1977).

Similar to findings in Janowitz and Fischer (2010), results of –E experiments show that male longevity was the same in all treatments (Figure 4.4) although –E+F focal males copulated significantly fewer times (Figure 4.2) and ejaculated significantly fewer sperm in their lifetime (Figure 4.3). Therefore, my findings from –E experiments do not demonstrate a clear trade-off between longevity and reproductive outputs in *E. kuehniella* in response to socio-sexual contexts, contradicting findings and predictions in many other studies (e.g., Kotiaho & Simmons 2003; Simmons & Kotiaho 2007; Hoefler 2008; Jordan & Brooks 2010; Papadopoulos et al. 2010; Wedell 2010; Bretman et al. 2013b; Scharf et al. 2013). However, as compared to CONT, although higher courtship cost in +F+E reduced focal males’ longevity (Figure 4.10), it did not lower their lifetime copulation frequency and fecundity (Figures 4.8 and 4.9). These findings suggest that pre-pairing exposure to mates also plays a role in adjustment of resource allocation by focal males, i.e. males allocate more resource for sperm production on the cost of longevity in response to perception of higher mating opportunity during their early adulthood, partially supporting the model by Abe and Kamimura (2015).

### 7.6 Female promiscuity in response to socio-sexual environment

Theories predict that a female-biased condition makes males parsimonious and females promiscuous (Abe & Kamimura 2015) but females should be choosier in the
male-biased sex ratio, leading to longer mating latency (Emlen & Oring 1977; Kvarnemo & Ahnesjo 1996). However, my results do not support these predictions.

In both –E and +E experiments, the mean mating latency was between 7 and 8 hours with no significant difference between treatments (Figures 5.1B and 5.7B). Similarly, there was no significant difference in mating latency in any given copulation between treatments except the first mating in +E experiment where mating latency was significantly shorter in +M than in +F, but that in neither +M nor +F was significantly different from that in CONT (Tables 5.1 and 5.4). If females are choosier in a male-biased environment as predicted by theories (e.g., Kvarnemo & Ahnesjo 1996; Tinghitella et al. 2013; Atwell & Wagner 2014; Judge et al. 2014; Abe & Kamimura 2015; Stoffer & Uetz 2015), then they should have fewer matings in such context. However, my results show otherwise, i.e., in both –E and +E experiments, females mated significantly more times in +M than in +F or in CONT (Figures 5.1A and 5.7A).

When I looked into the probability of mating in relation to socio-sexual environment, I found that the focal females in +M were more receptive to the courting males compared to those in +F and CONT, especially in the first two days of pairing (Figures 5.2 and 5.8). My findings strongly suggest that *E. kuehniella* females are more receptive rather than choosier under male-biased sex ratio. Furthermore, higher mating frequency in the focal females in +M should not be attributed to greater male effort in courtship because males made significantly less courtship display in male-biased socio-sexual environment (Figures 4.6B and 4.12B in Chapter 4).

### 7.7 Female calling behaviour under different socio-sexual contexts

In lepidopteran moths, females call and release sex pheromones to attract males for mating. Females are expected to call less under a male-biased sex ratio (Sadek et al. 2012) but more in a female-biased sex ratio (Stoffer & Uetz 2015). However, this notion is not supported by my findings where *E. kuehniella* females called significantly more when no additional individuals were present (CONT) than when they were exposed to either males (+M) or females (+F), with no difference between the latter two treatments (Figure 5.7D). It is thus suggested that perception of no conspecifics around
by the female may signal her the risk of losing opportunities to find a mate and trigger her to allocate more energy for calling.

The present study indicates that *E. kuehniella* females respond to socio-sexual environment very differently from their male counterparts which have significantly longer courtship display when additional females are present (Figures 4.6B and 4.12B in Chapter 4). It is likely that females reduce calling duration in response to increased local population density regardless of sex ratio. In other moth species *Grapholita molesta* (Busck) (Stelinski et al. 2006) and *Lobesia botrana* (Denis & Schiffermüller) (Harari et al. 2015), calling is shorter in female-biased sex ratio. These findings suggest that female calling in response to socio-sexual environment could be species specific.

7.8 Female resource allocation in response to socio-sexual environment

Despite the potential harms of multiple mating, females tend to increase the number of matings to maximize their reproductive fitness (Arnqvist & Nilsson 2000; South & Lewis 2011) through nutrient supply (e.g., ejaculates or nuptial gifts) (e.g., Wiklund et al. 1993; Wiklund et al. 1998; Billeter et al. 2012; Okada et al. 2013, 2015; Lee et al. 2014; Fan et al. 2015), sperm replenishment, genetic benefits (Arnqvist & Nilsson 2000; Wedell 2001; Parker & Birkhead 2013), or longevity (e.g., Wiklund et al. 1998; Wigby & Chapman 2004; Lee et al. 2014). However, under even sex ratio, re-mating does not increase fertility, fecundity or longevity in *E. kuehniella* females (Xu & Wang 2009a). In the present study, although +M and +F treatments had no effect on female longevity (Figures 5.3 and 5.9) and little impact on fecundity (Figures 5.5A and 5.11A), focal females had significantly higher fertility rate in +M than in +F regardless of whether pre-pairing exposure occurred (Figures 5.5B and 5.11B). This finding indicates that perception of additional males makes females more receptive so that they mate more times (Figures 5.1A and 5.7A) and fertilise more eggs (Figures 5.5B and 5.11B).

