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REPRODUCTIVE BEHAVIOUR OF
*EPHESTIA KUEHNIELLA* ZELLER
(LEPIDOPTERA: PYRALIDAE)

Jin Xu

2010
REPRODUCTIVE BEHAVIOUR OF

EPHESTIA KUEHNIELLA ZELLER

(LEPIDOPTERA: PYRALIDAE)

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Abstract

_Ephestia kuehniella_ is a pest of stored grain products. It also is widely used to rear parasitoids and predators. Prior to this study, little information was available on its reproductive behaviour. The fitness of _E. kuehniella_ decreases with the increase of rearing density; a density of 100 larvae/50g food is recommended to produce high quality insects. Females emerge earlier than males. Emergence peaks at dusk; calling, courtship and mating peak in the late part of the 1st scotophase following emergence; oviposition peaks in the early part of the 2nd scotophase following emergence. Newly emerged virgin females carry <5 mature eggs, and the egg load increase to ≈240 three days after emergence and remains unchanged thereafter. Male accessory gland secretions stimulate egg maturation; mated females produce ≈300 mature eggs. Males produce two types of sperm, eupyrene (nucleate) and apyrene (anucleate) sperm. After mating, it takes 11 h for most eupyrene and apyrene sperm to reach the spermatheca. The presence of eupyrene sperm in the spermatheca is the main factor that elicits oviposition. The highest fecundity can be achieved when both sexes are 1-d-old at mating compared to older insects; delaying mating for 7 d reduces female fecundity by 60%. There is no significant effect of parental age on offspring fitness. Virgin females live longer than mated ones because the former allocate less resource for egg production. Larger females have higher fecundity and larger males produce larger spermatophores. Larger parents have larger sons and daughters. Females prefer large and mid-aged males for mating. Males prefer large, young and virgin females for mating. Males strategically adjust ejaculate size according to the degree of sperm competition risks. Both sexes mate multiply where males can copulate up to 9 times and females up to 4 times in their lifetime. Larger and younger females are more likely to remate. Multiple mating does not increase female fecundity, fertility and longevity. Females discriminate against previous mates and strategically adjust oviposition to gain genetic benefit via increasing offspring genetic diversity. Using a chemosterilant, thiotepa, I determined that the last male to mate with a female sires most of her offspring. The last male sperm precedence may be due to sperm displacement at both sperm ejaculation and storage sites, where the 2nd male physically displaces the 1st male’s spermatophore with his own in the bursa copulatrix and triggers the female to dump ≈50% resident sperm in the spermatheca. Spermathecal contractions appear to be the mechanism for sperm ejection. The outcome of sperm displacement is the result of male×female interactions.
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CHAPTER 1
GENERAL INTRODUCTION

1.1 Introduction

Annual losses of grain in storage due to insect infestation have been estimated as about 10% ($\approx 90$ million tons) worldwide (Munro 1966; Hill 2002). Approximately 70 species of moths, primarily in the families Pyralidae, Tineidae, Oecophoridae, and Gelechiidae, are associated with infestations of stored products (Cox & Bell 1991). However, only a few species such as the Mediterranean flour moth ($Ephestia kuehniella$, Pyralidae), tobacco moth ($E. elutella$, Pyralidae), almond moth ($Cadra cautella$, Pyralidae), raisin moth ($C. figulilella$, Pyralidae), Indian meal moth ($Plodia interpunctella$, Pyralidae), and Angoumois grain moth ($Sitotroga cerealella$, Gelechiidae) are considered widely distributed and major pests of stored foods (Cox & Bell 1991; Sedlacek et al. 1996).

The Mediterranean flour moth $E. kuehniella$ Zell is a serious pest of stored products, particularly flour (Cox & Bell 1991; Sedlacek et al. 1996; Hill 2002; Rees 2003). This pest is likely to be found in any mill or warehouse where flour or other powdered cereal products are stored (Sedlacek et al. 1996). The larvae prefer powdered foods but also attack whole grains, bran, etc (Sedlacek et al. 1996). In heated mills $E. kuehniella$ may breed five generations a year (Scott 1984). Larvae spin silken threads wherever they go and bind with webbing particles of food on which they have been feeding. As a result, they spoil more than they consume in stored grain products (Cox & Bell 1991; Sedlacek et al. 1996). For example, webbing may block conveyers or spouts and other parts of the milling machinery, encouraging other insects and mites (Hebanowska et al. 1990; Hill 2002; Rees 2003). Larvae live inside the webbing and pupate in cocoons, which protect them against fumigants, contact insecticides, natural enemies, and loss of water (Hebanowska et al. 1990; Cox & Bell 1991; Sedlacek et al. 1996). Eggs hatch between 10°C and 31°C without any apparent influence of ambient humidity on duration of development or viability (Jacob & Cox 1977). $E. kuehniella$ has thus proved to be a severe and tough to control pest of stored grain products.
E. kuehniella is also widely utilised to laboratory rear parasitoids and predators for biological control of a number of key pests (e.g. De Clercq et al. 2005; Kim & Riedl 2005; Hamasaki & Matsui 2006; Paust et al. 2008) and research into behaviour, biochemistry and molecular biology (e.g. Corbet 1973; Rahman et al. 2004; Rahman et al. 2007; Jamoussi et al. 2009). Adult moths of this species do not need to feed before they lay their eggs (Norris 1932). The selective advantage of this feature has probably contributed to the success of the moth as a pest in the dry environment of warehouses and provender mills (Calvert & Corbet 1973). This is an easily-bred species as larvae can develop on simple food resources (e.g. wheat or maize flour) under a wide range of temperature (10 ~ 31°C) without any apparent influence of ambient humidity on developmental duration or survival (Moeiaety 1959; Jacob & Cox 1977; Hebanowska et al. 1990). Under 25 ~ 30°C, a colony of this species can continually provide adult moths for use without diapause, with a generation lasting for 50 days (Gonzalez Nicolas 1966). These features make this insect of great potential to serve as valuable material for laboratory research and commercial use.

1.2 Importance and Relevance of This Study

The study of the reproductive behaviour of an insect helps us understand its life history, behavioural ecology, sexual selection mechanisms and evolutionary ecology of senescence (Hughes & Reynolds 2005; Bonduriansky et al. 2008). It can also provide information useful for the design and implementation of innovative monitoring or control tactics, and for better use of this insect as research materials or hosts of parasitoids.

1.2.1 Sexual Selection

In addition to natural selection (Darwin 1859), Darwin (1871) proposed a second type of selection, sexual selection, as the basis of his theory of evolution and speciation. Darwin (1871) correctly realized that sexual selection could be mediated by intrasexual competition for mates (usually by male–male combat) or by intersexual choice of mates (usually by female choice of attractive males). Today, the theory of sexual selection through male competition has been widely accepted. However, his idea of female preference for male ornaments is still controversial although it has received new support in bird and fish (Majerus 1986), and in genetic models (reviewed in
Darwin (1871) based his theory of sexual selection on pre-copulatory mate competition and choice. However, Parker (1970) pointed out that sexual selection may continue after copulation via sperm competition between males for fertilization, or female post-copulatory manipulation of sperm use, also known as cryptic female choice (e.g. Birkhead & Møller 1993; Eberhard 1996; Simmons 2001). Studies have often suggested the importance of post-copulatory sexual selection in evolution but much less is known about its mechanisms.

In many polyandrous species, including lepidopteran insects, the last male to mate with a female often sires most of her offspring, a phenomenon called last male sperm precedence (reviewed in Silberglied et al. 1984; Simmons 2001; Friedlander et al. 2005). This phenomenon has been explained as the result of male’s and/or female’s influence on sperm use pattern for maximum reproductive success (reviewed in Danielsson 1998; Simmons 2001; Snook & Hosken 2004; Snook 2005). A number of mechanisms for last male precedence have been suggested and tested (reviewed in Xu & Wang 2010a). However, in all studied species it is still not clear whether these mechanisms work together, and to what extent each mechanism contributes to last male precedence.

The underlying mechanisms of sexual selection are often difficult to resolve (Andersson 1994; Simmons 2001). It has been noted that difficulties in resolving the mechanisms of sexual selection are particularly evident in insect species (Thornhill & Alcock 1983; Andersson 1994; Simmons 2001). In Lepidoptera, and several other groups of animals, males produce two types of sperm, eupyrene (nucleate) and apyrene (anucleate) spermatozoa (Meves 1903). Although both eupyrene and apyrene sperm reach the spermathecae of the inseminated females, only eupyrene sperm can fertilize eggs (Friedlander & Gitay 1972). The function of apyrene spermatozoa is still not clear although they have been argued to play a role in sperm competition, perhaps aiding transport of eupyrene sperm, or acting as cheap filler to deceive females about their sperm load (e.g. Cook & Wedell 1999; Simmons 2001).

Lepidopteran species have often been used as models for resolving the mechanisms of pre- and post-copulatory sexual selection (reviewed in Simmons 2001; Cordero 2005; Koshio et al. 2007; Oliver et al. 2009). In *E. kuehniella*, females emerge
earlier than males (Xu et al. 2008); individuals are highly variable in phenotypical features (Cerutti et al. 1992); both sexes mate multiply with males mating more times than females (Xu & Wang 2009b). These biological characteristics suggest that the quality of individual moths is highly variable and operational sex ratio is temporally and spatially dynamic in *E. kuehniella*, allowing both pre- and post-copulatory sexual selection to take place. Moreover, male reproductive systems and spermatogenesis of *E. kuehniella* have been studied in detail (e.g. Riemann & Thorson 1978; Wolf & Bastmeyer 1991). Therefore, *E. kuehniella* should be a good model for the investigation of sexual selection mechanisms.

### 1.2.2 Evolution of Ageing and Life Span

Classic evolutionary models interpret ageing, or senescence, as a cost of reproduction (Medawar 1952; Rose 1991; Hughes & Reynolds 2005) but evolutionary research has largely neglected the association between the evolution of ageing and sexual selection (Bonduriansky et al. 2008). One central question in evolutionary ecology of senescence is how individuals optimize resource allocation to their survival and reproduction. However, the physiological mechanisms by which this trade-off is controlled remain poorly understood at all biological levels (Zera & Harshman 2001; Harshman & Zera 2006). Most lepidopteran species do not feed on a protein source as adults; even in those nectar-feeding species, only a small amount of protein may be obtained by adults (Baker & Baker 1973). As a consequence, most materials for reproduction and survival must be obtained during the larval stage. Therefore, Lepidoptera should have evolved mechanisms for optimal resource allocation between survival and reproduction.

Females are believed to invest more in reproduction than males. Antagonistic coevolution between the sexes is thus driven when male adaptations to intrasexual competition and intersexual selection have detrimental effects on their mates (Parker 2006; Bonduriansky et al. 2008). Such male effects can increase ageing rate and shorten lifespan of females by physical damage (e.g. Crudgington & Siva-Jothy 2000; Rönn et al. 2007) or toxic male ejaculates (e.g. Chapman et al. 1995; Green & Tregenza 2009). While sexual conflict makes copulation costly in females, mating may also increase female fecundity and/or longevity by water and nutrients contained in ejaculates (Arnqvist & Nilsson 2000; Maklakov et al. 2005). Therefore, the trade-off between the
costs and benefits of matings, as well as between survival and reproduction in females can be more complex and is worth examining.

Females of many species often prefer to mate with older males (reviewed in Brooks & Kemp 2001) but why females do so is still highly controversial. The traditional “good genes” models assume that mating with older males should be beneficial for females because old age is a demonstration of a male's high genetic quality that enables him to survive (reviewed in Brooks & Kemp 2001). Nevertheless, the quality of gametes may decrease with male age due to the accumulation of deleterious mutations that may reduce their offspring fitness substantially (Hansen & Price 1995; Brooks & Kemp 2001). Therefore, the “good genes” models may be subject to re-examination.

1.2.3 Insect Manipulation

Pheromone-based mating disruption and sterile insect technique (SIT) have been successfully used in pest control world wide (e.g. Suckling et al. 1990; Caprio & Suckling 1995; Lo et al. 2000). However, whether or not these control tactics are successful in the control of an insect pest largely depends on our understanding of the reproductive behaviour of the pest (Cardé & Minks 1995; Michereff et al. 2004). For example, the relationship between age and reproduction is vital information for pheromone-based mating disruption that achieves control by preventing or delaying mating (Michereff et al. 2004; Wenninger & Averill 2006). Knowledge of mate choice in relation to morphological traits and age may be used to improve SIT based control effectiveness that relies heavily on a constant supply of insects with desirable characteristics such as optimal body size and age (Shelly et al. 2007). Moreover, the understanding of female multiple mating and sperm use patterns also is essential for the successful application of SIT (Kraaijeveld et al. 2005). The female sex pheromone has been identified for *E. kuehniella* and has been suggested for control applications (Trematerra 1994; Sieminska et al. 2009). Ayvaz et al. (2007) have developed a sterile insect technique for the control of this pest. However, mating behaviour of this insect has not been thoroughly investigated, making it difficult for optimal design and implementation of monitoring or control tactics for this pest.

The economical, constant and reliable supply of large numbers of high quality *E. kuehniella* is a pre-requisite for the use of this species as hosts of biological control
agents and research materials. Mass rearing is often used to produce high quantities of insects at the lowest cost and individual rearing may also be applied to obtain insects of high quality when necessary. In mass rearing systems, however, low performance and high mortality of the insects often occur due to food shortage (Cerutti et al. 1992; Sato et al. 2004), fecal and microbial contamination and cannibalism (Singh 1977; Stone & Sims 1992). Therefore, knowledge of the influence of rearing systems and larval density on adults’ performance is essential for the development of reliable rearing programmes. Information of parental effects (e.g. size, age at mating) on offspring performance also is important for the improvement of rearing programmes. For example, empirical studies have showed that paternal age has negative effects on progeny fitness in different species (reviewed in Prokop et al. 2007). Moreover, a good understanding of the reproductive behaviour and rhythms is needed for better use of this insect.

1.3 Aim and Objectives of This Study

The aim of this research is to provide accurate information on the reproductive behaviour of *E. kuehniella*, with three objectives:

1) To investigate aspects of the basic biology and population growth of *E. kuehniella*

2) To investigate the biotic and abiotic factors that affect the reproductive fitness of *E. kuehniella*

3) To investigate pre- and post-copulatory sexual selection, and reproductive senescence of *E. kuehniella*
CHAPTER 2
LITERATURE REVIEW

2.1 Introduction
This chapter reviews the current knowledge on reproductive behaviour relevant to my PhD studies on *Ephestia kuehniella* Zeller.

2.2 Classification of *Ephestia kuehniella*
In 1879 Zeller first described this species from an insect collected in Auckland, New Zealand. The classification for the species is:
- Order: Lepidoptera
- Superfamily: Pyraloidea
- Family: Pyralidae
- Subfamily: Phycitinae
- Genus: *Ephestia*
- Species: *kuehniella*

2.3 General Biology
*E. kuehniella* is found worldwide but not abundant in the tropic region (Hill 2002). The life cycle of *E. kuehniella* includes four stages: egg, larva, pupa and adult (Fig. 2.1). The complete life-cycle of this species takes about 50 days (Gonzalez Nicolas 1966).

2.3.1 Eggs
Pyralid eggs are usually flat and oval (Naumann 1991). The surface of the newly laid eggs of *E. kuehniella* is white in colour, and shining iridescent when observed by reflected light (Kamel 1969). Just before hatching the egg turns light yellow in color due to the development of the embryo which can be seen through the shell of the egg at this time (Brindley 1930). The egg is 500-550 µm long by 290-325
µm wide for *E. kuehniella* (Moreno et al. 1994) (Fig. 2.1b). The mean weight of a single egg is 0.023 mg (Brindley 1930). The eggs hatch in 96 hours at 30°C and 73% relative humidity (Brindley 1930).

![Image of *E. kuehniella* life cycle]

**Fig. 2.1** Life cycle of *E. kuehniella*. (a) adult moths in mating; (b) eggs; (c) mature (6th instar) larva and (d) mature (dark) and immature (brown) pupae. (Bars = 2 mm).

### 2.3.2 Larvae

Pyralid larvae usually do not have secondary setae; pro-thorax has 2 L setae; crochets are usually bi- or triordinal in a circle or mesal penellipse, and rarely uni- or biordinal in 2 transverse bands (Naumann 1991). *E. kuehniella* larvae are 0.866 mm long and 0.199 mm wide on average immediately after hatching and at this time they weigh 0.018 mg (Brindley 1930).

The newly hatched larvae are cream coloured and sparsely covered with long hairs. In general appearance they remain the same throughout their development. *E. kuehniella* larvae have six instars (Brindley 1930).
E. kuehniella larvae mainly feed on wheat flour but are recorded from a wide range of commodities and from dead insects (Hill 2002; Rees 2003). When reared on flour, E. kuehniella takes $41 \pm 2.4$ days (mean $\pm$ SD) to complete development through six instars at 30°C and 73% relative humidity (Brindley 1930). The mature (6th instar) E. kuehniella larva (Fig. 2.1c) is $12.6 \pm 0.78$ mm long and 27.8 mg in weight on average, and has a head capsule width of $1.110 \pm 0.032$ mm (Brindley 1930). The head capsule width has been used as a criterion to identify instars (Athanassiou 2006).

The incidence of diapause in E. kuehniella is influenced by both photoperiod and temperature. At 25°C, up to 50% of larvae enter diapause when reared in short photoperiods or continuous darkness while no diapause is detected at LD 14:10. At LD 14:10 no diapause occurs at 25 and 30°C but 12% of larvae enter diapause at 20°C (Cox et al. 1981).

2.3.3 Pupae

Pyralids pupate in silken cocoons or in larval shelters; pupae have pilifers, maxillary palps and antennae are long (Naumann 1991). Mature E. kuehniella larvae crawl to the surface of the material on which they have fed, and spin silk cocoons intermingled with particles of meal and flour for pupation. Pupae are pale green at the early stage and then turn to reddish brown on the dorsal side of the thorax (Fig. 2.1d). On the last day of development, pupae become dark in color (mature pupae) and emergence will occur within 24 hours. The average size of pupae is 9 mm long and 2.21 mm wide at thorax (Brindley 1930).

2.3.4 Adults

E. kuehniella is the largest member of the genus; 10-14 mm long when at rest, with wingspan being 20-25 mm; forewings are blue-grey with transverse dark wavy bars and a row of dark spots at the tip; hindwing are dirty white with fuscous veins (Fig. 2.1a). This species is sometimes distinct and recognizable without study of the genitalia (Hill 2002).

E. kuehniella females are nearly ready to mate and lay eggs when they emerge (Norris 1932; Calvert & Corbet 1973). The next day is a period of rapid maturation, when pheromone is synthesized, chorionation of the eggs proceeds, and some of the
chorionated eggs move from the ovarioles into the oviducts (Calvert & Corbet 1973). By the second dawn after emergence the concentration of pheromone is high, and most of the moths have ovulated; it is then that calling and mating begin for most females (Calvert & Corbet 1973).

*E. kuehniella* adults do not need to feed before they lay their eggs (Norris 1932). The selective advantage of this feature has probably contributed to the success of the flour moth as a pest in the dry environment of warehouses and provender mills (Calvert & Corbet 1973).

### 2.4 Reproductive Biology of Pyralidae

#### 2.4.1 Reproductive System

In the female Pyralidae the bursa copulatrix opens to the exterior on the ventral surface of the eighth abdominal segment so that the copulatory and oviducal apertures are situated on the eighth and ninth segments, respectively (Fig. 2.2). A pair of ovaries, each usually consisting of four ovarioles, open into the paired oviducts which join to form the common oviduct (Norris 1932). The common oviduct opens to the exterior on the ovipositor, which is believed to be formed of the fused ninth and tenth abdominal segments (Norris 1932). Entering into the dorsal side of a slightly inflated part of the common oviduct, is the vestibulum in which fertilization of the eggs takes place. A spirally coiled duct (spermathecal duct) initiates from the vestibulum leading to a small sac, the receptaculum seminis (spermatheca), where sperm are stored (Norris 1932). There are two pathways in the spermathecal duct, i.e. one is the main canal in which the sperm move from the vestibulum to the spermatheca, and the other is the fertilization canal in which the sperm move from the spermatheca to the vestibulum for fertilization (Lum et al. 1981; Suzuki et al. 1996). A tubular accessory gland, the spermathecal gland, opens into the spermatheca at its apex. Entering the vestibulum in the neighbourhood of the spermathecal duct is a second duct, the ductus seminalis, leading into the bursa copulatrix, into which the spermatophore is received at copulation (Norris 1932).

Male Pyralidae have an unpaired testis composed of eight follicles surrounded by a common “scrotum” (Norris 1932). Arising from the base of the testis are the paired vasa deferentia which are never fused (Fig. 2.3). Each vas deferens is more or less dilated near the middle of its course to form a vesicular seminalis in which sperm are
stored. The vasa deferentia open into the middle regions of the paired glands (i.e. ductus ejaculatorius duplex). The paired glands join basally to form the long ductus ejaculatorium simplex (i.e. unpaired gland). The terminal part of the ductus ejaculatorius simplex lies coiled up inside the chitinous aedeagus, forming, together with it, the penis (Norris 1932).

Fig. 2.2 A dorsal view of female reproductive organs of *E. kuehniella* (drawed based on Norris 1932). bc. Bursa copulatrix; ds. Ductus seminalis; ld. Lamina dentata; nbc. Neck of bursa copulatrix; ov. Ovary; ovp. Ovipositor; rct. Rectum; sc. Spermatheca; sd. Spermathecal duct; sg. Spermathecal gland; vlv. Vulva; vs. Vestibulum.

### 2.4.2 Mating Behaviour

The readiness of a pyralid female for mating is indicated by the “calling” position, which may be assumed as soon as an hour after emergence (Norris 1932). Dickins (1936) showed that *E. kuehniella* females produce a sex pheromone attractive to males (Dickins 1936), and she described the “calling” attitude, in which the abdomen protrudes between the wings with the tip everted, exposing the glands from which the
pheromone is released. *E. kuehniella* males in a wind tunnel respond to the female pheromone by flying upwind and making frequent crosswind casts (Traynier & Wright 1972). In a more confined situation, male moths exposed to the pheromone raise and lower their antennae alternately, become active and begin to vibrate their wings, orientate to the source of pheromone and may attempt to mate with it. Males of this species possess hairpencils (scent structures) located on the eighth abdominal segment (Corbet & Laifook 1977) but whether it produces sex pheromones reported in other species (Nishida et al. 1982; Mori et al. 1993; Teal & Oostendorp 1995) is still unknown.

**Fig. 2.3** A dorsal view of male reproductive organs of *E. kuehniella* (from Norris 1932). ae. Aedeagus; ag. Accessory gland; h. Horns of the ductus ejaculatorius; pg. Paired gland; t. Testis; ug. Unpaired gland; vd. Vas deferens; vs. Vesicula seminalis.

Phelan & Baker (1990) have studied 12 pyralid moth species and revealed two major behavioural patterns of courtship: (1) interactive and (2) simple. The former is characterized by a complex sequence in which, typically, a male approaches a pheromone-emitting female, engages in a head-to-head posture with the female, and then bring his abdomen over his head and strikes the female on the head and thorax. This action brings male abdominal scent structures into close proximity with the female
antennae. The male then attempts to copulate from the head-to-head position by a
dorsolateral thrust of the abdomen toward the female genitalia. Males of these species
possessed scent structures located either on the eighth abdominal segment, or in a costal
fold of the forewing, or both (e.g. Corbet & Laifook 1977). Courtship in the second
group is much more prosaic. After locating the female by response to her sex
pheromone, the male simply attempts to copulate by lateral abdominal thrusts under the
female wing, without behavioural embellishments. Males exhibiting simple courtship
have either no scent structures or structures that appear vestigial. The courtship of *E.
kuehniella* is similar to the simple courtship of *Amyelois transitella* and *Laetilia
coccidivora*; however, it also contains some behavioral elements found in the
interactive-type courtship (Phelan & Baker 1990). Males approach females with
fanning wings; 79% of approaches are from the rear. They position themselves parallel
to females, facing in the same direction (Phelan & Baker 1990). Upon contacting
females, males elevate their abdomens over their heads and extend their claspers and
attempt dorsolateral copulation without the abdominal headthump. Although
abdominal hairpencils are present, they appear vestigial because they are never
observed to be exposed during courtship (Phelan & Baker 1990). During this process,
females remain stationary with their abdomens in approximately the same position as
when calling occurs but with the pheromone gland retracted (Phelan & Baker 1990).

### 2.4.3 Insemination and Fertilization

During copulation the valves of the male clasp the lateral walls of the seventh
abdominal segment of the female while the uncus pushes under the overhanging part of
this segment and presses down very tightly upon the eighth tergite. The ovipositor is
completely retracted within the eighth segment. The aedeagus is thrust a short distance
up the neck of the bursa and the evaginated vesica penetrates into the bursa copulatrix
where the spermatophore is deposited. The transfer of one spermatophore per mating is
the rule (Norris 1932). Both eupyrene and apyrene sperm migrate from the
spermatophore to the ductus seminalis and then travel through the vestibulum and
thence to the spermatheca (Friedlander et al. 2005). It is not certain whether this
migration relies only on either sperm motility or contractions of the female
reproductive duct or on both (Friedlander et al. 2005). In order to fertilise eggs, sperm
must travel from the spermatheca downwards via the spermathecal duct to the
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vestibulum where fertilization takes place (Friedlander et al. 2005). In *Cydia pomonella*, sperm need between 3-6 hours to exit the spermatophore (Howell 1991). In most Lepidoptera oviposition begins < 24 h after mating.

2.5 Factors Affecting Reproductive Fitness

A growing number of empirical studies have demonstrated that insect reproductive performance is affected by biotic and abiotic factors. Most papers focus on the female reproductive output but neglect the male influence on the female reproductive fitness (Ellis & Steele 1982; Vahed 1998; Bergstrom et al. 2002; Tregenza & Wedell 2002). Some other studies have demonstrated that male mating history may influence female reproductive fitness (Linley & Hinds 1974; Gage & Cook 1994; Wedell 1996).

2.5.1 Effect of Age at Mating on Reproductive Fitness


More importantly, the study of age–reproductive performance relationship helps clarify the evolutionary ecology of senescence (see review in Section 2.8.6).

2.5.2 Effect of Body Weight on Reproductive Fitness

Body size is a key determinant of an organism’s ecological and physiological properties (Thornhill & Alcock 1983; Wickman & Karlsson 1989; Honek 1993). It is generally recognized that selection for higher fecundity favors larger females (Honek
Larger females often have more eggs available for laying and are able to regenerate eggs faster when required than smaller ones (Cloutier et al. 2000; Garcia-Barros 2000). A growing list of empirical studies in Lepidoptera has demonstrated a positive correlation between female pupal weight and fecundity (e.g. Marks 1976; Jones et al. 1982; Tammaru et al. 1996; Jimenez-Perez & Wang 2004a; Xu & Wang 2009a).

The reproductive advantages of being a large male are not as clear as those of large females. This may be because measurements of the reproductive success of males over their entire lifespan are extremely uncommon compared with females (reviewed in Simmons 1988; McElligott & Hayden 2000). Nevertheless, large size has been used as an indication of “good quality” in males, such as having better genes and more ejaculate supply over smaller ones (Phelan & Barker 1986; Bissoondath & Wiklund 1996). In some species of insects, larger males have a higher probability of obtaining mates (Mathis 1991; Savalli & Fox 1998; Sokolovska et al. 2000) and probably mate more often (Brockmann & Grafen 1989; Oneill et al. 1989; Alcock 1990).

2.5.3 Female Multiple Mating

Bateman’s principle, a well-established paradigm in evolutionary biology, suggested that while a male’s reproductive success should be limited only by the number of females he can inseminate, a female’s reproductive success should be largely independent of the number of matings she obtains (Bateman 1948). Contrary to these predictions, however, the majority of female insects mate multiply, most often with different males (polyandry) but also with the same male (repeated matings) (Arnqvist & Nilsson 2000). Mating is costly, such as the energy costs of sexual behaviour and the risks of predation, disease transmission, and injury inflicted by males (Daly 1978; Arnqvist & Nilsson 2000; Blanckenhorn et al. 2002). Therefore, the benefits of multiple matings in females must outweigh the costs to make the evolution of polyandry possible.

