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MECHANISMS OF THE DECLINE IN REPRODUCTIVE FITNESS WITH AGE IN THE MOTH *SPODOPTERA LITURA*

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ABSTRACT

Although several hypotheses have been proposed, the mechanisms underlying the decline in reproduction of insects with age remain unclear. In this study, the effect of aging on the reproductive performance of both sexes was studied in Spodoptera litura. Aging in both sexes significantly reduced reproductive fitness. Female aging had a significantly stronger effect than male aging on fecundity and fertility. This study clearly demonstrates that oviposition by virgin females is an important contributor to the decline in female fecundity with delayed mating. However, the reduction in fecundity in females mated late relative to females mated early was much greater than (almost three times) the number of eggs laid by virgin females before mating. What accounts for this additional loss in fecundity? To answer this question, egg production, oosorption, mating frequency and longevity in both sexes were analysed. The results suggest that S. litura females may emerge with extra nutrient reserves that can be allocated to adult survival and egg production; delayed mating may cause females to allocate more resources towards survival, leaving less for egg production after mating relative to females mated young. This mechanism is likely the key cause of the decline in fecundity due to aging. In addition, the fertility rate was significantly influenced by female, but not male, age at mating, suggesting that senescence degraded egg quality but not sperm quality. The results of this study also showed that the mating frequency of moth pairs was negatively correlated with male age at mating, which is likely the primary mechanism underlying the negative effects of male aging on female reproductive success.

Keywords: *Spodoptera litura*, Tobacco cutworm, Egg production, Mating delay, Mating frequency, Egg quality.

Contribution/ Originality

The paper's primary contribution is finding that senescence degraded egg quality but not sperm quality in the moth *Spodoptera litura*. Moreover, delayed mating in this insect may cause females to allocate more resources towards survival, leaving less for egg production after mating relative to females mated young.

1. INTRODUCTION

Aging, or senescence, is defined as the decline in reproductive fitness with age and the corresponding age-specific increase in mortality rate [1-3]. Studies of reproductive biology in Lepidoptera indicate that delayed mating in females generally has negative effects on the females' reproductive fitness [4-7]. In addition, empirical studies have shown that male age may also affect female reproductive performance [8-10]. However, other empirical studies have suggested that the senescence process influences females more than males [6, 10].

Delayed mating in females generally reduces fecundity and fertility. Several hypotheses have been proposed to explain the reduction of fecundity due to aging in females: (1) with increasing age in virgin females, fewer nutrients are allocated to the maturing ova and may even be reabsorbed from the ova [11-13], reducing the number of eggs that can potentially be fertilized; (2) females maintain an extra nutrient reservoir for survival and reproduction that they can use to produce more eggs if mated young, whereas delayed mating may require females to allocate more nutrients to survival, leaving less for the production of eggs after mating [14, 15]; (3) oviposition of unfertilized eggs by virgin females affects their overall fecundity [14-17]; (4) older females are less attractive to potential mates, reducing their mating opportunities [18-20]; and (5) senescence may reduce female ability to transport or store sperm [19], potentially reducing their fertility. However, it is still unclear whether and to what extent these factors explain the reduction in female fecundity with age.

The effects of aging on male reproductive fitness are also poorly understood. Delayed mating in males may influence male reproductive fitness by reducing sperm quality and quantity [17, 21], thus reducing female fertility and fecundity. In Lepidoptera, sperm production begins during the late larval and pupal stages, and the adult male emerges with largely functional sperm [22]. Sperm are released from the testis and are transported via the vas deferens to the duplex, where they are stored until mating [22, 23]. Sperm are released following a circadian rhythm [24, 25], and the number of sperm present in the duplex increases with the time until mating [26, 27]. Therefore, delayed mating in males may influence male reproductive fitness through a decline in sperm quality due to long-term storage [17, 21], thereby reducing female fertility and fecundity.

Pheromone-based mating disruption and the sterile insect technique (SIT) have been successfully used in pest control worldwide [28-30]. However, their success in controlling an insect pest largely depends on our understanding of its reproductive biology [5, 31, 32]. For example, an understanding of the relationship between age and reproduction is required for effective pheromone-based mating disruption, which achieves control by preventing or delaying mating [32, 33]. Knowledge of the relationship between age and reproductive performance allows the accurate assessment of the efficacy of pheromone-based mating disruption [31-33] and the optimal delay of mating of the target insects for achieving maximum control efficacy. Understanding the relationship between age and reproductive performance is also important in the implementation of the SIT, as it relies heavily on a constant supply of insects with desirable characteristics, such as optimal age [34].

The tobacco cutworm, *Spodoptera litura* (Lepidoptera: Noctuidae) is a serious polyphagous pest worldwide [35] and is a potential candidate for SIT [36]. The female sex pheromone in *S. litura* has been identified [37] and proposed for control applications [38, 39]. Importantly, *S. litura* larvae and adults are widely utilised in behaviour, physiology and molecular biology studies due to their large size and ease of breeding [15, 40, 41]. The longevity of adult moths of *S. litura* is approximately 10 d [42], and both sexes become sexually mature 24 h after emergence (1 day old) [43]. Virgin *S. litura* females lay approximately 450 unfertilized eggs before death, and no oosorption has been observed [15].

In the present study, we examined whether and to what extent mating delay in *S. litura* affects (1) reproductive fitness, (2) mating frequency, and (3) longevity in either sex. The age combination design also allowed us to determine which sex was more severely affected in these parameters. We also discuss the underlying mechanisms of the decline in reproductive fitness of males and females in relation to aging. This study provides valuable information for pest control and further investigations in this insect.

2. MATERIALS AND METHODS

2.1. Insects

S. litura were reared under a 14:10 h light:dark photoperiod at 26 ℃ and 60–80 % relative humidity. Larvae were reared on an artificial diet [44], and adult moths were fed a 10 % honey solution.

Pupae were sexed based on the morphology of the exterior paramera [45]. Male and female pupae were caged separately for later emergence to ensure virginity. Newly emerged moths (< 10 h old) were collected and weighed to an accuracy of 0.0001 g using an electronic balance (Sartorius Bp221S, Germany). Mean body weight (mean ± SD) was 158.2 ± 26.9 mg and 203.2 ±

25.8 mg for male and female moths, respectively. Only virgin moths of average body weight (i.e., within one SD of the mean) [15] were used in this study.

2.2. Influence of Age at Mating on Reproductive Performance of Both Sexes

The effect of age at mating on the reproductive performance of both sexes was studied by confining 238 individual breeding pairs of nine age combinations (Table 1) for the remainder of their lifespan in plastic boxes (25 cm long, 15 cm wide, 8 cm high), with one pair per box. Each box was provided with a 10 % honey solution as food and a paper strip (15 × 20 cm) folded in zigzag fashion as an oviposition substratum.

The eggs laid by each pair were collected daily and incubated in Petri dishes (8.5 × 1.5 cm). Eggs with black dots (larval heads) after 2 days of incubation were recorded as fertilized [43]. The number of hatched eggs was recorded 4 days after incubation. Male and female longevity was also recorded.

To determine whether age at mating of both sexes had any effect on mating frequency, we dissected each female upon her death and counted the number of spermatophores in her bursa copulatrix under a dissecting microscope (Olympus SZ2, Japan), as this number represents the number of copulations a female has achieved in this species [43].

For the treatments (age combinations) 1 × 1 and 6 × 1 (x-day-old female × x-day-old male), the numbers of unfertilized eggs laid before pairing and ovarian mature eggs at death were also recorded. To count the ovarian mature eggs at death, the ovaries were separated from the female's abdomen in saline on a plastic Petri dish (8.5 × 1.5 cm) under a dissecting microscope. The eggs in ovaries were stained with 1 % acetocarmine for 10 s [46]. The chorion of immature eggs absorbs the stain but that of mature eggs does not; therefore, unstained eggs were classified as mature and stained eggs as immature. In addition, mature eggs that are being resorbed will also stain, similar to immature eggs, as oosorption increases the permeability of eggs, although mature eggs undergoing oosorption are obviously larger than immature ones [46].

2.3. Statistical Analyses

A central composite design (CCD); i.e., response surface [47], was used to analyse the effect of age at mating on the mating frequencies (no. of spermatophores in females), fecundity (no. of eggs laid), fertility (no. of fertile eggs laid), hatchability (no. of hatched eggs), fertility rate (number of fertilized eggs laid/number of eggs laid), hatch rate (number of hatched eggs/number of fertilized eggs laid) and longevity of both sexes. The relationship between mate age and reproductive response is given by the polynomial equation: response = $\exp(\beta_0 + \beta_1 x_f + \beta_2 x_m + \beta_{11} x_f^2 + \beta_{22} x_m^2 + \beta_{12} x_f x_m)$, where β_0, β_1, β_2, β_{12}, β_{11} and β_{22} are model parameters, and x_f and x_m are female and male age at mating, respectively. Only significant terms identified after running the full regression models were retained in the final models. A log-likelihood ratio test [48] was then applied to determine whether the effect of age on reproductive fitness differed by sex.

The numbers of unfertilized eggs laid before pairing, eggs laid after pairing, ovarian mature eggs at death, total eggs produced (eggs laid before pairing + eggs laid after pairing + ovarian mature eggs at death); longevity and mating frequency between treatments 1 × 1 and 6 × 1 were analysed using an ANOVA.

The rejection level was set at $\alpha < 0.05$. All analyses were conducted using SAS 9.1 [49].

3. RESULTS

Both male and female age at mating significantly reduced female fecundity ($F_{4,127} = 4.77$, $P < 0.05$) (Fig. 1). According to the likelihood ratio test, female aging had a significantly stronger effect on fecundity than male aging ($\chi^2 = 119.77$, $DF = 2$, $P < 0.0001$). The effects of aging in both sexes on fertility and hatchability were similar to those on fecundity and are not reported here.

The fertility rate was significantly influenced by female age at mating ($F_{1,124} = 7.18$, $P = 0.0074$) (Fig. 2), whereas neither male age at mating nor the female–male age interaction had any effect ($P > 0.05$). However, neither male age at mating nor female age at mating had any effect on hatch rate ($P > 0.05$). The mean (± SE) hatch rate of all females was 90.7 ± 0.9 %.

Female longevity was significantly influenced by female age at mating and the female–male age interaction ($F_{2,129} = 6.80$, $P < 0.01$) (Fig. 3). Male longevity was significantly influenced by both male and female age at mating ($F_{4,127} = 13.52$, $P < 0.01$) (Fig. 4). The likelihood ratio test indicated that female age at mating had a significantly stronger effect on male longevity than did male age at mating ($\chi^2 = 650.45$, $DF = 2$, $P < 0.0001$).

The probability of mating frequency, evidenced by the number of spermatophores in females, was significantly influenced by male age at mating ($F_{1,130} = 4.73$, $P = 0.0297$) (Fig. 5), whereas neither female age at mating nor the female–male age interaction had any effect ($P > 0.05$).

Females of the 1 × 1 treatment laid significantly fewer eggs before pairing ($F_{1,29} = 15.64$, $P < 0.0001$) but more eggs after pairing ($F_{1,29} = 4.39$, $P < 0.05$) than those of the 6 × 1 treatment (Fig. 6). No significant difference in the number of ovarian mature eggs at death was found between these two treatments ($P > 0.05$). Overall, 1 × 1 females produced significantly more eggs over their lifetimes (eggs laid before pairing + eggs laid after pairing + ovarian mature eggs at death) ($F_{1,29} = 4.38$, $P < 0.05$) but lived shorter lives ($F_{1,29} = 10.08$, $P = 0.004$) than 6 × 1 females. No significant differences in mating frequency or male longevity were found between these two treatments ($P > 0.05$). No oosorption was found in any of the dissections.

4. DISCUSSION

Delayed mating generally reduces female reproductive fitness [10, 50]. In the present study, we also found that delayed mating in both sexes significantly reduced female reproductive success in *S. litura* (Figs 1, 2, 5 & 6). However, the likelihood ratio test showed that female aging had significantly stronger effect than male aging on fecundity and fertility, consistent with the prediction of previous studies; namely, that the senescence process influences females more than males [10, 51].

Although several hypotheses have been proposed, the mechanisms underlying the decline in female reproductive fitness with age are still unclear. Our previous study [15] showed that virgin females can lay some eggs before death. In the present study, 1 × 1 females did not lay any eggs before pairing, whereas 6 × 1 females laid 258 eggs before pairing (Fig. 6), indicating that the oviposition of unfertilized eggs by virgin females is an important contributor to the decline in female fecundity of females due to delayed mating. However, the loss in fecundity from the 1 × 1 treatment to the 6 × 1 treatment after pairing is 849 eggs, far more than 258 eggs laid before pairing. To elucidate this difference, we counted the numbers of spermatophores in females and the ovarian mature eggs at death and determined whether oosorption occurred in *S. litura.* The results (Fig. 6) revealed no significant difference in mating frequency between these two treatments and no oosorption, suggesting that mating frequency and egg reabsorbsion [11, 13] cannot explain the fecundity decrease in females with delayed mating. Lepidoptera species do not usually feed on protein sources as adults [52]; as a result, all of the materials necessary for reproduction and survival must be obtained during the larval stage. Therefore, Lepidoptera females should have evolved mechanisms for the optimal allocation of resources between survival and reproduction. In *S. litura*, female longevity is negatively correlated with egg production; i.e., the longer a female lives, the fewer eggs she lays over her lifetime [15]. Therefore, it is possible that *S. litura* females emerge with extra nutrient reserves that can be allocated to adult survival, egg production and egg maturation both before [14] and after [15] mating. Delayed mating may cause females to allocate more resources to survival, leaving less for egg production after mating, relative to females mated early [14, 15]. This process is likely the key cause of the decline in female fecundity with delayed mating. In the present study, we also found that the fertility rate was significantly influenced by female age at mating (Fig. 2), suggesting that senescence leads to degraded egg quality [27, 46] and/or diminished female ability to transport or store sperm [27].

In contrast, how aging influences male reproductive fitness remains poorly understood. Delayed mating in males may influence male reproductive fitness through a reduction in sperm quality and quantity due to longer storage and aging [17, 21], subsequently resulting in a reduction in female fertility and fecundity. However, the present study showed that female fertility rate was significantly influenced by female age at mating, whereas neither male age at mating nor the female–male age interaction had any effect, suggesting that aging in males may not affect sperm quality or quantity in *S. litura.* In the present study, we also found that mating frequency, as evidenced by the number of spermatophores in females, was significantly negatively correlated with male age at mating (Fig. 5), which is likely the primary reason male aging affected female reproductive success (Figs 1, 2 & 6). One possible reason for decreased mating success in old males may be due to diminished mating behaviour; courtship is vital for successful mating in *S. litura* [43]. Alternatively, decreased mating success in old males may be a consequence of mate choice. Traditionally, females are believed to be more choosy than males because they invest more in reproduction [53, 54]; therefore, females may be less likely to mate with old males.

Pheromone-based mating disruption has been effective in pest suppression [28-30], but the successful control of an insect pest largely depends on our understanding of its life history, particularly the effects of delayed mating [5]. