Doctorate Thesis (Abridged)

Studies on the reproductive investment in the adzuki bean borer, *Ostrinia scapularis*

（アズキノメイガの繁殖投資に関する研究）

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GENERAL INTRODUCTION

Introduction

Reproductive success is one of the important measurements of the fitness of insects. Male reproductive success depends on the number of females that he has inseminated, while female reproductive success depends on the number of eggs that she has produced (Bateman 1948; Trivers 1972). Natural selection drives both sexes of sexually reproducing organisms to maximize their reproductive success. However, this driving force may have different consequences on males and females because of the differences in their investment in gametes (Andersson 1994). Male produce a large number of small gametes (i.e. sperm), whereas females produce a small number of relatively large gametes (i.e. eggs). Thus, reproduction is costly– for females in particular. Since resources required for egg production is generally limited, females should strategically allocate reproductive investments. Accordingly, to date, many studies have been conducted on allocation of reproductive investments by females. However, reproduction can also be costly for males when they provide nutritious ejaculates or nuptial gifts to females during copulation (Hunt et al. 2004; McNamara et al. 2008). In these cases, males should also strategically allocate resources to maximize his reproductive success.

Males may adopt pre and post-copulatory reproductive strategies-to maximize their reproductive success. Pre-copulatory reproductive strategies may affect male mating success whereas post-copulatory reproductive strategies may influence male fertilization success under "sperm competition"–sperm from two or more males compete for a female's ova" (Triver1972; Parker 1998; Arnqvist & Rowe 2005; Wong & Candolin 2005; Parker & Pizzari 2010). Male pre-copulatory mating success is influenced by two mechanisms: female choice or male-male competition (Berglund et al. 1996; Hirai et al. 1978; Aragon

2009). In general, females try to maximize their fitness by mating with high quality males that can provide direct fitness benefits (e.g., greater fecundity) (Markow et al. 1978; Jimenez & Wang 2004) and indirect genetic benefits (e.g., higher offspring viability and son's mating success) (Kirkpatrick & Barton 1997; Andersson 1994). Therefore, male reproductive success may depend on female mate choice. In addition, prior to mating, males may produce courtship displays, which convey visual, acoustic, or chemical signals (Wyatt 2003; Kelley 2004; Johansson et al. 2005). These important signals may have an influence on male mating success.

Lepidopteran species are particularly interesting for exploring variation in male pre- and post-copulatory reproductive strategies. Males of lepidopteran species use various forms of courtship displays (e.g., visual, acoustic and olfactory stimuli) during courtship (Spangler 1984; Nakano et al. 2006; Costanzo & Monteiro 2007; Fisher et al. 2008). These male secondary sexual characteristics or signals are needed to make female receptive (Andersson et al. 2007). Moreover, males of many lepidopteran species provide a nuptial gift in the form of capsule, "spermatophore", which contains sperm and accessory gland nutrients (e.g., carbohydrate, protein, water) to females during copulation (Engebretson & Mason 1980; Marshall 1982, 1985). The production of spermatophore is known to be physiologically costly for males. This is evidenced by the observation that a certain period of time is required for producing another spermatophore (Bissoondath & Wiklund 1996a; Wedell & Karlsson 2003). As discussed below more in detail, spermatophore may function as "*paternal investment*" (i.e., increasing female reproductive output with male donated nutrients) and/or "*male mating effort*" (i.e., delaying female re-mating) (Simmons & Parker 1989; Eberhard 1996; Simmons 2001). Transferring a large spermatophore may provide advantages for males because the contents of spermatophore may increase female reproductive output, which in turn increases male reproductive success directly (Boggs & Gilbert 1979; Greenfield 1982; Wedell 1996). Moreover, transferring of a large spermatophore may reduce sperm competition to some extent because females that received a larger spermatophore are less likely to re-mate soon than females that received a smaller spermatophore (Oberhauser 1997; Wedell & Cook 1999; Marcotte et al. 2006; McNamara et al. 2009). Males transfer two types of sperm, nucleated, fertile "*eupyrene*" sperm and anucleated, non-fertile "*apyrene*" sperm (Meves 1902). Although the exact function of apyrene sperm has yet to be studied, it may have a role in sperm competition; males fill the female spermatheca with apyrene sperm, not eupyrene sperm to delay female re-mating because apyrene sperm may be cheaper for males to produce (Cook & Wedell 1999; Silberglied et al. 1984). In most lepidopteran species, spermatogenesis usually ends at the early developmental stage and adult males typically emerge with a limited amount of fertilizing sperm (Wedell et al. 2002). Nevertheless, sperm production can be limited for males because the number of sperm transferred by male declined with successive matings (Torres-Vila & Jennions 2005). Moreover, nutrients donation can be regarded as a reproductive investment of male. Several studies reported that male - donated nutrients are needed for egg production and somatic maintenance of females (Wiklund et al. 1998). Among nutrients, protein can be a limiting resource for males because most of the proteins come from larval feeding stage and males can obtain only a small amount of protein in the adult stage, especially for nectar - feeding species (Boggs 1981; O'Brien et al. 2002). Thus the protein contained in a spermatophore is also likely to be an important component of male resource allocation strategy.

Mating system can have profound effects on male reproductive strategies. In lepidopteran species, female mating systems range from strict *monandry* (mate only once) to strong *polyandry* (multiple mating), while multiple mating by males, *polygyny*, is common in many species. In many lepidopteran species, spermatophore remains in the "bursa copulatrix" (female reproductive organ), for an extended period of time, even until after death. Thus, female mating frequencies can be easily estimated by the analysis of the bursal contents of females (Drummond 1984). Males of polyandrous species have larger testes (Gage 1994), a higher ejaculate production capacity, and higher mating frequency compared with males of monandrous species (Svärd & Wiklund 1989). Moreover, males of highly polyandrous species produce larger spermatophore than males of monandrous species (Svärd & Wiklund 1989). Larger spermatophores have been suggested 1) to lengthen the duration of female refractory period (Oberhauser 1989, 1992; Wiklund et al. 1993; Kaitala & Wiklund 1994; Wiklund & Kaitala 1995; Bissoondath & Wiklund 1997), 2) to have a higher fertilization success (Wedell 1991), 3) to be advantageous in sperm competition because of a greater number of sperm (Svärd & Wiklund 1989), and to have more protein (Bissoondath & Wiklund 1995). In polyandrous mating system, females mate several times, so it is important for males to be able to re-mate and reproduce a spermatophore of sufficient size in a short period of time, in order to increase their fertilization success. In contrast, in monandrous species, females are no longer receptive after their first matings. As a result, the expected opportunities for future matings are uncertain for males of monandrous species.

Outline of thesis

Reproductive investment (RI), as a measurement of the resource allocated to reproduction, is important for understanding of life history evolution (Stearns 1992). Theory predicts that males should adjust their investment in current mating with the investment in future mating (Parker 1983; Bonduriansky 2001; Reinhold 2002). Moreover, it may be beneficial for males to mate discriminately (i.e. male mate choice) when costs associated with mating are high and when there is sufficient variation in female quality. Males can reduce their reproductive cost and increase their fitness by mating with females of high quality (Bonduriansky 2001; Kokko & Wong 2007). Such adjustment of male reproductive investment may impact on female reproductive fitness. Environmental factors are also important for individual fitness. Male investment is also affected by environmental factors (e.g. temperature, nutritional stress, diapause) (Fox et al. 2006; Wedell 1994; Sadakiyo & Ishihara 2012). These environmental factors are associated with the cost of males, which in turn cause decrease in male ejaculate investment to females. As a result, females are influenced directly by the environmental factors and indirectly by males. However, a few studies reported the environmental effects on male ejaculate investment.

The objective of my study is to investigate variation in reproductive investment in the adzuki bean borer, *Ostrinia scapulalis* (Lepidoptera: Crambidae). The adzuki bean borer is a monandrous species, i.e., almost all females mate only once throughout her life, whereas males are polygynous. Adult of this species may take water and some nectar as reported in a congener, *O. nubilalis* (Becker 1987). Like other *Ostrinia* species, males find conspecific females by following a female-derived sex pheromone and show a courtship display after landing close to the female (Haung et al. 1997; Nakano et al. 2006). During copulation, males transfer a "spermatophore" to females. *O. scapulalis* is a multivoltine species (i.e., one generation in late spring–early summer, and one generation in summer- autumn in the kanto region of Japan). In late summer (July-August), the mature larvae stop feeding and overwinter in diapause. They resume development in early spring and the adults of overwintering generation emerge in late spring.

In this study, I first investigated if males of *O. scapulalis* change their reproductive investments in courtship and nuptial gift as they age (Chapter 1). Due to a trade-off between current and future mating, males should increase their reproductive investment in the current mating when expected future matings become low (i.e. terminal investment hypothesis) (Clutton-Brock 1984). I showed that males increased the frequency of courtship displays towards the end of their life span, and accordingly, older males had higher mating success than younger ones. Moreover, I also found that males increased spermatophore investment as they age. Next, I examined the effect of male mating history i.e., virgin or mated, on their reproductive investment (spermatophore size and protein content) (Chapter 2). To exclude the effect male age on reproductive investment, I compared reproductive investment by virgin and mated males of the same age in this chapter. I also determined the effects of variation in male reproductive investment by male age and mating history on female fitness consequences. I found that spermatophore investment was costly for males, as spermatophore size decreased in the second mating. If the cost of reproduction in males is high and variation in female quality varies, male mate choice is likely to evolve (Bonduriansky 2001; Edward & Chapman 2011). In chapter 3, I examined whether male reproductive investment is changed in response to female quality. I also examined if the magnitude of male mate choice on female quality changes age dependently in this species. In chapter 4, I investigated the effects of diapause on post-diapause reproductive investment of males and females.

Chapter 1

AGE-RELATED MALE REPRODUCTIVE INVESTMENT IN COURTSHIP AND NUPTIAL GIFT

Abstract

Due to a trade-off between current versus future reproduction, costly reproductive investments should be increased toward the end of a lifespan when the probability of reproduction becomes low (terminal investment hypothesis). I investigated age-related changes in male reproductive investment toward courtship display and the spermatophore in three age classes (young, middle-aged, and old) of a monandrous moth, *Ostrinia scapulalis*. As predicted by the hypothesis, old males had more mating success than young and middle-aged males in no-choice tests. Two-choice tests revealed that middle-aged males had a higher success rate than young males because of their higher courtship frequency rather than any female preference for them. It was found that old males produced a larger spermatophore than young and middle-aged males, suggesting greater reproductive effort. The protein content of spermatophores also tended to increase with male age. Despite the age-related variation in spermatophore size and protein content, age did not affect female fecundity or longevity. A decrease in the number of sperm in the older males might counteract the nutritional benefit of larger spermatophores. Alternatively, fitness components other than longevity and fecundity may be influenced by male age.