In many insect species a female obtains enough sperm from a single mating to fertilize her full egg-load (Thornhill & Alcock 1983) but in many others the female needs to mate more than once to fully fertilize her eggs (Arnqvist & Nilsson 2000). In an extensive review, Ridley (1988) suggests that the general pattern for females to
remate is in order to obtain sperm supplies for polyandrous species. Nevertheless, there is relatively little information on the relationship between sperm numbers and female fertility. Linley & Hinds (1974) show that in Culicoides melleus female fertility is reduced when mating with males whose ejaculate size falls below a threshold (50% of that of a virgin male) due to successive matings. However, in Cadra cautella, males produce decreased numbers of sperm on successive matings but no obvious decrease in female fecundity and fertilisation success occurs with increased number of male copulations (McNamara et al. 2007).

During copulation, males of many insect species not only transfer sperm but also supply water and/or nourishment for females in the form of glandular secretions (Wilson et al. 1999; Arnqvist & Nilsson 2000; Maklakov et al. 2005). Studies in several insect species have found that nutrients from the spermatophore delivered from males to females at mating are incorporated into the soma and eggs of females (Boggs & Gilbert 1979; Boggs 1981; Boggs & Watt 1981; Greenfield 1982). Polyandry thus has been viewed as an adaptation whereby females can obtain these male-derived nutrients to increase egg production and somatic maintenance (Parker & Simmons 1989; Boggs 1990). Indeed, several studies have found a positive relationship between the number of matings and female fecundity (Pardo et al. 1995; Ward & Landolt 1995; Sakurai 1996; LaMunyon 1997; Wilson et al. 1999; Arnqvist & Nilsson 2000; Maklakov et al. 2005) and longevity (Wiklund et al. 1993). However, some other studies have found no such relationship (Kraan & Straten 1988; Svard & Wiklund 1988; Ono et al. 1995; Rodriguez 1998; Kawagoe et al. 2001; Xu & Wang 2009b). Whether or not a female relies on male-derived nutrients may depend on larval diet, adult diet and the number of yolked eggs at eclosion (Boggs 1990).

The recent literature shows that females may gain genetic benefits from copulating with multiple mates, and a number of hypotheses have been proposed to explain the evolution of polyandry in this light (Simmons 2005). For example, the genetic incompatibility hypothesis suggests that polyandry enables females to bias paternity towards males with genes that confer higher fitness or that are more compatible with the females’ genome; as a result, their offspring viability increases (e.g. Zeh & Zeh 1996, 1997; Tregenza & Wedell 1998; Konior et al. 2001; Pai & Yan 2002). Recent studies propose that polyandry can benefit females by enhancing the genetic diversity of their offspring (reviewed in Jennions & Petrie 2000; Cornell & Tregenza...
2007), which may reduce sib competition (e.g. Ridley 1993) and inbreeding costs (Cornell & Tregenza 2007), and enhance disease resistance (e.g. Tooby 1982).

2.5.4 Male Multiple Mating

In a landmark paper, Dewsbury (1982) shows that the cost of producing ejaculate, often considered to be very low, is nontrivial. Males may also invest nutrients in reproduction besides sperm. In some insect species, males provide females with a nuptial gift during courtship or copulation (Thornhill & Alcock 1983). Nutrients contained in the spermatophore have been found in the eggs and soma of females (Boggs 1981; Wiklund et al. 1993). However, most non-pollen feeding Lepidoptera do not feed on a protein source as adults, instead, sequester most of the protein needed for egg production and basal maintenance during their larval feeding stage (Gilbert 1972). Even in those nectar-feeding Lepidoptera, only small amounts of protein may be obtained in some nectars (Baker & Baker 1973). Male Lepidoptera therefore have a limited protein supply and are likely to incur substantial costs such as sperm depletion or reduced survival through mating (Wedell et al. 2002).

Male investment of gametes and seminal products can have important consequences for female fitness (Torres-Vila & Jennions 2005). Therefore, male copulation experience may have a profound impact on female reproductive success if male reproductive investment declines over consecutive copulations. Some studies show that females that mate with non virgin males have lower lifetime reproductive success than those that mate with virgin males while other studies do not find this relationship (see review of Torres-Vila & Jennions 2005). This inconclusive scenario may occur because male mating experience is affected by a number of factors. The size, quality and number of spermatophores delivered by males have been shown to be highly sensitive to such factors as male body size, age at mating, larval and adult feeding regime, mating order and the duration between consecutive matings (Torres-Vila et al. 1995).

2.6 Sexual Selection

2.6.1 Introduction

Charles Darwin (1859) bases his theory of evolution from natural selection on
the central theme of ‘the struggle for existence’, ‘the survival of the fittest’ and ‘the preservation of favoured races’. In addition to natural selection, he proposes a second type of selection, sexual selection. “This depends, not on a struggle for existence, but on a struggle between males for possession of the females; the result is not death to the unsuccessful competitor, but few or no offspring” (Darwin 1871).

With many fascinating research programmes, sexual selection has now become one of the most active areas in the field of evolutionary biology (reviewed in Andersson 1994; Andersson & Iwasa 1996; Simmons 2001; Andersson & Simmons 2006; Jones & Ratterman 2009). Sexual selection is so vast a subject that it is impossible to cover all (or even most) of its aspects in detail in this review. Thus, in this chapter I review the current knowledge on sexual selection relevant to my study.

2.6.2 Mechanisms and Models of Sexual Selection

Darwin (1871) hypothesizes two forms of sexual selection: (1) intrasexual competition for mates, usually by males, in which males battle either for direct access to females or for possession of some critical resource (like food or breeding sites) that females need, and (2) intersexual choice of mates, usually by females, in which females choose males directly, basing their choice on individual preference for male ornaments. The theory of sexual selection through male competition has been widely accepted. Darwin clearly understood that female preferences exist, but he has never compellingly explained why and how such preferences would evolve (Andersson & Simmons 2006; Jones & Ratterman 2009).

Now—150 years after Darwin’s foundation, the theory of sexual selection through female choice for male ornaments is still controversial, although this theory has received new support from two directions. First, male ornaments favoured by female choice have been showed in bird and fish (Majerus 1986). Second, the reasons why females prefer ornamented males is clarified by genetic models (reviewed in Andersson & Simmons 2006).

Showing how mating preferences evolve genetically is harder than showing that they exist. To demonstrate experimentally the connection between a morphological trait in males and mating preferences in females, the male trait is often physically manipulated. This becomes difficult to manipulate if it is a behavioural trait. Although
molecular genetic and genomic tools enable the detailed characterization of genes and their effects on behaviour (reviewed in Fitzpatrick et al. 2005), evidence for genetics of mating preferences still is scarce (Andersson & Simmons 2006; Jones & Ratterman 2009). Nevertheless, the sensory bias model of sexual selection posits that female mating preferences are by-products of the underlying physiology of their sensory systems, which have been molded by natural selection, and that males evolve traits that match those sensory system characteristics (reviewed Fuller et al. 2005).

Fisher’s (1958) genetic model explains why females prefer ornamented males: a female choosing a male with an attractive trait will have sons and daughters that can both carry alleles for the attractive trait. This idea has been formulated in major gene and quantitative genetic models (reviewed in Andersson & Simmons 2006). Moreover, Zahavi (1975) suggests that females use the elaborate sexual displays of males to assess their quality because such displays are costly and therefore cannot be easily faked. Only males of high quality can afford to support such a handicap to survival (e.g. a peacock’s elaborate trait). Hamilton & Zuk (1982) suggest that females prefer to mate with males with showy sexual displays because they are the healthiest and the most resistant to parasites.

Competition and mate choice, as directed by Darwin (1871), are two most important mechanisms of sexual selection and have attracted most interest. There are, however, also other important mechanisms of sexual selection that have received attention, particularly sperm competition (see review in Simmons 2001). Since 1970 when Parker pointed out its importance, sperm competition has been found in many animals, and corresponding processes have been found to occur in plants (Harvey & Bradbury 1991; Andersson & Iwasa 1996; Simmons 2001). Notably, several authors have suggested the importance of cryptic female choice although much less is known about its mechanisms than sperm competition (Birkhead & Møller 1993; Eberhard 1996; Simmons 2001)

2.6.3 Pre-copulation Sexual Selection

2.6.3.1 Mate Choice in Relation to Body Size, Age and Virginity

Selection on characters may vary greatly in different mating systems. It is often assumed that larger individuals of a species have a fitness advantage over smaller
individuals, especially among insects (Wickman & Karlsson 1989). The rationale behind this assumption is that larger individuals may be better competitors for food (Wilson 1975) or mates (Alcock 1998), or have greater survivorship or longevity (e.g. Sokolovska et al. 2000). Also, large individuals are able to produce more gametes, provide a larger nuptial gift, a larger territory or better oviposition sites in addition to their genetic quality (Cloutier et al. 2000; Garcia-Barros 2000). Furthermore, selection for higher fecundity favours larger females (Honek 1993) but the reproductive advantages of being a large male are not as clear. Males and females are often subject to different selection pressures, which can result in sexual size dimorphism (Fairbairn & Preziosi 1994). While natural selection favours large females that produce more eggs, sexual selection in the form of male–male competition or female choice can result in larger body size in males (e.g. Fairbairn 1997).

However, there are conflicting selection pressures operating on body size of both males and females (Schluter et al. 1991). Potential costs of large body size in animals include higher mortality rates due to longer juvenile development times, increased energy demands, increased conspicuousness to predators or parasites, and increased heat stress (Blanckenhorn 2000). Furthermore, larger individuals of a species may have a mating disadvantage if attaining large size is associated with late reproduction, or increased energy requirements. Other trade-offs may cause selection against large size in males. For example, large male size may increase the burden for females of species that carry males during long postcopulatory periods, resulting in female-biased sexual size dimorphism (Taylor et al. 1998). Furthermore, if flight is a requirement for successful mating (as in mayflies), large size associated with decreased agility could reduce male fitness as has been shown for other insects (Marden 1987; McLachlan & Allen 1987; Neems et al. 1990). Therefore, conflicting selection pressures especially on male size may result in no apparent large male advantage, or stabilizing selection for some optimal intermediate size with maximum lifetime fitness (Schluter et al. 1991; Neems et al. 1998; Preziosi & Fairbairn 2000; Stoks 2000).

Generally, young and virgin mates are preferred in mate choice because they ensure higher reproductive potential (Halliday 1983; Andersson 1994). For example, young females have a better reproductive output (Karalius & Buda 1995; Vickers 1997; Xu & Wang 2009a) and virgin males can produce larger spermatophore (Howell et al. 1978; Kaitala & Wiklund 1995; Bissoondath & Wiklund 1996). Some authors have
documented mating advantages associated with virgin (Lewis & Iannini 1995; Arnaud & Haubruege 1999) and young individuals (Yasui 1996; Xu & Wang 2009a). Nevertheless, there is evidence that for some species the classical sexual selection theory does not apply. For example, in the tortricid *Choristoneur rosaceana*, old females obtain more matings than young females (Delisle 1995). In the butterfly *Drosophila hydei* males prefer to mate with recently mated females (Markow 1985).

In addition, mate choice in relation to age is one of the key evolutionary processes shaping life span (see review in Section 2.8.6 for detail).

### 2.6.3.2 Mate Choice by Females between Novel and Previous Mates

A number of studies show that multiple copulations may allow females to replenish sperm supply and/or obtain nutritional resources from males (e.g. Arnqvist & Nilsson 2000; Simmons 2001; Jimenez-Perez et al. 2003; Wang & Davis 2006). In many species, females may seek multiple mates for material benefits because of potential resource depletion from previous mates (e.g. Lemaitre et al. 2009).

The genetic incompatibility model requires that sperm from multiple males are present at the site of fertilization (Simmons 2005), and the genetic diversity model necessitates that the offspring are fathered by multiple males (Jennions & Petrie 2000). Therefore, to gain genetic benefits from polyandry, females must have developed strategies to discriminate against previous mates in subsequent copulations (Zeh et al. 1998). However, so far only seven studies have explicitly tested whether females have any preference for new versus previous mates in their subsequent copulations. Discrimination against previous mates by females has been reported in four invertebrate (Bateman 1998; Zeh et al. 1998; Archer & Elgar 1999; Ivy et al. 2005) and two vertebrate (Eakley & Houde 2004; LaDage & Ferkin 2007) species. In a cannibalistic spider, however, females appear to have no discrimination against previous mates (Fromhage & Schneider 2005). Archer & Elgar (1999) suggest that hide beetle *Dermestes maculates* females choose new mates for subsequent copulations to gain the benefit of genetic diversity. In a follow-up study of Zeh et al.’s (1998) observations, Newcomer et al. (1999) obtain evidence that in the pseudoscorpion *Cordylochernes scorpioides*, females discriminate against previous mates for higher offspring viability.
2.6.4 In-copulation Sexual Selection

Sperm are produced in astronomical numbers in comparison with eggs, and there is good evidence that sperm competition is the force behind the evolution of so many tiny sperm (Parker 1982; Smith 1984; Simmons 2001). However, males cannot produce unlimited numbers of sperm as spermatogenesis inevitably incurs costs (Dewsbury 1982). As first argued by Dewsbury (1982), single sperm may be cheap, but as males transfer huge numbers of sperm, their ejaculate can inevitably be limited (Pitnick & Markow 1994; Cook & Gage 1995; Savalli & Fox 1999). Therefore, views such as "the male is generally eager to pair with any female" (Darwin 1871), and "mate quality would seldom be as important to a male as their mate number" (Williams 1975), need to be re-examined in the light of the costs of ejaculates.

In studies of mate choice, most authors (e.g. Thornhill 1983; Blanckenhorn et al. 2000; Fedina & Lewis 2007) concentrate on how females choose their mates with the belief that females invest more in reproduction than males. However, females usually vary in reproductive potential, and thus if sperm supply is limited, males should be expected to choose among available females (Wedell et al. 2002). Simmons (2001) suggests that males may employ two strategies to undertake mate choice: (1) pre-copulation mate choice, where males choose high quality females and reject low quality ones for copulation, and (2) in-copulation mate choice, where males allocate different amount of ejaculates to their mates depending on mate quality. The first strategy has been experimentally confirmed in many species (e.g. Goshima et al. 1996; Pizzari et al. 2003; Sato & Goshima 2007). The differential allocation of sperm by males is suggested as cryptic male choice (Parker et al. 1996; Simmons 2001; Engqvist & Sauer 2003; Rönn et al. 2008) similar in principle to the differential acceptance and/or use of sperm envisaged as cryptic female choice (Thornhill 1983; Eberhard 1996; Dixson 2002; Fedina 2007). Recent empirical studies have suggested that males can use both strategies to choose their mates in the fowl Gallus gallus (Pizzari et al. 2003), and in the stone crab Hapalogaster dentate (Sato & Goshima 2007).

Galvani and Johnstone (1998) have examined theoretically how ejaculation strategies should vary with female reproductive quality. Their optimality model predicts that males should invest an increasing amount of their ejaculates as the female reproductive quality increases, irrespective of the amount of remaining sperm they have. Body size and age are generally considered cues of female reproductive potential, with
large and young females tending to offer greater reproductive returns to males than small and aged ones (Arak 1988; Bonduriansky & Brassil 2005). It has been reported that males of several species prefer larger or younger mates (see review in Bonduriansky 2001), and there is good evidence that males provide larger ejaculates to larger females (see review in Wedell et al. 2002). However, these high quality females may pose a greater risk of future sperm competition than aged and smaller females (e.g. Gage 1998; Wedell & Cook 1999). Therefore, using an alternative sigmoid function Galvani and Johnstone (1998) predict that if sperm competition risk is high males will allocate smaller ejaculates to females of high quality, but when the risk is moderate they should allocate larger ejaculate to high quality females. Simmons (2001) suggests that sperm competition risk should be moderate if females can mate no more than twice in their life time, but high if females can mate more than four times.

Operational sex ratio is the ratio of sexually competing males to females that are ready to mate in a population (Emlen & Oring 1977). In the natural environment, operational sex ratio is temporally and spatially dynamic (Krupa & Sih 1993; Casula & Nichols 2003; Forsgren et al. 2004; Wang & Chen 2005), which may provide information about the probability and intensity of sperm competition in polygamous mating systems (Wedell et al. 2002). Theoretically (Wedell et al. 2002), males should produce a smaller ejaculate and conserve sperm for future matings in the female biased sex ratio where male mating opportunities and potential reproductive rates increase (e.g. Pitnick 1993). On the contrary, males may increase ejaculate size in the male biased sex ratio where the presence of rival males provides information about the increasing probability of sperm competition in polyandrous mating systems (e.g. Nicholls et al. 2001).

Theoretical (Parker 1990, 1998), comparative (Parker et al. 1997) and experimental (Martin et al. 1974; Gage & Morrow 2003) studies have suggested that the paternity is determined by the relative number of competing sperm in females from different males. In this scenario, males should transfer larger ejaculates strategically to females when having to compete with rival sperm (reviewed in Simmons 2001). For example, Cook & Gage (1995) demonstrate that the male pyralid moth *P. interpunctella* increases his ejaculate size in the presence of a rival spermatophore. However, Hodgson & Hosken (2006) proposed an untested scenario where the second male should reduce his ejaculate size because the ejaculate by the previous male has buffered
the hostile female tract.

**2.6.5 Post-copulation Sexual Selection**

Sperm competition and post-copulatory female choice are considered as important parts of sexual selection (Parker 1970). Males have evolved various strategies for sperm competition, including stimulating mating when females are not sperm limited, allocating more sperm to females to compete with rival sperm, and displacing sperm from previous males (reviewed in Danielsson 1998; Simmons 2001).

In the process of sperm reception, storage and release, females also have opportunities to bias sperm fate to favor the sperm of a particular male over those of others, a process known as cryptic female choice (Thornhill 1983; Eberhard 1996; Dixson 2002; Fedina 2007). Therefore, the outcome of sperm competition should be the result of male × female interactions (reviewed in Danielsson 1998; Simmons 2001).

In the Lepidoptera and many other animals, the last male to mate often achieves a higher fertilization rate (reviewed in Silberglied et al. 1984; Friedlander et al. 2005; Xu & Wang 2009b; Xu & Wang 2010b). In these species, sperm packed in a spermatophore are ejaculated to the bursa copulatrix (the ejaculation site) from which they migrate to the spermatheca (the storage site) before they can fertilize ova (Smith 1984; Birkhead 2000; Friedlander et al. 2005). Sperm migration from the spermatophore to the spermatheca lasts < 0.5 h to > 10 h depending on species (reviewed in Friedlander et al. 2005). Therefore, the sperm competition battle between males that copulate with the same female should first take place in the bursa copulatrix, and the duration of intermating intervals in females should be correlated with the outcome of sperm competition (reviewed in Danielsson 1998; Simmons 2001). For example, the shorter the intermating duration in the female is, the more likely the second male can displace the spermatophore of the first male in the bursa copulatrix before most sperm of the first male emigrate from the spermatophore, resulting in a higher P$_2$ (the proportion of eggs fertilized by the second of the two males to mate) (Retnakaran 1974; Drnevich et al. 2000; Takami 2007). These authors consider the spermatophore displacement in the bursa copulatrix as the mechanism, and the intermating duration as the function, of the second male sperm precedence. However, in all studied species it is still not clear how much this mechanism contributes to the last male precedence and what factors determine the intermating duration.
After sperm migrate from the spermatophore, the sperm competition process between males that copulate with the same female may continue in the spermatheca (Silberglied et al. 1984; Simmons 2001; Friedlander et al. 2005). Hypotheses over the sperm precedence mechanisms at the sperm storage site generally fall into two categories: (1) sperm displacement, where resident sperm from the first male are flushed out from the storage by the incoming ejaculate of the last male (Pair et al. 1977; Silberglied et al. 1984) or ejected from the storage by the female to accept the sperm from the last male (Villavaso 1975; Hellriegel & Bernasconi 2000), and (2) sperm incapacitation, where incoming seminal fluids interfere with fertilization capability of resident sperm (Price et al. 1999) or females kill the resident sperm using spermicide after subsequent matings (Hosken et al. 2001). Pair et al. (1977) report that the female tobacco budworm *Heliothis virescens* mated to a sterile male after an initial mating to a fertile male shows a 90% reduction in the stored sperm. Therefore, these authors assume that the incoming ejaculate of the last male flushes the resident sperm from the spermatheca. In the Oriental leafworm moth *Spodoptera litura*, however, the reduction in the stored sperm appears to have occurred prior to the arrival of the sperm from the second copulation (Etman & Hooper 1979), casting doubt on the validity of the flushing hypothesis. Although not observed directly, many authors (e.g. Villavaso 1975; Hellriegel & Bernasconi 2000) suggest that the resident sperm may be physically displaced from storage by females. In the boll weevil *Anthonomus grandis*, females whose spermathecal muscles are severed have 22% resident sperm displaced after a second mating compared to 66% displacement for normal females, suggesting that sperm displacement is subject to spermathecal muscular control (Villavaso 1975). However, how the sperm displacement occurs in the spermatheca still remains unclear. So far, there is still no direct evidence supporting the sperm incapacitation hypothesis.

In addition, Parker (1970) proposes the sperm stratification hypothesis to explain the nonrandom use of sperm from different males by females and the last male sperm precedence. This hypothesis suggests that the spermatheca is a blind sac so that the last sperm to enter the sac would be the nearest to the exit and more likely to fertilize eggs first and to fertilize more eggs. The nonrandom use of sperm has been experimentally tested by several authors (e.g. Lewis & Jutkiewicz 1998; Takami 2007), who report that twice-copulated females have higher $P_2$ in eggs laid earlier and lower $P_2$ in those laid later. However, there is still no direct evidence to support the explanation...
of the last male sperm precedence by the stratification hypothesis. Furthermore, many studies do not support this hypothesis (e.g. Price et al. 1999; Rafinski & Osikowski 2002; Carbone & Rivera 2003).

### 2.6.6 Sexual Selection and Evolution of Ageing and Life Span

Ageing, or senescence, is defined as a corresponding decline in reproductive performance and an age-specific increase in mortality rate (Medawar 1952; Rose 1991; Hughes & Reynolds 2005). The cost of reproduction is widespread in organisms: elevated reproductive rate is associated with reduced life span and, in some cases, accelerated ageing (see review in Bonduriansky et al. 2008). However, evolutionary research has largely neglected the association between the evolution of ageing and a key mode of selection on male and female reproductive strategies – sexual selection and sexual conflict (Bonduriansky et al. 2008).

One central question in evolutionary ecology of senescence is to understand how individuals optimize resource allocation to their survival and reproduction and the trade-off between gametes and soma, yet the physiological mechanisms by which this trade-off is controlled remain poorly understood at all biological levels (Zera & Harshman 2001; Harshman & Zera 2006). One supposed mechanism in female insects is nutrient recycling through oosorption, the resorption of nutrients from unfertilised oocytes, which is predicted to occur in response to environmental stress, such as lack of food and mates (Kotaki 2003; Wang & Horng 2004). The positive correlations between oocyte degradation and female longevity under nutritionally poor or mating delayed conditions suggest that longer female lifespan is the result of recoup resources from eggs through oosorption (Ohgushi 1996; Wang & Horng 2004).

Females are believed to be the limiting sex, producing relatively few but large (expensive) gametes, whereas males usually produce astronomical numbers of tiny (cheap) sperm. This dichotomy in reproductive investment between sexes has been generally recognized as the force behind the evolution of female’s choiceness (Darwin 1859; Trivers 1972) and male’s competition (Parker 1982; Smith 1984; Simmons 2001) for fertilizations. Antagonistic coevolution between the sexes was thus driven when male adaptations to intrasexual competition and intersexual selection have detrimental effects on their mates (Parker 2006; Bonduriansky et al. 2008). Such male detrimental
effects can increase ageing rate and shorten life span of females by direct effect, via physical damage from ‘traumatic insemination’ (e.g. Crudgington & Siva-Jothy 2000; Rönn et al. 2007), or by indirect effect, from toxic male ejaculates (e.g. Das et al. 1980; Chapman et al. 1995; Green & Tregenza 2009). For example, females of many insect species suffer fitness costs (reduced longevity and reproductive success) as a result of actions of male derived Acps (accessory gland proteins) (reviewed in Jin & Gong 2001; Lung et al. 2002; Gillott 2003). Acps mediate a variety of effects that benefit males, including stimulation of female egg production, reduction of female receptivity and promotion of male success in sperm competition (reviewed in Jin & Gong 2001; Lung et al. 2002; Gillott 2003). The female survival cost that arises from Acp transfer by males may be a side effect of Acp function (Chapman et al. 1995; Lung et al. 2002). This Acp-mediated mating cost is potentially large and is incurred in addition to reproduction costs, such as those that result from egg production (Patridge et al. 1987; Sgro & Partridge 1999). The side effect of Acps appears to be dose-dependent, i.e., a higher dose has greater effect than a lower dose (Chapman et al. 1995; Lung et al. 2002).

Male reproductive strategies are typically related to elevated mortality risks and stronger selection for short life span relative to females, resulting in ‘live fast, die young’ male life histories (Vinogradov 1998; Carranza & Pérez-Barbería 2007). Generally, the opportunity for and intensity of sexual selection are greater in males because males invest less to each offspring than females do. This frees up resources that males can use to compete for additional matings and thus males can benefit by sacrificing longevity for the possibility of enhanced mating success (Bonduriansky et al. 2008). Moreover, male-biased mortality rates are related to polygynous mating systems characterized by intense male sexual competition (Clutton-Brock & Isvaran 2007). The theory predicts stronger selection for short life in males than in females, which is supported by higher mortality rates in males within a broad range of taxa (e.g. Promislow 1992; Vieira et al. 2000). However, empirical studies suggest that sexual selection need not always promote ‘live fast, die young’ strategies in males (Bonduriansky et al. 2008). First, selection on male condition and performance may favour genes with positive pleiotropic effects on longevity and somatic maintenance (Williams & Day 2003; Bronikowski & Promislow 2005). Second, in species with age-dependent expression of secondary sexual traits, sexual selection may favour males
that can survive and maintain their soma long enough to attain a large body size, large weapons or signals, or high social rank (Clinton & Le Boeuf 1993; Kokko et al. 1999). In numerous species, mating success is correlated with age and many empirical studies have suggested that females often prefer to mate with older males (reviewed in Brooks & Kemp 2001). Therefore, the traditional view derived from “good genes” models of sexual selection assumes that mating with older males should be beneficial for females because old age is a demonstration of a male’s high genetic quality that enables him to survive (reviewed in Brooks & Kemp 2001). However, what actually functions for the fitness of a female’s offspring is not the quality of her partner but of his gametes. Gametes may actually decrease with male age due to the accumulation of deleterious mutations that may reduce their offspring fitness substantially (Hansen & Price 1995; Brooks & Kemp 2001). A number of empirical studies have showed that paternal age has negative effects on offspring fitness in different species (reviewed in Prokop et al. 2007). Therefore, the “good genes” models may be subject to re-examination.

While sexual conflict makes copulation costly in females, mating may also increase female fecundity and/or longevity by water and nutrients contained in ejaculates (Arnqvist & Nilsson 2000; Maklakov et al. 2005). Therefore, the trade-off between the costs and benefits of matings, as well as between lifespan and matings in females can be more complex. Our understanding of the evolution of female remating rate is limited (Arnqvist & Nilsson 2000; Jennions & Petrie 2000) although the evolutionary causes and consequences of female remating rate are at the heart of sexual selection (Maklakov et al. 2006).

Therefore, phenotypes are likely to vary in life span and ageing rate, and this variation may reflect secondary sexual trait expression and can therefore modulate the magnitude or even the sign of sexual selection and conflict, and likewise, sexual selection and conflict may also react upon life span and ageing (Bonduriansky et al. 2008).
CHAPTER 3
REPRODUCTIVE BIOLOGY OF *EPHESTIA KUEHNIELLA*

3.1 General Introduction

Reports on biological control of *Ephestia kuehniella* and use of this species as insect food or research materials are piling up. However, the basic reproductive biology of this species has not been thoroughly investigated, knowledge of which is important for the control of this pest using pheromones or sterile insect technique (SIT) and improvement of natural enemy production using this pest as food. This chapter reports growth, circadian rhythms and general reproductive parameters of *E. kuehniella*.

3.2 General Methodology

The materials, procedures, environmental conditions and definitions detailed in this section were used throughout the thesis.