Regardless whether focal females were exposed to other individuals in their early adulthood, with the increase of age their daily fecundity significantly increased and then significantly decreased in all treatments and CONT (Figures 5.6 and 5.12).
However, the daily fecundity patterns were different depending on whether pre-pairing exposure occurred. In –E experiment, +M females increased their daily fecundity significantly faster during their first half of life and decreased significantly faster compared to +F females (Figures 5.6). The opposite was the case in +E experiments (Figure 5.12). My results suggest that pre-pairing exposure alters females’ lifetime resource allocation patterns for oviposition. Although the underlying mechanisms are not clear, my results on the probability of copulation (Figures 5.2 and 5.8) and oviposition (Figures 5.4 and 5.10) under different socio-sexual environment may provide a clue for future investigation.

7.9 Virgin Male Response to Socio-Sexual Environment

My experiments on virgin males generated findings similar to those in Chapter 4. For example, virgin males produced significantly more eupyrene and apyrene sperm in +M than in +F (Figure 6.1). Furthermore, males were found to have substantial number of eupyrene and apyrene sperm at emergence (Figure 6.1), indicating that these sperm are produced before emergence and partially supporting the prediction that eupyrene spermatogenesis starts during or before pupation (Lai-Fook 1982; Lachance & Olstad 1988; Friedlander 1997; Friedländer et al. 2005). However, although newly emerged and 1-d-old virgin males produced similar number of apyrene sperm (Figure 6.1B), 1-d-old virgin males almost doubled the number of eupyrene sperm produced in 24 hours (Figure 6.1A), contradicting the above prediction. These findings demonstrate that eupyrene sperm are produced both before and after emergence in *E. kuehniella*. Comparison of findings in Chapter 4 and Chapter 6 suggests that sperm production of *E. kuehniella* males in response to the socio-sexual environment is independent of their mating state.

When I examined the correlation between sperm production and longevity in virgin males under different socio-sexual environment, I did not find any evidence that the increase of eupyrene sperm production in response to higher SC levels might reduce the longevity of virgin males (Figure 6.3), contradicting general predictions. However, in the presence of females, virgin males started courting significantly earlier (Figure 6.2A), performed significantly longer courtship (Figure 6.2B) and lived significantly
shorter (Figure 6.3) than in the presence of males or CONT. These findings strongly suggest that courtship instead of sperm production costs virgin male’s longevity in this species.

7.10 Virgin female response to socio-sexual environment

My study shows that virgin females responded to socio-sexual environment differently from virgin males. For example, virgin females started laying eggs earlier in the presence of either males or females than in CONT (Figure 6.4A) but they laid similar number of eggs daily or in lifetime across treatments (Figures 6.5A and 6.6). However, virgin females produced fewer eggs (total number of eggs produced: eggs laid + eggs unlaid) in +M than in +F (Figure 6.5C), suggesting that virgin females slow egg production under male-biased sex ratio. This finding at least partially supports the prediction that virgin females reduce reproductive investment during their early life for future opportunities of mating (e.g., de Clercq & Degheele 1997; Torres et al. 1997; Fauvergue et al. 2008; Steiner & Ruther 2009; Soares et al. 2011; Xu & Wang 2011).

Consistent with above findings in oviposition, +M and +F virgin females had shorter longevity as compared to CONT (Figure 6.8). These results support previous theoretical and empirical studies that higher reproductive rate may result in greater damage to the soma and thus accelerate senescence (McNamara et al. 2009; Kirkwood & Austad 2000; Salmon et al. 2001; Wang et al. 2001; Edward & Chapman 2011a; Travers et al. 2015).

7.11 Conclusion

My PhD studies have provided insight into the plasticity in reproduction and survival under dynamic socio-sexual environment for *E. kuehniella*. I demonstrate that males only respond to mean SC levels and eupyrene sperm are produced both before and after emergence. Lifetime reproductive fitness in males depends on the number of copulations they can achieve, rather than the number of sperm ejaculated in each copulation. Regardless of whether focal males are exposed to rivals or not during their early adulthood, copulation duration and sperm allocation are not positively correlated, indicating that copulation duration cannot be used as a correct estimate of sperm
allocation in *E. kuehniella*. Contradicting the prediction where, in the presence of rivals, males invest more in courting, my experiments reveal that males invest more in courtship in the presence of additional females, which reduces their lifetime copulation frequency and fecundity.