Introduction

Life-history theory predicts that animals should allocate their resources strategically to maximize their lifetime reproductive success (Williams 1966). A trade-off between current and future reproduction may occur because individual expendable resources are limited (Williams 1966; Stearns 1992). If the opportunity for future reproduction declines with age and their residual reproductive value decreases, individuals are expected to increase their reproductive investment toward the end of their life (i.e., terminal investment hypothesis; Clutton-Brock 1984). Males are regarded to invest heavily in reproduction through efforts such as bearing sexual signals, mate searching, intra-sexual competition, courtship display, parental care, and producing ejaculate (Simmons & Parker 1989; Magnhagen 1991; Wedell 2010). Consistent with the terminal investment hypothesis, a positive correlation between reproductive investment and male age has been reported in birds (Velando et al. 2006), insects (Fischer et al. 2008), frogs (Felton et al. 2006), and mammals (Willisch et al. 2012).

In lepidopteran species, courtship display behaviour is a significant predictor of male mating effort. Male courtship displays can take a variety of forms including acoustic and visual displays, and the release of chemicals, i.e., male sex pheromones (Spangler 1984; Nakano et al. 2006; Costanzo & Monteiro 2007; Lassance & Löfstedt 2009). Some studies have reported that older lepidopteran males invest more in courtship behaviour and/or sexual signals than younger males to improve their mating success. For example, in the tropical butterfly *Bicyclus anynana*, older males had considerably more mating success than younger males. The increased success was likely ascribable to greater motivation for mating (Fischer et al. 2008). Likewise, in the European corn borer *Ostrinia nubilalis*, older males, which released a more attractive sex pheromone during courtship, had a higher success rate than younger males (Lassance & Löfstedt 2009).

The reproductive effort in lepidopteran species is not limited to the pre-copulatory display. During copulation, males transfer a capsule called the “spermatophore” to females, which contains not only sperm but also nutrients such as protein, carbohydrate, mineral, and sugar (Engebretson & Mason 1980; Marshall 1982, 1985). These nutrients are mostly derived from larval resources (Hough & Pimentel 1978; Oberhauser 1989). Therefore, the production of spermatophores imposes a substantial physiological cost on males. The male-derived nutrients in spermatophores are known to enhance female reproductive output. In many lepidopteran species, a positive relationship between the size/number of spermatophores received at mating and female fecundity has been reported (reviewed by South & Lewis 2011). Among the nutrients in spermatophores, protein is potentially important for egg production (Wheeler 1996), given that females take in little protein from their adult diet, e.g., nectar (Baker & Baker 1973; O’Brien et al. 2002).

Lepidopteran males may strategically increase their resource investment in the spermatophore as they age, which will in turn affect female reproductive output. However, limited information is available on the evolution of their strategies for age-related resource allocation. Several studies have shown that spermatophore size increases with male age (Jones et al. 1986; Oberhauser 1988; Delisle & Bouchard 1995; Hughes et al. 2000; Milonas & Andow 2010), but these studies did not examine the variation in nutritional quality of the spermatophore. The reported effects of male age on female fitness are not consistent among lepidopteran species. For example, females of *Dahlica lichenella* achieved higher fecundity by mating with older males (Elzinga et al. 2011), while females that mated with intermediate males had higher fecundity than those that mated with younger or older males in *O. nubilalis* (Milonas & Andow 2010). Furthermore, in *Choristoneura rosaceana* and *Siederia listerella*, male age did not affect female fecundity and longevity (Delisle & Bouchard 1995; Elzinga et al. 2011).

In the present study, I examined the age-related variation in male reproductive investment in a moth, the adzuki bean borer *Ostrinia scapulalis* (Lepidoptera: Crambidae). I tested whether males increase investment in courtship displays and have higher mating success as they age. I also tested whether older males increase investment in the spermatophore, measured as its size and protein content, and whether the spermatophores produced by older males increase female fitness.

Materials and Methods

Insects

Cultures of *O. scapulalis* (E-type, Huang et al., 2002) used in this study were established from feral adult females collected at Matsudo City, Chiba Prefecture (35.7°N, 139.9°E) in September 2010 or at Osaki City, Miyagi Prefecture (38.5°N, 140.9°E) in July 2011 in Japan. The field-collected females were confined individually in small 30-ml plastic cups supplied with cotton pad soaked with water to let them lay eggs (the first generation, G1). Twenty-one wild females collected at Osaki in 2011 were used to investigate the monandry/polyandry of this species. After allowing females to lay eggs, I checked the number of spermatophore residues in the female's bursa copulatrix. The number of spermatophores in a female is a good indicator of the number of matings because males transfer a single spermatophore per copulation and most spermatophores persist in recognizable form (Drummond 1984; Cordero 1999; Milonas & Andow 2010).

Egg masses were transferred into a plastic bottle (8 cm in diameter, 12 cm in height) containing an artificial diet for insects (Silkmate ^{TM}; Nosan Crop., Yokohama, Japan) and maintained in a rearing room at 23 ± 1°C and 80 ± 10% relative humidity with a 16:8 (light-dark) photoperiod until pupation. Pupal weight was used as a substitute for adult body weight in this study. Mean pupal weight (mean ± SE) was 69.8 ± 0.8 mg and 76.9 ± 1.0 mg for males and females, respectively. The weighed pupae were kept individually in small plastic cups until adult emergence to ensure virginity and for determining age.

Newly emerged male and female moths (0-day-old) were collected daily and transferred into 430-ml plastic cups provided with water. I first checked the longevity of males under laboratory conditions to define three age classes (young, middle-aged, and old) for males. From the survival curve (Fig. 1.1), I selected 1-, 3-, and 6-day-olds as young, middle-aged, and old males, respectively. All experiments were carried out in the rearing room described above. Insects were used only once for each trial. All behavioral experiments were started after the 4th h in the scotophase (D4) because females of *O. scapulalis* are known to actively emit pheromones at this hour (Huang et al. 1997). The second-generation (G2) adults of the Osaki population were exclusively used for all experiments except for the two-choice test experiment. In the two-choice test, the G2 adults of both the Matsudo populations were also used in addition to the Osaki population.

Two-choice Test

In the two-choice test, a 3-day-old virgin female was allowed to choose her mate from two males of different age. The male-age combinations used were 1- and 3-day-olds, and 3- and 6-day-olds. Two males of different age were anesthetized with CO_2 and marked with a different coloured permanent pen on the basal portion of the right forewing. During the scotophase of the following day, the two males were introduced into a nylon mesh cage (24 × 24 × 24 cm) containing a 3-day-old virgin female. When a female did not show calling behaviour (i.e., emit pheromone) within 30 min, although rare (< 10%), she was replaced with another. The sample size was 88 pairs for the 1- and 3-day-old combination, and 55 pairs for the 3- and 6-day-olds. In this test, I used the Matsudo and Osaki populations of *O. scapulalis*, but insects from a single population were used for each triplet (two different-aged males and one female).

The mating behaviour of moths was observed continuously with the aid of a 5-W red light bulb until either male successfully copulated. When a male perceived a female’s pheromone, he promptly initiated courtship. The male usually approached from the rear of the female fanning his wings. Then, the male courted the female by vibrating his uprightly raised wings and subsequently extending his genital claspers with the abdomen curved towards the calling female (see Nakano et al. 2006). The male succeeded in copulation when the female accepted him, but the female often flew away or walked away during the courtship. The numbers of male courtships and female escape behaviour (the sum of flying away and walking away when males courted) were counted. The frequency of female escape behaviour (number of escape behaviour/ number of male courtships) was used as an indicator of female preference. Male-male competition was not observed.

No-choice Test

In the no-choice test, a single 3-day-old female was paired with a single male from three age classes (1-day: $n = 104$, 3-day: $n = 43$, 6-day: $n = 38$). A pair was introduced into a 150-ml steel wire screen cage and observed for copulation. The mated females were used in the subsequent analyses of the spermatophore. To examine the difference in the duration of copulation in relation to age, we randomly chose pairs with 1-day-old males ($n = 17$) and 6-day-old males ($n = 6$), and checked the mating pairs every 15 min until the completion of copulation.

Spermatophore Size and Protein Content

To examine the effect of male age on the size and protein content of spermatophores, mated females were dissected to remove the bursa copulatrix, in which a spermatophore was stored. In this analysis, the bursa copulatrix containing a spermatophore was considered a ‘spermatophore’ because the presence of a membranous bursa copulatrix is known to have little effect on the measurement of the size and protein content of the spermatophore (Marshall 1985, Bissoondath & Wiklund 1995). The bursa copulatrix containing the spermatophore was cleaned with tissue paper before being photographed using a digital camera (DP12, Olympus, Japan) connected to a microscope (SZX-12, Olympus). The dimension of the roundish region of the bursa copulatrix (see Fig. 1.2) was measured with the software Image-J (http://rsb.info.nih.gov/ij/). The area of this region (2 dimensional) was raised to the 1.5th power to use as an indicator of spermatophore volume (3 dimensional). The 'spermatophores' were placed individually in plastic microtubes and stored at –20°C for 1–2 weeks until used for the protein analysis. The protein assay was performed as described by Bissoondath & Wiklund (1995). In brief, the spermatophore was homogenized in 200 μl of 0.1% Triton-X solution using a plastic pestle and the homogenate was left for 5 days at 4°C. On the day of analysis, samples were centrifuged at 3000 rpm for 5 min at 17°C, and 10-μl aliquots of the supernatant were each mixed with 200 μl of dye reagent (Bradford 1976; Bio-Rad, Japan) in wells of a 96-well microplate. The concentration of solubilized protein was estimated from the $\mathrm{OD}_{595\ \mathrm{nm}}$ measured using a microplate reader (Model 550, Bio-Rad, Japan) with bovine serum albumin as the standard. A total of 145 males (1-day: *n* = 76, 3-day: *n* = 33, 6-day: *n* = 36) that successfully mated in the no-choice test were used in this experiment.

Female Fecundity and Longevity

To examine the effect of male age on female fecundity and longevity, a total of 141 males were newly selected from each age class (1-day: *n* = 66, 3-day: *n* = 28, 6-day: *n* = 47) and paired to 3-day-old virgin females as described for the no-choice test. The mated females were placed individually in 430-ml plastic cups supplied with a few droplets of water and allowed to oviposit. Females of this species generally lay 30–150 eggs in a mass (12 egg masses per female on average). Eggs were collected every third day by cutting the plastic where an egg mass was laid. Females were transferred into new plastic cups provided with a few droplets of water to allow them to lay more eggs. The dimensions of the egg masses laid by each female were measured as described for spermatophores. I summed the area of the masses for each female, and used the value as an indicator of female fecundity. I also recorded female longevity by checking the females daily.

Statistical Analyses

Data were analysed using the statistical software package R version 2.12.1 (R Development Core Team, 2010). In the two-choice test, mating success in an age group was compared with 50% (i.e., null hypothesis) by the chi-square test. The effect of age on the number of copulation attempts made by males was analysed using a generalized linear mixed model (GLMM) with Poisson error distribution. Male age was entered as an explanatory variable, and pair identification as a random factor. I also analysed the effect of male age on the frequency of female escape behaviour using GLMM. In this model, I entered male age and the number of copulation attempts as explanatory variables, and pair identification as a random factor. In the no-choice test, the difference in the mating success of different-aged males was analysed by the chi-square test with Bonferroni correction. The duration of copulation was compared for 1-and 6-day-old males using the Wilcoxon rank-sum (Mann-Whitney's U) test.