3.2.1 Materials

*Test cylinders:* Transparent plastic cylinders (8 cm diameter × 10 cm height, LabServ, Auckland) were used for rearing and experiments on reproductive behaviour. The same cylinders internally lined with porous plastic sheets (aperture diameter = 0.15 mm) were used for oviposition. Cylinders were covered with two layers of nylon mesh (aperture diameter = 0.2 mm).

*Glass tubes:* Glass tubes (2 cm diameter × 7.5 cm height) covered with one layer of nylon mesh (aperture diameter = 0.2 mm) were used for temporary rearing of individual adults between experiments.

*Dissecting microscope:* An Olympus SZ III (Japan) dissecting microscope with transmitted light fitted with a micrometer eyepiece was used for dissecting, measurement and egg counting.

*Phasecontrast microscope:* An Olympus BX51 (Japan) phasecontrast microscope with a micrometer eyepiece was used for sperm counting and measurement.
Electronic scale: A METTLER TOLEDO AG135 (Switzerland) balance with a readability of 0.00001 g accuracy was used for weighing pupae and male ejaculates.

3.2.2 Procedures

Egg incubation: Eggs were collected daily and incubated in plastic Petri dishes (8.5 × 1.5 cm).

Fertility assessment: Three-d-old eggs were observed under the dissecting microscope to determine egg fertility. Eggs with black dots (larval heads) were recorded as fertile (Xu et al. 2007).

Adult weight assessment: Adult weight was significantly positively correlated with pupal weight in both sexes (Fig. 3.6, Section 3.5). Therefore, pupal weight was considered adult body weight in this thesis. Mean pupal weight (mean ± SD) was 22.8 ± 1.9 mg and 25.1 ± 2.3 mg for male and female, respectively (Section 3.5). I categorized pupal weight as average, light (< 1 SD from the mean), or heavy (> 1 SD from the mean). To reduce the random error derived from the variance of body weight, insects of average pupal weight are used for all following experiments unless stated otherwise.

3.2.3 Environmental Conditions

Standard Conditions: 25 ± 1°C and 70 ± 10% RH, with a photoperiod of 14:10 h light:dark (lights on from 10:00 to 24:00 and off from 24:00 to 10:00).

Reversed Photoperiod Conditions: The same temperature, RH and photoperiod as above but lights on from 22:00 to 12:00 and off from 12:00 to 22:00.

3.2.4 Definitions

Fecundity defined as the total number of eggs laid.

Fertility defined as the total number of fertilized eggs laid.

Fertility rate is the ratio of fertility vs fecundity.
3.2.5 Statistical Analysis and Reported Values

All analyses were made using SAS 9.1 (SAS Institute, Cary, NC, U.S.A.) (SAS 2006). Rejection level was set at \( \alpha < 0.05 \). Unless stated otherwise all reported values are means \( \pm \text{SE} \).
3.3 Growth and Reproduction of *Ephestia kuehniella* under Different Larval Densities

3.3.1 Introduction

The economical production of a large number of high quality *E. kuehniella* is a pre-requisite for the use of this species as insect food or research materials.

Previous studies show that crowding may affect biological fitness of insects (Peters & Barbosa 1977). In *E. kuehniella*, larval crowding has impacts upon mortality (Bell 1976; Cerutti et al. 1992), adult size (Ulyet & Merwe 1947; Cerutti et al. 1992) and fecundity (Ulyet & Merwe 1947; Cerutti et al. 1992). However, the comparison of biological parameters of the New Zealand strain of *E. kuehniella* reared in different densities has not been reported, making it difficult to determine the optimal rearing density for research and mass production of biological control agents. In this study, experiments were carried out to determine the overall performance of this insect in four larval densities.

3.3.2 Materials and Methods

3.3.2.1 Insects

Laboratory colonies were maintained in plastic cylinders, each filled with 100 g of a standard diet (43.5% wholemeal wheat flour, 43.5% maize meal, 3.0% yeast and 10% glycerine), in the Entomology and IPM Laboratory of Massey University (Palmerston North, New Zealand). Adults were not given food or water as they do not feed (Norris 1934). To start the colonies, three pairs of moths were introduced into each cylinder to lay eggs, which were then deposited onto the standard diet (Karalius & Buda 1995). Two crumpled paper towels (25 × 25 cm) were placed in each cylinder for pupation. Ten cylinders were used.

3.3.2.2 Rearing Densities

Eggs were collected from 20 pairs of moths in a plastic container (20×16×10 cm) lined with two porous plastic sheets (20×5 cm) as an oviposition surface. To make densities of 100, 500, and 1000 neonate larvae, I introduced 116, 578 and 1157 newly laid eggs (< 24 h old) on to 50 g of the standard diet in a plastic cylinder, respectively.
because egg-hatching rate is 86.4 ± 0.7% (Xu et al. 2007). Each cylinder was provided with two crumpled paper towels (25×25 cm) for pupation and considered to be a replicate. The cylinders were covered with two layers of nylon mesh. Ten replicates were performed for densities of 100 and 1000 larvae and eight replicates for that of 500 larvae.

For the density of 1 neonate larva (individually reared), a newly laid egg (< 24 h old) was inoculated on to 2 g standard diet in each of 580 glass vials (2 cm diameter × 7.5 cm height). A crumpled paper towel (6×6 cm) was placed in each vial for pupation, and the vial was covered with a layer of nylon mesh. These vials were divided into groups of 116 vials, giving 5 replicates.

The final larvae densities of the 4 treatments were 1 larva per 2 g food per vial; or 100, 500 or 1000 larvae per 50 g food per cylinder.

### 3.3.2.3 Survival Rate and Reproductive Output

The emerged moths were collected and sexed daily and the pupation rate (number of pupae/number of neonate larvae), emergence rate (number of emerged moths/number of pupae) and survival rate (number of emerged moths/number of neonate larvae) were recorded.

To determine the effect of larval density on fecundity and fertility, 51, 43, 43 and 47 pairs of newly emerged moths (< 12 h old) were randomly collected from densities of 1, 100, 500 and 1000 larvae, respectively. Each pair was caged for life in a plastic cylinder, internally lined with porous plastic sheets for oviposition, and covered with two layers of nylon mesh. Eggs were collected daily and incubated in Petri dishes (8.5×1.5 cm). Total eggs were counted for each pair, and those with black dots (larval heads) after 3 days (egg development period of this colony was 4-5 days) of incubation were recorded as fertile.

### 3.3.2.4 Statistics

A goodness-of-fit test was used to test the distribution of data before analysis. The fertility data were not normally distributed even after transformation and thus were analysed using the nonparametric Kruskal-Wallis test followed by Dunn’s procedure for multiple comparisons (Zar 1999). Other data were normally distributed and
analysed using ANOVA followed by a Tukey's studentized range test. The percentage data were arcsine transformed prior to analysis.

### 3.3.3 Results

With the increased larval density in *E. kuehniella* the emergence (*DF* = 3, 28; *F* = 38.72; *P* < 0.0001) and pupation (*DF* = 3, 28; *F* = 149.29; *P* < 0.0001) rates significantly decreased (Fig. 3.1). The survival rates were also significantly different between density treatments (*DF* = 3, 28; *F* = 113.26; *P* < 0.0001), with 73% of neonate larvae surviving to adults in the density of 100, compared to 91% in individually reared larvae (Fig. 3.1). When the larval density increased to 500 and 1000, only 50% and 10% larvae, respectively, became adults.

The fecundity and fertility of resultant adults of *E. kuehniella* significantly decreased with the increasing larval density (*DF* = 3, 165; *F* = 34.82; *P* < 0.0001 for fecundity and *DF* = 3; *χ²* = 63.9; *P* < 0.0001 for fertility) (Fig. 3.2). However, these parameters were not significantly different between the density of 1 and 100 larvae (*P* > 0.05) (Fig. 3.2), indicating that the insects reared under the density of 100 larvae can still produce adults of the highest fecundity and fertility. To obtain 100 adults, the calculated time consumption of individual rearing was > 2 h while that of the 100 larval density was < 10 min.

![Fig. 3.1](image_url) Mean emergence, pupation and survival rates at four larval densities in *E. kuehniella*. For each parameter, columns with different letters are significantly different (*P* < 0.05).
3.3.4 Discussion

Crowding generally reduces insect survival and reproductive fitness (Peters & Barbosa 1977). The low survival rate at high larval density may be due to food shortage (Cerutti et al. 1992; Sato et al. 2004), fecal and microbial contamination and cannibalism (Singh 1977; Stone & Sims 1992). The lower reproductive output of adult from high density may be the result of less resources they obtained during the larval stage due to food shortage (Peters & Barbosa 1977; Cerutti et al. 1992; Sato et al. 2004).

This study suggests that although the overall performance of *E. kuehniella* decreased with the increase of larval density, a rearing density of 100 neonate larvae per 50 g food per cylinder is highly recommended to produce a satisfactory quantity and quality of *E. kuehniella*, particularly when the cost of labour is taken into consideration. This rearing density was thus used for the following experiments throughout the thesis.
3.4 Emergence, Sexual Maturation and Reproductive Rhythms of *Ephesia kuehniella*

3.4.1 Introduction

Circadian rhythms influence many aspects of insect biology, fine-tuning life functions to the temperature and light cycles associated with the solar day (Giebultowicz 2000). Moreover, variations in circadian rhythmicity can reduce direct competition between species that share the same resources, and synchronise mating activities to ensure genetic isolation of sibling species (Saunders 1982).

Only a few papers have briefly described the emergence (Bremer 1926; Moëiaety 1959) and reproductive behaviour (Calvert & Corbet 1973) of this pest. In the present study, the details of emergence and subsequent adult reproductive activity patterns of *E. kuehniella* were investigated. This information will enhance our ability to better manipulate the population of *E. kuehniella* and provide vital information for further investigations on reproductive behaviour, particularly sexual selection and sperm competition in this species.

3.4.2 Materials and Methods

3.4.2.1 Adult Emergence

To determine daily adult emergence rates and circadian emergence rhythm of this insect, *E. kuehniella* larvae were reared under the density of 100 neonate larvae per 50 g food per cylinder as outlined in Section 3.3.2.2 in separate rearing rooms set with:

1. normal photoperiod – lights on from 10:00 to 24:00 and off from 24:00 to 10:00 and
2. reversed photoperiod room – lights on from 22:00 to 12:00 and off from 12:00 to 22:00. For each room, 10 cylinders of 116 eggs were set up and the number of emerged adults was recorded daily.

To detect the circadian emergence on an hourly basis, the number of emerged adults was recorded hourly during the photophase in the normal photoperiod room and the scotophase in the reversed photoperiod room from the 8th to the 11th day after the first emergence. Data were pooled and presented. The number of adults that emerged from the normal photoperiod room was recorded daily at 10:00 to determine the daily emergence pattern.
3.4.2.2 Adult Activity Patterns

Late pupae (when they turned dark) were collected from the crumpled paper towels and kept individually in glass vials (2 cm in diameter × 7.5 cm in height) until adult emergence to ensure virginity. The emerged moths were sexed and kept in the same glass vials before being used for experiments.

To observe adult activity patterns on a 24 h basis, 20 males and 20 females adults (< 12 h old, virgin) randomly selected from each of the above-mentioned two rooms were individually paired in above-mentioned cylinders and maintained in their original rearing room for their lifespan. Each plastic cylinder with a pair of moths was lined with a multipore plastic film as an oviposition substrate and was covered with the same plastic film secured with a rubber band. Behaviour observations were made during the photophase in the normal photoperiod room and scotophase in the reversed photoperiod room, and illumination during the scotophase was provided by a 30W red light tube. Activity of both sexes was observed every 10 min by quickly scanning all pairs and recording the following: courtship – the male jumping and fanning his wings over or around the female or if the male exposed his genitalia trying to engage the female’s genitalia; calling – the female protruding her abdomen between the wings with the tip everted (Dickins 1936); mating – the two insects engaged by the tip of the abdomen; oviposition – the female protruding her ovipositor to find oviposition site or to lay eggs. The total number of times each activity was recorded in a particular hour in the first four days after emergence is presented. Most E. kuehniella mating and oviposition occur during the first 4 days after emergence (Section 4.4).

3.4.2.3 Statistics

The number of adults emerged during the photophase and scotophase was compared using the paired-sample \( t \) test (Zar 1999). A Mann-Whitney two-sample two-tailed rank test (Zar 1999) was used to determine whether daily emergence differed between sexes.

3.4.3 Results

3.4.3.1 Emergence

The Mann-Whitney rank test indicates that adult females emerged significantly
earlier than males \((DF = 1; U_0 = 45.04; P < 0.001)\) (Fig. 3.3). About 60\% of females had emerged during the first 8 days of emergence while only 44\% of males emerged during this period. Adult emergence occurred throughout the 24 h cycle in both sexes and peaked 3 hours before lights off (Fig. 3.4). The number of adults that emerged during the scotophase \((25.1 \pm 1.0\%)\) was significantly lower than that during the photophase \((74.9 \pm 1.0\%)\) \((DF = 1, 5; t = 14.59; P < 0.001)\).

**Fig. 3.3** Daily emergence of female and male *E. kuehniella* adults.

**Fig. 3.4** Circadian adult emergence rhythms of *E. kuehniella* (lights on at 10:00 and off at 24:00).
3.4.3.2 Activity patterns

Circadian rhythms of adult activities during the first 4 days after emergence are shown in Fig. 3.5. Adult activity took place mostly during the scotophase, and calling, courtship and mating continued to the early hours of the photophase (Fig. 3.5a-c). Two days after emergence, both calling and courtship showed an obvious peak in the last
hour of the scotophase. All paired insects performed the first mating within the first day after emergence and peaked at midnight but remating peaked at dawn on the subsequent 3 days (Fig. 3.5c). Females started to lay eggs 1 day after the first mating, during the night, and oviposition activity peaked during the first few hours of the scotophase and declined sharply afterwards (Fig. 3.5d).

3.4.4 Discussion

These results suggest that *E. kuehniella* is a protogynous species because females emerged significantly earlier than males and all paired females and males mated on the emergence day. Protogyny may be a mechanism that has evolved to reduce inbreeding (Rhainds et al. 1999) because early emerged females are less likely to mate with their brothers. Norris (1934) found that some mature eggs were already present at the time of adult emergence in *E. kuehniella*, and Calvert & Corbet (1973) showed that the male and female reproductive systems of this insect became mature soon after emergence. Therefore, pairing *E. kuehniella* on the emergence day may result in highest fecundity and fertility.

Calling and courtship peaks were always followed by the mating peak, suggesting that female calling and male courtship are essential for successful matings in this species. Therefore, using sex pheromone for mating disruption or mass trapping in *E. kuehniella* appears to be control tactics worth investigating.

Oviposition did not occur until the second night after emergence. This may be because *E. kuehniella* females have a long ductus seminalis (the duct connecting between the bursa copulatrix and spermatheca) (Norris 1932) and sperm migration from the spermatophore to the spermatheca needs quite a few hours (Section 3.6.3).

On the circadian basis, both emergence and reproductive activities of *E. kuehniella* were highly rhythmic. It is suggested that the end of photophase (emergence peak) and the start of scotophase (oviposition peak) are optimal time to collect fresh moths and eggs, respectively, for research or natural enemy rearing.

This study has provided the foundation for the better handling of *E. kuehniella* and future studies of its reproductive behaviour, particularly sexual selection and sperm competition.
3.5 Influence of Mating on Egg Maturation, Oviposition and Female Longevity

3.5.1 Introduction

Behavioural changes after mating are widespread phenomena in insects. In many species of insects, mating stimulates egg maturation and oviposition, and reduces female life span (see review in Jin & Gong 2001; Green & Tregenza 2009).

In this section, I report how mating and male ejaculates affected egg production, egg laying and female longevity in *E. kuehniella*.

3.5.2 Materials and Methods

3.5.2.1 Insects

Insects were reared under the density of 100 neonate larvae per 50 g food per cylinder (Section 3.3.2.2).

3.5.2.2 Relationship between Pupal and Adult Weight

Mature pupae (when they turned dark) were collected from the crumpled paper towels and weighed using an electronic balance. The weighed pupae were kept individually in glass tubes until adult emergence to ensure virginity and age. To minimize weight changes over time, only those insects that emerged within 12 h after pupal weighing were used for experiments. The emerged moths were sexed and weighed as above.

3.5.2.3 Influence of Mating on Female Egg Production and Longevity

To determine whether and how mating influenced female longevity and egg production, I set up five treatments: (1) virgin females (treatment vf, n = 30), (2) virgin females mated once to virgin males (vf×vm, n = 26), (3) virgin females mated once to males that had copulated once in the same scotophase (vf×cm, n = 21), (4) virgin females injected with spermatophore extract (spm, n = 20), and (5) virgin females injected with saline (PBS) (sal, n = 20). Mating or injection was conducted in the first scotophase after emergence. Females were reared individually in the cylinders after treatments and their fecundity (no. of eggs laid) and longevity were recorded. Dead
females were dissected to count ovarian mature eggs.

My preliminary observations showed that males of this species could copulate twice within the 10 h scotophase but their second ejaculates in the same scotophase are smaller than the first ones (1/3 of the first ejaculates in weight) and are unfertile (can not fertilize any eggs). Dissection showed that in the second mating the male only transfers some secretions and a morphologically incomplete or abnormal spermatophore with no sperm. To obtain females for treatment (3), I allowed 1-d-old virgin females to copulate with 1-d-old males that had copulated once previously within the same scotophase.

Spermatophore extract preparation and injection followed the methods outlined in Karube & Kobayashi (1999) with minor modifications. Briefly, 20 spermatophores were removed from females immediately after mating and 20 µl of PBS was added and homogenized. After centrifugation (10,000 rpm for 7 min at 4°C), the supernatant was moved into a new tube (0.5 ml) and stored on ice. A virgin female was injected with 1.0 µl (equivalent to 1.0 spermatophore) supernatant for treatment (4) and with 1.0 µl PBS for treatment (5). The solution was injected into the female abdominal cavity through the intersegmental membrane (Jin & Gong 2001).

To count the number of ovarian mature eggs in females after death, I dissected them 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). Stained eggs were classified as immature and unstained eggs as mature and presumed to be available for oviposition.

Longevity of virgin males was also recorded (n = 30).

3.5.2.4 Influence of the Presence of Sperm in Spermathecae on Oviposition

Above mating and injection experiments suggest that a fertile ejaculate (can fertilize eggs) rather than male accessory gland secretions is the main trigger of oviposition (see Fig. 3.7 in Results). In the present experiment, I tested whether the presence of sperm in spermathecae covaried with egg laying (e.g. Thibout 1979) and whether apyrene and eupyrene sperm differentially influenced egg laying.
Reproductive Biology

The chemosterilant, thiotepa (C$_6$H$_{12}$N$_3$OP), has been used to sterilize male insects (Nabi & Harrison 1984a, b; Thakur & Kumar 1987; Xu & Wang 2010b). The sterilized males still produce sperm that fertilize the eggs but those fertilized by sterilized males can not hatch (Section 5.4). Thiotepa can negatively affect spermatogenesis and sperm motility at a relatively high dose (Thakur & Kumar 1987; Nejad et al. 2008). In *E. kuehniella*, about 10 percent of males treated with 2.5% thiotepa aqueous solution (by dipping their heads in the solution for 10 s) can not transfer fertile ejaculate (no eupyrene sperm reach spermathecae) to females (this study). In the present study, 130 1-d-old virgin females were mated once to 2.5% thiotepa-treated males and reared individually in the cylinders after mating. Oviposition started in the subsequent scotophase (refer as the first oviposition scotophase) after mating with about 50% of eggs laid in the first oviposition scotophase (Fig. 3.8 in Results of this study). The number of eggs laid in the first oviposition scotophase was recorded. Females were dissected after the end of the first oviposition scotophase to check the presence of sperm in the spermathecae. Thirty virgin females were used as control.

3.5.2.5 Process of Egg Maturation in Virgin and Mated Females

To determine the process of egg maturation in virgin and mated females, I set up 2 treatments: (1) virgin females without mating (treatment vf), (2) virgin females mated once to males that had copulated once in the same scotophase (vf×cm). Mating was conducted at the first scotophase after emergence as above. Females were individually reared after treatment and eggs laid were recorded until dissection to count mature eggs in ovaries. Females from treatment (1) were subdivided into four groups that were dissected at 1, 3, 7, and 11 days after treatment, respectively (mean longevity for virgin females is 11 d). Females from treatment (2) were subdivided to three groups that were dissected at 1, 3, and 7 days after treatment, respectively (mean longevity for mated females is 7 d). Thirteen females were dissected for each group.

Mature or immature eggs were determined by 1% acetocarmine staining as above. Oosorption increases permeability of eggs and thus mature eggs that are being resorpted may also be stained like immature eggs (Edwards 1954). However, mature eggs are obviously larger than immature ones (Edwards 1954).
3.5.2.6 Statistics

The relationship between pupal and adult weight was analysed using a linear regression. Data on weight, number of eggs laid, total mature eggs (no. of eggs laid + ovarian mature eggs) and longevity were analysed using an analysis of variance (ANOVA) followed by Tukey's studentized range test. Data on oviposition in response to sperm presence in spermathecae were not normally distributed even after transformation and thus were analyzed using the nonparametric Kruskal-Wallis test followed by Dunn’s procedure for multiple comparisons (Zar 1999).

3.5.3 Results

3.5.3.1 Relationship between Pupal and Adult Weight

Female pupae are significantly heavier than male ones ($DF = 1, 123; F = 41.1; P < 0.0001$). Mean pupal weight (mean ± SD) was 22.8 ± 1.9 mg and 25.1 ± 2.3 mg for male and female, respectively. Female adults were also significantly heavier than males ($DF = 1, 123; F = 197.2; P < 0.0001$). Mean adult weight (mean ± SD) was 15.3 ± 2.3 mg and 21.6 ± 2.7 mg for male and female, respectively.

Adult weight was significantly positively correlated with pupal weight in both sexes ($DF = 1, 60; F = 192.0; P < 0.0001$ for males and $DF = 1, 60; F = 634.0; P < 0.0001$ for females) (Fig. 3.6).

![Graphs showing relationship between pupal and adult weight](image)

**Fig. 3.6** Relationship between pupal and adult weight in males (a) and females (b) of *E. kuehniella*. 

_reproduced from the original text_
3.5.3.2 Influence of Mating on Female Egg Production and Longevity

The longevity of virgin males and females was $11.6 \pm 0.4$ and $11.2 \pm 0.3$ d, respectively. No significant difference in longevity was found between sexes ($DF = 1, 57; F = 0.88; P = 0.35$).

Un-injected and saline-injected virgin females live significantly longer than mated females and virgin females injected with spermatophore extract ($DF = 4, 112; F = 26.85; P < 0.0001$) (Fig. 3.7). Tukey's studentized range test did not find significant difference in longevity among females that mated with virgin and recently mated males and virgin females injected with spermatophore extract ($P > 0.05$) (Fig. 3.7).

Females that mated with virgin males laid significantly more eggs in their lifetime than virgin females, females that mated with recently mated males, and virgin females injected with saline or spermatophore extract ($DF = 4, 112; F = 68.68; P <$
0.0001) (Fig. 3.7). No significant difference was found among unmated females, females mated with recently mated males and virgin females injected with saline or spermatophore extract ($P > 0.05$) (Fig. 3.7).

Mated females and virgin females injected with spermatophore extract produced significantly more mature eggs (eggs laid + ovarian mature eggs at death) in their lifetime than virgin females and virgin females injected with saline ($DF = 4$, $112$; $F = 9.82$; $P < 0.0001$) (Fig. 3.7). No significant difference in numbers of mature eggs produced was found among mated females and virgin females injected with spermatophore extract ($P > 0.05$) (Fig. 3.7).

Virgin females laid a few eggs daily during their life span while females that mated with virgin males laid $> 90\%$ of their eggs load in the first five days after emergence (Fig. 3.8).

### 3.5.3.3 Influence of the Presence of Sperm in Spermathecae on Oviposition

In the 130 females that mated with thiotepa-treated males, dissection after the first oviposition scotophase shows that 114 had both apyrene and eupyrene sperm in their spermathecae, 12 had apyrene but no eupyrene sperm in spermathecae, and in the remaining 4 females there was no sperm in spermathecae. Females that had both types of sperm in their spermathecae laid significantly more eggs in the first oviposition scotophase than those that had no sperm or only had apyrene sperm in their spermathecae ($DF = 3$; $\chi^2 = 93.09$; $P < 0.0001$) (Fig. 3.9). No significant difference was found between females that had no sperm and those that had apyrene sperm in their spermathecae ($P > 0.05$).

### 3.5.3.4 Process of Egg Maturation and Resorption

Newly emerged virgin females (< 10 min old) carried only $2.3 \pm 0.5$ mature eggs but this number increased to $200.1 \pm 6.1$ 24 h after emergence.

Mated females had significantly more total mature eggs than virgin females ($DF = 6$, $83$; $F = 11.91$; $P < 0.0001$) (Fig. 3.10). In virgin and mated females, the total number of mature eggs peaked 3 d after emergence and remained unchanged thereafter.

No oosorption was found in all dissections.
**Fig. 3.8** Oviposition pattern of virgin females (a) and females that mated once with virgin males (b) in *E. kuehniella*.

**Fig. 3.9** Influence of the presence of sperm in spermathecae on oviposition in *E. kuehniella*. Treatments A+E+, A+E− and A−E− refer to females that had both apyrene and eupyrene sperm, only had apyrene sperm and had no sperm in spermathecae after mated with thiotepa-treated males, respectively. Treatment vf-1 refers to virgin females. Bars with different letters are significantly different (*P* < 0.05).
3.5.4 Discussion

Three kinds of stimuli could affect egg production and egg laying in female insects: (1) mechanical stimulation by males during mating (e.g. Obara et al. 1975; Sugawara 1981), (2) male accessory gland secretions (e.g. Yi & Gillott 1999), and (3) presence of the fertile eupyrene spermatozoa in the spermatheca (e.g. Thibout 1979). My study clearly indicates (Fig. 3.7 & 3.9) that presence of the fertile eupyrene sperm in the spermatheca, rather than mechanical stimulation by males during mating or male accessory gland secretion, is the main factor that elicits oviposition in *E. kuehniella*. Positive covariation between the eupyrene sperm in spermathecae and egg laying was also found in the leek-moth (Giebultowicz et al. 1991) and the gypsy moth (Giebultowicz et al. 1991). Furthermore, in the silkmoth, *Bombyx mori*, the eupyrene sperm were not effective unless they reached the vestibulum (Karube & Kobayashi 1999).

The present study suggests that male accessory gland secretions stimulate egg maturation in *E. kuehniella* and this process is independent of the presence of sperm because females injected with spermatophore extract produced significantly more eggs than controls (Fig. 3.7). A number of proteins isolated from male accessory gland secretions have been showed to stimulate egg maturation (e.g. Yi & Gillott 1999; Heifetz et al. 2001). These male accessory gland proteins (Acps) have target receptors
within the female reproductive tract and haemolymph (Ottiger et al. 2000; Yapici et al. 2008).

My study shows that virgin females lived significantly longer than mated ones. The nutrient recycling hypothesis suggests that female insects may conduct nutrient recycling through oosorption to enhance longevity, which is supported by the positive correlations between oosorption and female longevity (Ohgushi 1996; Wang & Horng 2004; Kotaki 2005). However, the present study did not find such correlations in *E. kuehniella* females. Lum (1983) also reported that other pyralid female moths, *P. interpunctella* and *Cadra cautella*, do not carry out nutrient recycling through oosorption to enhance their longevity. Therefore, mating may reduce female longevity in *E. kuehniella*.

Sexual conflict theory predicts that three mating derived effects may shorten female longevity: (1) physical injuries incurred from ‘traumatic insemination’ (e.g. Crudgington & Siva-Jothy 2000; Rönn et al. 2007), (2) toxic male ejaculates (e.g. Das et al. 1980; Chapman et al. 1995; Green & Tregenza 2009), and (3) resource relocation (Jiao et al. 2006; Wenninger & Averill 2006). Females of a wide range of invertebrates suffer from physical injuries incurred from spiky male genitalia (Crudgington & Siva-Jothy 2000; Rönn et al. 2007) or other forms of traumatic insemination (Stutt & Siva-Jothy 2001; Tatarnic et al. 2006; Kamimura 2007). In insects, however, such physical injuries are most prevalent in the Heteropteran infraorder Cimicomorpha (see review in Tatarnic et al. 2006) but not reported in moths. My dissection in this study shows that *E. kuehniella* males do not have such spiky genitalia and mated females have no obvious injuries in their genital tracts. Therefore, the longevity reduction in *E. kuehniella* females may not be the result of physical injuries incurred from mating.

The side effect of male derived Acps may reduce longevity and reproductive success of females in many insect species (see review in Jin & Gong 2001; Lung et al. 2002) and the side effect is dose-dependent, i.e., the higher the dose is, the stronger the effect may be (Chapman et al. 1995; Lung et al. 2002). Whether or not *E. kuehniella* females also suffer from such toxic male ejaculates is unknown. However, the current study shows that females that received a full ejaculate (from mating with a virgin male) had similar egg production and longevity to those that received a reduced ejaculate (1/3 of a full ejaculate in weight, from mating with a recently mated male). Moreover, females that mated more than once also had similar egg production and longevity to
those that mated only once (Section 4.4). These results suggest that the effect of male derived factors on female egg production and longevity is not dose-dependent and that male ejaculates in this species may not be toxic to females.

Fig. 3.7 indicates that mated or spermatophore extract injected females produced more eggs and lived shorter than un-injected or saline injected virgin ones in *E. kuehniella*. According to the disposable soma model (Kirkwood & Rose 1991; Kirkwood & Austad 2000), ageing occurs because resources allocated to reproduction are unavailable for investment in somatic repair, making individuals or populations that invest more in reproduction likely incur faster ageing and shorter life span. Therefore, the longevity reduction in mated and spermatophore extract injected *E. kuehniella* females is likely caused by the stimuli derived from male accessory factors that induce resources to be allocated to ova after mating or injection. Section 4.2 shows that females with delayed mating had fewer eggs produced but lived longer than normally mated females, suggesting that females use more resources for survival (to wait for mating) and allocate fewer resources for egg production. This result thus supports the disposable soma model in *E. kuehniella*.
3.6 Ejaculation, Sperm Movement and Sperm Storage

3.6.1 Introduction

After insemination sperm usually need to migrate to the sperm storage site before they can fertilize ova; the structure of sperm storage site varies dramatically across taxa, particularly in insects (Smith 1984; Birkhead 2000). In Lepidoptera, sperm packaged in a spermatophore are transferred to the bursa copulatrix during copulation; sperm then migrate from the spermatophore to the spermatheca before they can fertilize eggs (Friedlander et al. 2005). Knowledge of ejaculation, sperm migration in female reproductive duct and dynamics of sperm storage helps us understand the mechanisms of sperm use patterns (Birkhead 2000).

3.6.2 Materials and Methods

3.6.2.1 Insects

Insects used in this experiment were reared under the density of 100 neonate larvae per 50 g food per cylinder (Section 3.3.2.2). Insects were weighed and categorized as in Section 3.5.

The procedure used for counting sperm is described in detail by Koudelova & Cook (2001) and Watanabe & Hachisuka (2005).

3.6.2.2 Effect of Male Age and Bodyweight on Ejaculation

These experiments were designed to explore whether and to what extent male age and bodyweight affected ejaculate size, information of which is essential for experimental designs in the following studies of sperm allocation and competition. In the first experiment, I set up three treatments (1-d-old male × 1-d-old female, 4-d-old male × 1-d-old female, and 7-d-old male × 1-d-old female) with 15 replicates for each treatment. Virgin insects of average weight were used for this experiment. For each replicate, a male and a female were allowed to mate in a plastic cylinder. Females were dissected under a stereo microscope immediately after copulation to count sperm in the spermatophore.

In the second experiment, I performed three treatments (light male × average
female, average male × average female, and heavy male × average female) with 15 replicates for each treatment. One-day-old virgin insects were used for this experiment. Mating and sperm count were as in the first experiment.

3.6.2.3 Spermatophore Formation and Sperm Transfer during Copulation

This experiment was designed to determine the process of spermatophore formation and sperm transfer during copulation. Virgin females were allowed to mate with virgin males at the scotophase. Mated females were caged individually after mating. Females were dissected at 0, 15, 30, 45, 70, 90, and 120 min from the commencement of copulation to record the spermatophore formation and count sperm in the spermatophore with 8 females used at each time point. One-day-old virgin insects with average weight were used.

The spermatophore deposition position in the bursa copulatrix in above once mated females was examined. Because females of this species can mate more than once (Section 3.4), I also examined deposition position of two spermatophores in females that mated twice successfully with two males in one scotophase.

3.6.2.4 Sperm Migration and Dynamics of Sperm Storage

To record the migration of sperm from the spermatophore (in the bursa copulatrix, Fig. 3.11b) to the spermatheca (Fig. 3.11d) and the dynamic of sperm storage in the spermatheca, I allowed virgin and average weight females to mate once with virgin and average weight males at the beginning of the scotophase (referred as the 1st scotophase and the following photophase as the 1st photophase) and caged mated females individually after mating. Females were then dissected at 0, 1, 2, 3, 4, 5, 8, 11, 14 and 21 h, and 2, 3, 4 and 5 d after mating to count sperm in the spermatophore and the spermatheca with 8 females used at each time point.

My preliminary experiments show that oviposition was delayed for 2 or 3 days if mated females were transferred to 24:0 h (light:dark) cycle in *E. kuehniella*. To test whether the reduction of the number of sperm in the spermatheca was associated with oviposition, I reared once-mated females in the 24:0 h (light:dark) for sperm counts at 2, 3, 4 and 5 d after mating as above (n = 8 for each day).
3.6.2.5 Statistics

Data on the effect of male age and bodyweight on sperm number were analyzed using an analysis of variance (ANOVA) followed by Tukey's studentized range test. Data on dynamics of sperm storage in spermathecae between females under 24:0 and 14:10 h photoperiods were analyzed using a Mixed procedure (SAS 2006).

3.6.3 Results

3.6.3.1 Effect of Male Age and Bodyweight on Ejaculate Size

The mating duration of *E. kuehniella* was 115 ± 3.3 min (from 1-d-old average male ×1-d-old average female).

Males ejaculated significantly more sperm with the increase of age at their first mating (*DF* = 2, 41; *F* = 10.95; *P* = 0.0002 for apyrene and *DF* = 2, 41; *F* = 90.16; *P* < 0.0001 for eupyrene sperm) (Fig. 3.12).

Larger males ejaculated significantly more eupyrene sperm (*DF* = 2, 41; *F* = 3.49; *P* = 0.039) than smaller ones at their first mating; however, male weight had no effect on the number of apyrene sperm ejaculated (*DF* = 2, 41; *F* = 0.27; *P* = 0.76) (Fig. 3.13).

3.6.3.2 Spermatophore Formation and Sperm Transfer during Mating

The process of spermatophore formation and sperm transfer is summarized in Table 3.1 and Figs 3.11 & 3.14.

The spermatophore weighed 0.434 ± 0.031 mg and was filled with male accessory gland secretions and apyrene and eupyrene sperm. Apyrene sperm were fully pre-dissociated while eupyrene sperm were still in bundles (Fig. 3.11e) at the time when they were transferred to females in *E. kuehniella*. After mating, it took up to 2 h for the bundles to reach a full dissociation. Eupyrene sperm were longer and thicker than apyrene sperm (Fig. 3.11f).

Dissection shows that after copulation the spermatophore opening (on the end of the spermatophore neck) was attached to the opening of the ductus seminalis (through the wall of bursa copulatrix) that leads to the spermatheca (Fig. 3.11b). After the second copulation, the spermatophore opening of the first male was pushed away.
from the ductus seminalis opening and the spermatophore opening of the second male took the position (Fig. 3.11c).

**Fig. 3.11** (a): spermatophore in formation; (b): bursa copulatrix with one spermatophore; (c): bursa copulatrix with two spermatophores; (d): spermatheca and spermathecal gland; (e): eupyrene sperm bundles and dissociated apyrene sperm; (f): apyrene (shorter) and eupyrene (longer) sperm. ae. Aedeagus; apy. Apyrene sperm. bc. Bursa copulatrix; ds. Ductus seminalis; dso. Opening of ductus seminalis; eup. Eupyrene sperm bundle. h. Horns of the ductus ejaculatorius; hs. Horns of the spermatophore; ld. Lamina dentata; nbc. Neck of bursa copulatrix; o1. Opening of spermatophore from the 1st male; o2. Opening of spermatophore from the 2nd male; pn. Penis; s1. Sac of the spermatophore from the 1st male; s2. Sac of spermatophore from the 2nd male; sc. Spermatheca; sg. Spermathecal gland; ug. Unpaired gland. Bars = 0.2 mm.
3.6.3.3 Sperm Migration and Storage

Sperm in the spermatophore declined quickly after copulation, and about 90% sperm moved out of the spermatophore 4 h after copulation (Fig. 3.15).

Both types of sperm reached spermathecae simultaneously 4 h after copulation (Fig. 3.16). The number of apyrene sperm in the spermatheca increased fast and peaked 8 h after copulation (Fig. 3.16a) while eupyrene sperm peaked 11 h after copulation (Fig. 3.16b). Under the photoperiod of 24:0 h (light:dark), the number of both apyrene and eupyrene sperm declined significantly more slowly than under 14:10 h (light:dark) from 21 h to 5 d after copulation ($DF = 4, 50; F = 12.09; P = 0.0012$ for apyrene and $DF = 4, 50; F = 5.74; P = 0.021$ for eupyrene sperm) (Fig. 3.16).

A small number of sperm were found in the spermathecal accessory gland (apyrene plus eupyrene sperm < 100 on average) (Fig. 3.17a). Similar pattern is shown under 24:0 h (light:dark) (Fig. 3.17b).

![Graph](image)

**Fig. 3.12** Apyrene (a) and eupyrene (b) sperm ejaculated by males of different age. For each parameter, bars with different letters are significantly different ($P < 0.05$).
Fig. 3.13 The number of sperm ejaculated in the sermatophore in relation to male bodyweight in his first mating. For each parameter, bars with different letters are significantly different ($P < 0.05$).

Table 3.1 Process of spermatophore formation (n = 8 at each time point)

<table>
<thead>
<tr>
<th>Time from the start of copulation (min)</th>
<th>Spermatophore formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No spermatophore</td>
</tr>
<tr>
<td>15</td>
<td>Male transferred gelatinous mass to the bursa copulatrix; no sign of spermatophore</td>
</tr>
<tr>
<td>30</td>
<td>Spermatophore started to form in male internal sac</td>
</tr>
<tr>
<td></td>
<td>Spermatophore neck was partially in male internal sac and partially in female bursa copulatrix (Fig. 3.11a); sac (1/4 as big as the complete one) was in female tract and sperm started to be transferred</td>
</tr>
<tr>
<td>45</td>
<td>Spermatophore neck was partially in male internal sac and partially in female bursa copulatrix; sac was 1/2 as big as the complete one and sperm transfer was still ongoing</td>
</tr>
<tr>
<td>70</td>
<td>Complete spermatophore was properly lodged in the bursa cupulatrix (Fig. 3.11b)</td>
</tr>
<tr>
<td>90</td>
<td>Mating pairs separated</td>
</tr>
</tbody>
</table>
Fig. 3.14 Apyrene (a) and eupyrene (b) sperm transferred during mating in *E. kuehniella*.

Fig. 3.15 Changes in the number of apyrene (a) and eupyrene (b) sperm in the spermatophore after the end of copulation.
Fig. 3.16 Changes in the number of apyrene (a) and eupyrene (b) sperm in the spermatheca after copulation.

Fig. 3.17 Changes in the number of apyrene and eupyrene sperm in the spermathecal accessory gland after copulation. (a). 14:10 (light:dark); (b). 24:0 (light:dark).
3.6.4 Discussion

The process of spermatophore formation in *E. kuehniella* is similar to that reported for other Lepidoptera (e.g. Holt & North 1970; Ferro & Akre 1975; Seth et al. 2002).

The time needed for sperm to migrate from the spermatophore to the spermatheca varies considerably among lepidopteran species (from < 0.5 h to > 10 h) and is associated with the length of the reproductive duct and dissociation rate of sperm bundles (Friedlander et al. 2005). *E. kuehniella* females have a long ductus seminalis (the duct connecting between the bursa copulatrix and spermatheca) (Norris 1932), which may be the reason why the sperm need at least 4 h after copulation to reach the spermatheca (Fig. 3.16) although at this time about 90% of sperm have moved out of the spermatophore (Fig. 3.15). This study shows that apyrene sperm were fully pre-dissociated while eupyrene sperm were still in bundles (Fig. 3.11b) at the time they were transferred to females. It took up to 2 h for the eupyrene bundles to be fully dissociated, which may explain why eupyrene sperm reach the plateau in the spermatheca 3 h after apyrene sperm. A long ductus seminalis may be evolved to promote sperm competition by favoring the ‘vigorous’ sperm that could reach the spermatheca and fertilize eggs (Keller & Reeve 1995).

In many insect species, male age and bodyweight affect the ejaculate size (e.g. Bissoondath & Wiklund 1996; Ferkau & Fischer 2006; Lehmann & Lehmann 2009). The positive relationship between male body mass and ejaculate size found in *E. kuehniella* and other species (Bissoondath & Wiklund 1996; Lehmann & Lehmann 2009) supports sexual selection models where sexual traits are predicted to link to genetic factors and be expressed in proportion to the condition of their bearer (Andersson 1994; Johnstone 1995). Empirical studies suggest that when reserves collected by larvae limit reproduction, the proportional increase of reserves with body size should be paralleled by an increase in reproductive effort, possibly resulting in increased ejaculate production by larger males (Boggs 1981; Wiklund & Kaitala 1995). The increase in ejaculate size with male age is consistent with that reported for other species (e.g. Lehmann & Lehmann 2000; Wedell & Ritchie 2004; Lehmann & Lehmann 2009). The age-related differences in ejaculate size reflect adaptive plasticity in male effort spent in a current mating and resources left for future matings (Simmons 1995). Such an adjustment is commonly seen as strategic investment in insects (Wedell

My present study demonstrates that the second male pushes the first male’s spermatophore away when producing his own and positions the opening of his spermatophore against the opening of the ductus seminalis (Fig. 3.11c). Spermatophore displacement is also found in other species (reviewed in Danielsson 1998; Simmons 2001) and some authors suggest that it may function in sperm competition (Retnakaran 1974; Drnevich et al. 2000; Takami 2007). Whether and how this mechanism contributes to sperm competition in *E. kuehniella* was explored in Section 5.5.
CHAPTER 4
FACTORS AFFECTING REPRODUCTIVE FITNESS IN
EPHESTIA KUEHNIELLA

4.1 General Introduction

Insect reproductive performance is affected by biotic and abiotic factors. Understanding the factors affecting reproductive fitness is important not only in terms of behavioural ecology of the species, but also from an applied perspective (Cloutier et al. 2000).

Most previous studies focus on the female reproductive output but neglect the male influence on the female reproductive fitness (Ellis & Steele 1982; Vahed 1998; Bergstrom et al. 2002; Tregenza & Wedell 2002). However, some other studies have demonstrated that males may influence female reproductive fitness (Linley & Hinds 1974; Gage & Cook 1994; Wedell 1996).

This chapter reports the impact of age, bodyweight and multiple mating of both sexes on the reproductive fitness of E. kuehniella.

4.2 Effect of Age at Mating on Reproductive Fitness in E. kuehniella

4.2.1 Introduction

Mating and oviposition in insects must occur within a limited period because the physiology of both sexes changes over time. Therefore, the age of insects at mating influences their reproductive performance and population growth. In the natural environment, the population density and operational sex ratio is temporally and spatially dynamic (Krupa & Sih 1993; Casula & Nichols 2003; Forsgren et al. 2004; Wang & Chen 2005). Mating delay is predicted to occur in response to environmental stress, such as lack of mates (Kotaki 2003; Wang & Horng 2004).

Previous studies show that mating delay generally shortens oviposition period and reduces fecundity and fertility. This reduction in reproduction may be the results of oviposition of unfertilized eggs by virgin females (e.g. Foster et al. 1995), the reduction
of mating success due to the lack of attractiveness in old females (e.g. Proshold 1996), oocyte degradation (Ohgushi 1996; Wang & Horng 2004), or the reception of sperm of low quality and quantity from old males (Unnithan & Paye 1991; Rogers & Marti. 1994; Vickers 1997).

It is important to understand the mating process, the factors controlling it, and its effect on reproductive potential, if the mass rearing programs of insects are needed to provide insects of high quality or quantity (Cloutier et al. 2000). Knowledge of the relationship between age and reproduction thus will allow us to better understand the population dynamics of a species and to maximize its mass rearing in the laboratory (Michereff et al. 2004). A number of empirical studies have showed that paternal age has negative effects on progeny fitness in various species (reviewed in Prokop et al. 2007). For example, Price & Hansen (1998) report that paternal age negatively affects egg-hatching rate and larval survival in Drosophila melanogaster. Whether and how paternal age affects progeny fitness in E. kuehniella is still unknown, information of which is useful in mass rearing programs.

Pheromone-based mating disruption has proven to be successful in a number of pests of commercial crops (e.g. Cardé & Minks 1995; Calkins 1998; Spohn et al. 2003; Jung et al. 2006; Leskey et al. 2009). This control technique can prevent mating in some females and delay it in others (Ellis & Steele 1982; Vickers 1997). However, whether or not the disruption tactic is successful in the control of an insect pest largely depends on our understanding of the reproductive behaviour of the pest, particularly the relationship between age and reproduction (Cardé & Minks 1995; Michereff et al. 2004; Wenninger & Averill 2006). The female sex pheromone has been identified for E. kuehniella and has been suggested for control applications based on mating disruption (Trematerra 1994; Sieminska et al. 2009).

The aim of the present section was to determine (1) whether and to what extent age at mating affected the reproduction and longevity in E. kuehniella, and (2) whether parental age at mating affected offspring’s fitness in this species.

4.2.2 Materials and Methods

4.2.2.1 Insects

Insects were obtained form the colony reared under the density of 100 neonate
larvae per 50 g food per cylinder (Section 3.3.2.2). Adults of average bodyweight were used in this study. The longevity of virgin moths of *E. kuehniella* was about 11 d in both sexes (Section 3.5). Therefore, 1-d-old insects were defined as young and 4-d-old as mid-aged and 7-d-old as old in this study.

### 4.2.2.2 Influence of Age at Mating on Female Reproductive Performance and Offspring’s Fitness

The effect of age on fecundity and fertility was studied by confining 238 individual breeding pairs of moths of nine age combinations (Table 4.1) for the duration of their lifespan in plastic cylinders. Virgin females may lay a few eggs daily before mating (Section 3.5). Daily fecundity of females before pairing was recorded by cageing females individually in cylinders until paired with males. Daily fecundity and fertility after pairing were recorded as described in Section 3.3. I also counted the number of spermatophores in their bursa copulatrix when they died. The number of spermatophores found in the bursa copulatrix of a female represents the number of copulations a female has achieved in this species (Section 4.4).

**Table 4.1** Age combinations of pairs and sample size used to assess the effect of age on reproductive fitness in *E. kuehniella*

<table>
<thead>
<tr>
<th>Age (d) combinations of pairs</th>
<th>No. of pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males Females</td>
<td></td>
</tr>
<tr>
<td>1 1</td>
<td>26</td>
</tr>
<tr>
<td>1 4</td>
<td>25</td>
</tr>
<tr>
<td>1 7</td>
<td>27</td>
</tr>
<tr>
<td>4 1</td>
<td>25</td>
</tr>
<tr>
<td>4 4</td>
<td>23</td>
</tr>
<tr>
<td>4 7</td>
<td>28</td>
</tr>
<tr>
<td>7 1</td>
<td>28</td>
</tr>
<tr>
<td>7 4</td>
<td>28</td>
</tr>
<tr>
<td>7 7</td>
<td>28</td>
</tr>
</tbody>
</table>

Results of Section 3.5 suggest that the longevity of female *E. kuehniella* may be associated with resource allocation between soma and ova. The disposable soma model (Kirkwood & Austad 2000) assumes that mating delay may make females have to use more resources for survival and thus allocate less for egg production. To test this hypothesis, I also recorded and compared the egg production (eggs laid + ovarian
mature eggs at death) between two age combinations, 1d×1d and 1d×7d (male×female) (only females that received at least one spermatophore were used). Mature eggs were counted as described in Section 3.5.

To test whether and how age at mating affected offspring’s fitness, newly hatched larvae (< 24 h old) from three parental age combinations, 1d×1d, 4d×4d and 7d×7d (male×female), were randomly selected and reared in plastic cylinders, respectively. For each cylinder or a replicate, 50 larvae were introduced onto the 50 g standard diet contained, and reared under the same conditions as described in Section 3.3. Ten cylinders were set up for each of the three parental age combinations. Mature pupae were collected and weighed as described in Section 3.5. The weighed pupae were kept individually in above-mentioned glass tubes until adult emergence to determine sex. Survival rate (no. of adult moths/no. of larvae introduced) was recorded.

4.2.2.3 Statistics

I used a central composite design (CCD), i.e. response surface (Box & Draper 1987), to determine whether mating delay affected fecundity (no. of eggs laid after pairing), fertility, fertility rate (fertility/fecundity), egg-hatching rate of fertilized eggs (hatched eggs/fertility), longevity and the number of copulations. The CCD assumes a functional relationship between independent variables (ages) and response variables (e.g., fecundity, fertility). The estimated value of the response variable is given by the polynomial equation: $y = \beta_0 + \beta_1 x_f + \beta_2 x_m + \beta_3 x_s + \beta_{11} x_f^2 + \beta_{22} x_m^2 + \beta_{12} x_f x_m$, where $\beta_0, \beta_1, \beta_2, \beta_3, \beta_{11}, \beta_{12}$ are model parameters, $x_f$ and $x_m$ are female and male age, and $x_s$ is the number of copulations (this term is used to test whether remating increased female fecundity and fertility, only those mated at least once were used), respectively. The term $x_s$ (the number of copulations) was removed from the equation when the equation was used to determine the effect of mate age (independent variable) on the number of copulations (response). Only significant terms, after running the full regression models, were kept in the final models. A log likelihood ratio test (McCullagh & Nelder 1989) was applied to determine whether age of sexes had different effect on the reproductive fitness. A generalized linear model using SAS’ PROC GENMOD Procedure (SAS 2006) with normal distribution and log link function (McCullagh & Nelder 1989) was used for above analysis.
Eggs laid before and after pairing, ovarian mature eggs in dead females and total mature eggs (eggs laid + ovarian mature eggs at death) between 1d×1d and 1d×7d (male×female) age combinations, and offspring’s survival rate and pupal weight between parental age combinations were analysed using ANOVA followed by Tukey’s studentized range test.

4.2.3 Results

4.2.3.1 Effect of Age at Mating on Reproductive Fitness

Mating delay significantly reduced the number of spermatophores produced (the number of copulations) in *E. kuehniella* (*DF* = 2, 234; *F* = 9.62794; *P* < 0.001; *R*² = 0.17062; Fig. 4.1a). The likelihood ratio test shows that male ageing had significantly more effect on the number of spermatophores produced than female ageing (*DF* = 2; *χ*² = 58.74; *P* < 0.001; Fig. 4.1b).

The number of copulations (β₃xₛ) had no significant effect on any reproductive parameters recorded and thus was not reported here.

Mating delay significantly reduced the fecundity of *E. kuehniella* (*DF* = 4, 232; *F* = 6.8048; *P* < 0.001; *R*² = 0.1274; Fig. 4.2a). According to the likelihood ratio test, male ageing had significantly more effect on fecundity than female ageing (*DF* = 2; *χ*² = 47.24; *P* < 0.001; Fig. 4.2b).

Mating delay significantly reduced the fertility of *E. kuehniella* (*DF* = 3, 233; *F* = 7.6585; *P* < 0.001; *R*² = 0.14063; Fig. 4.3a). The likelihood ratio test shows that ageing affected females significantly more severely than males in terms of fertility (*DF* = 2; *χ*² = 81.70; *P* < 0.001; Fig. 4.3b).

Only mating delay in females significantly reduced the fertility rate (*DF* = 1, 186; *F* = 20.96; *P* < 0.001; *R*² = 0.18235; Fig. 4.4) in *E. kuehniella*. Age at mating of sexes had no effect on egg-hatching rate of fertilized eggs (*DF* = 1, 186; *F* = 1.07; *P* > 0.05), with the mean hatch rate being 93.0 ± 0.6%.

4.2.3.2 Effect of Mating Delay on Female Egg Production

Females of treatment 1d×1d (male×female) laid significantly fewer eggs before pairing (*DF* = 2, 45; *F* = 7.62; *P* = 0.008) but more eggs after pairing (*DF* = 2, 45; *F* =
Factors Affecting Reproductive Fitness

26.32; P < 0.0001) than those of 1d×7d (male×female) (Fig. 4.5). Females of 1d×1d (male×female) had fewer ovarian mature eggs at death ($DF = 2, 45; F = 12.36, P = 0.009$) but more total mature eggs (eggs laid + ovarian mature eggs at death) ($DF = 2, 45; F = 7.39; P = 0.002$) than females of 1d×7d (male×female) (Fig. 4.5).

$$y = \exp(0.8902 + (-0.0618)x_f + (-0.1258)x_m)$$

![Graph showing predicted number of spermatophores](image)

**Fig. 4.1** Effect of mating delay on the number of copulations females achieved in *E. kuehniella*: (a) Mean number of spermatophores found in females in pairs of different age combinations, and (b) predicted sex and age effect on the number of spermatophores found in females.
Fig. 4.2 Effect of mating delay on fecundity: (a) Mean number of eggs laid by females in pairs of different age combinations, and (b) predicted sex and age effect on the number of eggs laid.
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\[ y = \exp(5.9689 + (-0.1638)x_f + (-0.1083)x_m + 0.0161x_f x_m) \]

**Fig 4.3** Effect of mating delay on fertility: (a) Mean number of fertile eggs laid by females in pairs of different age combinations, and (b) predicted sex and age effect on the number of fertile eggs laid.
$y = \exp(4.6184 + (-0.0437)x_f)$

**Fig. 4.4** Effect of mating delay on fertility rate.

**Fig. 4.5** Influence of mating delay on female egg production. For each parameter, bars with different letters are significantly different ($P < 0.05$).

### 4.2.3.3 Effect of Age at Mating on Male and Female Longevity

Female longevity significantly increased with the increase of their age at mating ($DF = 1, 232; F = 68.23; P < 0.001; R^2 = 0.36637$) (Fig. 4.6) but male age at mating or female–male age interaction had no effect on female longevity ($P > 0.5$). Male longevity significantly increased with the increase of female age at mating but decreased with female–male age interaction ($DF = 2, 231; F = 4.05; P < 0.05; R^2 = 0.03344$) (Fig. 4.7). The number of copulations had no effect on longevity of both sexes ($P > 0.05$).
Factors Affecting Reproductive Fitness

\[ y = \exp(1.9713 + 0.0607x_f) \]

![Graph showing predicted female longevity based on female age.](image1)

**Fig. 4.6** Influence of mating delay on female longevity.

\[ y = \exp(2.375 + 0.0246x_f + (-0.0028)x_f x_m) \]

![Graph showing influence of mating delay on male longevity.](image2)

**Fig. 4.7** Influence of mating delay on male longevity.

### 4.2.3.4 Effect of Parental Age on Performance of Offspring

Parental age at mating had no effect on the survival rate \((DF = 2, 27; F = 0.12; P = 0.8841; \text{Fig. 4.8})\) and pupal weight \((DF = 2, 81; F = 0.35; P = 0.7037 \text{ for male and } DF = 2, 62; F = 1.41; P = 0.2527 \text{ for female; Fig. 4.9})\) of their offspring in *E. kuehniella*. 
Factors Affecting Reproductive Fitness

![Graph showing offspring survival rate](image)

**Fig. 4.8** Effect of parental age on offspring survival rate in *E. kuehniella*.

![Graph showing offspring pupae weight](image)

**Fig. 4.9** Effect of parental age on offspring pupae weight in *E. kuehniella*.

### 4.2.4 Discussion

Similar to previous reports in many species (e.g. Jimenez-Perez & Wang 2003; Michereff et al. 2004; Fitzpatrick 2006), my study indicates that mating delay in both sexes significantly reduced reproductive fitness in *E. kuehniella* (Figs 4.1-4.4).