Contradicting the previous prediction that a female-biased condition makes males parsimonious and females promiscuous but females should be choosier in the male-biased sex ratio, my results show that perception of additional males makes females more receptive so that they mate more times and fertilise more eggs. Focal females call more when they are lonely than when either males or females are present, suggesting that perception of no conspecifics around by the focal females may signal them the risk of losing opportunities to find a mate and trigger them to allocate more energy for calling. Although virgin females lay similar number of eggs in all treatments, they start oviposition earlier and live shorter in the presence of conspecific males or females, supporting previous predictions that higher reproductive rate may accelerate senescence. Virgin females produce fewer eggs (total number of eggs produced: eggs laid + eggs unlaid) under male-biased than female-biased sex ratio, suggesting that they reduce reproductive investment during their early life for future opportunities of mating.

Future work should focus on the evolution of age- and state-dependent life history strategies in this species. For example, understanding the evolutionary nature of the plasticity observed in the present study may contribute to novel knowledge on adaptation and its role in the development of behaviour-based pest management measures.
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Flirtation reduces males’ fecundity but not longevity

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Theory predicts that due to limited resources males should strategically adjust their investment in reproduction and survival. Based on different conceptual frameworks, experimental designs, and study species, many studies support while others contradict this general prediction. Using a moth Ephesia kuehnleinii whose adults do not feed and thus have fixed resources for their lifetime fitness, we investigated whether males adjusted their investment in various life activities under dynamic socio-sexual environment. We allowed focal males to perceive rivals or additional females without physical contact. We show that males do not adjust the number of sperm they transfer to mates in a given copulation at different immediate or both immediate and mean sperm competition levels. Contrary to general predictions, our results demonstrate that cues from additional females increase males’ investment in courtship and reduce their lifetime number of copulations and sperm ejaculated, whereas cues from rivals have no effect on these parameters. Males have similar longevity in all treatments. We suggest that the sex pheromone produced by multiple females overstimulates males, increasing males’ costly flirtations, and reducing their lifetime copulation frequency and fecundity. This finding offers a novel explanation for the success of mating disruption strategy using sex pheromones in pest management.

KEY WORDS: Courtship, ejaculation, Ephesia kuehnleinii, sex pheromone, sperm competition.

Sperm competition (SC) theory (Parker 1970) seeks evolutionary stable strategy (ESS) for ejaculate allocation (e.g., Parker et al. 1997; Wedell et al. 2002; Parker and Pezzi 2010; Trivers & Willard 1973; Parker et al. 2011a; delBazo-Trillo 2011; Parker et al. 2013) that predicts that with increasing risk of sperm competition (SC) males gain fitness by increasing ejaculation and that with increasing intensity of sperm competition (SCI) the male ESS is to reduce investment. In these models, female promiscuity is proposed to determine SCR and SCI levels and pre copulatory energetic costs in males. So far, most empirical studies (e.g., Wedell and Cook 1995; Trivers & Willard 1973; Parker et al. 2013a; Xu and Wang 2014) have supported while some (e.g., Cook and Gage 1995; Raman and Stockley 2007; Worthington et al. 2013; Ziviani et al. 2013a) contradicted these predictions.

Most evolutionary biologists have investigated female mating frequency and male ejaculate allocation separately assuming fixed levels of the opposite sex’s strategies (Alonzo and Pezzi 2010; Abe and Kaminura 2015). However, these strategies of sexes are likely to covary (Abe and Kaminura 2015). Recently, by incorporating dynamic reproductive strategies of both sexes Requena and Alonso (2014) predict that male ejaculate allocation and female promiscuity are negatively correlated, and Abe and Kaminura (2015) foresee that female-biased conditions make males parasitome and females promiscuous. Two these most recent models support some components and contradict others of the previous SC models. The differences in predictions between models may result from different conceptual frameworks (e.g., Requena and Alonso 2014), experimental designs (e.g., Engqvist and Rehnqvist 2005), and for life history strategies of study species. Parameters for life history strategies may include whether adults feed, how often both sexes mate, whether ejaculation and sperm storage sites are different, how females manipulate the use and storage of sperm from different males, and whether males produce a spermatophore (a capsule enclosing spermatozoa) during mating as examples.
Engqvist and Reinhold (2005) point out that many empirical experiments used to test theoretic predictions about SCR and SCI may not be appropriately designed. They illustrate four common pitfalls (references cited herein): (1) ignoring the difference between immediate and mean SCR and SCI levels—the accurate test for the first set of predictions concerning immediate SCR and SCI should be to test for differences in allocation of the males’ present sperm reserves at a given mating and for the second set of predictions concerning mean SCR and SCI to test for differences in sperm production before mating; (2) incorrect manipulation of immediate SCI—the number of males nearby should be different from the number of competing ejaculates, and thus, introducing more males will always increase immediate SCR, which has a positive rather than negative effect on ejaculate size; (3) ignoring past and future risk in studies of immediate SCR and SCI—males should take the females’ past mating history and future mating probability into account when estimating immediate SCR and SCI; and (4) ignoring the impact of sex ratio—similar to point (1) above, a relatively long-term manipulation of sex ratio to study immediate-SCR is inappropriate. Several additional points are also worth mentioning. First, although females may play important roles in male reproductive plasticity (Breitman et al. 2011a; Requeño and Alonso 2014; Abe and Kamimura 2015), they are often conditionally, as virgin females reared in groups) before mating, and the impact of such treatment on SC prediction is not considered (e.g., Price et al. 2012; Mootz et al. 2013; Breitman et al. 2013b; Worthington et al. 2013). Second, the effect of different life history strategies of study species on research outcomes (and thus predictions) is rarely considered in SC studies (Ingley et al. 2010). Third, in many SC studies authors measure various parameters for only one mating event rather than life-time reproductive activities (e.g., Breitman et al. 2009; Breitman et al. 2010; Lise et al. 2012; Price et al. 2012; Breitman et al. 2013a; Breitman et al. 2013b; Worthington et al. 2013). Fourth, copulation duration is usually considered an accurate measure of sperm allocation (e.g., Breitman et al. 2011a; Price et al. 2012; Mootz et al. 2013) but these two parameters are not negatively correlated in some species (e.g., Gildhurst and Patridge 2003, the present study). Fifth, sperm production mediated by mean SCR and SCI levels may increase in both male-biased and female-biased conditions because males may gain reproductive fitness by increasing sperm production in response to rivals (Wedell et al. 2002; Parker and Pizzari 2010) and additional mates (Abe and Kamimura 2015).

One of the earlier examples of costs of sexual activity to males is made by Patridge and Fairagher (1981). Today, it is an accepted notion that reproduction is costly for males (Scharf et al. 2013). The ultimate cost of reproduction for males is investment in current reproduction at the expense of future reproduction and longevity (Scharf et al. 2013). The first trade-off lies at the foundation of sperm allocation models assuming a limited amount of sperm and time, which should be optimally distributed (e.g., Wedell et al. 2002; Parker and Pizzari 2010; Lizé et al. 2012; Price et al. 2012; Pizzari et al. 2013; Xu and Wang 2014). The second trade-off predicts that sexual activities shorten male longevity (e.g., Kotiaho and Simmons 2003; Simmons and Kotiaho 2007; Hoefler 2008; Jordan and Brooks 2010; Papadoyannis et al. 2010; Wedell et al. 2010; Brennan et al. 2013b; Xu and Wang 2014) although male longevity and reproduction may not always be traded off against each other (Hanninen and Pitcher 2010). Preemptory courtship displays by males are considered costly across taxa including humans (e.g., Coote and Bradtge 1996; Chilton-Iloek and Langley 1997; Holback and Wagner 1997; Kotiaho and Simmons 2003; Hunt et al. 2004; Simmons and Kotiaho 2007; Hoefler 2008; Papadoyannis et al. 2010; Wedell et al. 2010; Gernick and Kaitzib 2014). It is generally accepted that females are choosier in the male-biased sex ratio, leading to higher male pre-copulatory expenditure, than in the female-biased sex ratio, resulting in lower male pre-copulatory expenditure (Emlen and Oring 1977). So far, few studies have quantified the cost of male courtship displays and its trade-off against other fitness currencies, particularly the number of mates inseminated and sperm transferred by mates in the lifespan, in response to dynamic socio-sexual environment (Scharf et al. 2013).

Our study species, Ephesia kuehneltella, is a moth and an important pest of stored products. Until recently, very little was known about why this moth pests are static and reproductive. We chose Ephesia kuehneltella for our study because it is a model system for study. Although its species has been documented to have a codominant sex ratio, these males exhibit a high degree of plasticity in response to environmental cues and can produce either male or female offspring. We are interested in understanding the costs and benefits of reproduction and how these are affected by environmental factors. We are also interested in understanding the mechanisms underlying these plastic responses, as well as how they interact with other factors that influence the male’s reproductive success, such as mate availability and competition. We hope to shed light on these questions by using a combination of experimental and field studies.
Materials and Methods

INSECTS

A colony of *E. kuehniella* was established from the larvae in infested flour collected at Turfs Poultry, Pukka, New Zealand, in 2013. Newly emerged adults were paired in transparent plastic cylinders (8 cm diameter × 10 cm height) for egg laying. Cylinders were covered with a plastic lid that had a hole (3 cm diameter) in the middle covered with two layers of cloth mesh (2.8 aperture per mm). Two-hundred newly hatched larvae were transferred into the cylinders containing 50 g of standard diet (43.5% whole meal wheat flour, 43.5% maize meal, 3% yeast, and 10% glycine). Two crumpled paper towels (25 × 25 cm) were placed into each cylinder for pupation. Mature pupae were collected from the paper towels, sexed (males have two bumps separated by a narrow groove on the last abdominal segment) and weighed using an electronic balance (Mettler Toledo AG135, Switzerland) with a readability of 0.00001 g. Pupal weight was categorized into three groups: light (< mean - 1 SD), average (mean ± 1 SD), or heavy (> mean + 1 SD). Pupae of average weight (25.1 ± 2.1 mg for males and 24.8 ± 2.4 mg for females) were used for experiments. These average weight pupae were individually kept in glass vials (2 cm diameter × 7.5 cm height) until adults were 1 day old before experiments. Breeding colony was maintained and all experiments were carried out at 25 ± 1°C and 60 ± 10% RH with a photoperiod of 14:10 h (L:D).

EXPERIMENTAL DEVICE

A device consisting of 15 experimental containers and an air-divider was constructed for experiments. The experimental container was made of two identical transparent plastic cylinders (8 cm diameter × 10 cm length) connected to each other with a Parafilm on external walls. The two cylinders were separated with a metal mesh (2.8 aperture per mm) in between, allowing free air movement. One cylinder was used as the mating chamber where a male and a female were held and another for accommodating rival males or additional females. The mating chamber had a lid at the end with a hole (3 cm diameter) in the middle that was covered with a fabric (cloth) mesh (2.8 aperture per mm).

The air from a compressed air tank was filtered through activated charcoal, measured via an airflow meter, humidified by passing through distilled water, and then blown into the air-divider, a bigger transparent plastic cylinder (15 cm diameter × 20 cm height), from which the air was equally divided into 15 silicone pipes, each of which was connected to a neighboring chamber. The air was blown through the neighboring chamber to the mating chamber and then out through the hole at the end of the mating chamber. The air speed was set to allow the air in all 15 experimental containers to be replaced once per minute. All containers were placed horizontally on an experimental bench.
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All experiments were carried out during the scotophase and a red light (Sylvania, F36W/Red, Holland) was used for illumination under the above environmental conditions.

EFFECT OF SOCIO-SEXUAL CONTEXT ON REPRODUCTION AND LONGEVITY OF MALES

To determine whether and how perception of the presence of rivals and additional mates by the focal male in the mating chamber affected his longevity and lifetime reproductive performance, we set up three treatments using the above described experimental device where the focal male perceived: (1) rivals (PLUS MALES)—one male and one female in the mating chamber and five males in the neighboring chamber, (2) additional females (PLUS FEMALES)—one male and one female in the mating chamber and five females in the neighboring chamber, and (3) neither rivals nor additional females (CONTROL)—one male and one female in the mating chamber and no insects in the neighboring chamber. Our experimental design allowed the focal male in the mating chamber to perceive rivals or additional mates in the neighboring chamber via chemical and/or acoustic cues (see Introduction) but did not allow him to come into contact with the latter. Insects were introduced into their chambers one hour before the onset of the scotophase. Insects quickly settled and remained still until the start of the scotophase. All insects used for this experiment were 1 day old and virgin except the focal males that were 1 day old and virgin only at the start of the experiments (see below). Fifteen replicates were performed for each treatment (only 14 replicates for PLUS MALES due to accidental death of a focal male). To avoid the effect of chemical residues left on experimental containers, we used three sets of 15 containers, each for one treatment.

Observations commenced immediately after the scotophase started. For each mating chamber, the female was immediately removed after the termination of copulation and dissected under a microscope (Olympus SZ HH, Japan). The number of sperm transferred by the male was counted according to Koulakov and Cook (2001) (see Supplementary Material). The total number of sperm ejaculated by the male in his lifetime was recorded as male fecundity. Insects in the neighboring chambers were removed after copulations were complete in all containers about one hour into the photophase. One hour before the onset of the next scotophase we offered the focal male a female and introduced five or no insects into the neighboring chamber according to treatment. We repeated the procedure until the death of the focal male. As a result, the focal male was exposed to PLUS MALES or PLUS FEMALES treatment for 12 hours a day (one hour before the scotophase + 10 hours in the scotophase + one hour in the photophase). Copulation duration (the period from the genital connection to disconnection) of each mating, lifetime number of copulations achieved and sperm ejaculated by the focal male and the longevity of the focal male were recorded.

EFFECT OF SOCIO-SEXUAL CONTEXT ON MATING LATENCY AND COURTSHIP DURATION

Males perform courtship display by fanning their wings when they encounter or perceive calling females (Tramontana and Pavian 1995; Xu et al. 2008; Xu and Wang 2009b). Therefore, we used wing-fanning duration as an index of courtship duration by males. To determine mating latency (period from the onset of scotophase to the commencement of copulation) and courtship duration under different socio-sexual context, we set up three treatments as in the previous experiment and recorded the behavior of the focal males using a TECHNIQ (QV-3034, Singapore) camera. Recording was made between the start of the scotophase and commencement of copulation. Videos were reviewed, and the mating latency and wing-fanning duration were recorded with a stopwatch. Twelve replicates were carried out for each treatment. All insects used for this experiment were 1 day old and virgin.