The effect of male age on spermatophore size was examined using a linear model (model 1). Male and female pupal weights were included as covariates to control the effects of these variables. The two covariates were entered in all subsequent models. The effect of male age on the protein content of the spermatophore was also examined using a linear model (model 2), in which spermatophore size was entered as a covariate to control the effect of this variable. The effect of male age on female fecundity was analysed using a linear model (model 3) as well, while the effect of male age on female longevity was examined using a Cox regression model (model 4). When male age was found to significantly influence the objective variable in these models (model 1–4), pairwise comparisons between age classes were conducted with the Tukey's *post hoc* tests.

Results

Number of Female Matings

All wild-caught females (n = 21) had one spermatophore, showing that they mated only once.

Two- choice Test

In the choice test between 1- and 3-day-old males, the rate of copulation among 3-day-old males was significantly greater than 50% (χ^2 = 5.27, p = 0.02, Fig. 1.3a): 3-day-old males were more successful (67%) than 1-day-old males (32%). Although insects from the Matsudo and Osaki populations were used in these experiments (see “Two- choice Test” section in Materials and Methods), there was no significant difference between the populations (p = 0.11). In the choice between 3-and 6-day-old males, neither copulation rate was significantly different from 50% (3-day: 58%, 6-day: 42%, χ^2 = 0.08, p = 0.78, Fig. 1.3a). There was no significant difference between the populations (p = 0.18).

The number of courtship displays (times ± SE) performed by 3-day-old males (1.22 ± 0.20) was significantly greater than that performed by 1-day-old males (0.64 ± 0.18) (p = 0.007, z = −2.7, Table 1.1). The incidence of female escape behaviour increased significantly with an increase in the number of courtship displays (z = 10.3, p < 0.001). When this relationship was taken into consideration, the frequency of female escape behaviour provoked by 1-day-old males (32.3 ± 10.3%) was not significantly different from that provoked by 3-day-old males (31.7 ± 6.5%)(z = 0.49, p = 0.62, Table 1.1). Meanwhile, the number of courtship displays performed by 3-day-old males (1.50 ± 0.41) was not significantly different from that of 6-day-old males (1.00 ± 0.29) (z = –1.25, p = 0.21, Table 1.1). The frequency of female escape behaviour provoked by 3-day-old males (37.3 ± 11.7%) was not significantly different from that provoked by 6-day-old males (59.2 ±

14.0%)($z = 0.99$, $p = 0.32$, Table 1.1), when the positive relationship between the incidence of female escape behaviour and male courtship ($z = 5.7$, $p < 0.001$) was controlled.

No-choice Test

Mating success in the three age groups differed significantly ($\chi^2 = 7.79$, $p = 0.02$, Fig. 1.3b): 73%, 77%, and 95% of males copulated successfully, respectively. Pairwise comparisons showed that 6-day-old males had a significantly higher success rate than 1- and 3-day-old males ($p < 0.001$, Fig. 1.3b). There was no significant difference in mating success between 1-day-olds and 3-day-olds ($p = 0.69$, Fig. 1.3b). Irrespective of male age, the body weight of males that succeeded in copulating was not significantly different from that of males that failed (Welch's *t* test, 1-day: $p = 0.98$, 3-day: $p = 0.09$, 6-day: $p = 0.56$). Six-day-old males copulated for a significantly longer time period than 1-day-old males (1-day: 89.7 ± 7.8 min, 6-day: 177.2 ± 22.8 min; Mann-Whitney's U-test, z = 27.6, p < 0.001)

Spermatophore Size and Protein content

Male age had a significant influence on spermatophore size ($p < 0.001$, model 1 in Table 1.2, Fig.1.4a). Spermatophores produced by 6-day-old males were significantly larger than those produced by 1- and 3-day-old males by 18% and 14%, respectively ($p <0.001$, Fig. 1.4a). Three-day-old males produced 3% larger spermatophores than 1-day-old males, but the difference was not significant ($p = 0.067$, Fig. 1.4a). Spermatophore size was positively related to the male's pupal weight ($p < 0.001$, model 1 in Table 1.2) and also to the female's pupal weight ($p < 0.001$, Table 1.2). Male age had a significant influence on the protein content of the spermatophore (Mean ± SE for 1-day-old males: 72.8 ± 1.5 µg, 3-day-old males: 86.3 ± 3.1 µg, and 6-day-old males: 84.7 ± 2.7 µg; $p < 0.0074$, model 2 in Table 1.2; Fig. 1.4b). Pairwise comparisons revealed a significant difference between 1- and 3-day-old males and between 1- and 6-day-old males ($p < 0.03$; Fig. 1.4b). Protein content was also significantly related to female pupal weight ($p = 0.043$, Table 1.2) but not to male pupal weight ($p = 0.43$, Table 1.2). Protein content was positively related to spermatophore size [$p = 0.03$ by linear model (model 2 in Table 1.2); $r = 0.37$, $p < 0.001$ by single regression; Fig. 1.5].

Female Fecundity and Longevity

Fecundity tended to be greater among the females mated with 6-day-old males than those mated with 1- and 3-day-old males (Fig. 1.6a), but the differences were not statistically significant ($p = 0.09$, model 3 in Table 1.3). Fecundity was positively related to female pupal weight ($p < 0.001$, Table 1.3), but not to male pupal weight ($p = 0.48$, Table 1.3). No significant difference was found in the longevity of females mated with different-aged males (1-day: 8.7 ± 0.2 day, 3-day: 8.5 ± 0.4 day, 6-day: 9.2 ± 0.2 day, $p = 0.84$, model 3 in Table 1.3, Fig. 1.6b). Female longevity was positively related to female pupal weight ($p < 0.001$, model 4 in Table 1.3), but not to the pupal weight of male mates ($p = 0.51$, Table 1.3).

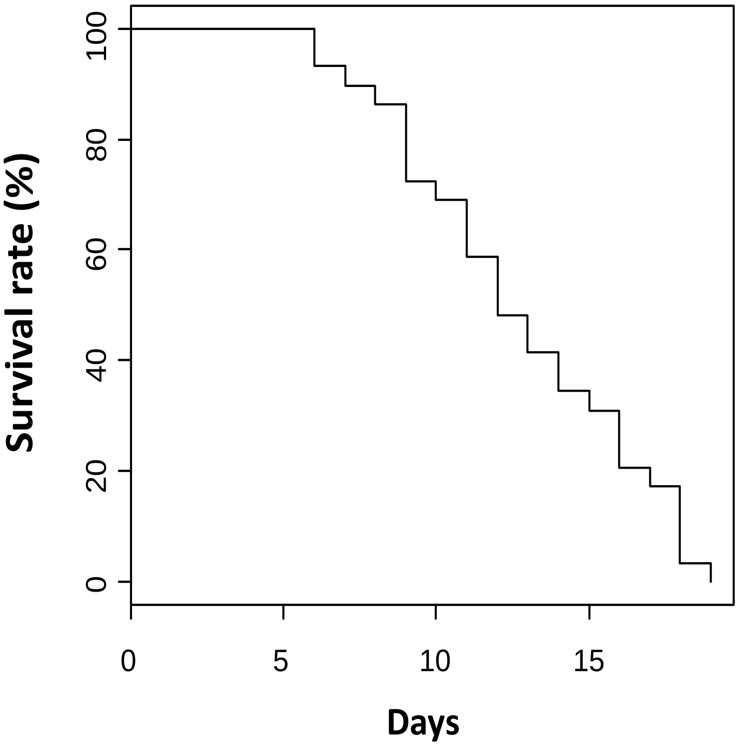

Fig. 1.1: Survival curve of male *Ostrinia scapulalis* moths under laboratory conditions.

Fig. 1.2: The area of the roundish part of the bursa copulatrix containing a spermatophore (bounded by a broken line) was measured and used as “spermatophore” size.

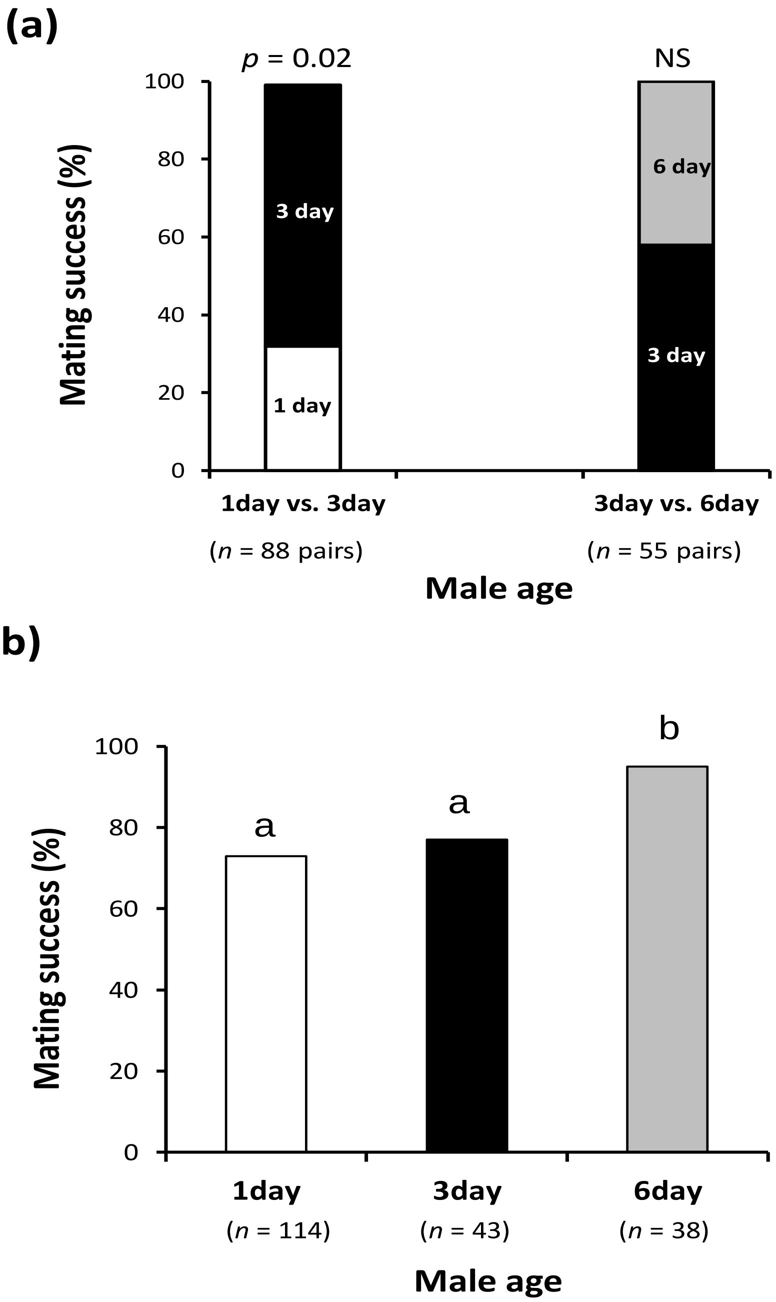

Fig. 1.3: Rate of mating success among different-aged males in the (a) two-choice test and (b) no-choice test. The difference in mating success was analysed by chi-square test. Bars with the same letter are not significantly different. NS: not significant.