The current study demonstrates that (1) females laid some eggs before mating (Fig. 4.5, also see Fig. 3.8 in Section 3.5), (2) some old females did not mate after pairing (Fig. 4.1), and (3) female ageing reduced fertility rate. These factors may contribute to the reduction of reproductive fitness in females with delayed mating.
Moreover, when mating is delayed, females may have to use more resource for survival and allocate less for egg production (see review in Zera & Harshman 2001), e.g. females with delayed mating produced fewer total mature eggs (eggs laid + ovarian mature eggs at death) (Fig. 4.5) (also see Section 3.5 for further discussion). The negative effect of female ageing on fertilization may be attributed to egg degradation due to reduced resource allocation (reviewed in Zera & Harshman 2001) and senescence also may diminish female ability to transport or store sperm (Proshold 1996). My study indicates that female longevity increased linearly with the increase of their age at mating (Fig. 4.6), which may also be due to resource relocation between ova and soma (see review in Zera & Harshman 2001). Delayed mating has been shown to increase adult longevity in many moth species, and unmated females especially tend to live longer than mated ones (reviewed in Wenninger & Averill 2006). These results confirm that the disposable soma model (Kirkwood & Rose 1991; Kirkwood & Austad 2000) also applies to E. kuehniella (see Section 3.5 for detail).

My study also shows that male ageing significantly decreased female fecundity and fertility, particularly when old males were paired with young females (Figs 4.2 & 4.3). However, this study also shows that male ageing had no significant effect on fertility rate. Furthermore, males of this species can mate up to nine times in their lifetime and generally can fertilize all female eggs in each mating (see section 4.5). Therefore, the reduction on female fecundity and fertility due to male ageing may be because young females are reluctant to mate with old males (Fig. 4.1) (also see Section 5.2) rather than ageing reduced sperm fertilization ability (e.g. Unnithan & Paye 1991; Rogers & Marti. 1994; Vickers 1997). As a result, ageing is more likely to reduce male attractiveness and thus reduce their potential in mate selection (also see Section 5.2 for further discussion) in E. kuehniella. This study showed male longevity significantly increased with the increase of female age at mating (Fig. 4.7), which may be also because males are reluctant to mate with old females (Fig. 4.1) (also see Section 5.2) and thus cost less in reproduction (e.g. spermatophores, courtship), probably due the reduction of sex pheromone releasing in aged females (Calvert & Corbet 1973).

Central to the existing senescence theory is the assumption that the effects of ageing are confined to a single generation, i.e. there is no persistent senescent phenotypic effect transferred to offspring (Priest et al. 2002). However, numerous studies in human beings and other mammals have showed that old parents have
negative effects on offspring fitness (reviewed in Parsons 1964; Prokop et al. 2007). In insect, studies on *Drosophila* species demonstrate that age of both maternal (Hercus & Hoffmann 2000; Kern et al. 2001) and paternal (Price & Hansen 1998) is negatively correlated with hatch rate of eggs and larval-to-adult viability, suggesting that parental effects could play a fundamental part in the evolution of ageing. However, in some species other than *Drosophila*, studies have demonstrated that there is no effect of maternal age on offspring fitness (McIntyre & Gooding 1998; Mohaghegh et al. 1998; Moore & Harris 2003). In my study, I have not detected any significant effect of parental age on offspring fitness by comparing fertility rate, egg-hatching rate of fertilized eggs, offspring survival and pupal weight. Therefore, further exploration in the context of the evolution of senescence in terms of parental age effects is needed from organisms with diverse life histories and reproductive biology.

This study showed that the best reproductive performance can be achieved when both sexes were 1-d-old compared to older insects. If mating disruption delays female mating until she is 4 d old (assuming that she mates with a male of the same age), her fertility would decrease from 276.5 ± 12.5 fertile eggs to 233.9 ± 21.9 fertile eggs, a reduction of 15.4%; and if mating delays until she is 7 d old, her fertility would decreased to 107.2 ± 17.8 fertile eggs, a 61.2% reduction. It is thus suggested that it is necessary to delay female mating for 7 d to achieve a significant control.
4.3 Effect of Body Weight on Reproductive Fitness in *E. kuehniella*

4.3.1 Introduction

Body size is a key determinant of an organism’s ecological and physiological properties (reviewed in Wickman & Karlsson 1989; Honek 1993). It is widely accepted that selection for higher fecundity is a major evolutionary force that selects for larger body size (directional selection) in most organisms (Andersson 1994; Klingenberg & Spence 1997; Blanckenhorn 2000). Nevertheless, organisms do not increase in size continuously (Roff 1981; Blanckenhorn 2000; Thompson & Fincke 2002) because selection for large body size is eventually counterbalanced by opposing selective forces, such as higher mortality rates due to longer juvenile developmental times, resulting in stabilized selection for optimal intermediate size with maximum lifetime fitness (e.g. Peckarsky et al. 2002). However, counterbalancing selection favoring smaller body size is often masked by the good condition of the larger organism and is therefore less obvious, particularly when the evidence for selection favoring larger body size is overwhelming (Blanckenhorn 2000).

The aim of this section was to determine whether and how conflicting selection pressures act on body size in *E. kuehniella* by testing two hypotheses (1) selection for higher reproductive success favors larger individuals (Honek 1993) and (2) selection for higher survival favors smaller individuals (Peckarsky et al. 2002). To test these hypotheses, I carried out a series of experiments in the laboratory to determine whether larger individuals of sexes have higher fecundity, larger parents have larger progeny, and larger progeny suffer higher mortality rates during juvenile stage.

4.3.2 Materials and Methods

4.3.2.1 Insects

Insects were reared under the density of 100 neonate larvae per 50 g food per cylinder (Section 3.3.2.2). Insects were weighed and categorized as in Section 3.5.

4.3.2.2 Effect of Body Weight of Both Sexes on Female Reproductive Output and Offspring Fitness

The effect of pupal weight on female fecundity and fertility was studied by
confining 255 breeding pairs of 1-d-old moths individually for the duration of their lifespan in plastic cylinders. A complete factorial block design was used for this experiment, where each sex (factor) had three different pupal weights: light, average and heavy. Thus, this experimental design produced nine treatments (3 female weights × 3 male weights) of breeding pairs (Table 4.2). Fecundity and fertility were recorded as described in section 3.3.

Table 4.2 Number of *E. kuehniella* breeding pairs in different bodyweight combinations

<table>
<thead>
<tr>
<th>Male class</th>
<th>Female class</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light</td>
<td>Light</td>
<td>29</td>
</tr>
<tr>
<td>Light</td>
<td>Average</td>
<td>30</td>
</tr>
<tr>
<td>Light</td>
<td>Heavy</td>
<td>29</td>
</tr>
<tr>
<td>Average</td>
<td>Light</td>
<td>29</td>
</tr>
<tr>
<td>Average</td>
<td>Average</td>
<td>30</td>
</tr>
<tr>
<td>Average</td>
<td>Heavy</td>
<td>28</td>
</tr>
<tr>
<td>Heavy</td>
<td>Light</td>
<td>25</td>
</tr>
<tr>
<td>Heavy</td>
<td>Average</td>
<td>25</td>
</tr>
<tr>
<td>Heavy</td>
<td>Heavy</td>
<td>30</td>
</tr>
</tbody>
</table>

To test whether parental bodyweight affected offspring weight and survival, newly hatched larvae (< 24 h old) from three size combinations, light×light, average×average and heavy×heavy (male×female), were randomly selected and reared in plastic cylinders, respectively. For each cylinder or a replicate, 50 larvae were introduced onto the 50 g standard diet are contained and reared under the same conditions as described in Section 3.3. Ten cylinders were set up for each of the three weight combinations. Mature pupae were collected and weighed as Section 3.5. The weighed pupae were kept individually in glass tubes until adult emergence to ensure sex. Survival rate (no. of adult moth/no. of larvae introduced) was recorded.

4.3.2.3 Statistics

Data on the effect of body weight on female fecundity and fertility were analyzed using a two-way analysis of variance (ANOVA) followed by Tukey's
studentized range test. Offspring survival rate and pupal weight were analysed using a one-way analysis of variance (ANOVA) followed by Tukey's studentized range test. Data on survival rate were arcsine transformed prior to analysis.

### 4.3.3 Results

My results show that neither male weight nor female–male weight interaction had any effect on female lifetime fecundity ($DF = 2, 252; F = 0.3; P = 0.7375$ for male weight, and $DF = 4, 250; F = 1.71; P = 0.1474$ for female-male interaction) and fertility ($DF = 2, 252; F = 0.43; P = 0.652$ for male weight, and $DF = 4, 250; F = 1.46; P = 0.2147$ for female-male interaction). However, heavy females had significantly higher fecundity and fertility than light and average females (Table 4.3).

**Table 4.3** Reproductive output of *E. kuehniella* females of different weights*

<table>
<thead>
<tr>
<th>Output</th>
<th>Female weight</th>
<th>$F$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heavy</td>
<td>Average</td>
<td>Light</td>
</tr>
<tr>
<td>Fecundity</td>
<td>333.6±13.6A</td>
<td>278.6±12.4B</td>
<td>271.6±9.6B</td>
</tr>
<tr>
<td>Fertility</td>
<td>324.6±14.0a</td>
<td>268.8±13.0b</td>
<td>256.1±10.4b</td>
</tr>
</tbody>
</table>

* numbers with different letters in rows are significantly different ($P < 0.05$).

![Fig. 4.10](image) **Fig. 4.10** Effect of parental body weight on offspring weight in *E. kuehniella*. For each parameter, bars with different letters are significantly different ($P < 0.05$)
Factors Affecting Reproductive Fitness

Fig. 4.11 Effect of parental body weight on offspring survival rate in *E. kuehniella*. For each parameter, bars with different letters are significantly different (*P* < 0.05)

Heavier parents have significantly heavier offspring than lighter ones (*DF* = 2, 143; *F* = 56.07; *P* < 0.001 for male offspring and *DF* = 2, 117; *F* = 41.43; *P* < 0.001 for female offspring; Fig. 4.10). However, offspring of heavy parents had lower survival rate than those of average and light parents (*DF* = 2, 27; *F* = 5.48; *P* = 0.01; Fig. 4.11) in *E. kuehniella*.

4.3.4 Discussion

Similar to many empirical studies in other insect species (e.g. Marks 1976; Jones et al. 1982; Tammaru et al. 1996; Jimenez-Perez & Wang 2004a), my study demonstrates that female fecundity and fertility significantly increased with her body weight. Consistent to Jimenez-Perez & Wang’s (2004a) work on *Cnephasia jactatana*, the present study shows that male pupal weight or female–male weight interaction had no effect on female reproductive output in *E. kuehniella*. These results support the notion that natural selection for higher fecundity is a major evolutionary force that selects for larger body size in females (Andersson 1994; Klingenberg & Spence 1997; Blanckenhorn 2000).

Further study on mate choice in relation to bodyweight in *E. kuehniella* shows that sexual selection also selects for larger body size in male and female *E. kuehniella* (see Section 5.2 for detail).

The exact nature and even existence of the balance between natural and sexual
selection are still controversial (Kirkpatrick 1987). As Schluter et al. (1991) have suggested, there are conflicting selection pressures operating on body size of both sexes in many organisms. The present experiments on offspring performance show that heavy individuals have lower survival rate than average and light ones in *E. kuehniella*. It is suggested that natural selection for higher survival might reduce body size in *E. kuehniella*. It is often assumed that organisms have to grow for longer time or grow faster to achieve a larger size. Longer prereproductive period increases cumulative mortality due to predation, parasitism and starvation, giving nonzero mortality rates at all times (Roff 1980; Steams & Koella 1986). Faster growth also is likely to increase mortality rate because of higher metabolic demands under resource limitation (Gotthard et al. 1994; Blanckenhorn 1998). Moreover, *E. kuehniella* is a protogynous species—females emerge earlier than males (Section 3.4). As a consequence, larger males of this species may have a mating disadvantage due to late reproduction because of possible longer juvenile development stage (Blanckenhorn 2000).

In conclusion, *E. kuehniella* provides an example of an insect species in which conflicting selection pressures operates on body size in both sexes. While selection for higher fecundity favors large size, selection for higher survival favors small size. Such counterbalancing selection may lead to the selection of optimal intermediate size with maximum lifetime fitness, or no increase in size continuously.
4.4 Female Multiple Mating in *E. kuehniella*

4.4.1 Introduction

Elucidating the factors controlling polyandry is important not only in terms of evolutionary behaviour and ecology of the species, but also from an applied perspective. For example, because the effectiveness of the sterile insect technique is negatively correlated with female multiple mating rate (female remating increases her chance of mating with a wild male), knowledge of polyandry may help us determine the overflooding rate (the ratio of sterile males to wild females) (Kraaijeveld et al. 2005).

Permanent pairs of *E. kuehniella* can mate more than once (Section 3.4). In this section, I tested whether (1) females mated multiply with different males, (2) females mated multiply to replenish sperm supply for higher fertility, (3) females mated multiply to obtain nutritional resources for higher fecundity and/or greater longevity, and (4) females mated multiply to gain genetic benefits.

4.4.2 Materials and Methods

4.4.2.1 Insects

Insects were reared under the density of 100 neonate larvae per 50 g food per cylinder (Section 3.3.2.2). Average weight insects were used in this study.

4.4.2.2 Influence of Recopulation on Female Lifetime Reproductive Output and Daily Oviposition Patterns

To determine whether recopulation with the same or different males affected lifetime female reproductive output and daily oviposition patterns, I set up six treatments: (1) a female copulated once with a virgin male (M-1), (2) a female copulated twice with the same male (SM-2), (3) a female copulated twice with different males, first with a virgin male and then with another once-copulated male (DM-2), (4) a female copulated twice with different virgin males (DMV-2), (5) a female was provided with a virgin male in each scotophase until death (DMV-P), and (6) a female was permanently paired with the same male until death (SM-P). The once-copulated females and males were obtained by allowing virgin moths to copulate in the plastic cylinders (one pair per cylinder) in the first scotophase following emergence. The
second copulation was allowed in the second scotophase by pairing the females and males from the beginning to the end of the scotophase. I recorded the number of copulations that females achieved in treatments (5) and (6).

Following copulation, I removed the male and allowed the female to oviposit in the cylinder. I then recorded fecundity, fertility and the number of hatched eggs. I also recorded longevity and oviposition period of the female. Dead females were dissected to determine the number of spermatophores in their bursa copulatrix.

### 4.4.2.3 Remating Preference Between Novel and Previous Mates

Results of above experiments (Section 4.4.2.2) show that oviposition patterns differed significantly between treatments during the first three oviposition scotophases, depending on whether females encountered males and whether they encountered new or previous males at the beginning of the first oviposition scotophase (Fig. 4.13). These differences could be due to sexual conflict if they are controlled by males, or to female strategy for genetic benefits if they are governed by females. To clarify these questions, I set up two experiments to test whether individuals of each sex discriminated between new and previous mates, and what the remating preference of each sex between new and previous mates: (1) mate choice by females, in which once-copulated females were allowed to choose between new and previous mates (present simultaneously) for a second mating, and (2) mate choice by males, in which once-copulated males were allowed to choose between new and previous mates (present simultaneously) for a second mating.

The once-copulated females and males were obtained by allowing virgin moths to copulate in the plastic cylinders (one pair per cylinder) in the first scotophase following emergence. Copulated pairs were separated and individually maintained in the cylinders for 14 h before their use in the following experiments at the beginning of the second scotophase: (1) in mate choice by females, a once-mated female was caged with her previous mate and a new mate (which had the same mating history and similar body weight ($\pm$ 1mg) to the previous mate) in a cylinder simultaneously, and (2) in mate choice by males, a once-mated male was caged with his previous mate and a new mate (which had the same mating history and similar body weight ($\pm$ 1mg) to the previous mate) in a cylinder simultaneously. The previous and new mates were randomly marked with different trace color powder (Magruder Color Company, USA). The mark
did not influence mate choice (Binominal test, \( P > 0.05 \)). Mating events were observed during the whole scotophase until copulation occurred.

**4.4.2.4 Influence of Female Recopulation Treatments on Offspring Fitness**

I collected eggs laid in the first 4 days after first copulation (> 90% of eggs were laid in the first four days after the first copulation, Section 3.5) from treatments M-1, SM-2, DM-2 and DMV-2 and incubated them in Petri dishes for 5 days to allow hatching to occur. I randomly selected 50 neonate larvae from each of the four treatments and reared them on 50 g of a standard diet in a plastic cylinder. For each treatment, 10 cylinders of insects were set up.

I collected and weighed mature pupae and kept the weighed pupae individually in glass tubes until adult emergence. I recorded survival rate (number of adults emerged/number of larvae introduced).

**4.4.2.5 Statistics**

Females that copulated twice in treatments SM-2, DM-2 and DMV-2 and those that copulated in treatments M-1, SM-P and DMV-P were used for analysis. Data on lifetime fecundity, fertility, number of hatched eggs, fertility rate (no. of fertilized eggs laid/no. of eggs laid), hatch rate (no. of hatched eggs/no. of eggs laid), oviposition period and female longevity were analysed using a multivariate analysis of variance (MANOVA). MANOVA is superior to analysis of variance (ANOVA) for these data because the above seven fitness measures of females (multiple dependent variables) are expected to be correlated, and MANOVA allows effects on both overall dependent variables and each dependent variable to be tested (Scheiner 2001). Prior to MANOVA, I square transformed the data for lifetime fecundity, fertility and number of hatched eggs (Zar 1999), square-root transformed the data for oviposition period and longevity, and arcsine transformed the data for fertility rate and hatch rate.

Data on offspring survival rate (no. of adults emerged/no. of neonate larvae introduced) and pupal weight were analysed using ANOVA. Prior to ANOVA, I arcsine transformed the data on offspring survival rate.

Data on daily fecundity and fertility patterns (see Results, Fig. 4.7) were not normally distributed even after transformation and thus analysed using the
nonparametric Kruskal–Wallis test followed by Dunn’s procedure for multiple comparisons (Zar 1999).

A Fisher’s exact test was used to analyse mate preference between novel and previous mates.

4.4.3 Results

4.4.3.1 Female Remating Patterns

Females that were permanently paired with the same males (treatment SM-P) copulated up to four times (mean ± SE = 1.92 ± 0.18 times) and 62.5% of them copulated at least twice (n = 24; Fig. 4.12a). Females that had access to a new virgin male daily for their life span (treatment DMV-P) copulated up to four times (mean ± SE = 1.96 ± 0.17 times) and 77.8% of them copulated at least twice (n = 27; Fig. 4.12b). Dissections reveal that the number of copulations recorded was equal to the number of spermatophores found in female bursa copulatrix in both SM-P and DMV-P treatments.

4.4.3.2 Influence of Recopulation on Female Lifetime Reproductive Output and Offspring Fitness

There was no significant difference in fecundity, fertility, number of hatched eggs, fertility rate, hatch rate, oviposition period or longevity regardless of whether females copulated once, twice with the same males or different males, or were provided with new males daily, or permanently paired with the same males (MANOVA: DF = 35, 561; F = 1.01; P = 0.46; Table 4.4). In addition, I found no significant difference in offspring survival or pupal weight for the different categories of females (Table 4.4). Therefore, neither multiple copulation nor polyandry significantly affected lifetime reproductive output and offspring performance in *E. kuehniella* females.

4.4.3.3 Influence of Recopulation on Daily Oviposition Patterns

Daily fecundity and fertility from different treatments in the first four oviposition scotophases are shown in Fig. 4.13. In the first oviposition scotophase, females that copulated twice with different males (treatments DM-2 and DMV-2) laid significantly fewer eggs than females that copulated once (M-1) or females that
copulated twice with the same males (SM-2) (fecundity: $DF = 3; \chi^2 = 14.66; P < 0.0001$; fertility: $DF = 3; \chi^2 = 12.76; P < 0.0001$). In the second oviposition scotophase, however, DM-2 and DMV-2 females laid significantly more eggs than M-1 and SM-2 females (fecundity: $DF = 3; \chi^2 = 17.50; P < 0.0001$; fertility: $DF = 3; \chi^2 = 16; P < 0.0001$).

More than 80% of eggs were laid in the first two oviposition scotophases in all treatments (Fig. 4.13). In the third oviposition scotophase, once-copulated females laid significantly fewer eggs than twice-copulated females (fecundity: $DF = 3; \chi^2 = 4.72; P = 0.0039$; fertility: $DF = 3; \chi^2 = 4.43; P = 0.0057$). In the fourth oviposition scotophase, there was no significant difference in fecundity ($DF = 3; \chi^2 = 1.88; P = 0.138$) or fertility ($DF = 3; \chi^2 = 1.41; P = 0.2449$) between treatments.

4.4.3.4 Female Remating Preference Between Novel and Previous Partners

In the experiment of mate choice by females, 29 females copulated a second time and they also significantly preferred new to previous mates for mating (Fisher’s exact test: $P < 0.0001$; Fig. 4.14). In the experiment of mate choice by males, however, males ($n = 31$) significantly preferred previous to new mates for mating (Fisher’s exact test: $P < 0.0001$; Fig. 4.14).

**Fig. 4.12** Copulation states in female *E. kuehniella* over time: (a) permanently paired (SM-P) and (b) exposed to a virgin male each day (DMV-P).
Table 4.4 Influence of recopulation on female lifetime reproductive output, longevity and offspring performance in *E. kuehniella*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± SE</th>
<th></th>
<th></th>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M-1</td>
<td>SM-2</td>
<td>DM-2</td>
<td>DMV-2</td>
<td>DMV-P</td>
<td>SM-P</td>
<td>F</td>
<td>P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fecundity (no of eggs laid)</td>
<td>318±21</td>
<td>348±15</td>
<td>349±20</td>
<td>311±16</td>
<td>314±12</td>
<td>329±13</td>
<td>$F_{5,153} = 1.28$</td>
<td>0.28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fertility (no of fertilized eggs laid)</td>
<td>307±24</td>
<td>339±16</td>
<td>327±22</td>
<td>300±19</td>
<td>300±13</td>
<td>315±14</td>
<td>$F_{5,153} = 1.07$</td>
<td>0.38</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hatched eggs</td>
<td>301±23</td>
<td>332±16</td>
<td>319±18</td>
<td>293±15</td>
<td>291±13</td>
<td>305±21</td>
<td>$F_{5,153} = 1.12$</td>
<td>0.35</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fertility rate (%)</td>
<td>92.0±2.4</td>
<td>94.6±2.2</td>
<td>91.8±2.2</td>
<td>96.2±2.7</td>
<td>95.0±2.4</td>
<td>94.5±2.5</td>
<td>$F_{5,153} = 0.93$</td>
<td>0.46</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hatch rate (%)</td>
<td>90.3±3.0</td>
<td>92.6±2.9</td>
<td>89.5±2.4</td>
<td>93.6±1.6</td>
<td>92.0±1.6</td>
<td>91.6±2.3</td>
<td>$F_{5,153} = 1.29$</td>
<td>0.27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oviposition period (d)</td>
<td>3.8±0.2</td>
<td>3.9±0.1</td>
<td>4.0±0.1</td>
<td>3.6±0.2</td>
<td>4.1±0.1</td>
<td>4.1±0.2</td>
<td>$F_{5,142} = 0.80$</td>
<td>0.55</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female longevity (d)</td>
<td>7.5±0.2</td>
<td>7.5±0.2</td>
<td>7.9±0.2</td>
<td>7.7±0.2</td>
<td>7.7±0.2</td>
<td>7.5±0.2</td>
<td>$F_{5,142} = 0.87$</td>
<td>0.50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Offspring survival rate (%)</td>
<td>88.0±2.7</td>
<td>90.8±1.6</td>
<td>87.4±2.0</td>
<td>88.6±2.2</td>
<td></td>
<td></td>
<td>$F_{3,36} = 0.47$</td>
<td>0.71</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female offspring pupal weight (mg)</td>
<td>24.3±0.5</td>
<td>24.3±0.4</td>
<td>24.7±0.4</td>
<td>24.5±0.4</td>
<td></td>
<td></td>
<td>$F_{3,117} = 1.26$</td>
<td>0.29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male offspring pupal weight (mg)</td>
<td>28.0±0.5</td>
<td>27.6±0.5</td>
<td>27.0±0.4</td>
<td>28.3±0.5</td>
<td></td>
<td></td>
<td>$F_{3,117} = 0.18$</td>
<td>0.91</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Factors Affecting Reproductive Fitness

Fig. 4.13 Daily (a) fecundity and (b) fertility patterns in relation to copulation treatments in *E. kuehniella*. Within the same oviposition scotophase, different letters above bars denote significant differences between treatments (*P* < 0.05).

![Graph showing fecundity and fertility patterns](image)

Fig. 4.14 Recopulation preference in *E. kuehniella*. In each experiment, different letters above bars denote significant difference between treatments (*P* < 0.05).

![Graph showing recopulation preference](image)

4.4.4 Discussion

Polyandry is increasingly recognized as a pervasive feature of many species, but its adaptive significance is still being debated (Zeh & Zeh 2001). The hypotheses proposed to explain the evolution of polyandry generally fall into two categories: (1) to gain material benefits and (2) to obtain genetic benefits (Zeh & Zeh 2001). In addition,
Parker (1984) proposed the convenience hypothesis to explain multiple copulations in females (i.e. females may copulate multiply simply because the costs of persistent harassment from males outweigh the costs of additional copulations).

It has been widely reported that females gain direct material benefits from multiple copulations either with the same or different mates, with repeat-mating females gaining from sperm replenishment (e.g. Hunter et al. 1993; Wang & Davis 2006) or from receiving more nutritional resources for egg production (e.g. Wilson et al. 1999; Jimenez-Perez et al. 2003). In the present study, females that copulated once and those that copulated more than once with the same or different males laid similar numbers of fertilized eggs, suggesting that *E. kuehniella* females recopulate for reasons other than sperm replenishment. Furthermore, the fact that the number of copulations did not significantly affect the fecundity or longevity of *E. kuehniella* females does not support the direct material benefit hypotheses. However, the diet that I fed the experimental insects might be of much higher quality than is typical of what moths encounter in nature. Therefore, if *E. kuehniella* females are nutritionally stressed in the wild, as are females in the mosquito *Aedes aegypti* (Klowden & Chambers 1991), then the nutritional hypothesis cannot be completely ruled out.

I have found no evidence for the convenience hypothesis (Parker 1984) in *E. kuehniella*, which is consistent with results of other experiments of the current study. First, no forced copulations have been observed in *E. kuehniella* (Section 3.4). Second, female *E. kuehniella* choose between mates for the first and subsequent copulations (Section 5.2). Finally, even when female *E. kuehniella* are provided with a virgin male each day for 6 days, they only copulate about twice on average (Fig. 4.12b).

Reports on polyandry for genetic benefits have been increasing in the recent literature (e.g. Zeh & Zeh 1996; Newcomer et al. 1999; Tregenza & Wedell 2002; Ivy & Sakaluk 2005; Garcia-Gonzalez & Simmons 2007; Hughes et al. 2008; Dunn et al. 2009). The genetic benefits from polyandry based on genetic incompatibility model requires that sperm from multiple males are present at the site of fertilization (Simmons 2005), and the genetic diversity model necessitates that the offspring are fathered by multiple males (Jennions & Petrie 2000). It is not possible for females to gain any genetic benefits from polyandry if there is a complete first-male sperm precedence (e.g. Elgar 1998). Therefore, to gain genetic benefits from polyandry, females must have developed strategies to discriminate against previous mates and control sperm use or
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egg laying to promote sperm competition or offspring diversity (Zeh et al. 1998; Archer & Elgar 1999).