STATISTICAL ANALYSIS

A goodness-of-fit test (Shapiro-Wilk test, SWT; UNIVARIATE procedure) was used to test the distribution of data residuals calculated by a general linear model (GLM, GLM procedure). Data on the mean number of sperms transferred in a mating (Fig. 1A, Tables SI, S2) and total number of sperms transferred in lifetime (Fig. 1B) were normally distributed (SWT: W = 0.9647 - 0.9447, P < 0.05) and thus analyzed using an analysis of variance (ANOVA, GLM procedure) followed by Tukey's Studentized multiple range test. Data became normally distributed (SWT: W = 0.9507 - 0.9885, P > 0.05) after square-root transformation for the mean number of copulations (Fig. 2) and after log transformation for the mean copulation duration (Fig. 2; Table S3) and mean wing-fanning duration (Fig. 3). Those transformed data were then analyzed using ANOVA followed by Tukey’s Studentized multiple range test. Male survival was analyzed using a Lifetest (LIFTEST procedure) (Fig. 3).

Data on mating latency (Fig. 3) were not normally distributed even after transformation (SWT: W = 0.7533 - 0.8441, P < 0.05) and thus analyzed using a likelihood ratio test (LRT) of a generalized linear model (GLM, GIMMIX procedure). Data on the relationships between copulation duration/sperm ejaculated and successive copulations were analyzed using a generalized linear mixed model (GLMM, GIMMIX procedure) because they were not normally distributed (SWT: W = 0.8673 - 0.9259, P < 0.05). The slopes of linear lines were compared using an analysis of covariance (ANCOVA) with treatments as covariates in the GLMM model (Fig. 4). All analyses were done using SAS 9.13. Rejection level was set at α < 0.05. Reported values were means ± SE.
Results

Effect of Immediate Sperm Competition Levels on Ejaculate Allocation and Copulation Duration

Figure 1A shows sperm allocation in the first copulation under different SC levels. The number of eupyrene and anypyrene sperm transferred by males was not significantly different between treatments although males allocated slightly more eupyrene sperm in PLUS MALES treatment (ANOVA: \( F_{2,41} = 1.80, P = 0.2826 \) for eupyrene; \( F_{2,41} = 0.45, P = 0.6837 \) for anypyrene). Copulation duration in the first copulation was also similar for all treatments (ANOVA: \( F_{2,41} = 1.32, P = 0.2786 \)) (Table S3).

Effect of Both Immediate and Mean Sperm Competition Levels on Lifetime Trade-offs

The total mean number of eupyrene and anypyrene sperm transferred by males in their lifetime was significantly lower in PLUS FEMALES than in PLUS MALES and CONTROL (ANOVA: \( F_{2,41} = 7.40, P = 0.0018 \) for eupyrene; \( F_{2,41} = 4.85, P = 0.0131 \) for anypyrene) (Fig. 1B).

In their lifetime males in PLUS FEMALES copulated significantly fewer times and had significantly shorter copulations than in other treatments (ANOVA: \( F_{2,41} = 6.81, P = 0.0028 \) for number of copulation; \( F_{2,41} = 3.75, P = 0.0254 \) for copulation duration) (Fig. 2). However, the male longevity was similar in all three treatments (Lifetext: \( a^2 = 2.43, P = 0.2961 \)) (Fig. 3).
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Figure 3. Male survival under different SC levels. Lines with the same letters are not significantly different ($P > 0.05$).

We show that sperm transferred declined (Fig. 4A, B) and copulation duration increased (Fig. 4C) with successive copulations. There was no significant difference in slopes between treatments (ANOVA: $F_{2,17} = 2.39, P = 0.0947$ for eyepiece; $F_{2,17} = 1.18, P = 0.3110$ for eyepiece; $F_{2,17} = 0.58, P = 0.5634$ for copulation duration).

EFFECT OF SOCIO-SEXUAL CONTEXT ON MATING LATENCY AND COURTSHIP DURATION

In all treatments focal males fanned their wings before copulation occurred. Most wing fanning took place in the last few hours of the scotophase. In PLUS FEMALEIS focal males often performed their wing fanning over the metal mesh between the two chambers.

Our data show that socio-sexual context had no effect on mating latency (LRT, GLM: $\chi^2 = 0.51, P = 0.7737$) (Fig. 5). However, the focal males in PLUS FEMALEIS performed wing fanning five to eight times longer than those in CONTROL and PLUS MALES (ANOVA: $F_{2,20} = 34.35, P < 0.0001$) (Fig. 5). The wing fanning duration was not significantly different between CONTROL and PLUS MALES (Fig. 5).