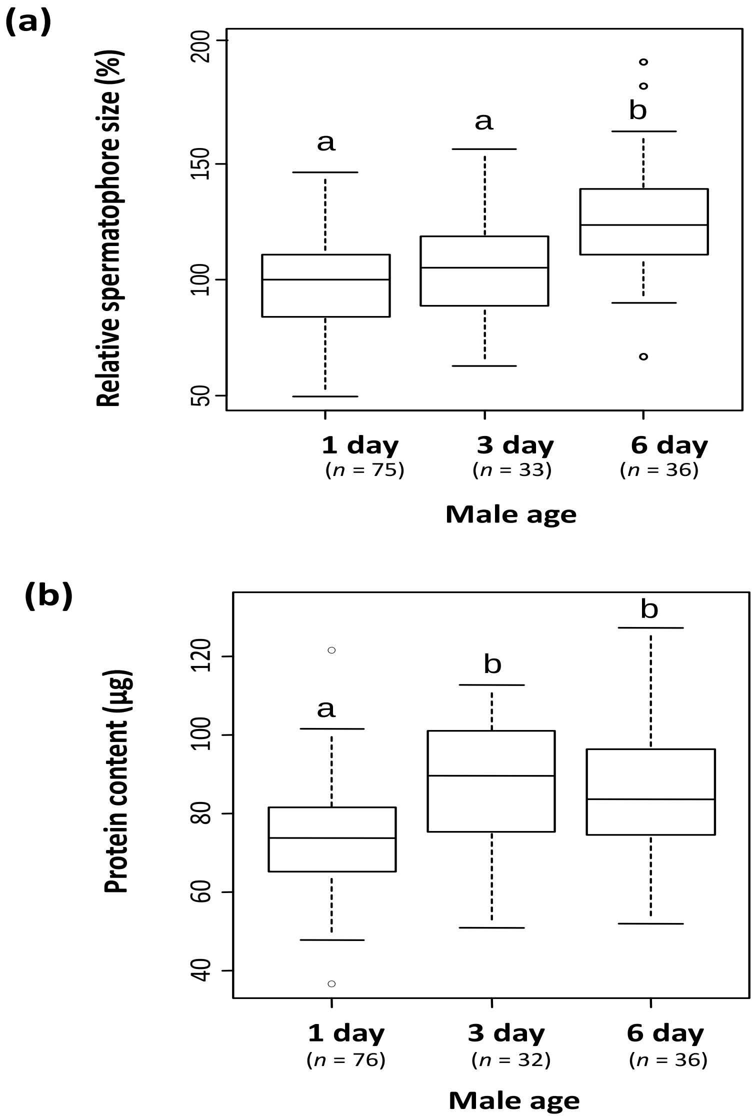

Fig. 1.4: Relationship between male age and (a) spermatophore size and (b) the amount of protein (µg) in the spermatophore. Box-and-whisker plots show the median, the first and third quartiles, and the maximum and minimum data points within 1.5-fold of the difference between the first/third quartile and median. Circles denote outliers. Boxplots with the same letter are not significantly different.

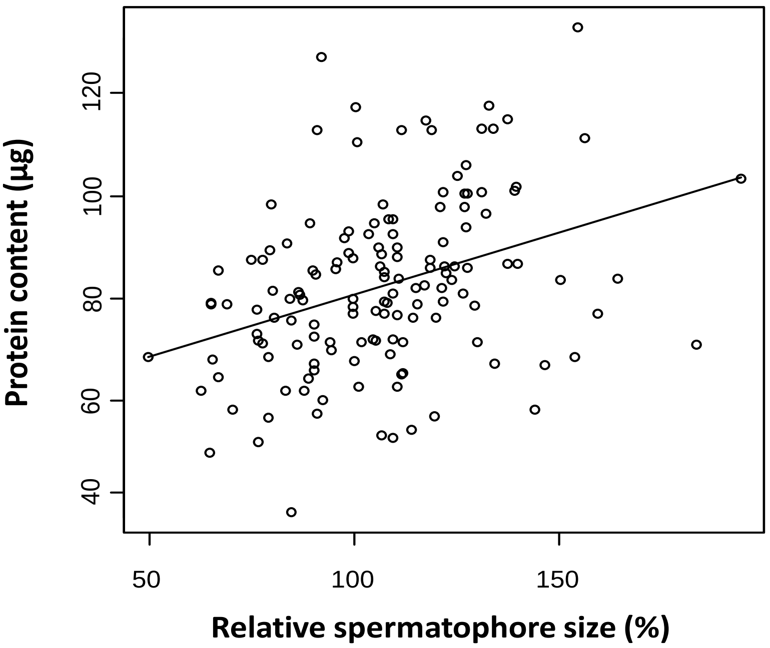

Fig. 1.5: Relationship between spermatophore size and the amount of protein (µg) in the spermatophore ($r = 0.37$, $p < 0.001$).

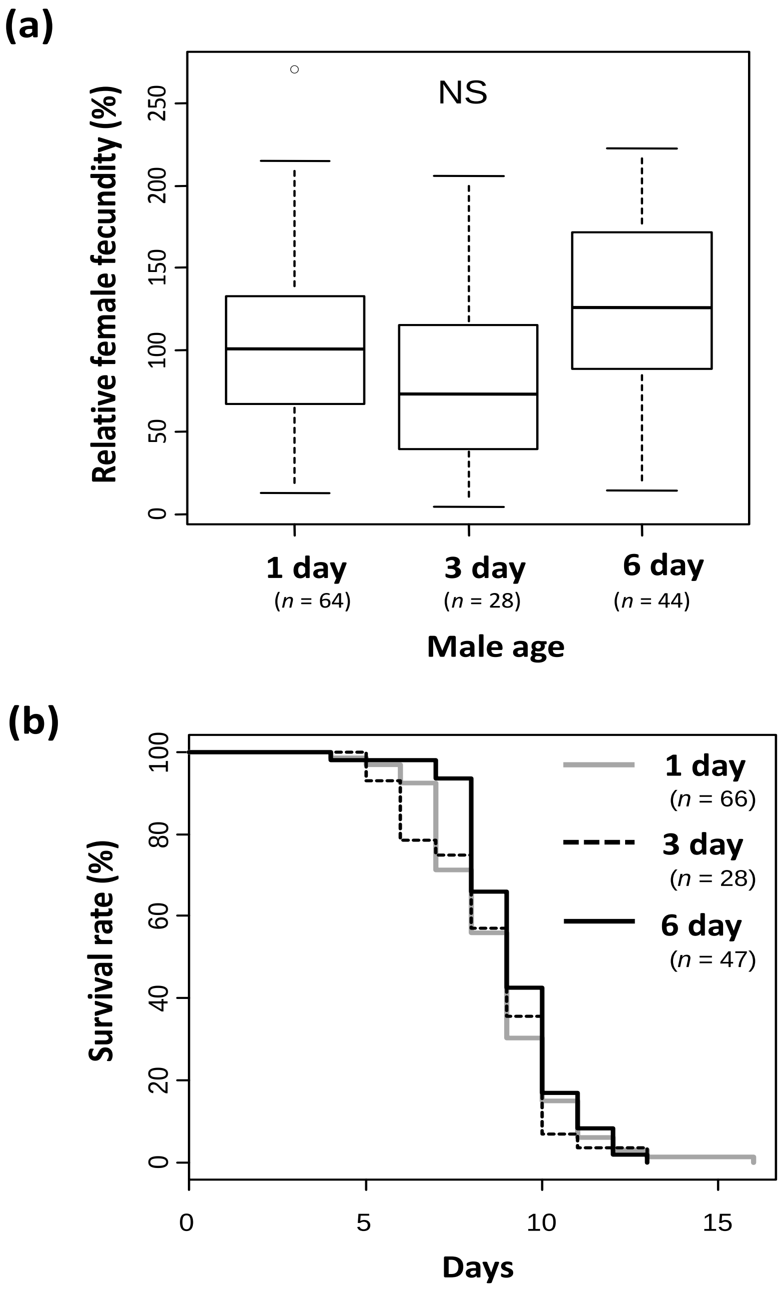

Fig.1.6: Relative fecundity (a) and survival curve (b) of females mated with different-aged males. See the legend to Fig. 1.4 for box-and-whisker plots. Results of statistical analyses are shown in Table 1.3. NS: not significant.

Table 1.1: Results of behavioural observations in the two-choice test

Behaviour	**1 day vs. 3 day (*n* = 45)**			**3 day vs. 6 day (*n* = 16)**		
	1 day	**3 day**	***p***	**3 day**	**6 day**	***p***
Number of male courtships	**0.64 ± 0.18**	**1.22 ± 0.20**	**0.006**	**1.50 ± 0.41**	**1.00 ± 0.29**	**0.21**
Frequency of female escape behaviour (%)	**32.3 ± 10.3**	**31.7 ± 6.5**	**0.62**	**37.3±11.68**	**59.2 ± 14.0**	**0.32**

Mean ± SE. Statistical analyses were conducted using GLMM with Poisson error distribution.

Table 1.2: Results of a linear model analysis of the effect of male age on spermatophore size and protein content

	Spermatophore size (model 1)			**Protein content (model 2)**		
	$F_{3,140} = 35.1$, $p < 0.001$, adjusted $R^2 = 0.42$			$F_{4,139} = 8.17$, $p < 0.001$, adjusted $R^2 = 0.17$		
	Coefficient	***t***	***p***	**Coefficient**	***t***	***p***
Male age	**43.17**	**7.24**	**< 0.001**	**6.4×10^{-3}**	**2.72**	**0.0074**
Male pupal weight	**0.66**	**0.12**	**< 0.001**	**-3.5×10^{-5}**	**–0.79**	**0.43**
Female pupal weight	**0.53**	**0.09**	**< 0.001**	**7.5×10^{-5}**	**2.05**	**0.043**
Spermatophore size	**-**	**-**	**-**	**6.3×10^{-5}**	**2.20**	**0.030**

Male and female pupal weights were entered as covariates in model 1, whereas spermatophore size was also entered as a covariate in model 2.

Table 1.3: Results of a linear model analysis of the effect of male age on female fecundity, and Cox regression model analysis of the effect of male age on female longevity

	Female fecundity (model 3) $F_{3,132} = 25.5$, $p < 0.001$, adjusted $R^2 = 0.35$			**Female longevity (model 4)** $Wald_3 = 14.4$, $p = 0.002$, adjusted $R^2 = 0.10$		
	Coefficient	***t***	***p***	**Coefficient**	***z***	***p***
Male age	**31594**	**1.73**	**0.09**	**-8.8×10^{-3}**	**–0.21**	**0.84**
Male pupal weight	**305**	**0.71**	**0.48**	**-6.8×10^{-4}**	**–0.66**	**0.51**
Female pupal weight	**2422**	**8.18**	**< 0.001**	**-2.6×10^{-3}**	**–3.81**	**< 0.001**

Male and female pupal weights were entered as covariates in model 3 and model 4.

Discussion

The higher mating success of older males has been reported in many animals including *O. nubilalis* (Lassance & Löfstedt 2009), a congener of *O. scapulalis*. In the two-choice test, significant differences in mating success and courtship frequency were observed between 1-day-old and 3-day-old males but not between 3-day-old and 6-day-old males (Fig 1.3a, Table 1.1). These results alone may appear to suggest sexual immaturity among 1-day-old males rather than an increased reproductive effort made by older males. However, the result of the no-choice test, that 1-day-old males achieved a mating success comparable to 3-day-old males (Fig. 1.3b), clearly excludes this possibility. The results of the no-choice test also suggest that 6-day-old males made greater reproductive effort than 3-day-old males (Fig. 1.3b), although 6-day-old and 3-day-old males achieved a similar success rate under competitive conditions (two-choice test, Fig 1.3a).

The frequency of female escape behaviour provoked by 1-day-old and 3-day-old males was not significantly different (Table 1.1). This result indicates that the higher mating success of 3-day-old males was attributable to an increased frequency of courtship, not to a female preference for 3-day-old males; however, this result differs from that of a previous study using *O. nubilalis*, in which the male-age-dependent variation in mating success was attributed to female choice (Lassance & Löfstedt 2009). The reason for the absence of female preference for older males in *O. scapulalis* is unclear. However, some models suggest that female preference for older males may not evolve if the cost incurred by deterioration or decrease of sperm in older males outweighs the "good genes" benefits from them (Brooks & Kemp 2001; Beck & Promislow 2007; Johnson & Gemmell 2012).

I found that old (6-day-old) males produced a larger spermatophore than 1- and 3-day-old males (Fig. 1.4a and Table 1.2). Spermatophore production imposes a substantial cost to lepidopteran males (Oberhauser 1989). In *O. scapulalis*, 6-day-old males probably have a diminished chance of future mating considering the increased mortality after 6 days even under optimal laboratory conditions (Fig. 1.1). Selection may favour the strategic age-dependent allocation of limited resources in the spermatophore. The increased investment in the spermatophore by older males is consistent with previous studies conducted using several other species (Jones et al. 1986; Oberhauser 1988; Delisle & Bouchard 1995; Hughes et al. 2000) including *O. nubilalis* (Milonas & Andow 2010). Protein content positively correlated with spermatophore size (Fig. 1.5). However, the correlation was not strong, and the relationship between spermatophore size and male age was not fully consistent with that between protein content and male age (Fig. 1.4a, b). In this study, we found that older (6-day-old) males copulated for a longer period than 1-day-old males. Prolonged copulation is sometimes explained as a male adaptive mechanism for reducing the risk of sperm displacement (Parker 1970). However, in this monandrous species, it is not necessary for males to guard females from rivals. The increased copulation duration in 6-day-old males of *O. scapulalis* may be simply attributable to the senescence-related physiological constraints of transferring ejaculates (Hughes et al., 2000) or to producing a larger spermatophore.

The lifetime resource allocation strategy may vary among individuals because of variation in resource acquisition due to genetic and/or environmental factors (Kokko 1997; Brooks & Kemp 2001). For example, male crickets reared on a high-quality diet are known to invest heavily in courtship songs at an earlier stage and live shorter than males reared on a low-quality diet (Hunt et al. 2004). My experimental approach identifies the ‘average’ strategy in a population, but cannot detect the variation in strategy among individuals. Moreover, the male strategy for allocation across successive matings remains unclear in our study because we used only virgin males. Spermatophore size in *O. scapulalis* probably decreases with multiple matings as in many other lepidopteran species (South & Lewis 2011). In the field, older males are likely to have already experienced copulations. Individual-based investment across successive matings needs to be examined to further understand the male strategy.

I found that males produced a larger spermatophore with more protein when they mated with heavier females (Table 1.2). Males probably gain from size-dependent allocation because heavier females had higher fecundity (Table 1.3). I also found that heavier males produced a larger spermatophore. A positive relationship between male weight and spermatophore size is reported in several other species (Wiklund & Kaitala 1995; Bissoondath & Wiklund 1996b). Heavier males probably have more resources to afford to females.

Age of the male mates did not affect the female's fecundity and longevity in this species (Fig. 1.6a and Table 1.3). This result suggests that females did not receive any benefit in terms of fecundity and longevity from mating with males that produced larger and protein-rich spermatophores. Similar results were reported for *Choristoneura rosaceana* (Delisle & Bouchard 1995) and *Siederia listerella* (Elzinga et al. 2011). In contrast, in *O. nubilalis*, Milonas & Andow (2010) found a higher fecundity of females mated with 3-day-old males, despite that the size of their spermatophores was intermediate. Why didn't the larger spermatophores produced by older males increase female fitness? One possible reason is an age-related decline in the number of sperm. In *O. nubilalis*, testes size was found to decrease with age (Milonas & Andow 2010). If older males of *O. scapulalis* produce fewer sperm than younger males, this may counteract the nutritional benefit of a larger spermatophore. The larger spermatophore produced by older males could delay the remating or reduce the receptivity of females, as reported in other lepidopteran species (e.g., Wedell & Cook 1999; Marcotte et al. 2006; McNamara et al. 2009). However, these effects seem to be irrelevant in this species because females mate only once. Meanwhile, we did not examine fitness components other than longevity and fecundity, for example, the total number of eggs hatched (fertility) and oviposition period. Fertility and the oviposition period may be dependent on the age of male mates as reported in other species (Jones & Elgar 2004; Avent et al. 2008; Liu et al. 2010; Milonas & Andow 2010; Elzinga et al. 2011).

In summary, older males of *O. scapulalis* had a higher mating success because of their higher courtship frequency, and they produced a larger spermatophore with more protein than younger males. The greater reproductive investment of older males is consistent with the terminal investment hypothesis.

Chapter 2

DIFFERENTIAL RESOURCE ALLOCATION BY VIRGIN AND MATED MALES AND ITS CONSEQUENCE EFFECT ON FEMALE REPRODUCTIVE FITNESS

Chapter 3

FEMALE CONDITION-DEPENDENT ALLOCATION OF NUPTIAL GIFTS BY MALES

Abstract

The males of many insect species transfer a spermatophore, i.e. a proteinaceous capsule containing sperm, to females during copulation, and this may also function as a nuptial gift. If production of the spermatophore is costly and variations in the quality of females are large, males may strategically allocate their investment based on the quality of the mate in order to maximize their own reproductive success. I examined the size and protein content of spermatophores transferred to females of different ages and body sizes, and also to water-deprived and water-replete females in the moth *Ostrinia scapulalis*. Males transferred a spermatophore of a smaller size or with less protein to older females, smaller females, and water-deprived females. These results indicated that *O. scapulalis* males manipulated their reproductive investment based on the conditions of the mate. I also demonstrated that older males varied their resource allocation to a greater extent in response to female conditions than younger males. Thus, resource allocation by males in this species is modulated by both female conditions and the age of the males.

Introduction

Females are generally considered to be more choosy about the quality of mates than males (Darwin 1874, Bateman 1948, Trivers 1972, Williams 1975), and this has been attributed to differences in the investment involved in a single gamete; females produce a few large ova while males produce countless and relatively cheap sperm (Bateman 1948, Trivers 1972). However, increasing evidence has shown that the male reproductive investment imposes a substantial cost on the males of diverse taxa (Birkhead 2000). Even though it may be cheap to produce a single sperm, the production of a large number of sperm is not (Dewsbury 1982; Van Voorhies 1992; Gage & Cook 1994; Pitnick et al. 1995; Olsson et al. 1997). Furthermore, the male reproductive investment is not limited to the production of sperm: it includes nuptial gifts, territorial guarding, and parental care. When the male investment is costly and variations in the quality of females are large, the males of polygynous species may strategically allocate an investment in females that provides the greatest reproductive returns, thereby saving resources (Bonduriansky 2001, Edward and Chapman 2011). As predicted, the males of many species provide more resources such as nuptial gifts to larger females (Gage 1998; MacDiarmid & Butler 1999; Reading & Backwell 2007, Lupold et al. 2011; Nandy et al. 2012), younger females (Weislo 1992; McDonald & Borden 1996), and females in a better nutritional condition (Engqvist & Sauer 2002, 2003; Nandy et al. 2012). This strategic resource allocation by males is referred to as cryptic male choice (Bonduriansky 2001).

Males may also modulate the level of choosiness depending on their own age (Bonduriansky 2001; Engqvist & Sauer 2002; Dukas & Baxter 2014). The life-history theory predicts that older males invest more resources in low-quality females because they have fewer chances of future matings than younger males (Galvani & Johnstone 1998;

Kvarnemo & Simmons 1998; Thomas et al. 1998; Bonduriansky 2001; Engqvist & Sauer 2002; Nandy et al. 2012). For example, older males of the scorpionfly *Panorpa cognate* have been shown to decrease their sensitivity in providing nuptial gifts to the quality of females because they have fewer chances of future matings than younger males (Engqvist & Sauer 2002).

The males of Lepidoptera transfer a capsule called a "spermatophore" to females during copulation. It contains not only sperm, but also nutrients such as proteins, carbohydrates, mineral, and sugars, which may increase the female reproductive output or contribute to female somatic maintenance (Engebretson & Mason 1980; Marshall 1982, 1985). The production of spermatophores imposes a substantial physiological cost on males (Wedell & Cook 1999). Among the nutrients provided in spermatophores, protein is potentially important for egg production (Wheeler 1996). Protein is generally a limited resource for adult moths because their food, nectar, contains very little protein (Baker & Baker 1973; O'Brien et al. 2002).

The main objective in the present study was to determine whether males manipulated their investment in spermatophore depending on the quality of the mate. I specifically tested the prediction that males would produce a smaller spermatophore containing less protein when they mated with older and smaller females and females under physiological stress (e.g. water-deprived). Furthermore, older *O. scapulalis* males are known to invest more in reproduction than younger males (chapter 1), which suggests that males allocate their resource strategically across their lifetime. Thus, males may also change their response to the quality of females based on their own age. Therefore, I investigated whether the magnitude of manipulation varied with the age of males.

Materials and Methods

Insects

O. scapulalis females, most of which had mated, were caught in a field in Matsudo, Chiba Prefecture, Japan (35.7°N, 139.9°E) in July 2011. All experimental insects were maintained in the laboratory as described in chapter 1.

Female conditions

All experiments were conducted under the same laboratory conditions as described in chapter 1. I conducted two experiments (experiments 1 and 2) to examine the effects of the quality of females. In experiment 1, I examined whether males allocated less resources to old females than to young females. As the average female longevity is ca. 9 days (chapter 1), I used 3-day-old- and 7-day-old females to represent 'young' and 'old'. I also examined the interaction between male age (1-day-old young males and 3-day-old middle-aged males) and female age. Thus, I conducted a 2 × 2 factorial design experiment (3-day-old female × 1-day-old male (n = 71), 7-day-old female × 1-day-old male (n = 56), 3-day-old female × 3-day-old male (n = 44), 7-day-old female × 3-day-old male (n = 51)). A previous study reported that females begin emitting sex pheromones in the fourth hour of scotophase (D4) (Huang et al. 1997). Accordingly, female and male couples were singly placed in a steel wire screen cages (150 ml) at D4. Wet tissue papers were placed on the bottom of the cages to provide water to the insects. The cages were checked for the occurrence of mating every 30 min with the aid of a 5-W red light bulb during scotophase.

In experiment 2, I examined whether males allocated less resources to females deprived of water than to those provided with water (control). Three-day-old virgin males and females were used in this experiment. Newly emerged female moths in the water-deprived group (n = 60) were kept in 430-ml plastic cups individually without being provided with water until mating, while females in the control group (n = 45) were provided with water. The mating experiment was conducted as described above.

I used insects of various weights in both experiments because I also wanted to examine the effects of body weights of both sexes on resource allocation by males.

Spermatophore size and protein content

Detailed methods for analyses of spermatophores are described in chapter 1.

Statistical analyses

All statistical analyses were performed using the statistical software package R version 2.12.1 (R Development Core Team, 2010). I examined the effects of female age on male resource allocation by linear models in experiment 1. I used spermatophore size or protein content as an objective variable. I entered female age, male age, female weight, male weight, the interaction between male age and female age, and the interaction between male age and female weight as explanatory variables. I examined the effect of female treatment (with or without water) on male resource allocation by linear models in experiment 2. I used spermatophore size or protein content as an objective variable, and entered treatment, female weight, and male weight as explanatory variables.

Results

Experiment 1 (female age)

Fifty-six percent (40/71) and 71% (40/56) of 1-day-old males successfully copulated with 3-day-old and 7-day-old females, respectively, with no significant difference being observed between the percentages (Fisher's exact test, $p = 0.10$). Eighty-four percent (37/44) and 73% (37/51) of 3-day-old males successfully copulated with 3-day-old and 7-day-old females, respectively, with no significant difference being observed between the percentages (Fisher's exact test, $p = 0.22$). Three-day-old males produced a larger spermatophore than 1-day-old males ($p < 0.0001$, Table 3.1, Fig. 3.1a). Spermatophore size was positively related to female and male weights ($p = 0.026$ and $p < 0.0001$, respectively, Table 3.1). However, female age did not affect spermatophore size ($p = 0.125$, Table 3.1, Fig. 3.1a). The interaction between female age and male age and between male age and female weight were not significant ($p = 0.904$ and 0.139, respectively, Table 3.1).

Males invested more protein when they copulated with younger females than older females ($p < 0.0001$, Table 3.1). Three-day-old males invested more protein than 1-day-old males ($p < 0.0001$, Table 3.1). The interaction between female age and male age ($p = 0.0030$, Table 3.1) indicated that males differentially allocated protein depending on both their own age and female age. While three-day-old males that copulated with 7-day-old females provided 12.3% less protein than those that copulated with 3-day-old females (linear model, female age: $F_{1,70} = 38.6$; $p < 0.0001$; female weight: $F_{1,70} = 16.5$; $p = 0.0001$; male weight: $F_{1,70} = 2.80$; $p = 0.098$; Fig. 3.1), 1-day-old males that copulated with 7-day-old females provided only 4.4% less protein than those that copulated with 3-day-old females (linear model, female age: $F_{1,76} = 7.41$; $p = 0.008$; female weight: $F_{1,76} = 2.60$; $p = 0.11$; male weight: $F_{1,76} = 4.64$; $p = 0.034$; Fig. 3.1b). Protein content was positively related to female and male weights ($p < 0.0001$ and $p = 0.0079$, respectively, Table 3.1). Interaction between male age and female weight was marginally significant ($p = 0.063$, Table 3.1), and male age-specific analyses revealed a positive relationship between protein content and female weight in 3-day-old males, but not in 1-day-old males (see above for statistical values; Fig. 3.2).

Experiment 2 (water access)

Eighty-two percent (37/45) and 73% (42/60) of 3-day-old males successfully copulated with water-deprived and water-replete females, respectively, with no significant difference being observed between the percentages (Fisher's exact test, $p = 0.18$). Males transferred a 7.4% smaller spermatophore to water-deprived females than control females ($p = 0.0080$, Table 3.2, Fig. 3.3a). Spermatophore size was positively related to both female and male weights ($p = 0.026$ and $p < 0.0001$, respectively, Table 3.2). On the other hand, no significant differences were observed in the protein content in the spermatophores between those transferred to water-deprived females and those transferred to water-replete females ($p = 0.12$, Table 3.2, Fig. 3.3b). Protein content was not related to female or male weight ($p =$ 0.17 and 0.17, respectively, Table 3.2).

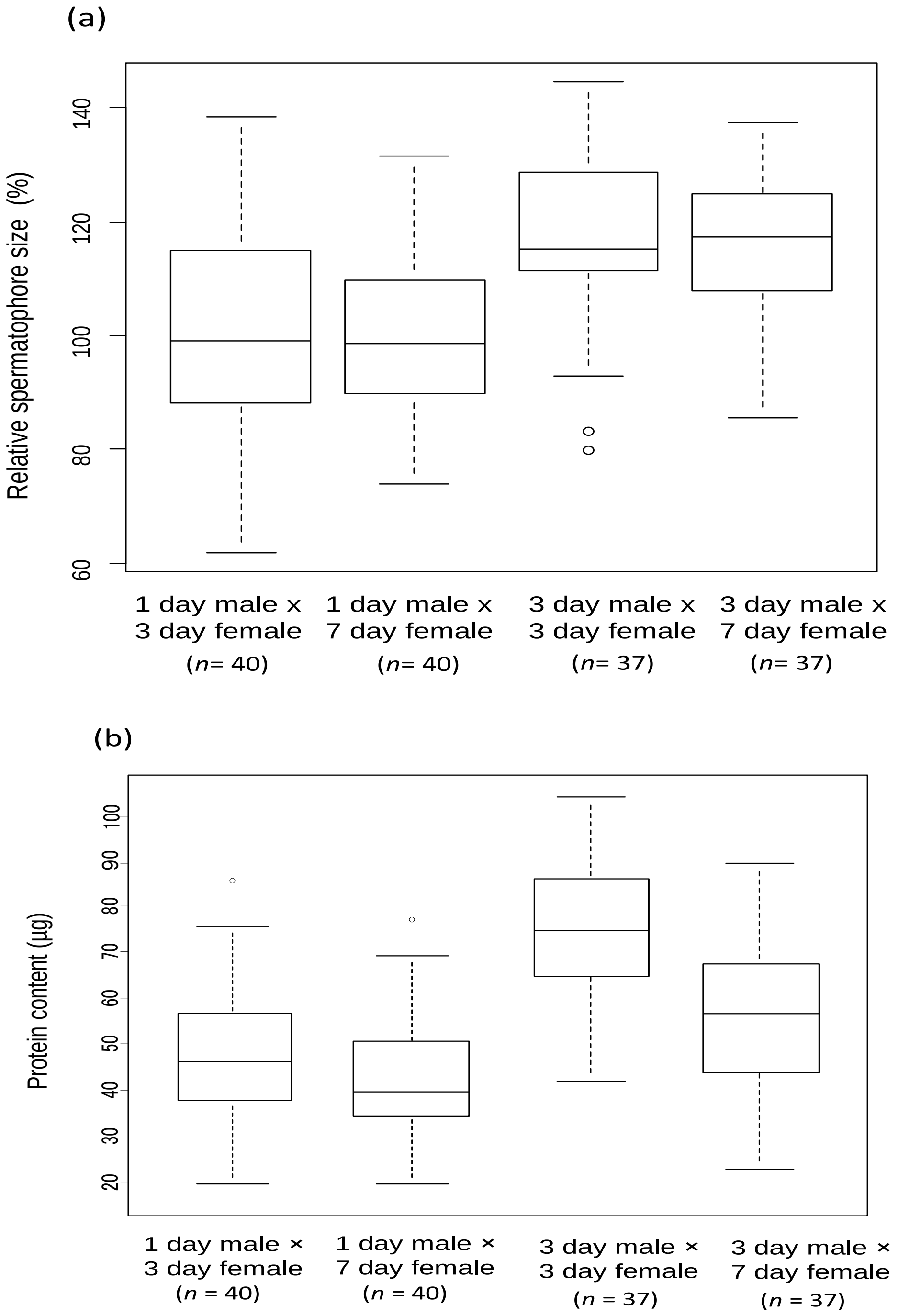

Fig. 3.1: Relative spermatophore size (a) and protein content in a spermatophore (b) produced by 1-day-old or 3-day-old males that copulated with 3-day-old or 7-day-old females

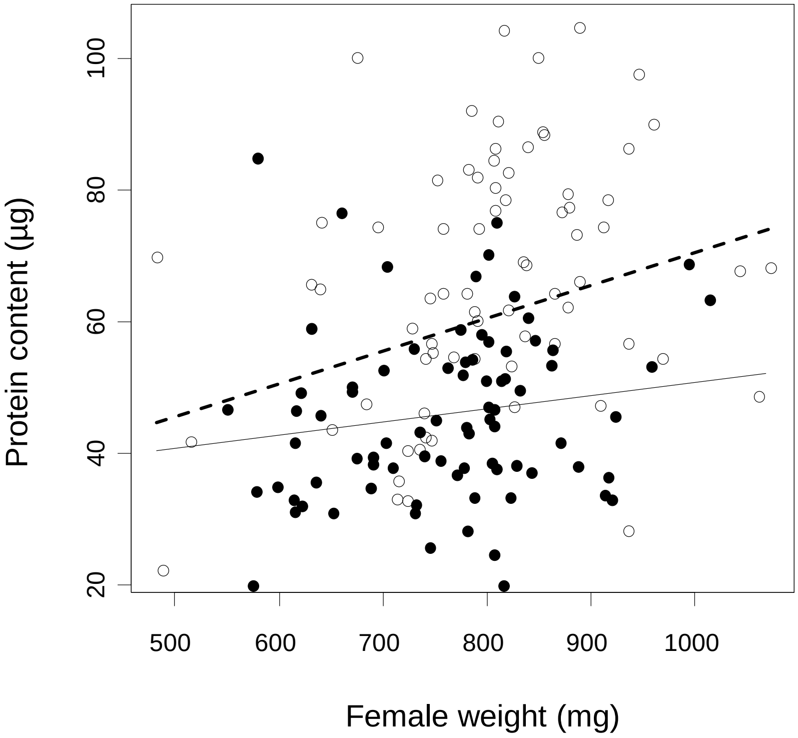

Fig. 3.2: Relationship between female weight (mg) and protein content (µg) in the spermatophore transferred to females of different ages. Solid symbols, old (7-day) females; open symbols, young (3-day) females. The lines are from least-squares regressions. Solid line, old females; dashed line, young females.