My present study demonstrates that female *E. kuehniella* significantly prefer new to previous mates for subsequent copulations. Similar mate preference patterns have been reported in six other animal species (Bateman 1998; Zeh et al. 1998; Archer & Elgar 1999; Eakley & Houde 2004; Ivy et al. 2005; LaDage & Ferkin 2007). In their study of the cricket *Gryllodes sigillatus*, Ivy et al. (2005) suggested that females mark males with their own unique chemical signatures during copulation, enabling females to recognize previous mates in subsequent encounters and to avoid recopulation with them. In the guppy *Poecilia reticulate*, Eakley & Houde (2004) found that females discriminated against their previous mates using colour patterns. Copulations of *E. kuehniella* take place exclusively during the night (Xu et al. 2008), suggesting that females in this species are unlikely to use visual cues to recognize mates. In the mating system of many pyralid species, mate location, courtship and copulation are achieved through the use of female (e.g. Gries et al. 1998; Wakamura & Arakaki 2004; Kawazu et al. 2007) and male (e.g. McLaughlin 1982; Royer & McNeil 1992; Burger et al. 1993; Sasaerila et al. 2003) sex pheromones. A female sex pheromone was identified for *E. kuehniella* (Kuwahara et al. 1971). Although the male sex pheromone has not been reported for this species, a male sex pheromone was identified in *E. elutella* (Phelan et al. 1986). Therefore, it is highly likely that *E. kuehniella* females use chemical cues to recognize and discriminate against previous mates. However, the exact underlying mechanism for the olfactory mate recognition and discrimination is still unknown for this species and warrants further study.

In *E. kuehniella*, the first copulation occurs in the first scotophase after emergence, and oviposition starts in the second scotophase (Section 3.4). Here I refer to the second scotophase after emergence as the first oviposition scotophase. Results of the present study show that oviposition patterns differed significantly between treatments during the first three oviposition scotophases, particularly during the first two oviposition scotophases, depending on whether females encountered males and whether they encountered new or previous males at the beginning of the first oviposition scotophase (Fig. 4.13): (1) once-copulated females that encountered no male (treatment M-1) or a previous male (treatment SM-2) at the beginning of the first oviposition scotophase laid significantly more eggs in this scotophase than those that
encountered a new male (treatments DM-2 and DMV-2) in the same period, and (2) females in treatments DM-2 and DMV-2 laid significantly more eggs in the second oviposition scotophase than those in treatments M-1 and SM-2 in the same period. These differences could be due to sexual conflict (Arnqvist & Rowe 2005) if they are controlled by males, or to female strategy for genetic benefits if they are governed by females. In the present study, I have found no evidence that differences in oviposition pattern were attributed to sexual conflict. First, all experimental females received the same treatment in the first scotophase (i.e. they copulated once with virgin males). Second, oviposition patterns differed only after females were treated differently in the second scotophase (i.e. no males, previous males or new males). Oviposition usually occurs in the first half of the scotophase, and courtship and copulation take place in the second half of the scotophase (Section 3.4). Therefore, the differences had already taken place before these females mated the second time, minimizing the possibility that male control or sexual conflict was responsible for the differences. Third, there was no significant difference in female life span parameters between treatments (Table 4.4).

My studies show that polyandry in *E. kuehniella* was clearly controlled by the female where females were able to distinguish and choose between new and previous mates, suggesting that differences in oviposition patterns are also controlled by the female and may be attributed to the female strategy to gain genetic benefits. For maximal genetic benefits, *E. kuehniella* females that encountered new males in the first oviposition scotophase strategically saved eggs until the next scotophase to allow sperm from subsequent copulations with new mates to fertilize these eggs. Similarly, females of the hide beetle *D. maculates* delay oviposition until they have copulated several times to obtain sperm from different mates for genetic benefits (Archer & Elgar 1999). However, in the present study, once-copulated females that encountered previous males or no males in the first oviposition scotophase did not delay oviposition to wait for new mates. The difference between the two species in this regard may be attributed to their very different oviposition periods, i.e. the oviposition period for *E. kuehniella* is only about four days (scotophases) with > 80% of eggs having been laid in the first two oviposition scotophases (Fig. 4.13) while *D. maculates* females have > 40 days to lay their eggs (Archer & Elgar 1999). Therefore, it would be too risky for *E. kuehniella* females to delay oviposition if they are unlikely to encounter new mates in the near future.
The genetic incompatibility hypothesis (Zeh & Zeh 1996, 1997, 2001) assumes that females benefit from polyandry for higher offspring viability. In the present study, however, the offspring produced by *E. kuehniella* females mated to the same or different males had similar viability in terms of egg hatching success, offspring survival rate and body weight, suggesting that polyandry does not increase offspring viability in this species. Similarly, in the frog *Crinia georgiana* (Byrne & Roberts 2000) and seed bug *Nysius huttoni* (Wang & Davis 2006), females do not obtain any benefit from polyandry in terms of offspring viability.

Recent studies suggest that polyandry can benefit females by enhancing the genetic diversity of their offspring (reviewed in Jennions & Petrie 2000; Cornell & Tregenza 2007). Above discussion has demonstrated that *E. kuehniella* females discriminate against previous mates and strategically adjust their oviposition patterns depending on whether they encounter new or previous mates after the first copulation. Further study shows *E. kuehniella* females allow multiple males to fertilize their eggs (Section 5.4). These results suggest that *E. kuehniella* females gain benefit from polyandry through producing offspring fathered by different males and, thus, increase the genetic diversity of their offspring. According to Yasui (1998), offspring of a half sib family are twice as diverse as offspring from a full sib family; greater genetic diversity within a brood may raise offspring fitness by reducing full sib competition and increasing half sib cooperation. Full sib competition occurs when full sibs compete with each other more intensively than do half sibs, because different genotypes can better partition the limited resources and reduce competition (e.g. Robinson 1992; Ridley 1993). Half sib cooperation occurs when individuals that vary genotypically interact in a cooperative manner. For example, disease transfer is reduced when different genotypes have different susceptibilities to pathogens or parasites so that half sib progeny are less likely to infect one another (e.g. Tooby 1982). Using a new model, Cornell & Tregenza (2007) suggested that genetic diversity may be a means of inbreeding avoidance when offspring live together and sib copulations occur often. *Ephestia kuehniella* is a stored-product pest with limited dispersal ability (Rees 2003); each female produces over 300 eggs, which are laid locally within a brief period (> 80% eggs are laid in the first two scotophases; this study), suggesting that sib competition and copulations may be very common in this species. Therefore, offspring genetic diversity should be extremely important for *E. kuehniella*, and polyandry probably
benefits *E. kuehniella* females by increasing genetic diversity of their offspring.

My study found that male *E. kuehniella* significantly prefer previous to new mates for subsequent copulations, which coincides with the results found in neotropical pseudoscorpion, *Cordylochernes scorpioides* (Zeh et al. 1998). It is still poorly known why these males like to do so. One possible explanation may be that males can increase their reproductive success by minimizing the opportunity for postcopulatory sexual selection (Zeh et al. 1998). Empirical studies have showed males may use prolonged copulation (McLain 1989) or postcopulatory courtship (King & Fischer 2005) as post-insemination guarding tactics. In *E. kuehniella*, females like to mate multiply but usually mate no more than twice (Fig. 4.12). Remating thus should be the most effective way for mate guarding. The difference between males and females in their propensity to remate with the same mate may reflect a conflict between the sexes, with males seeking to minimize sperm competition and females actively keeping open the opportunity for sperm competition and female choice of sperm by discriminating against previous mates (Zeh et al. 1998).
4.5 Male Multiple Mating in *Ephestia kuehniella*

4.5.1 Introduction

Sperm production is costly and limited (Dewsbury 1982; Pitnick & Markow 1994; Cook & Gage 1995; Savalli & Fox 1999). Males may also invest nutrients in reproduction besides sperm (Boggs & Watt 1981; Wiklund et al. 1993). Lepidoptera usually do not feed on a protein source as adults, instead sequester most of the protein needed for egg production and basal maintenance during their larval stage (Gilbert 1972; Baker & Baker 1973). Male Lepidoptera therefore have a limited protein supply and are likely to incur substantial costs such as sperm depletion or reduced survival through mating (Wedell et al. 2002). As a consequence, male copulation experience may have a profound impact on female reproductive success if male reproductive investment declines over consecutive copulations. There are a lot of empirical studies of effects of male mating experience on female reproduction (see review of Torres-Vila & Jennions 2005) but data on male life time reproductive investment and success, and affect of male mating experience on ejaculate size are scarce.

There has been no report on how male mating history influences the reproductive fitness of *E. kuehniella*. In this section, I investigated the lifetime reproductive investment and reproductive success of male *E. kuehniella* and tested whether and how male mating experience affected ejaculate size and female reproductive fitness.

4.5.2 Materials and Methods

4.5.2.1 Insects

Insects were reared under the density of 100 neonate larvae per 50 g food per cylinder (Section 3.3.2.2). Average weight insects were used in this study.

4.5.2.2 Impact of Male Mating Experience on Ejaculate Size

This experiment was designed to test whether sperm supply declined over successive copulations by males.
Section 3.5.2 showed that some males (40%) of this species could copulate twice within the 10 h scotophase but their second ejaculates < 10 h after the first copulation were smaller (1/3 of the first ejaculate in weight) and unfertile (could not fertilize any eggs). Therefore, males of this species need at least 10 h after a copulation to form another viable spermatophore. In the present experiment, a 1-d-old virgin male was allowed to copulate with a 1-d-old virgin female, and then offered a 1-d-old virgin female every 24 h until he died. Fifteen males were tested. Mating duration was recorded. Females were dissected under a stereo microscope immediately after copulation to count the sperm in spermatophores.

To test the relationship between the number of sperm in the spermatheca and male mating history, ten males were allowed to mate once each day with virgin females as above. After mating, females were individually reared for 11 h (to allow sperm to migrate from spermatophore to spermatheca, see Section 3.6 for detail) before being dissected to count the sperm in spermathecae.

4.5.2.3 Impact of Male Mating Experience on Female Reproductive Fitness

This experiment was designed to test whether male copulation history affected female reproductive output. For each replicate, a male was allowed to mate once with a virgin female each day until he died as described above (Section 4.5.2.2). Twenty males were used. The copulated females were caged individually for their lifespan in the plastic cylinders. Female fecundity and fertility were recorded as described in Section 3.3.

4.5.2.4 Statistics

The difference between treatments in female fecundity and fertility, mating duration, sperm number in spermatophores or spermathecae were analyzed using an analysis of variance (ANOVA) followed by Tukey's studentized range test.

4.5.3 Results

Males that had access to a new virgin female daily for their lifespan mated up to 9 times successfully (produced viable offspring) with the mean number of matings being 8.31 (± 0.21).
Mating duration (Fig. 4.15) increased significantly with the increase of male mating experience ($DF = 1, 124; F = 451.94; P < 0.0001$) while the number of apyrene and eupyrene sperm ejaculated by males significantly decreased over successive copulations ($DF = 8, 110; F = 43.93; P < 0.0001$ for apyrene and $DF = 8, 111; F = 8.88; P < 0.0001$ for eupyrene sperm) (Fig. 4.16). The number of sperm that reached spermathecae also significantly decreased with the increase of male mating experience ($DF = 3, 35; F = 15.13; P < 0.0001$ for apyrene and $DF = 3, 34; F = 16.76; P < 0.0001$ for eupyrene sperm) (Fig. 4.17).

On average, a male in his lifetime ejaculated 752,160 ($\pm$ 59,472) apyrene and 69,364 ($\pm$ 9,794) eupyrene sperm ($n = 15$).

However, male copulation history had no significant effect on female fecundity ($DF = 8, 161; F = 1.14; P = 0.3382$) and fertility ($DF = 8, 161; F = 0.91; P = 0.5099$) (Fig. 4.18); females receiving an average of 153,000 apyrene and 11,000 eupyrene sperm produced the same number of offspring as those receiving an average of 29,000 apyrene and 3,400 eupyrene sperm.

On average, a male in his lifetime fertilized 2642 ($\pm$ 104) eggs ($n = 20$).

![Mating duration under different insemination status of male E. kuehniella. Bars with different letters are significantly different ($P < 0.05$).](Fig. 4.15)
Fig. 4.16 Number of apyrene (a) and eupyrene (b) sperm ejaculated by males with different mating history in *E. kuehniella*. For each parameter, bars with different letters are significantly different (*P* < 0.05).

Fig. 4.17 Number of apyrene (a) and eupyrene (b) sperm in spermathecae of females inseminated by males with different mating history in *E. kuehniella*. For each parameter, bars with different letters are significantly different (*P* < 0.05).
Factors Affecting Reproductive Fitness

Fig. 4.18 Number of eggs and number of fertile eggs laid by female *E. kuehniella* under different insemination status.

4.5.4 Discussion

A male’s reproductive success primarily depends on the number of females he can inseminate (Simmons 2005). Multiple matings are common in lepidopteran males but male remating rate varies between species. For example, *C. jactatana* males can mate 6 times (Jimenez-Perez & Wang 2004b) while *Cydia pomonella* males 18 times (Howell et al. 1978).

*E. kuehniella* adults are nocturnally active (Section 3.4). The present study shows that 40% of males can copulate twice within the same scotophase but these second matings can not fertilize any eggs. However, if the second mating occurs in the subsequent scotophase after the first mating, it can fertilize eggs normally (Fig. 4.18). Therefore, males of this species need a recovery period for at least 24 h between successive matings. Similar results have been found in *C. jactatana* (Jimenez-Perez & Wang 2004b) and *Lobesia botrana* (Torresvila et al. 1995). Furthermore, like many other studies in the Lepidoptera (Howell et al. 1978; Kaitala & Wiklund 1995; Bissoondath & Wiklund 1996), the current study demonstrates that male mating duration significantly increased with the increase of male mating experience but the ejaculate size significantly decreased over successive copulations. These facts generally support the hypothesis that sperm production is costly and limited (Dewsbury 1982; Pitnick & Markow 1994; Cook & Gage 1995; Savalli & Fox 1999) and follows a circadian rhythm (Giebultowicz & Brooks 1998).
Unlike many other moth species such as Zeiraphera canadensis (Carroll 1994) and C. jactatana (Jimenez-Perez & Wang 2004b), E. kuehniella male mating history had no significant effect on female fecundity and fertility (Fig. 4.18). From a meta-analysis Torres-Vila et al. (2004) shows that the reproductive fitness of females in species with low female re-mating rates is less negatively impacted by smaller male reproductive investments than that in highly polyandrous species. Female reproductive output is affected by sperm number, oviposition duration and male derived nutrition (Unnithan & Paye 1991; Jimenez-Perez & Wang 2004b; Snook & Hosken 2004). Ephestia kuehniella females do not obtain male nutritional investment for fecundity (Section 4.4). They lay all their eggs within a short period after mating (about 4 days, Section 4.4), reducing the chance of sperm loss during storage due to sperm ageing or female hostile condition (Snook & Hosken 2004). This may explain why male copulation history had no significant effect on female fecundity and fertility in this species. In contrast, the reduction of female reproductive output due to male mating history in C. jactatana may be because females have a long oviposition period (15 d) and male derived nutrition can enhance female fecundity (Jimenez-Perez & Wang 2004b).
CHAPTER 5
SEXUAL SELECTION OF EPHESTIA KUEHNIELLA

5.1 General Introduction

Sexual selection (Darwin 1871) is one of the most active areas in behavioural and evolutionary ecology because it directly influences the reproductive fitness of animals. Individuals select their partners because potential mates vary in quality, quantity and availability. Sexual selection may occur through intrasexual selection where males or females compete for mates or through intersexual selection where females or males choose their mates with certain characteristics (Thornhill & Alcock 1983; Jennions & Petrie 1997). Sexual selection may occur pre- or post-copulation or even during copulation (Parker 1970; Simmons 2001).

The understanding of mate preference and discovering of traits selected by males and females could provide information necessary for the implementation of behaviour-based control tactics. Sterile insect technique (SIT) has been successfully used in pest control world-wide including New Zealand (e.g. Suckling et al. 1990; Caprio & Suckling 1995; Lo et al. 2000) and has been suggested for the control of E. kuehniella (Ayvaz et al. 2007). The SIT consists of mass rearing a pest species, irradiating it to cause sterilization, and releasing millions of sterilized insects to mate with the pest in the wild either before an outbreak becomes significant or once an outbreak is reduced with other methods (Knipling 1955). One SIT issue recognized since the beginning (Knipling 1955) is whether sterilized males can successfully attract and mate with wild females. Thus, whether or not this technique is successful in the control of an insect pest largely depends on our understanding of the mating behaviour of the pest (Knipling 1955; Cardé & Minks 1995). The difficulties with SIT may be reduced in some cases if the factors that contribute to male mating success and female preference in mate choice are better known. For example, many empirical studies have showed that male mating success is correlated with his age at mating and body size (see review in Brooks & Kemp 2001) and thus it is expected that releasing sterilized males of optimal body size and at their optimal age is more likely to achieve higher control efficacy. Indeed, Shelly et al. (2007) showed that releasing mid-aged sterilized males
had significant higher control efficacy than releasing younger ones in the Mediterranean fruit fly, *Ceratitis capitata*.

This chapter reports pre- and in-copulatory mate choice in relation to age, size, mating history and sex ratio, and post-copulatory mate choice in association with sperm use pattern and sperm displacement in *E. kuehniella*.

5.2 Pre- and In-copulation Mate Choice of *E. kuehniella*

5.2.1 Introduction

One of the most obvious mechanisms through which sexual selection acts is mate choice (Andersson 1994; Andersson & Iwasa 1996). Despite the costs individuals incur from mate choice, discrimination among potential partners is advantageous because it can lead to increased offspring production, viability, or offspring mating success (Parker 1970; Simmons 2001). The focus of recent research has been to determine the traits that individuals use when discriminating among potential mates and such decision-making processes are mostly studied using simultaneous choice experiments, where a female or a male is presented with at least two different mates (reviewed in Schafer & Uhl 2005). However, it has been argued that there are at least two ways in which individuals can successively choose among mates and mate with the mate of higher quality: (1) they can assess and choose mates with direct comparison (simultaneous choice) and (2) they can compare successive mates with one another (sequential choice) (Schafer & Uhl 2005). A few empirical studies have demonstrated that females are more likely to mate with attractive males in sequential encounters without direct comparison (see review in Gibson & Langen 1996; Schafer & Uhl 2005).

Traditionally, females are believed to be choosier than males because females invest more in reproduction (Darwin 1859, 1871; Trivers 1972). However, increasing evidence shows that sperm production in males is nontrivial (Dewsbury 1982; Pitnick & Markow 1994; Cook & Gage 1995; Savalli & Fox 1999), and females can be highly variable in reproductive potential (Pizzari et al. 2003; Jimenez-Perez & Wang 2004a; Sato & Goshima 2007), making males choosy also. Similar to other studies in many other species (e.g. Pitnick & Markow 1994; Cook & Gage 1995; Savalli & Fox 1999), sperm production is costly and limited in *E. kuehniella* males (Section 4.5). To achieve the maximal life time reproductive return, not only should *E. kuehniella* males choose high quality females to mate, they also have to allocate different amounts of ejaculates
to their mates depending on mate quality and risks of sperm competition (Simmons 2001).

In *E. kuehniella*, male and female reproductive fitness is affected by age, bodyweight and mating history (Chapter 4). In this section I explicitly investigated the mate choice of both sexes and male ejaculate allocation of *E. kuehniella* in relation to body size, mating experience and age at mating, under simultaneous and/or sequential choice.

### 5.2.2 Materials and Methods

#### 5.2.2.1 Insects

Insects were reared under the density of 100 neonate larvae per 50 g food per cylinder (Section 3.3.2.2). Bodyweight was categorized as in Section 3.5. Insect age was categorized as in Section 4.2.

#### 5.2.2.2 Mate Choice in Relation to Age at Mating, Virginity and Body Size

To test whether males and females performed mate choice based on their partners’ age, I allowed a 1-day-old moth (selector) to choose from three potential partners (selectees) of different ages (1-, 4- and 7-day-old) for copulation. For each replicate, I released a selector and three selectees into a plastic cylinder and observed mating events during the entire scotophase until copulation occurred. Virgin insects of average weight were used in this experiment. I performed 36 replicates for male selectors and 47 replicates for female selectors. Selectees were marked as described in Section 4.4.2.3.

Results from Section 4.3 indicated that all males and most females accepted a second mating in the subsequent scotophase after the first mating. To test whether males and females performed mate choice based on their partners’ mating history, I released a 1-day-old virgin moth (selector) and two potential partners (one 2-day-old virgin moth and one 2-day-old once-mated moth (mated at 1-day-old)) into a plastic cylinder and observed mating events during the entire scotophase until copulation occurred. Insects of average weight were used in this experiment. I performed 26 replicates for male selectors and 27 replicates for female selectors. Selectees were marked as described in Section 4.4.2.3.
To test whether *E. kuehniella* males and females discriminated between partners based on their body weight, I caged a virgin selector (male or female of a specific weight class) in a plastic cylinder with three potential virgin mates (light-, average-, and heavy-weight) and recorded mating events during the entire scotophase until copulation occurred. All insects used in this experiment were 1-d-old virgin moths. A total of 126 males and 129 females were used as selectors (Tables 5.3 and 5.4). Selectees were marked as described in Section 4.4.2.3.

### 5.2.2.3 Sperm Allocation in Relation to Female Age, Body Size and Mating Status

To test whether males allocated different numbers of sperm to females of different age, I set up three female age treatments (1-d-old male × 1-d-old female, 1-d-old male × 4-d-old female, and 1-d-old male × 7-d-old female) with 20, 18, and 18 replicates, respectively. In each replicate, I allowed a male and a female to copulate in a plastic cylinder during the scotophase. All insects used were of average weight and virgin. Females were dissected under a stereo microscope immediately after copulation. The numbers of eupyrene and apyrene sperm in the spermatophore were counted.

To test whether males allocated different numbers of sperm to females of different body size, I undertook three female body weight treatments (average male × light female, average male × average female, and average male × heavy female) with 20 replicates for each treatment. All insects used were 1-day-old and virgin. Females were immediately dissected after copulation to count the sperm in the spermatophore.

To test whether the presence of a rival spermatophore in the bursa copulatrix affected the male ejaculate size, I set up two treatments: (1) virgin males copulated with virgin females (n = 17), and (2) virgin males copulated with once-copulated females (copulated once with other virgin males previously within the same scotophase) (n = 17). Females were immediately dissected after copulation to count the sperm in the spermatophore. For treatment (2), sperm were counted in the spermatophore from the second male.

### 5.2.2.4 Effect of Sex Ratio on Male Ejaculates

To determine whether the presence of rival males or potential mates affected male ejaculates, I set up three mating treatments: (1) one male and one female were allowed to mate in the presence of rival males (male-biased sex ratio), (2) one male and
one female were allowed to mate in the presence of potential female mates (female-biased sex ratio), and (3) one male and one female were allowed to mate in the absence of other males or females (even sex ratio). For male-biased mating, three rival males (virgin and < 3-d-old, randomly selected) were released to the cylinder once copulation had commenced in the previously released pair. Similarly, for female-biased mating, three potential female mates (virgin and < 3-d-old, randomly selected) were released to the cylinder once copulation had commenced in the previously released pair. Females were dissected immediately after copulation to count sperm in spermatophores. All insects used for mating were 1-d-old virgin moths with average body weight. Thirty replicates were used in each treatment.

5.2.2.5 Influence of Body Size of Both Sexes on Female Remating

This experiment was designed to test whether female and male bodyweight affected female remating rate. In the first scotophase, 151 virgin females (randomly selected from the colony, 1-d-old) were randomly paired with 151 virgin males (randomly selected from the colony, 1-d-old) for mating with one pair in one cylinder. Mated females were reared individually. In the second scotophase, these once-mated 151 females were paired randomly with another 151 virgin males (randomly selected from the colony, 1-d-old) from the start to the end of the scotophase until copulation occurred. Females mated with the second males were recorded. Body weight of all used insects was recorded.

5.2.2.6 Influence of Female Age at First Mating on Her Remating

To test whether female age at the first mating affected her remating rate, virgin females of different age (27 from 1-d-old, 22 from 4-d-old, 22 from 7-d-old) were allowed to mate once with 1-d-old virgin males. Mated females were reared individually. In the second scotophase, these once-mated females were paired with novel 1-d-old virgin males from the start to the end of the scotophase until copulation occurred. Insects of average bodyweight were used. Females mated or not with the second males were recorded.
5.2.2.7 Influence of Male Ejaculate Size on Female Remating

In this section, I tested whether larger ejaculates could delay or reduce female remating rate. The above experiments (Section 5.2.2.4) showed that males transferred significantly more sperm to females under male-biased ratio than under female-biased ratio (Fig. 5.4). Therefore, I allowed virgin females to mate once under male-biased ratio (n = 90) and female-biased ratio (n = 90) at the beginning of the scotophase as described in Section 5.2.2.4. All insects used were virgin with average body weight. Once the first copulation had completed, the mated females were paired with new 1-d-old virgin males (which had similar body weight to the first males, ± 1mg) for mating by one pair per cylinder. Observations were conducted from pairing to the end of scotophase under red light. The number of females that remated was recorded.

5.2.2.8 Statistics

The Marascuilo procedure of the nonparametric analysis (Daniel 1990) was used to assess the effect of age, bodyweight and virginity on mate choice, and the effect of age on female remating rate.

The difference between treatments in the number of apyrene and eupyrene sperm ejaculated by males were analysed using ANOVA followed by Tukey’s studentized range test.

The Logistic procedure was used to analyze the effect of bodyweight on female remating. The relationship between female remating probability and bodyweight of their own and their partners was given by the equation: 
\[
y/(1-y) = \exp(\beta_0 + \beta_1 x_f + \beta_2 x_{m1} + \beta_3 x_{m2} + \beta_4 x_{m2-m1}),
\]
where \(\beta_0, \beta_1, \beta_2\) and \(\beta_4\) are model parameters, \(x_f\) is female body weight, \(x_{m1}\) and \(x_{m2}\) are the first and second male’s bodyweight, \(x_{m2-m1}\) is the weight difference between the second and first males (2nd male – 1st male), and \(y\) is female remating probability (the probability of females that mate the second time), respectively. Only significant terms, after running the full regression models, were kept in the final models.

A Fisher’s exact test was used to analyse the effect of ejaculate size on female remating rate.

Data on intermating duration were analysed using ANOVA.
5.2.3 Results

5.2.3.1 Mate Choice in Relation to Age at Mating, Virginity and Body Size

When offered females of three different ages, males significantly preferred 1-day-old females to 4- or 7-day-old females for copulation but did not appear to demonstrate any preference between 4- and 7-day-old females (Table 5.1). However, female *E. kuehniella* significantly preferred 4-d-old males to 1- or 7-d-old males for mating but did not appear to have any preference between 1- and 7-day-old males (Table 5.1).

When offered virgin and mated potential partners (selectees), the male selector significantly preferred virgin to mated females while female selectors did not have any preference over selectees’ mating history (Table 5.2).

Both *E. kuehniella* males and females selected their mates based on both their own and their mates’ bodyweight and showed similar selection pattern (Tables 5.3 & 5.4). Heavy and average selectors significantly preferred heavy selectees, whereas light selectors did not have preference over selectee weight class when they selected mates.

**Table 5.1** Mate choice in relation to age in *E. kuehniella*

<table>
<thead>
<tr>
<th>Selector</th>
<th>Selectee</th>
<th>DF</th>
<th>U₀</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-d-old</td>
<td>4-d-old</td>
<td>7-d-old</td>
<td>n</td>
</tr>
<tr>
<td>Male (1 d)</td>
<td>36 A</td>
<td>13 B</td>
<td>17 B</td>
<td>66</td>
</tr>
<tr>
<td>Female (1 d)</td>
<td>11 a</td>
<td>28 b</td>
<td>8 a</td>
<td>47</td>
</tr>
</tbody>
</table>

* numbers with different letters are significantly different (*P* < 0.05).

**Table 5.2** Mate choice in relation to virginity in *E. kuehniella*

<table>
<thead>
<tr>
<th>Selector</th>
<th>Virgin</th>
<th>Mated</th>
<th>DF</th>
<th>U₀</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>24 A</td>
<td>2 B</td>
<td>1</td>
<td>18.62</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Female</td>
<td>12 a</td>
<td>15 a</td>
<td>1</td>
<td>0.33</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

* numbers with different letters are significantly different (*P* < 0.05).
Table 5.3 Effect of body weight on mate selection by *E. kuehniella* females*

<table>
<thead>
<tr>
<th>Female weight class (Selector)</th>
<th>Male weight class (Selectee)</th>
<th>( U_0 )</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heavy</td>
<td>20 a</td>
<td>9 ab</td>
<td>6 b</td>
</tr>
<tr>
<td>Average</td>
<td>35 a</td>
<td>15 b</td>
<td>4 b</td>
</tr>
<tr>
<td>Light</td>
<td>13 a</td>
<td>15 a</td>
<td>12 a</td>
</tr>
</tbody>
</table>

* numbers with different letters are significantly different (\( P < 0.05 \)).