Discussion

Surprisingly, our findings in the present study generally do not support any of our three hypotheses. SC theory (Wedell et al. 2002; Parker and Pizzari 2010) predicts that SC levels would determine the copulation duration and escalate allocation of the focal male where the male increases sperm allocation to his mate and prolongs the copulation in the presence of rivals, and the opposite is the case in the presence of additional females. For the first set of the SC prediction, we tested the effect of immediate SC levels by comparing the treatments for the first copulation before which time all insects were kept individually from the pupal stage. We show that the focal male allocated similar number of eyepiece and eyepiece sperm from his reserves (Fig. 1A) to the mate and had similar copulation duration regardless of presence or absence of immediate SC effect. These findings phenotypically contradict the predictions by the SC model (Wedell et al. 2002; Parker and Pizzari 2010), sperm use and storage model (Roquero and Alonso 2014) and sperm economy model (Jae and Kamiruru 2015). Although not tested directly, we argue that the lack of phenotypic response to immediate socio-sexual context could result from female-male interactions (or conflicts). For example, with elevated SC levels males may attempt to increase sperm allocation (Wedell et al. 2002; Parker and Pizzari 2010) but females may intend to mate again for genetic diversity via the last sperm precedence mechanism (Xu and Wang 2010) and thus accept fewer sperm, with reduced SC levels males may attempt to save sperm to inseminate more mates (Wedell et al. 2002; Parker and Pizzari 2010) but females may attempt to collect more sperm (Roquero and Alonso 2014). Alternatively, male’s response to SC levels may depend on a long time frame so that the immediate perception of rivals during the mating scotophase may give a poor measure (L.A. Parker, pers. comm.). However, in a differently designed experiment using L. barchichiella where rivals or additional mates are released to a mating pair immediately after copulation commences (thus all insects have physical contact and the test of courtship cost to the focal male is not allowed), Xu and Wang (2014) report a significant decrease of escalate allocation in the presence of additional mates and increase of that in the presence of rivals, phonotropically supporting the SC theory.

For the second set of the SC prediction (both immediate and mean SC levels) that is the hardest nut to crack (Engqvist and Reinhold 2005), we look at all copulation events during the focal males’ lifetime where focal males had perceived different socio-sexual environments for about 12 h a day since the first mating event. We have not found any difference in sperm allocation (Tables S1 and S2) and copulation duration (Table S3) in any given copulation between treatments except for the second copulation, where the focal male transferred significantly more eyepiece sperm in PLUS MALUS than in CONTROL and PLUS
Figure 4. Number of eupyrene (x > 256) and aepyrene (x < 3000) sperm ejaculated by males and mating duration (MD) in relation to mating number (MN) under different SC levels. (A) Eupyrene sperm: CONTROL, ln(eupyrene) = 3.5357 - 0.0576MN (F_{4,14} = 18.13, P < 0.0001); PLUS MALES, ln(eupyrene) = 3.7899 - 0.0063MN (F_{4,14} = 5.42, P = 0.0041); PLUS FEMALES, ln(eupyrene) = 3.5098 - 0.6038MN (F_{4,14} = 8.00, P = 0.0072). (B) Aepyrene sperm: CONTROL, ln(aepyrene) = 1.0273 - 0.1244MN (F_{4,14} = 39.11, P < 0.0001); PLUS MALES, ln(aepyrene) = 2.1652 - 0.1618MN (F_{4,14} = 65.81, P < 0.0001); PLUS FEMALES, ln(aepyrene) = 3.0658 - 0.1358MN (F_{4,14} = 65.81, P = 0.0002). (C) Mating duration: CONTROL, ln(MD) = 0.8115 + 0.0045MN (F_{4,14} = 5.92, P = 0.0176); PLUS MALES, ln(MD) = 0.9881 + 0.0076MN (F_{4,14} = 4.62, P = 0.0432); PLUS FEMALES, ln(MD) = 0.5716 + 0.1584MN (F_{4,14} = 5.06, P = 0.0099). All original data were used for analysis but only mean ± SE values were presented.

FEMALES (Tables S1), and fifth copulation, where the copulation duration was significantly longer in PLUS FEMALES than in CONTROL and PLUS MALES (Tables S3). Although the discrete patterns in these two copulations cannot be explained using any model at the present, our findings phenotypically contradict the SC predictions. In a test of the effect of mean SC levels on sperm allocation in a cricket, Worthington et al. (2013) also indicate that males do not prudently adjust the number of sperm they transfer to mates based on mean SC levels, agreeing to our findings. However, as Bielemann et al. (2010) and Parker (2015, per. comm.) point out, the length of exposure to rivals may be critical in determining male responses to the mean SC levels. The focal male’s exposure to rivals for 12 hours a day in the present study may not be sufficient to trigger a measurable response of the male to mean SC levels. Furthermore, in a study of D. melanogaster Bielemann et al. (2011b) show that males assess their socio-sexual environment using any two cues from sound, smell, and touch. As reviewed in the Introduction, the focal male can detect the presence of other individuals via chemical and/or acoustic cues without physical contact in E. kuehnii. However, whether physical contact is important for E. kuehnii males to respond to rivals remains to be determined.
FLIRTAION REDUCES MALES’ FECUNDITY BUT NOT LONGEVITY