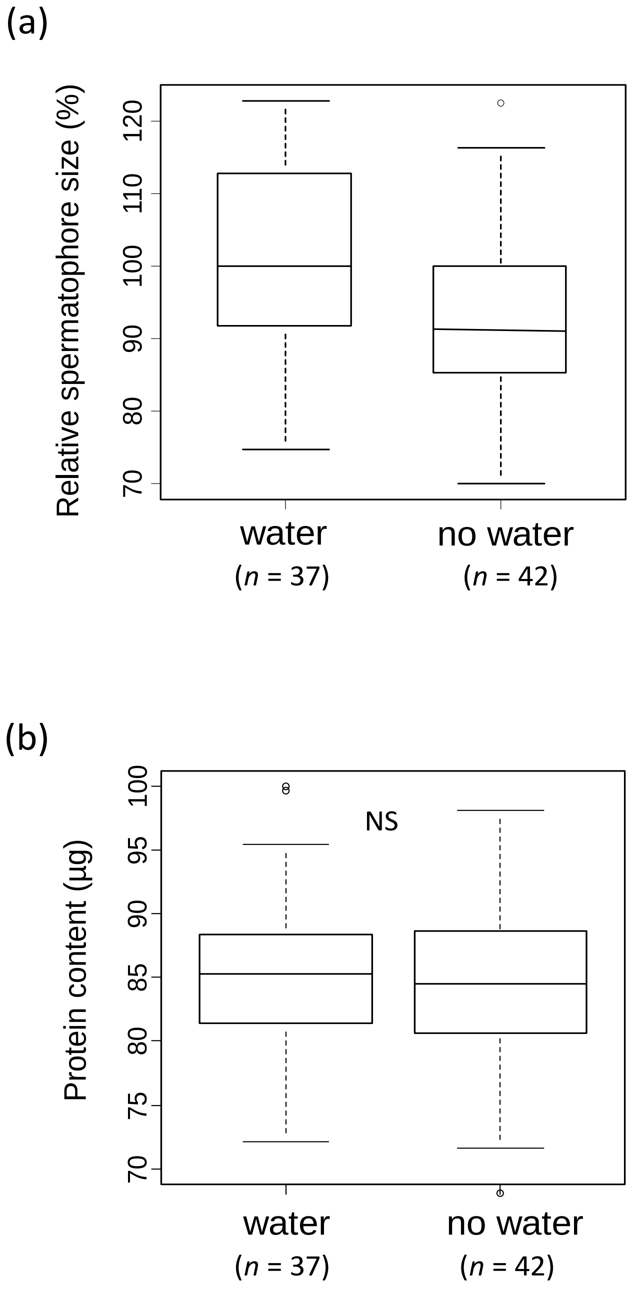

Fig.3. 3: Relative size of the spermatophore (a) and protein content (µg) (b) transferred to females provided with or without water.

Table 3.1: Results of linear model analysis of the effects of female age on spermatophore size and protein content

	Spermatophore size $F_{6,147} = 27.1, p < 0.001$, adjusted $R^2 = 0.51$			Protein content $F_{6,147} = 25.1, p < 0.001$, adjusted $R^2 = 0.49$		
	Mean SS	*F*	*p*	Mean SS	*F*	*p*
Female age	22.9	2.38	0.125	0.089	42.5	< 0.0001
Male age	483	50.2	< 0.0001	0.140	67.4	< 0.0001
Female age × Male age	0.14	0.015	0.904	0.019	9.13	0.003
Female weight	48.9	5.09	0.026	0.038	18.1	< 0.0001
Male weight	660	68.6	< 0.0001	0.015	7.25	0.0079
Male age × Female weight	21.2	2.21	0.139	0.0073	9.13	0.063

Table 3.2: Results of linear model analysis of the effects of female treatment (provided with or without water) on spermatophore size and protein content

	Spermatophore size $F_{3,75} = 17.0, p < 0.001$, adjusted $R^2 = 0.38$			Protein content $F_{3,75} = 2.67, p = 0.054$, adjusted $R^2 = 0.06$		
	Mean SS	F	p	Mean SS	F	P
Treatment	66.0	7.43	0.008	0.0068	2.54	0.12
Female weight	46.0	5.18	0.026	0.0052	1.94	0.17
Male weight	255	28.8	< 0.0001	0.005	1.88	0.17

Discussion

Female fecundity has generally been positively related to body size (Honek 1993) and inversely related to age (Xu & Wang 2009). A strong correlation between female body size and fecundity has also been reported in *O. scapulalis* (chapter 1). The fecundity of the old (7-day-old) females of *O. scapulalis* is expected to be low because they were less likely to survive until the completion of oviposition given that their mean longevity is ca. 9 days (chapter 1). In addition to body size and age, environmental and physiological stresses may also affect the fecundity of females. Water-deficiencies have previously been shown to severely depress the fecundity of the congener *O. nubilalis* (Kira et al. 1969). In the present study, the males of *O. scapulalis* transferred 1) a spermatophore containing less protein to older females than to younger females, 2) a smaller spermatophore containing less protein to smaller females than to larger females, and 3) a smaller spermatophore to water-deprived females than to water-replete females. All these results are consistent with the hypothesis that *O. scapulalis* males may increase or decrease their investment in the spermatophore depending on the expected fecundity of the mate.

The production of spermatophores imposes physiological costs on males. For example, part of the protein in a spermatophore is produced by breaking down flight muscles (Stjernholm & Karlsson 2006) because the required amount of protein cannot be obtained from the diet in adult Lepidoptera. This breaking down of flight muscles may decrease the efficiency of mate searching. Moreover, the production of a spermatophore has been reported to reduce male longevity in some lepidopteran species (Cordero 2000; Ferkau & Fisher 2006) Thus, adjustments to the spermatophore size and its protein content based on the conditions of the mates may represent an adaptive strategy by males to save their limited resources for future matings and maximize their expected fertilization success. In polyandrous species, larger or younger females are generally more likely to re-mate because they may be more attractive to males (Gage 1998). Males allocate more resources to such females in an attempt to increase confidence in their paternity by maximizing the female sexual refractory period and success in future sperm competitions (Bretman et al. 2009; Barbosa 2011). However, this may not be applicable to *O. scapulalis*, which is monandrous (chapter 1).

The strategic allocation of resources by males may occur at the precopulatory step, similar to some species such as the orange tip butterfly, *Anthocaris cardamines* (Wiklund & Forsberg 1986) and the antler fly *Protopiophila litigat*e (Bonduriansky & Brooks 1998). However, no significant relationship was found between female quality and mating success in O. scapulalis.

Not only males, but also females may play active roles in the transfer of ejaculate. Females of the scorpionfly *P. cognate* and cucumber beetle *Diabrotica undecimpunctata* have been suggested to manipulate sperm transfer by muscle contraction (Tallamy et al. 2002; Vermeulen et al. 2008). If this is true for *O. scapulalis*, old, small, or water-deprived females may not be able to assist in ejaculate transfer due to their poor physiological conditions, thereby resulting in a smaller spermatophore with less protein. Further experiments including the experimental manipulation of female muscle contraction are needed to determine the role of females in ejaculate transfer.

I showed that the mature (3-day-old) males of *O. scapulalis* allocated more resources to females than younger (1-day-old) males. The life-history theory predicts that the reproductive investment should be increased when males become older and the chance of future mating opportunities decreases (Williams 1966). The greater investment of mature males than young males is consistent with this prediction. This theory further predicts that males should be less prone to selectiveness and conserve reproductive resources toward the end of their lifespan (Engqvist & Sauer 2002; Reinhold et al. 2002). However, I found that mature males were sensitive to female age and body weight. This result suggested that mature males may be more selective than young males, which appears to contradict this prediction and previous findings in other species (Engqvist & Sauer 2002). The younger males of *O. scapulalis* are known to have lower mating success than older males (chapter 1). Younger males may be willing to invest more resources in females with a low reproductive potential than older males. In the present, I did not examine individual variations in resource allocation strategies throughout the life-span of this moth. However, given that the longevity of *O. scapulalis* males is highly variable (6-18 days, chapter 1), resource allocation at a certain time point may differ among individuals. Although I need to examine individual-based allocations to elucidate mating strategies throughout the life span of this moth, my results suggest that males change their strategy of resource allocation as they age.

In conclusion, the males of *O. scapulalis* provided more nutrients to larger, younger, and healthier females. Although males did not choose mates prior to copulation, they allocated their limited resources to females with a high reproductive potential, thereby maximizing their total reproductive success. Furthermore, the sensitivity of males to female conditions changed in a manner that was dependent age. These results highlight the importance of considering the conditions of both females and males as determinants of the amount of investment transferred to females.

Chapter 4

EFFECTS OF DIAPAUSE ON POST-DIAPAUSE REPRODUCTIVE INVESTMENT

GENERAL DISCUSSION

Several studies focused on reproductive investment in females because investments by females are more important for reproductive fitness. Like females, the study of male reproductive investment is required for understanding of evolutionary biology. Male reproductive success depends on the increasing number of females that he can inseminate throughout his lifetime (Bateman 1948; Trivers 1972). Nevertheless, reproduction can be costly for males when they provide large nutritious ejaculates or nuptial gifts to the females during copulation (Hunt et al. 2004; McNamara et al. 2008). As a result, males should adjust strategically their reproductive investment (Parker 1983; Bonduriansky 2001; Reinhold 2002) with respect to their conditions (e.g., age, size and mating history), the quality of their mates and environmental conditions in order to reduce their reproductive cost.

In chapter 1, I found that older *Ostrinia scapulalis* males had higher mating success and courtship activities than younger males because of their higher courtship frequency. The higher mating success of older males has been reported in many animals (Felton et al. 2006; Velando et al. 2006; Fischer et al. 2008; Willisch et al. 2012) including *O. nubilalis* (Lassance & Löfstedt 2009), a conger of *O. scapulalis.* Moreover, I found that older males produced a larger spermatophore with more protein than younger males. Spermatophore production imposes a substantial cost to lepidopteran males (Oberhauser 1989). Selection may favour the strategic age-dependent allocation of limited resources in the spermatophore because older males probably have diminished chance of future mating considering their increased mortality (Fig. 1.1). The increase in investment in the spermatophore by older males is consistent with previous studies conducted using several other species (Jones et al. 1986; Oberhauser 1988; Delisle & Bouchard 1995; Hughes et al. 2000) including *O.*

nubilalis (Milonas & Andow 2010). The greater reproductive investment of older males is consistent with the terminal investment hypothesis. Despite the age-related variation in spermatophore size and protein content, male age did not affect female fecundity or longevity. A decrease in the number of sperm in the older males might counteract the nutritional benefit of larger spermatophore. Additionally, other fitness components (e.g. fertility) than fecundity and longevity may be influenced by male age.

The male strategy for allocation of resources across successive matings remained unclear in the study in chapter 1 because only virgin males were used. In chapter 2, I investigated the effect of male mating history on male spermatophore investment and female fitness consequences. A male's previous mating experience may influence not only its ability to obtain subsequent matings but also their reproductive investment (Schlaepfer & McNeil 2000; Milonas et al. 2011; Bissoondath & Wiklund 1995; 1996; Torres-Vila & Jennions 2005; Vande Velde et al. 2011). Spermatophore size and protein content of the same males decreased from first to second mating. For males of the same age, transferred spermatophore size and protein content was higher in virgin males (3-day-old) than in mated males (3-day-old), indicating that mating history per se rather than male age resulted in decreased in spermatophore investment. Males of monandrous species should allocate most of their resources only in their first matings because the number of male life-time matings should be low and operational sex ratio (OSR) is always male-biased as females are no longer receptive after the first mating. Moreover, female fecundity and longevity were not affected by male mating history, that is, whether she mated with virgin or mated males, within the same male or males of the same age. The amount of male resource investments became less in the second mating one day after the first mating, but probably, they are still sufficient to fertilize the entire egg complement of females. Although spermatophore size and protein amount declined after the first mating, males might be able to produce more or constant amount of other components of spermatophore (e.g., sperm and other nutrients), which are also important for female reproduction in second mating (Engebretson & Mason1980; Marshall 1982, 1985). In chapter 2, I found that spermatophore size decreased in the second mating, suggesting that cost of reproduction is significantly high for males.