Table 5.4 Effect of body weight on mate selection by *E. kuehniella* males*

<table>
<thead>
<tr>
<th>Male weight class (Selector)</th>
<th>Female weight class (Selectee)</th>
<th>( U_0 )</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heavy</td>
<td>22 a</td>
<td>6 b</td>
<td>8 b</td>
</tr>
<tr>
<td>Average</td>
<td>32 a</td>
<td>6 b</td>
<td>13 b</td>
</tr>
<tr>
<td>Light</td>
<td>18 a</td>
<td>10 a</td>
<td>11 a</td>
</tr>
</tbody>
</table>

* numbers with different letters are significantly different (\( P < 0.05 \)).

5.2.3.2 Sperm Allocation in Relation to Female Age, Body Size and Mating Status

Males allocated significantly more apyrene (\( DF = 2, 50; F = 9.71; P = 0.0003 \)) and eupyrene (\( DF = 2, 50; F = 13.17; P < 0.0001 \)) sperm to 1-d- and 4-d-old females than to 7-d-old ones (Fig. 5.1).

However, males only adjusted the number of apyrene sperm ejaculated depending on female weight, with significantly more apyrene sperm being allocated to heavy females (\( DF = 2, 59; F = 13.49; P < 0.0001 \)) but similar number of eupyrene sperm to females of all weight categories (\( DF = 2, 59; F = 0.91; P = 0.4095 \)) (Fig. 5.2).

There was no significant difference in copulation duration between the first and second copulations in females (\( DF = 1, 26; F = 1.91; P = 0.18 \)), with the mean copulation duration being 112 ± 3 min. Males ejaculated similar number of apyrene (\( DF = 1, 31; F = 0.0; P = 0.98 \)) and eupyrene (\( DF = 1, 31; F = 0.20; P = 0.66 \)) sperm to virgin and once-copulated females (Fig. 5.3). Furthermore, no significant difference was found in the ratio of apyrene : eupyrene sperm between treatments (\( DF = 1, 31; F = 0.0 \)).
0.77; \( P = 0.39 \)), with the ratio of apyrene : eupyrene sperm being about 14 : 1.

**Fig. 5.1** Number of apyrene (a) and eupyrene (b) sperm ejaculated by male *E. kuehniella* to 1-, 4- or 7-d-old females. For each parameter, bars with different letters are significantly different (\( P < 0.05 \)).

**Fig. 5.2** Effect of female pupal weight on the number of apyrene (a) and eupyrene (b) sperm ejaculated by male *E. kuehniella*. For each parameter, bars with different letters are significantly different (\( P < 0.05 \)).
Fig. 5.3 Number of apyrene (a) and eupyrene (b) sperm transferred by virgin males to virgin or once mated females. For each parameter, bars with different letters are significantly different ($P < 0.05$).

Fig. 5.4 Number of apyrene (a) and eupyrene (b) sperm ejaculated by male *E. kuehniella* under different sex ratios. For each parameter, bars with different letters are significantly different ($P < 0.05$).

5.2.3.3 Effect of Sex Ratio on Male Ejaculates

Males under male-biased or even sex ratio ejaculated significantly more apyrene ($DF = 2, 70; F = 5.37; P < 0.0068$) and eupyrene ($DF = 2, 70; F = 10.8; P < 0.0001$) sperm to females than under female-biased sex ratio (Fig. 5.4). No significant
difference was found between male-biased and even sex ratio ($P > 0.5$).

### 5.2.3.4 Influence of Bodyweight of Both Sexes on Female Remating

The Logistic procedure analyses indicate that female remating probability significantly increased with the increasing of the bodyweight of their own and bodyweight difference between males (2nd male – 1st male) ($n = 151$), Global likelihood ratio test for $\beta = 0$: $\chi^2 = 14.33$; $P = 0.0008$) (Fig. 5.5).

$$y/(1-y) = \exp(-4.9025 + 0.1783x_f + 0.1108x_{m2-m1})$$

**Fig. 5.5** Predicted female remating probability in response to female bodyweight and bodyweight difference between males (2nd male – 1st male) in *E. kuehniella.*

### 5.2.3.5 Influence of Female Age at Mating on Her Remating

Female remating rate significantly decreased with the increase of their age at first mating (Marascuilo procedure: $DF = 2$; $U_0 = 8.36$; $P = 0.02$) (Fig. 5.6).

### 5.2.3.6 Influence of Male Ejaculate Size on Female Remating

Of 90 females that received large ejaculates in the first mating (from mating under male-biased sex ratio), 34 mated the second time with the new mates in the same scotophase (remating rate = 34/90 = 37.8%), with the intermating duration being 115.7 ± 14.1 minutes. Of 90 females that received small ejaculates (from mating under
female-biased sex ratio), 37 mated with the new mates in the same scotophase (remating rate = 37/90 = 41.1%), with the intermating duration being 65.5 ± 9.1 minutes. Females that mated in male-biased sex ratio had significantly longer intermating duration than those that mated in female-biased sex ratio (ANOVA: $DF = 1, 68; F = 9.21; P = 0.003$). No significant difference on remating rate between treatments (Fisher’s exact test: $P = 0.38$).

![Graph showing percentage of females that mated the second time in relation to their age at first mating in E. kuehniella. Bars with different letters are significantly different ($P < 0.05$).](image)

**Fig. 5.6** Percentage of females that mated the second time in relation to their age at first mating in *E. kuehniella*. Bars with different letters are significantly different ($P < 0.05$).

### 5.2.4 Discussion

My results show that *E. kuehniella* males preferred younger females for copulation when given a choice. This strategy allows males to gain the maximum reproductive return as younger females have higher reproductive potential (Section 4.2). However, females of this species significantly preferred 4-d-old males to 1- or 7-d-old males for mating. This preference is unlikely for direct benefit (higher fecundity and fertility) or indirect (genetic) benefit (higher offspring fitness) because age of males has no significant effect on female fecundity, fertility, egg-hatching rate, and offspring’s survival and body weight (Section 4.4 & 4.5). Calvert & Corbet (1973) show that male responsiveness to female sex pheromone peaked two days after emergence and declined 5 days after emergence in *E. kuehniella*. This could be the reason why 4-d-old males have higher mating success than 1- or 7-d-old ones in this species.

Mating history in males and females has different consequences. Male’s preference for virgin to mated females for mating may be his strategy to reduce sperm competition. Unlike some other species, such as *C. jactatana* (Jimenez-Perez & Wang
2004c) where females prefer virgin to non-virgin males for mating, my study shows that *E. kuehniella* females do not discriminate their partners in terms of virgin or not. This difference may be due to the fact that male mating history has negative effect on female reproductive fitness in *C. jactatana* (Jimenez-Perez & Wang 2004b) while this does not have significant effect on female fecundity and fertility in *E. kuehniella* (Section 4.5).

My results show that both sexes of *E. kuehniella* prefer larger mates to mate. Moreover, females were significantly more likely to remate if encountering males that were larger than their previous mates. Body size is generally considered a cue of the female reproductive potential with large females tending to offer greater reproductive returns to males than small ones (Arak 1988; Bonduriansky & Brassil 2005). In *E. kuehniella*, male preference to mate with larger females is likely to gain direct benefit because female fecundity and fertility increased with her weight (Section 4.3). Large size may also be a cue of good quality in males, such as having better genes and more sperm supply over smaller ones (Phelan & Barker 1986; Bissoondath & Wiklund 1996). In *E. kuehniella* (Section 3.6) and many species of Lepidoptera, the size of ejaculate transferred during first matings is positively correlated with male body size (reviewed in Bissoondath & Wiklund 1996), suggesting that females mated to large males may gain direct benefit in terms of more sperm and nutrition. For example, Wiklund & Kaitala (1995) show that in *Pieris napi* larger males inseminated larger ejaculates to females and that females receiving more male invested nutrients had higher fecundity. However, *E. kuehniella* males do not provide nutrition (Section 4.4), and they generally transfer more sperm than necessary in one mating to fertilize all eggs, suggesting that female’s preference for large males for mating is not to gain direct benefit. Fisher’s (1958) genetic model explains why females prefer ornamented males: a female choosing a male with an attractive trait will have sons and daughters that can both carry alleles for the attractive trait. In *E. kuehniella*, a female choosing a large mate will have large offspring (Section 4.3) and thus she will gain indirect genetic benefit because her large sons and daughters possess higher fitness in pre-copulatory mate choice (Tables 5.3 & 5.4), and probably also in post-copulatory mate choice (e.g. Kempenaers et al. 1992; Keller & Reeve 1995).

My study also shows that larger and younger females were significantly more likely to remate than smaller and older ones. This phenomenon has also been reported
in other insect species (Van Dongen et al. 1999; Wedell & Cook 1999; Jimenez-Perez et al. 2003; Schafer & Uhl 2005). Heavy females, with an inherently greater egg-laying capacity, are able to produce more eggs and so they need to obtain more sperm and male derived factors from remating to achieve their maximum reproductive gains (Shapiro et al. 1994). Male preference for larger mates may also play a role in the probability of female remating (Schafer & Uhl 2005; this study). Given the cost of mating, female remating rate would be expected to evolve to the minimum necessary to achieve full egg-fertility (Chapman et al. 1995). One mating is enough to achieve full fertilization in females of *E. kuehniella* (Section 4.4 & 4.5). Older females remating less than younger ones may be a strategy to save time and energy for egg-laying. Moreover, the decreasing of female attractiveness with ageing may also contribute to the low remating rate in old females.

Although sperm competition has resulted in the evolution of sperm so small and so numerous, the nontrivial cost of sperm production and the varying intensity of sperm competition have also promoted prudent ejaculate allocation by males between matings (Dewsbury 1982; Pitnick & Markow 1994; Wedell et al. 2002). A tradeoff between opportunities to remate and sperm competition is predicted to shape optimum ejaculate allocation at a given mating (Wedell et al. 2002). Similar to previous reports in other species (Pitnick 1993; Nicholls et al. 2001), my study indicates that in *E. kuehniella* males allocated (1) significantly more sperm of both types to females under male-biased sex ratio than under female-biased sex ratio, (2) significantly more sperm of both types to 1- and 4-day-old females than to 7-day-old ones, and (3) significantly more apyrene sperm to heavy females than to average and light ones but similar number of eupyrene sperm to females of all three weight categories. These results generally support the sperm competition theory (Parker 1982, 1990) which predicts that males experiencing higher levels of sperm competition risk are selected to increase investment in sperm production. Parker (1982, 1990) suggests that the mechanisms of sperm competition may follow a 'loaded raffle', i.e. relative to their rivals males, the more sperm a male inseminates into a female, the more likely he is to fertilize her eggs. Generally, males ejaculate much more apyrene than eupyrene sperm (Silberglied et al. 1984; Cook & Gage 1995) during mating and the number of apyrene may make up as much as 99% of the sperm ejaculated to females, leaving just 1% capable of
fertilization (Baker 1996). It has been suggested that more apyrene sperm may fill the spermatheca and delay female receptivity (Cook & Wedell 1999).

On the contrary, when males face low risk of sperm competition, they should produce a smaller ejaculate and conserve sperm for future matings (Galvani & Johnstone 1998). Wedell et al.’s (2002) hypothesis and Galvani & Johnstone’s model (1998) suggest: given the diverse factors affecting optimal ejaculate characteristics, males are expected to exhibit prudence in ejaculate allocation to maximise their overall lifetime reproductive success. Sperm production is costly and a very small ejaculate (from a male that had mated 8 times previously) still can fully fertilize all eggs of a female in *E. kuehniella* (Section 4.5). It is expected that *E. kuehniella* males will inseminate more females and thus gain more offspring in their lifetime under female-biased matings because of sperm saving.

Sperm competition theory (Parker 1982) and empirical study (Cook & Gage 1995) predict that males are selected to increase ejaculate size to achieve a higher paternity in the presence of rival sperm. However, Hodgson & Hosken (2006) propose an opposite scenario whereby the second male should reduce his ejaculate size because the ejaculate by the previous male has buffered the hostile female tract. In the present study I found no evidence to support either of these hypotheses, because *E. kuehniella* males ejaculate a similar number of sperm of both types to females regardless of whether there is already a rival spermatophore in the bursa copulatrix.

As have been mentioned above, previous studies in other insect species suggest that transfer of more sperm to females may delay or reduce female remating (Gromko et al. 1984; Cook & Wedell 1999). In the present study, transferring more sperm by *E. kuehniella* males does marginally delay female remating but does not reduce female remating rate. Therefore, delay in reamating or reduction in remating rate can not perfectly explain the evolution of why *E. kuehniella* males have to allocate more sperm to females under higher risk of sperm competition. Theoretical (Parker 1990, 1998), comparative (Parker et al. 1997) and experimental (Martin et al. 1974; Gage & Morrow 2003) studies have suggested that the paternity is determined by the relative number of competing sperm in females from different males. In *E. kuehniella*, the more sperm are transferred, the more sperm will reach female spermathecae (Section 4.5) and sperm competition outcome is determined by the relative number of competing sperm in spermathecae (Section 5.5).
5.3 Development of Method for Sperm Use Pattern Measurement

5.3.1 Introduction

Sperm competition, which occurs when sperm from more than one male compete for a given set of eggs (Parker 1970), represents an important component of sexual selection and is recognized as a key factor in the evolution of so many tiny sperm (Parker 1982; Simmons 2001). When two males copulate with the same female, the measurement of the proportion of eggs fertilized by each male has been used to help elucidate sperm competition mechanisms (e.g. Boorman & Parker 1976; He et al. 1995; Harano et al. 2008).

There are three main approaches used for determining sperm precedence in insects: sterile male technique (Parker 1970), selective breeding for diagnostic morphological traits (e.g. Suzuki et al. 1996), and the use of DNA markers (e.g. Dierkes et al. 2008). The sterile male technique using radiation (e.g. He et al. 1995; Vermette & Fairbairn 2002; Harano et al. 2008) is the most common approach. However, sterilization using radiation is likely to be restricted by the equipment availability and cost. Chemosterilization may thus be a cheaper and easier alternative for sterilization of male insects in sperm competition studies.

The chemosterilant, tepa (N,N',N"'-triethylenephosphoramide; Chemical formula: C₆H₁₂N₃OP), has proved to be effective in pest control based on sterile insect technique (Fernando 1970) and in study of sperm competitiveness in insects (Snow et al. 1970) and other animals (Beil et al. 1976; Wall et al. 1985). However, thiotepa (N,N',N"'-triethylenetriphosphoramide; Chemical formula: C₆H₁₂N₃PS), an analog of tepa, has been found to be the most effective sterilant in a number of insect species without obvious adverse effect on mating and longevity (Nabi & Harrison 1984a, b; Thakur & Kumar 1987). It reacts with DNA by forming cross-links with guanine or adenine and thereby inhibits DNA synthesis and consequent cell division (van Maanen et al. 2000). Therefore, thiotepa-treated males should still produce sperm that fertilize the eggs but those eggs fertilized by sterile males can not hatch (e.g. Nabi & Harrison 1984a).

In the application of chemosterilants both dose and treatment methods need considering to ensure full sterilization and no adverse effect on male copulation and fertilization (Fernando 1970; Tan & Mordue 1977; Haynes et al. 1981; Nabi & Harrison
So far little is known about whether chemosterilants can be reliable markers for the study of sperm competition and precedence.

In the present study I dipped male *E. kuehniella* adults in aqueous solution of thiotepa of different concentration and evaluated the potential of this chemical in male sterilization and sperm precedence determination, with four objectives: (1) to determine the effective dose for full sterilization, (2) to test whether the effective dose affected male copulation ability and female fecundity, (3) to examine whether the effective dose affected sperm transfer, motility and fertilization, and (4) to evaluate whether thiotepa could be used as a reliable marker for sperm competition study.

### 5.3.2 Materials and Methods

#### 5.3.2.1 Insects

Insects were reared under the density of 100 neonate larvae per 50 g food per cylinder (Section 3.3.2.2). Insects of average body weight were used in this study.

#### 5.3.2.2 Determination of Optimal Thiotepa Dose for Complete Sterilization of Males and Effect of Treatment on Male Copulation Ability and Female Fecundity

Thiotepa is toxic to humans (van Maanen et al., 2000). All operations were made under a fume cupboard and protective glasses and rubber gloves worn when this chemical was handled. Three thiotepa (SIGMA-ALDRICH, Co., USA) doses (5.0%, 1.0% and 0.5% aqueous solution) were used to treat male *E. kuehniella* adults. Newly emerged male moths (< 12 h old) were light anaesthetized with carbon dioxide and then treated by dipping their heads in the thiotepa solution for 10 s. Treated males were kept individually in above mentioned glass tubes for 24 hours before being paired with females. Each male was paired with a virgin female in a plastic cylinder from the start to the end of the first scotophase to allow copulation. Copulation events were recorded by hourly observation as the copulation duration of this species is 2 h. Fifteen replicates were performed for each dose. Twenty-six untreated males were used as controls.

Immediately after copulation females were caged individually for their lifespan in the same plastic cylinders. Eggs were collected daily and incubated in Petri dishes (8.5 × 1.5 cm). The number of eggs laid (fecundity), the number of fertilized eggs laid (fertility) and egg hatch rate (no. of hatched eggs/no. of eggs laid) were recorded.
Unfertilized eggs are yellow and transparent and shriveled after 48 hours incubation but eggs fertilized by either treated or untreated males are white, opaque and not shriveled after 48 hours incubation. Egg-hatching occurred after 4-5 days’ incubation. All males treated with 5% thiopeta died < 24 h after treatment and were thus excluded from copulation tests.

My preliminary experiments revealed that eggs fertilized by males treated with 1.0% thiopeta in their first copulation failed to hatch. Furthermore, multiply copulated *E. kuehniella* males could fertilize full load of eggs in females (Section 4.5). To test whether the sterilization effect on males was lasting after the first copulation, I allowed the above 1.0% thiotepa-treated males to copulate for a second (n = 15) and third times (n = 15) with 1-d-old virgin females with an interval of 24 h between copulations. Fecundity, fertility and hatch rate were recorded as above.

Whether treated and untreated males had different copulation ability in the above experiments was also recorded.

### 5.3.2.3 Effect of Thiopeta Treatment on Sperm Transfer and Motility

These experiments were designed to test whether thiopeta treatment affected sperm transfer and motility. Males were treated with 1.0% thiopeta and allowed to copulate 24 h after treatment as above.

In the first experiment, 18 females were allowed to copulate with treated males and then dissected under a stereo microscope immediately after copulation. The numbers of eupyrene and apyrene sperm in the spermatophore were counted. Thirty-two females that copulated with untreated males were used as controls.

Sperm in spermatophores need to move to the spermathecae before they can fertilize eggs (Friedlander et al. 2005). In the second experiment, 15 females were allowed to copulate with treated males five hours into the scotophase. Copulated females were individually caged for 14 hours and then dissected to count the number of sperm in the spermatheca before the start of the second scotophase (oviposition will start in the 2nd dark period, Section 3.4). Fifteen females that copulated with untreated males were used as controls.
5.3.2.4 Measurement of Sperm Precedence

To determine whether thiotepa could be used as a reliable marker for sperm precedence measurement in the study of sperm competition, I allowed females to copulate twice in the same scotophase with 1.0% thiotepa-treated and untreated males in different orders, and then examined the offspring paternity through the measurement of hatch rate.

Four treatments were set up (Table 5.6): (1) females first copulated with treated males and then with untreated ones (T-U); (2) females first copulated with untreated males and then with treated ones (U-T); (3) females copulated twice, each with a different treated male (T-T), and (4) females copulated twice, each with a different untreated male (U-U). First copulation was allowed at the beginning of the scotophase and the copulated females were separated from males and individually caged for 4.5 hours before the second males were introduced. The minimum 4.5 hour interval between two copulations was chosen because 4 hours after copulation almost all sperm of the first male have moved from the spermatophore to the ductus seminalis that leads to the spermatheca (Section 3.6). The above twice-copulated females were caged individually for their lifespan to measure hatch rate as above. The success of both matings was verified by examining the dead female moths for the presence of two spermatophores (Section 4.4).

The proportion of eggs fertilized by sperm from each male was calculated using the formula developed by He et al. (1995):

\[
P_2 = \frac{1}{\sqrt{kh} + 1}
\]

\[
k = \frac{(X_{U-U} - X_{F-U})}{(X_{T-U} - X_{T-T})}
\]

\[
h = \frac{(X_{U-T} - X_{T-T})}{(X_{U-U} - X_{U-T})}
\]

where \(P_2\) = proportion of eggs fertilized by sperm of the second male, \(X_{U-U}\) and \(X_{T-U}\) = egg hatch rate of U-U and T-U treatments, respectively; \(X_{T-T}\) and \(X_{U-T}\) = egg hatch rate of T-T and U-T treatments, respectively.

5.3.2.5 Statistics

Data on fecundity, fertility, and number of sperm and ratio of sperm (apyrene/eupyrene) in the spermatophore and spermatheca were analyzed using an analysis of variance (ANOVA) followed by Tukey’s studentized range test. Data on
hatch rate were analyzed using the nonparametric Kruskal-Wallis test followed by Dunn’s procedure for multiple comparisons (Zar 1999).

5.3.3 Results

5.3.3.1 Determination of Optimal Thiotepa Dose for Complete Sterilization of Males and Effect of Treatment on Male Copulation Ability and Female Fecundity

Table 5.5 summarizes the effect of thiotepa on *E. kuehniella* male reproductive performance and fecundity, fertility and hatch rate of their mates. Almost all males copulated and inseminated their mates. Females that copulated with untreated or treated males laid similar number of eggs ($DF = 4, 80; F = 0.74; P = 0.57$) and fertile eggs ($DF = 4, 80; F = 0.84; P = 0.53$). However, eggs laid by females copulated with treated males had significantly lower hatch rate than those laid by females that copulated with untreated males ($DF = 4, 80; \chi^2 = 68.02; P < 0.0001$).

Results also show that 1.0% thiotepa completely sterilized males and the sterilization effect remained in their second and third copulations (Table 5.5).

Table 5.5 Effect of thiotepa treatment on reproduction in *E. kuehniella*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>% of males</td>
</tr>
<tr>
<td>Control</td>
<td>26</td>
<td>96</td>
</tr>
<tr>
<td>1st copulation by males treated with 0.5% thiotepa</td>
<td>15</td>
<td>100</td>
</tr>
<tr>
<td>1st copulation by males treated with 1.0% thiotepa</td>
<td>15</td>
<td>100</td>
</tr>
<tr>
<td>2nd copulation by males treated with 1.0% thiotepa</td>
<td>15</td>
<td>100</td>
</tr>
<tr>
<td>3rd copulation by males treated with 1.0% thiotepa</td>
<td>15</td>
<td>100</td>
</tr>
</tbody>
</table>

*Proportion of copulated females that laid fertilized eggs.
*For each parameter, numbers with different letters in column are significantly different ($P < 0.05$).

5.3.3.2 Effect of Thiopeta Treatment on Sperm Transfer and Motility

Males treated with 1.0% thiotepa transferred similar number of apyrene ($DF = 1,$
47; $F = 0.00; P = 0.98$) and eupyrene ($DF = 1, 47; F = 0.33; P = 0.57$) sperm to females in comparison with untreated males (Fig. 5.7). The ratio of apyrene/eupyrene sperm transferred was also similar between treated and untreated males ($16.6 \pm 1.8$ for untreated males, $17.4 \pm 1.9$ for treated males; $DF = 1, 46; F = 1.03; P = 0.32$).

Females copulated with treated or untreated males had similar number of apyrene ($DF = 1, 28; F = 0.42; P = 0.52$) and eupyrene ($DF = 1, 27; F = 0.25; P = 0.62$) sperm in their spermathecae 14 hour after copulation (Fig. 5.8). The ratio of apyrene/eupyrene sperm in the spermathecae was not significantly different between females copulated with treated and those with untreated males ($8.8 \pm 1.6$ for females copulated with untreated males, $8.5 \pm 1.2$ for females copulated with treated males; $DF = 1, 27; F = 0.02; P = 0.89$).

![Fig. 5.7 Effect of thiotepa treatment on the number of sperm in spermatophores in E. kuehniella.](image)

5.3.3.3 Sperm Precedence Estimation

Females in all four treatments had similar fecundity ($DF = 3, 67; F = 0.83; P = 0.48$) and fertility ($DF = 3, 67; F = 0.34; P = 0.80$) (Table 5.6).

Hatch rates were significantly different between treatments ($DF = 3; \chi^2 = 58.65; P < 0.0001$) (Table 5.6). It is indicated that sperm from thiotepa-treated males competed effectively with those from untreated males for fertilization. Based on He et al.’s (1995) method, the $P_2$ value equaled 0.86 for $E. kuehniella$, indicating that 86% offspring of the twice-copulated females were fathered by the second males and 14% by the first males.
Fig. 5.8 Effect of thiotepa treatment on the number of sperm in spermathecae 14 h after copulation in *E. kuehniella.*

Table 5.6 Fecundity, fertility and hatch rate of four treatments*

<table>
<thead>
<tr>
<th>Treatments</th>
<th>n</th>
<th>No. of eggs laid</th>
<th>No. of fertilized eggs laid</th>
<th>Hatch rate%</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-U</td>
<td>17</td>
<td>311±21 A</td>
<td>302±22 A</td>
<td>82.5±3.6 a</td>
</tr>
<tr>
<td>U-T</td>
<td>16</td>
<td>298±22 A</td>
<td>283±23 A</td>
<td>12.4±4.4 b</td>
</tr>
<tr>
<td>T-T</td>
<td>19</td>
<td>309±14 A</td>
<td>301±16 A</td>
<td>0.0±0.0 c</td>
</tr>
<tr>
<td>U-U</td>
<td>20</td>
<td>337±17 A</td>
<td>315±26 A</td>
<td>95.9±0.7 d</td>
</tr>
</tbody>
</table>

*For each parameter, numbers in column with different letters are significantly different (*P* < 0.05).

5.3.4 Discussion

Previous studies show that insects could be treated using chemosterilants in several ways, for example, dipping fruit fly pupae in chemosterilant solutions after which the mouthparts of the emerging adults touched the residue on the outside surface of puparial case (Fernando 1970), feeding moth adults with liquid food baited with chemosterilants (Snow et al. 1970), and injecting water-soluble chemosterilants into moth abdomen or applying acetone-soluble ones to the ventral surface of the abdomen (Tan & Mordue 1977). My preliminary attempts to treat pupae by dipping failed to achieve male sterilization and hence no details are reported here. Injection and abdominal treatment involving solvents can significantly reduce insect fitness (Tan &
Sexual Selection

Mordue 1977) including *E. kuehniella* (Section 3.5). For these reasons, I decided to dip adult moths. Snow et al.’s (1970) work suggested that the water-soluble thiotepa can be transported from mouthparts to testes. Although *E. kuehniella* adults do not feed, they have well-developed mouthparts, suggesting that they may ingest at least water. Therefore, in the present study I dipped heads of male adults in thiotepa aqueous solution to sterilize males, assuming that the chemical could be ingested from mouthparts.

Sperm competition studies using sterile male technique (Parker 1970) have generally assumed that sperm transferred by sterilized males can compete with sperm from normal males (e.g. Parker 1970; Danielsson 2001; Seth et al. 2002). Many studies have showed that males treated by optimal doses (lowest dose which induces complete sterilization) of gamma ray often retain normal mating behaviour and competitiveness in procuring mates (e.g. Parker 1970; Danielsson 2001; Seth et al. 2002; Vermette & Fairbairn 2002). However, radiation measures may destroy germinal tissue, resulting in reduced sperm production and eventual aspermia (Seo et al. 1990; Gilchrist & Partridge 2000). Chemostrilants can also cause high mortality in insects even at the dose level that does not fully sterilize males (Tan & Mordue 1977). It has been reported that thiotepa can negatively affect spermatogenesis in mice (Nejad et al. 2008) and sperm motility in fruit fly (Thakur & Kumar 1987). Therefore, it is essential to determine effective treatment doses that completely sterilize males with minimal negative effects on male copulation ability and sperm production and quality (Parker 1970).

In the present study, treatment of adult moths using 1.0% thiotepa completely sterilized *E. kuehniella* males but did not significantly affect male copulation ability and female reproductive potential, sperm transfer, motility and fertilization (Table 5.5; Figs 5.7 & 5.8). Furthermore, the reverse copulation treatments (T-U and U-T) clearly showed the last male precedence in *E. kuehniella* (Table 5.6), i.e., \( X_{T-U} \approx (100 - X_{U-T}) \times X_{U-U} \), indicating that sperm from thiotepa-treated and untreated males have very similar or the same competitive ability for fertilization (Parker 1970). It is thus strongly suggested that the male treatment using 1.0% thiotepa solution is an effective and reliable method for sperm competition studies in *E. kuehniella* and probably other species.

Results of this study support the notion that offspring of twice-copulated females appear to be mostly fathered by the last males (reviewed in Silberglied et al.
1984; Friedlander et al. 2005). In the present study all sperm of the first male had moved from the spermatophore to the ductus seminalis that leads to the spermatheca when the second copulation was allowed. It is possible that the last copulation had in some way influenced the movement of sperm from the first male to the spermatheca and even induced females to displace sperm of the first male that had reached spermatheca (e.g. Villavaso 1975). However, the exact mechanism underlying the sperm competition battle in *E. kuehniella* remains unknown and warrants further investigation.
5.4 Mechanisms of Last Male Precedence of *Ephesia kuehniella*

5.4.1 Introduction

Polyandry has evolved to allow different males to fertilise a female’s eggs, simultaneously allowing females to gain additional nutrients (reviewed in Arnqvist & Nilsson 2000; Simmons 2001) and/or genetic benefits (reviewed in Simmons 2005; Cornell & Tregenza 2007; Ivy 2007). In many polyandrous species, including lepidopteran insects, the last male to mate with a female often sires most of her offspring, a phenomenon called “last male sperm precedence” (reviewed in Silberglied et al. 1984; Simmons 2001; Friedlander et al. 2005). This phenomenon has been explained as the result of males and/or females influencing sperm use for maximum reproductive success (reviewed in Danielsson 1998; Simmons 2001; Snook & Hosken 2004; Snook 2005). On one hand, males have evolved various strategies to gain sperm precedence, such as stimulating mating when females are not sperm limited, allocating more sperm to females to compete with rival sperm, displacing sperm from previous males, and inducing females to favor their sperm (reviewed in Danielsson 1998; Simmons 2001; Gillott 2003; Hotzy & Arnqvist 2009). On the other hand, in the process of sperm reception, storage and release, females have opportunities to manipulate the fate of sperm from different males to maximize possible genetic benefits, a process known as cryptic or post-copulatory female choice (Thornhill 1983; Eberhard 1996; Dixson 2002; Fedina 2007). For example, females may dump aged sperm from storage (Villavaso 1975; Hellriegel & Bernasconi 2000; Snook & Hosken 2004) and eliminate sperm bearing somatic mutations (Jones et al. 2000; Siva-Jothy 2000) to favor younger and healthier sperm. Therefore, genetic benefits appear to be a primary force behind the evolution of polyandry and post-copulatory female choice over the cost of matings (reviewed in Simmons 2005; Cornell & Tregenza 2007; Ivy 2007).

Similar to many other species in the Lepidoptera (reviewed in Silberglied et al. 1984; Friedlander et al. 2005), the last male to mate achieves a higher fertilization rate in *E. kuehniella* (Section 5.3). As reviewed in Section 2.6.5, last male precedence may be due to (1) the second male increased his ejaculate size in the presence of a rival sperm (Cook & Gage 1995), (2) the second male displaced the spermatophore of the first male in the bursa copulatrix before most sperm of the first male emigrate from the spermatophore (Retnakaran 1974; Drnevich et al. 2000; Takami 2007), (3) resident
sperm from the first male are flushed out from the storage organ by the incoming ejaculate of the last male (Pair et al. 1977; Silberglied et al. 1984) or ejected from the storage organ by the female to accept the sperm from the last male (Villavaso 1975; Hellriegel & Bernasconi 2000), and (4) the last sperm to enter the sac would be the nearest to the exit and more likely to fertilize the eggs first and to fertilize more eggs as the spermatheca is a blind sac (Parker 1970).

Section 5.3 (Fig. 5.6) has showed the presence of rival sperm did not influence male ejaculate size, suggesting the last male precedence is not because the second male transferred more sperm to females. In this section, I test the other three potential hypotheses to elucidate the mechanisms of the last male precedence in *E. kuehniella*.

### 5.4.2 Materials and Methods

#### 5.4.2.1 Insects

Insects were reared under the density of 100 neonate larvae per 50 g food per cylinder (Section 3.3.2.2). Insects of average body weight were used in this study.

#### 5.4.2.2 Effect of Second Copulation on Sperm Storage in Spermathecae

This experiment was designed to determine how the female’s copulation with the second male influenced the storage of the first male’s sperm in the spermatheca. Section 3.6 showed that sperm did not reach the spermatheca until 4 h after copulation (Fig. 3.15); apyrene and eupyrene sperm in the spermatheca peaked 8 h and 11 h after copulation (Fig. 3.16), respectively. Therefore, in this experiment, I allowed the female to copulate the first time at the beginning of the scotophase and then kept her individually for 8 h before the second male was introduced to her. Females that copulated with the second males within 10 min after paired were used for sperm counting in spermathecae in the following three treatments: (1) females were dissected to count the sperm < 1 min after the second copulation commenced (treatment ‘8h+1min’), (2) females were dissected 3 h after the second copulation commenced (the second copulation had completed at this time, i.e. 1 h after the end of the second copulation; the duration of the second copulation is ca. 2 h, see results of this study) (treatment ‘8h+3h’), and (3) females were dissected 13 h after the second copulation commenced (treatment ‘8h+13h’). Once-copulated females that were individually maintained for 8 and 11 h after copulation were used as controls. Fifteen females were
used for each treatment and control.

5.4.2.3 P₂ and Sperm Use Patterns

I used thiotepa to treat males for sperm use pattern estimation (Section 5.3).

To test whether intermating duration had any impact on P₂, I allowed females to copulate first with thiotepa-treated males and then with untreated ones. Fifty-six females were used for this experiment. The first copulation was allowed at the beginning of the scotophase, and immediately after copulation the first males were removed and the second males introduced. The duration between the two copulations was recorded. Twice-copulated females were individually maintained for their lifespan. Eggs were collected daily and incubated in Petri dishes (8.5 × 1.5 cm). The number of eggs laid (fecundity) and the number of hatched eggs were recorded daily, which allowed me to estimate sperm use patterns over time by twice-copulated females.

Thiotepa treatment did not significantly influenced the male copulation ability, sperm transfer, motility and fertilization, and sperm from males treated by thiotepa competed equally with sperm from untreated males to fertilize female eggs in *E. kuehniella* (Section 5.3). Section 5.3 also showed that no eggs hatched (0.0%) in females mated to two thiotepa treated males (mated twice in one scotophase as conducted in the present study), whereas almost all eggs hatched (96%) in females mated to two untreated males in this species. Therefore, in the present study, I use the egg-hatching rate of a female as her P₂ (Fig. 5.11).

5.4.2.4 Statistics

Data on the number of apyrene and eupyrene sperm in the spermatheca were analyzed using an ANOVA followed by Tukey's studentized range test. Because the variance of response changes with the mean, the effect of intermating duration on P₂ was analyzed using a generalized linear model with gamma distribution and log link function (McCullagh & Nelder 1989). Hatch rate between 50% of eggs laid earlier and 50% of eggs laid later during the oviposition period was compared using a paired-sample t-test (Zar 1999).
5.4.3 Results

5.4.3.1 Effect of Second Copulation on Sperm Storage in Spermathecae

The numbers of sperm in the spermathecae were significantly different between treatments ($DF = 4, 49; F = 4.56; P = 0.003$ for apyrene and $DF = 4, 49; F = 15.43; P < 0.0001$ for eupyrene sperm) (Fig. 5.9). The number of apyrene and eupyrene sperm in spermathecae in treatment ‘8h+3h’ (3 h after commencement of, or 1 h after the completion of the second copulation) was significantly lower than that in treatment ‘8h+1m’ (1 min after the commencement of the second copulation) and in controls ‘8h’ and ‘11h’ ($P < 0.05$). These results indicate that the first male’s sperm in the spermatheca in treatment ‘8h+3h’ were not flushed out by the second male’s ejaculate, because at that time the sperm from the second male had not reached the spermatheca (Fig. 3.16). Furthermore, 11h after the completion of the second copulation (treatment ‘8h+13h’) both types of sperm in the spermatheca significantly increased with the number of eupyrene being significantly greater than that in all treatments and controls ($P < 0.05$). On the basis of the sperm migration experiment reported above and this experiment, I expect that all sperm in the spermatheca in treatment ‘8h+3h’ were from the first male after sperm reduction due to the second copulation, and those in treatment ‘8h+13h’ were a mixture from both males. In this scenario, 75% of eupyrene sperm in the spermatheca 11 h after the completion of the second copulation were from the second male.

During dissection I found that the spermatheca contracted vigorously (Fig. 5.10 a-d). A contraction cycle was initiated from the middle of the spermatheca followed by the quick shrinking in the anterior half of the spermatheca (near the ductus seminalis) (Fig. 5.10 a-c), and then the spermatheca returned to its original shape (Fig. 5.10 d). Each contraction cycle took ca. 2 s or less. It appears that the spermatheca can eject sperm by such movement.

5.4.3.2 Sperm Use Patterns in Females Mated Twice in One Scotophase

Paternity tests in twice-copulated females showed that $P_2$ significantly declined with the increasing intermating duration ($DF = 1, 54; \chi^2 = 11.13; P = 0.0008$) (Fig. 5.11). Furthermore, there was no difference in hatch rate between 50% of eggs laid earlier and 50% of eggs laid later during the oviposition period ($DF = 1, 53; t = 1.4; P = 0.17$).
Fig. 5.9 Effect of the second copulation on sperm numbers in the spermatheca. For each parameter, columns with different letters are significantly different ($P < 0.05$).

Fig. 5.10 Spermatheca in action of constriction (from (a) to (d) is a constriction cycle). sg. Spermathecal gland; ah. Anterior half of spermatheca (near ductus seminalis); ph. Posterior half of spermatheca (near spermathecal accessory gland); sc. Spermatheca. (Bars = 0.2 mm).

Fig. 5.11 Effect of intermating duration on $P_2$. 

$y = -0.0392x + 0.9693$

$R^2 = 0.2643$
5.4.4 Discussion

The spermatophore displacement hypothesis (reviewed in Danielsson 1998; Simmons 2001) suggests that the sperm competition battle between males that copulate with the same female should first take place in the bursa copulatrix, and the duration of intermating intervals in females should be correlated with the outcome of sperm competition. In *E. kuehniella*, sperm migration from the spermatophore to the spermatheca lasts > 10 h (Section 3.6). Detailed dissection showed that the second male pushes the first male’s spermatophore away when producing his own and positions the opening of his spermatophore against the opening of the ductus seminalis (Fig. 3.11). As a result, the sperm from the first male are prevented from moving to, and those from the second male are facilitated to migrate to the spermatheca (Figs 5.9 & 5.11). Although the processes are different, spermatophore displacement in the bursa copulatrix has been reported in other species (Retnakaran 1974; Drnevich et al. 2000; Takami 2007). However, if such spermatophore displacement occurs after all or most sperm from the first male have already moved to the ductus seminalis, the second male will not benefit much from this mechanism. Therefore, the outcome of spermatophore displacement in the bursa copulatrix depends on the duration between the two copulations. For example, in *E. kuehniella*, second male paternity significantly decreases with the increase of intermating duration (Fig. 5.11). In this scenario, the intermating duration may be determined by two factors: (1) how quickly the second males can persuade previously mated females to mate, and (2) when copulated females accept males’ mating attempts. On one hand, a second male should be able to fertilize more eggs if he succeeds in copulating with a mated female sooner, thus displacing the spermatophore of the previous male when fewer sperm have moved to the ductus seminalis. On the other hand, the once-mated female should benefit from genetic diversity (see Section 4.4 for detail) if she delays the second mating to allow some sperm from the first male to fertilize her eggs. However, the once-mated female remates sooner if she encounters a male that is larger than her previous mate (see Section 5.2 for detail), probably for good gene benefit (see review in Simmons 2005). Therefore, the intermating duration could be the product of mate choice and sexual conflict between the sexes in *E. kuehniella*.

My study indicates that 60% of females accept the second copulation < 24 h after the first copulation (Section 4.4), 40% do so < 8 h after the first copulation.
(Section 5.3), and only 15% mate again < 4 h after the first copulation (this Section). Therefore, on most occasions when *E. kuehniella* females accept a second copulation, most sperm from the first spermatophore have already moved to the ductus seminalis and reached the spermatheca (Fig. 2.2). Consequently, the sperm displacement mechanism in the bursa copulatrix alone can not explain the second male paternity precedence in this species.

Therefore, mechanisms outside the bursa copulatrix must be involved in facilitating second male paternity precedence in *E. kuehniella*. Here I clearly demonstrate that after the second copulation the sperm from the first male already stored in the spermatheca are significantly reduced and largely displaced by those from the second male (Fig. 5.9). Furthermore, just before oviposition occurs in the second scotophase about 75% of eupyrene sperm in the spermatheca are from the second male (treatment ‘8h+13h’, Fig. 5.9). In accordance with the sperm component in the spermatheca at this time (Fig. 5.9), $P_2$ is also about 75% (Fig. 5.11), suggesting that offspring paternity is directly related to the relative number of sperm of the two males in the spermatheca.

Sperm displacement within spermathecae is thought to be common (Eberhard 1996) but how this occurs is still poorly understood. Several hypotheses have been proposed to explain this phenomenon: (1) ejaculates from the second males may flush the stored sperm out of the spermatheca (Silberglied et al. 1984), (2) stored sperm may be digested in the spermatheca (Barnett et al. 1995), (3) stored sperm may move into the spermathecal accessory gland and be digested there (Hosken et al. 2001; Watanabe & Hachisuka 2005), and (4) stored sperm may be physically ejected from the spermatheca by the female (Villavaso 1975; Hellriegel & Bernasconi 2000; Snook & Hosken 2004). My study does not support the flushing hypothesis because the sperm loss has already occurred before the sperm from the second male reach the spermatheca (Fig. 5.9). Similar to Watanabe & Hachisuka’s (2005) study on the swallowtail butterfly *Papilio xuthus*, we found no evidence for sperm digestion in spermathecal accessory glands and/or spermathecae because only a very small number of sperm move into the glands over time (Fig. 3.17), and in all my dissections I have not found any half-digested sperm (such as morphologically changed or damaged sperm, e.g. Eady 1994). So far, muscular manipulations by the female are the only verified mechanism by which female insects influence sperm storage (Villavaso 1975; Hellriegel & Bernasconi 2000).
Lum et al. (1981) also suggest that stored sperm move out of the spermatheca by controlled spermathecal contractions in a pyralid moth. In the present study, I have observed vigorous spermathecal contractions in *E. kuehniella*, which appear to be able to eject sperm (Fig. 5-10 a-d).

Sperm displacement within spermathecae may be caused by post-copulatory female choice (Pizzari & Birkhead 2000), male copulatory manipulation (Cordoba-Aguilar 1999), or both (Snook & Hosken 2004). In *Drosophila*, sperm dumping is believed to be under female control (Arthur et al. 1998), although males may have some effect (Civetta 1999; Civetta & Clark 2000). In the present study, stored sperm in spermathecae decline more slowly in females kept under a 24:0 h light:dark cycle which inhibits oviposition as compared to those under 14:10 h which promotes egg laying (Fig. 3.16). This result suggests that *E. kuehniella* females have some control over sperm release, probably by spermathecal contractions (Lum et al. 1981).

However, how and to what extent males affect stored sperm displacement in or dumping from spermathecae is still poorly understood. Males may induce females to eject stored sperm through sensory exploitation during copulation. For example, during copulation male damselfly *Calopteryx haemorrhoidalis asturica* stimulates the cuticular plates in the female genital tract that bear mechanoreceptive sensilla, resulting in ejection of stored sperm from the spermatheca (Cordoba-Aguilar 1999). During the two hour long copulation, male *E. kuehniella* might also mechanically stimulate the female reproductive tract and increase spermathecal contractions for stored sperm ejection.

Male accessory gland proteins (AGPs) may stimulate egg production, reduce female receptivity and promote male success in sperm competition (see review in Jin & Gong 2001; Yapici et al. 2008; Fricke et al. 2009). It is also possible that AGPs could trigger ejection of stored sperm by increasing spermathecal contractions. Like other closely related moth species (e.g. McNamara et al. 2008), *E. kuehniella* males transfer seminal products before depositing the spermatophore in the bursa copulatrix (Section 3.6). However, whether these products from the second male are associated with stored sperm ejection is unknown.

Finally, my experiments on sperm use patterns and *P₂* determination demonstrate that the sperm from two males are freely mixed in the spermatheca and
randomly used for fertilization. Therefore, last male sperm precedence is not the result of sperm stratification in this species.

Based on the established facts, I suggest that last male sperm precedence is facilitated by sperm displacement operating at both sperm ejaculation and storage sites in *E. kuehniella* females. The outcome of sperm displacement before and after sperm storage appears to be the result of male × female interactions.
CHAPTER 6
GENERAL DISCUSSION AND CONCLUSION

6.1 Introduction

In this thesis, I report the reproductive biology and sexual selection of *E. kuehniella*, providing an insight into the life story of this species.

In this chapter, I summarise and discuss my main findings and their relevance to the behavioural and evolutional biology of *E. kuehniella* and to the development of management measures for this insect.

6.2 General Reproductive Biology

The overall performance of *E. kuehniella* decreased with the increase of larval density. A rearing density of 100 larvae per 50 g food per jar is highly recommended to produce *E. kuehniella* of satisfactory quantity and quality.

This study shows that *E. kuehniella* is a protogynous species (females emerge earlier than males), which may have evolved to reduce inbreeding because early emerged females are less likely to mate with their brothers (Rhainds et al. 1999). Emergence and reproductive activities of *E. kuehniella* are highly rhythmic. Calling and courtship peaks are always followed by the mating peak, suggesting that female calling and male courtship are essential for successful matings in this species. Therefore, using sex pheromone for mating disruption or mass trapping in *E. kuehniella* appears to be a control tactic worth investigating. This study suggests that the end of photophase (emergence peak) and the start of scotophase (oviposition peak) are optimal times to collect fresh moths and eggs, respectively, for research or natural enemy rearing.

In Lepidoptera and many other insect species, sperm packed in a spermatophore are ejaculated to the bursa copulatrix from which they migrate to the spermatheca before they can fertilize ova (Friedlander et al. 2005). After mating, it takes 4 h for sperm to first reach the spermatheca and another 7 h for most of sperm to get into the spermatheca in *E. kuehniella*. This may be attributed to the long ductus seminalis in this
species. A long ductus seminalis may have evolved to promote sperm competition by favoring the ‘vigorous’ sperm that could reach the spermatheca and fertilize eggs (Keller & Reeve 1995). As a consequence, females of this species generally do not lay fertile eggs until the next scotophase after mating.

This study clearly indicates that presence of the fertile eupyrene sperm in the spermatheca (e.g. Thibout 1979), rather than mechanical stimulation by males during mating (e.g. Sugawara 1981) or male accessory gland factors (e.g. Yi & Gillott 1999), is the main factor for eliciting oviposition in *E. kuehniella*. However, my study confirms that male accessory gland factors enhance female egg maturation in *E. kuehniella* and this process is independent of the presence of sperm.

Both sexes can mate in the first scotophase following emergence. The best reproductive performance can be achieved when both sexes were 1-d-old at mating comparing to older insects; delaying mating for 7 d reduces female fecundity by 60%. It is thus suggested that it is necessary to delay females’ mating for more than 7 d after emergence to achieve a good control.

Both sexes mate multiply with males mating up to 9 times and females up to 4 times. Female remating increases her chance of mating with a wild (fertile) male and thus decreases the effectiveness of the sterile insect technique (Kraaijeveld et al. 2005). However, the negative effects of remating on SIT may be ameliorated by increasing the overflooding rate (the ratio of sterile males to wild females), or releasing sterile males after mass trapping using female sex pheromones to remove wild males.

### 6.3 Multiple Mating and Sexual Selection

Consistent with many other species (Dewsbury 1982), sperm production is nontrivial in *E. kuehniella* males. Females may also incur cost from copulation, such as energy costs and disease transfer (Arnqvist & Nilsson 2000). Therefore, both sexes have evolved various strategies to choose mates and control sperm investment or fertilization for maximum reproductive success over the cost of matings (reviewed in Simmons 2001; Snook & Hosken 2004).

My study shows that *E. kuehniella* males prefer to mate with young, large and virgin females to gain direct benefit in terms of more offspring (Halliday 1983; Andersson 1994) as these females have higher reproductive potential. However, these
high quality (larger and younger) females also represent a greater risk of sperm competition because they are more likely to remate than lower quality ones. Sperm competition theory (Parker 1982, 1990) predicts that males experiencing higher levels of sperm competition risk are selected to increase ejaculate size to win sperm competition. On the contrary, males should produce a smaller ejaculate and conserve sperm for future matings to achieve maximum lifetime reproductive success when facing low risk of sperm competition (Galvani & Johnstone 1998). As a consequence, *E. kuehniella* males strategically ejaculate more sperm to females when mating with high quality females or under male-biased sex ratio than mating with low quality ones or under female-biased sex ratio.

My study indicates that *E. kuehniella* females prefer large and mid-aged males for mating regardless of male mating history. However, females that mate with larger and mid-aged males do not gain higher fecundity or fertility. In this species, a female choosing a large mate will have large offspring and thus she will gain indirect genetic benefit because her large sons and daughters possess higher reproductive fitness (e.g. Fisher 1958). Females’ preference for mid-aged males for mating may be because those males are more sensitive to female pheromone than younger or older ones (Calvert & Corbet 1973), rather than because females choose them for higher fecundity or “good gene” (Brooks & Kemp 2001). Females’ non-discrimination between virgin and non-virgin males for mating may be because male mating history generally does not affect female reproductive output in this species.

A male’s reproductive success primarily depends on the number of females he can inseminate (Simmons 2005). Females have also evolved to allow different males to fertilise their eggs from which they gain additional nutrients (reviewed in Arnqvist & Nilsson 2000) and/or genetic benefits (reviewed in Cornell & Tregenza 2007). Multiple mating in *E. kuehniella* females does not significantly increase their fertility, fecundity and longevity. However, females discriminate against previous mates, and adjust their oviposition patterns depending on whether they encounter new or previous mates after the first copulation and encourage multiple males to fertilize their eggs. These results suggest that *E. kuehniella* females may mate multiply for genetic benefit in terms of offspring diversity (Cornell & Tregenza 2007). Offspring diversity theory suggests that polyandry benefits females by reducing sib competition (e.g. Robinson 1992), disease transfer (e.g. Tooby 1982), and inbreeding cost (Cornell & Tregenza 2007).
*kuehniella* is a stored-product pest with limited dispersal ability (Rees 2003); each female produces over 300 eggs, which are laid locally within a brief period (> 80% eggs are laid in the first two scotophases), suggesting that sib competition and copulations may be very common in this species. Therefore, offspring genetic diversity should be extremely important for *E. kuehniella*.

Furthermore, *E. kuehniella* females are more likely to remate when encountering males are larger than their previous mates. Therefore, in addition to offspring diversity, polyandry may also benefit *E. kuehniella* females in terms of “good gene” from selecting better males in their subsequent matings (Keller & Reeve 1995).

Similar to many other species (reviewed in Xu & Wang 2010a), the last male to mate achieves a higher fertilization rate in *E. kuehniella*. This phenomenon has been explained as the result of males’ and/or females’ influence on sperm use for maximum reproductive success (reviewed in Xu & Wang 2010a). The last male sperm precedence in *E. kuehniella* may be due to sperm displacement at both sperm ejaculation and storage sites, where the second male physically displaces the first male’s spermatophore with his own in the bursa copulatrix and triggers the female to dump resident sperm in the spermatheca. Similar to other species (Drnevich et al. 2000; Takami 2007), the effect of spermatophore displacement on sperm precedence depends on the duration between the two copulations where $P_2$ decreases with the increase of intermating duration. However, on most occasions (> 85%) when *E. kuehniella* females accept a second copulation, most sperm from the first spermatophore have already moved to the ductus seminalis and reached the spermatheca. Consequently, the sperm displacement mechanism in the bursa copulatrix alone cannot explain the second male precedence in this species. Just before oviposition occurs in the second scotophase about 75% of eupyrene sperm in the spermatheca of twice-mated females are from the second male. In accordance with the sperm component in the spermatheca at this time, $P_2$ is also about 75%, suggesting that offspring paternity is directly related to the relative number of sperm of the two males in the spermatheca and sperm displacement within spermathecae should be the primary mechanism for last male precedence in *E. kuehniella*.

The mechanisms behind the sperm displacement in spermathecae are still poorly known. Although not observed directly, some authors (e.g. Villavaso 1975;
Hellriegel & Bernasconi 2000) suggest that the resident sperm may be physically displaced from storage by females. I have observed vigorous constricting movements of spermathecae in *E. kuehniella*, which appear to be able to eject sperm. Sperm displacement within spermathecae may be triggered by post-copulatory female choice, male copulatory manipulation, or both (Snook & Hosken 2004). In *Drosophila*, sperm dumping is believed to be under female control (Arthur et al. 1998) although males have some effect (Civetta & Clark 2000). My study shows that *E. kuehniella* females have some control over sperm release, probably by spermathecal contractions (Lum et al. 1981). However, how and to what extent males affect stored sperm displacement in or dumping from spermathecae is still poorly understood. *E. kuehniella* males might mechanically stimulate the female reproductive tract (e.g. Cordoba-Aguilar 1999) or transfer male accessory gland factors to stimulate spermathecal contractions and thus trigger ejection of stored sperm from spermathecae. Whether male accessory gland factors from the second male are associated with stored sperm ejection remains unknown and warrants further investigation.

### 6.4 Resource Allocation between Ova and Soma

Resource allocation between survival and reproduction is a central question in evolutionary ecology of senescence but the physiological mechanisms by which this trade-off is controlled remain poorly understood (Harshman & Zera 2006). One supposed mechanism in female insects is nutrient recycling through oosorption (Kotaki 2003; Wang & Horng 2004). My study shows virgin females live significantly longer than mated females but no obvious oosorption was found in all females. However, female longevity significantly negatively correlated with egg production in this species. According to the disposable soma model (Kirkwood & Austad 2000), ageing occurs because resources allocated to reproduction are unavailable for investment in somatic repair, making individuals or populations that invest more in reproduction likely incur faster ageing and shorter lifespan. Therefore, the longevity reduction in mated *E. kuehniella* females is probably because females allocate more resources to ova than soma after mating for higher fecundity under the stimuli derived from male accessory factors. Unmated or mating-delayed females may allocate more resources to soma than ova to wait for mating.
6.5 Thiotepa-based Sterile Technique and Sperm Use Pattern Measurement

Chemosterilants have often been used to sterilize insects and such chemosterilization technique has long been suggested to be used in sperm competition studies (Snow et al. 1970; Beil et al. 1976; Wall et al. 1985). However, little is known about whether chemosterilants can be reliable markers for the sperm use pattern measurement. In my study, dipping heads of male moths in 1% thiotepa solution results in complete sterilization, i.e. their sperm still fertilize eggs but those eggs do not hatch. The sterilization treatment does not significantly affect male copulation ability, female fecundity, and sperm transfer, motility and fertilization. Reverse copulation treatments clearly show that sperm from thiotepa-treated and untreated males have the same competitive ability for fertilization. Therefore, thiotepa is an effective and reliable marker for sperm competition studies in E. kuehniella and probably other species.

6.6 Conclusion

In this thesis I have reported and discussed my main findings of the reproductive behaviour in the Mediterranean flour moth E. kuehniella. The work has provided a much firmer basis of knowledge of this insect than existed hitherto, and a more rounded perspective of the reproductive biology in the species. Such knowledge is vital, as noted in the thesis, to appraising prospects for further investigation of the reproductive biology and sexual selection, pest management, and use of this increasingly important insect.
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