When we look at our findings longitudinally, we have found that in all three treatments, ejaculate size decreased linearly (Fig. 4A, B) over successive copulations, fitting the model on reproductive output declines with age of adults having fixed resources obtained during the immature stages (Ragon and Parker 1986). Furthermore, copulation duration increased linearly in successive copulations (Fig. 4C). These results demonstrate a negative correlation between sperm allocation and copulation duration that contrasts findings in many other studies (e.g., Price et al. 2012; Most et al. 2013). During copulation in *E. kuchteliae*, the male produces a spermatophore in his internal sac and pushes it to the female’s bursa (Xu and Wang 2011). A 24-h recovery period between copulations is necessary for a male to be able to deliver spermatophore filled with sperm to a female; if intermating periods are shorter, he can still transfer a spermatophore with no or few sperms (Xu and Wang 2011). This feature may explain why copulation duration and sperm number are not positively correlated over successive copulations. If the mean SC levels play a role in both sperm production and allocation, then we expect that the increase in copulation duration and decrease in ejaculate size over successive copulations would be faster in PLUS MALES than in PLUS FEMALES. However, ANCOVA show that the slopes of regression lines in both copulation duration and sperm depletion were not significantly different between treatments, indicating that the mean SC levels do not phenotypically affect sperm production and allocation. Again, this could result from male–female interactions and/or insufficient male exposure to SC levels as discussed above. When testing this set of SC prediction by measuring the focal male longevity, number of copulations, and number of offspring sired, Xu and Wang (2014) demonstrate that with elevated SC levels, the focal male lives shorter, inseminates fewer mates and sires fewer offspring, phenotypically supporting the SC theory. However, their experimental design does not allow to test whether courtship effort by, and rivals’ physical harassment of, focal males affect their longevity fitness. Therefore, the fitness decline observed by these authors could be due to physical conflict, or perhaps stress, that has little to do with the physiological costs of spermatogenesis and sperm allocation.

When we examine lifetime reproductive fitness in different socio-sexual environments, we have found that the focal male had significantly lower number of sperm transferred (Fig. 1B) and fewer copulations (Fig. 2) in PLUS FEMALES than in PLUS MALES and CONTROL, the latter two of which were not significantly different. These findings contrast Xu and Wang (2014) and cannot be explained by the SC prediction. Our data indicate that the lifetime number of sperm transferred by the focal male was in fact the function of his lifetime number of copulations, contradicting the prediction by the sperm economy model: female-biased conditions make males promiscuous and females promiscuous, that is both sexes mate more times under female-biased conditions (Abc and Kamina 2015). In a previous study on *E. kuchteliae* Xu and Wang (2009b) demonstrate that (1) a male can inseminate up to nine females in his lifetime, ejaculating between 3400 and 11,000 eupyrene sperm per copulation, and (2) once-mated females produce the same number of offspring (fertility) regardless of the number of eupyrene sperm received within the above range. This study shows that the number of females a male can mate during his life determines his lifetime reproductive fitness. In the present study, females received on average of 4100–10,500 eupyrene sperm in a given copulation (Table S1), falling into the range reported by Xu and Wang (2009b). It is strongly suggested that the reduced number of copulations by the focal male in PLUS FEMALES treatment (Fig. 2) leads to a decline in his lifetime reproductive fitness. Similar to findings in Janowicz and Fischer (2010) we show that male longevity was the same in all treatments (Fig. 3). This result suggests that longevity and reproduction are not traded off against each other in *E. kuchteliae* in response to socio-sexual contexts, contradicting findings and predictions in many other studies (e.g., Kotiah and Simmons 2003; Simmons and Kotiaho
Increasing empirical evidence shows that precopulatory courtship displays by males are costly across taxa including humans (e.g., Conforti and Pratridge 1996; Chittock-Brock and Langley 1997; Hoback and Wagner 1997; Kotaiko and Simmons 2003; Hunt et al. 2004; Simmons and Kotaiko 2007; Hochler 2008; Papadopoulos et al. 2010; Wedell 2010; Breitman et al. 2013b; Scharr et al. 2013).

Hochler (2008) notes that the number of spermatophores transferred decreases as the time from female copulation decreases, a finding that is consistent with the idea that males invest more in courtship when they are closer to the end of the mating season. This is because males are more likely to be able to transfer spermatophores before the female becomes unresponsive to courtship signals.

In summary, E. kuehniella males do not adjust the number of sperm they transfer to females in a given mating based on their relative age or the number of spermatophores transferred. Instead, they adjust their courtship behavior based on the age of the female, with older females receiving fewer spermatophores and younger females receiving more spermatophores.

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KAMRIZ ESPANDI ET AL.


Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Table S1. Mean number of spermatozoa (×10^6) sperm ejaculated by males in subsequent matings under different SC levels.

Table S2. Mean number of spermatozoa (×10^6) sperm ejaculated by males in subsequent matings under different SC levels.

Table S3. Mean copulation duration (hours) in subsequent matings under different SC levels.
STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate’s Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate’s contribution as indicated below in the Statement of Originality.

Name of Candidate: Kambiz Esfandi

Name/Title of Principal Supervisor: Professor Qiao Wang

Name of Published Research Output and full reference:
Flirtation reduces males’ fecundity but not longevity.


In which Chapter is the Published Work: Part of Chapter four

Please indicate either:

• The percentage of the Published Work that was contributed by the candidate:
  and / or

• Describe the contribution that the candidate has made to the Published Work:
  Experiments, data analysis and draft writing

Kambiz Esfandi 31/10/2017
   Candidate’s Signature  Date

Qiao Wang 31/10/2017
   Principal Supervisor’s signature  Date