If the cost of reproduction in males is high and variation in female quality varies, male mate choice is likely to evolve (Bonduriansky 2001; Edward & Chapman 2011). In chapter 3, I examined whether male reproductive investment changed in response to female quality (size, age, water-deprived). I found that males transferred a spermatophore of a smaller size or with less protein to older females, smaller females, and water-deprived females. These results indicated that *O. scapulalis* males manipulated their reproductive investment based on the conditions of the mate. Female fecundity has generally been positively related to body size (Honek 1993) and inversely related to age (Xu & Wang 2009). A strong correlation between female body size and fecundity has also been reported in this species (chapter 1). In addition to body size and age, environmental and physiological stresses may also affect the fecundity of females. Water deficiencies have previously been shown severely depresses the fecundity of the congener *O. nubilalis* (Kira et al. 1969). *O. scapulalis* males may increase or decrease their investment in the spermatophore depending on the expected fecundity of the mate. Male reproductive investments often change with age in this species (Chapter 1). For this reason, I also reexamined if age-dependent variation in male mate choice based on female quality occur. Theory predicts that males should be less prone to choosiness and conserve reproductive resources towards the end of their lifespan (Engqvist & Sauer 2002; Reinhold et al. 2002). In chapter 3, I found that mature males were more responsive to female age and body weight. This suggested that mature males may be more selective than young males, which appears to contradict this prediction and previous findings in other species (Engqvist & Sauer 2002). Although we need to examine individual-based resource allocations to elucidate mating strategies throughout the lifespan, the present results suggest that males change their strategy of resource allocation as they age.

Environmental factors are important for individual fitness. Male investment is also affected by environmental factors (e.g. temperature, nutritional stress, diapause, etc.) (Fox et al. 2006; Wedell 1994; Sadakiyo & Ishihara 2012). These environmental factors are associated with the cost of males, which in turn cause decrease in male ejaculate investment to females. As a result, females are influenced directly by the environmental factors and indirectly by males. However, the environmental effects on male ejaculate investment have been largely ignored. In chapter 4, I focused on the effect of environmental factors on reproductive investment in both males and females. I tested the effects of diapause on post-diapause males and females reproductive investment. I have shown that *O. scapulalis* males and females suffered from the cost of diapause. The cost of diapause differed between males and females. It affected post-diapause reproduction in females, but not in males. There was no relationship between diapause duration (short and long) and post-diapause reproduction in both males and females. My results suggest that significant decline in post-diapause female reproductive output was due to her own cost of diapause but not influenced by cost of diapause male.

In chapter 1 & 2, variation in the quality of male nuptial gift in response to their age and mating history did not affect female reproductive fitness in terms of fecundity. The other fitness consequences of variation in quality of male nuptial gift for female and offspring fitness remain to be tested. On the other hand, variation in male conditions and reproductive investments may influence female reproductive investment, reflecting females to adjust their resource allocations in relation to their partner quality (Cunningham & Russell 2000; Goncalves et al. 2010; Pischedda et al. 2011). When mating with younger and experienced males whose allocate lesser amount of resources, compared to older and virgin males, females may increase their overall fecundity by investing more resources per offspring in order to compensate for the cost on their fitness. Contrary to this prediction, no differences in female reproductive investment are also predicted when mating with high or low quality males. That is, female may produce constant number of eggs in her life-time even if she receives a high quality of ejaculate invested by male. In the future, to fully understand reproductive biology of *O. scapulalis*, studies focused on variation in egg size and number for female reproductive investment in response to male quality are needed.

In this thesis, I reported and discussed my findings of the reproductive investment in adzuki bean borer, *Ostrinia scapulalis*. My studies show that males did not increase their reproductive success simply by maximizing the number of females he inseminates. Males allocated their resources strategically depending on their own conditions, quality of their mate or environmental conditions in order to increase their reproductive success. Such knowledge noted in my thesis should provide many fruitful avenues for further investigations of male reproductive strategy in evolutionary biology.

Summary

Studies on the reproductive investment in the adzuki bean borer, *Ostrinia scapulalis*
（アズキノメイガの繁殖投資に関する研究）

Reproductive success is one of the important measurements of the fitness of insects. The study of reproductive success is required for understanding of evolutionary biology. Several studies focused on reproductive investment in females because investments by females are more important for reproductive fitness. Like females, the study of male reproductive investment is required for understanding of evolutionary biology. Male reproductive success depends on the increasing number of females that he can inseminate. Nevertheless, reproduction can be costly for males when they provide nutritious ejaculates or nuptial gifts to the females during copulation. As a result, males should use a wide variety of strategies to allocate their resource carefully to reduce their reproductive cost.

Lepidopteran species are particularly interesting for exploring variation in male pre- and post-copulatory reproductive strategies. Males use various form of courtship displays (e.g., visual, acoustic and olfactory stimuli, etc.) during courtship to attract females. Moreover, males of many Lepidopteran species provide a nuptial gift in the form of capsule, called "spermatophore" that contains sperm and accessory gland nutrients (e.g., carbohydrate, protein, water, etc.) to females during copulation. The production of spermatophore is known to be physiological costly for males because of the time required to recover before producing another spermatophore. Evolutionary theory predicts that it may function as "*paternal investment"* (i.e., increasing female reproductive output with male donated nutrients) and/or "*male mating effort"* (i.e., delaying female re-mating). Males transfer two types of sperm, nucleated, fertile "*eupyrene*" sperm and anucleated, non-fertile "*apyrene*" sperm. The production of sperm can be limited for males because the number of sperm transferred by male declined with successive matings. Several studies reported that male donated nutrients are needed for egg production and somatic maintenance of females. Among nutrients, protein can be a limiting resource for males because most of the proteins come from larval feeding stage and males can obtain small amount of protein as adult stage, especially for

nectar feeding species in Lepidoptera. Thus, the protein contained in a spermatophore is also likely to be an important component of male resource allocation strategy.

The adzuki bean borer, *Ostrinia scapulalis* (Lepidoptera: Crambidae), is a monandrous species as almost all females mate only once throughout her life whereas males are polygynous. Like other *Ostrinia* species, males find conspecific females by following a female-derived sex pheromone and show a courtship display after landing close to the female. During copulation, males transfer a nuptial gift called "spermatophore" containing sperm and nutrients to females. *O. scapulalis* is a multivoltine (i.e.one generation in spring, two or three generation in autumn), a facultative diapause mediated by short day length and low temperature and enters diapause as a mature fifth-instar larva. The mature larvae stop feeding and enter diapause in late summer and emerge in spring and resume their development. In this study, I investigated variation in reproductive investment of *O. scapulalis.* I first investigated if males of *O. scapulalis* change their reproductive investments in courtship and nuptial gift as they age. Next, I examined the effect of male mating history, i.e., virgin or mated, on their reproductive investment. Then, I examined whether male reproductive investment is changed in response to female quality. Finally, I investigated the effects of diapause on post-diapause reproductive investment of males and females.

Age-related male reproductive investment in courtship display and nuptial gift

Due to a trade-off between current and future reproduction, costly reproductive investments should be increased towards the end of a lifespan when the probability of reproduction becomes low (terminal investment hypothesis). I investigated age-related changes in male reproductive investment towards courtship display and the spermatophore in three age classes (young, middle-aged and old) of *O. scapulalis*. As predicted, old males had higher mating success than young and middle-aged males in no- choice tests. Moreover, two-choice tests revealed that middle-aged males had a higher success rate than young males because of their higher courtship frequency rather than any female preference for them. It was found that old males produced a larger spermatophore than young and middle-aged males, suggesting greater reproductive effort. The protein content of spermatophores also tended to increase with male age. Despite the age-related variation in spermatophore size and protein content, age did not affect female fecundity or longevity. A decrease in the number of sperm in the older males might counteract the nutritional benefit of larger spermatophores. Alternatively, fitness components other than longevity and fecundity may be influenced by male age. The male strategy for allocation of resources across successive matings (mating history) remained unclear in this chapter because only virgin males were used.

Differential allocation of nuptial gift by virgin and mated males

Re-mating by males has profound effect on male resource allocation and female fitness consequences in several lepidopteran species. Here, I investigated the effect of male mating history on male spermatophore investments and female fitness in *O. scapulalis.* My results showed that spermatophore size and protein content produced by the same males decreased from first mating (virgin males) to second mating (mated males). Males are expected to allocate most of their resources only in their first matings and saving large amount of resources for expected future matings may be disadvantage because the numbers of male life-time matings should be low and operation sex ratio (OSR) is always male-biased as females are no longer to re-mate after first mating. Spermatophore size and nutrient contents are varied depending on the number of days between matings. In the present study, one day interval between first and second mating was not sufficient to be able to produce the second spermatophore as large as the first one. For males of the same age, transferred spermatophore size and protein content was higher in virgin males (3-d-old) and in mated males (3-d-old), indicating that mating history per se rather than male age resulted in decrease in spermatophore investment. However, female fecundity and longevity were not affected by male mating history, that is, whether she mated with virgin or mated males, within the same male or males of the same age. Although spermatophore size and protein content declined after first mating, males might be able to produce more or constant amount of other components of spermatophore (e.g. sperm and other nutrients), which are also important for female reproduction, in second mating. These results suggest that there is a cost of mating to males although males can increase their reproductive fitness by re-mating.

Female condition-dependent allocation of nuptial gift by males

The males of many insect species transfer a spermatophore, i.e. a proteinaceous capsule containing sperm, to females during copulation, and this may also function as a nuptial gift. If production of the spermatophore is costly and variations in the quality of females are large, males may strategically allocate their investment based on the quality of the mate in order to maximize their own reproductive success. I examined the size and protein content of spermatophore transferred to females of different ages and body sizes, and also to water-deprived and water-replete females in *O.scapulalis*. Males transferred a spermatophore of a smaller size or with less protein to older females, smaller females, and water-deprived females. These results indicated that *O. scapulalis* males manipulated their reproductive investment based on the conditions of the mate. I also demonstrated that older males varied their resource allocation to a greater extent in response to female conditions than younger males. Thus, resource allocation by the males of this species was modulated by both female conditions and the age of the males.

Effects of diapause on post-diapause reproductive investment in male and female

Diapause is a strategy for many insect species to overcome adverse environmental conditions. However, diapause is associated with costs because stored metabolic resources are consumed during diapause, resulting in subsequent influence on post-diapause development and reproduction. I investigated effect of diapause on post-diapause reproductive investment of both males and females in a multivoltine moth, *O. scapulalis*. Post-diapause males and females were smaller and achieved lower mating success than non-diapause individuals. Post-diapause females had lower fecundity and shorter longevity than non-diapause females. However, post-diapause males transferred a similar number of eupyrene and apyrene sperm as non-diapause males. Alternatively, we found that fecundity and longevity of females mated with diapause males and non-diapause males were not significantly different. There was no trade-off between diapause duration (short and long) and post-diapause reproductive investments in both males and females. My results suggest that significant decline in post-diapause female reproductive output was due to her own cost of diapause but not influenced by cost of diapause male.

My study showed that males did not increase their reproductive success simply by maximizing the number of females that he can inseminate. Males allocated their resources strategically depending on their own condition, their mate quality and environmental conditions to increase their reproductive success. Such knowledge noted in this study should provide many fruitful avenues for further investigations of male reproductive strategy in evolutionary biology.

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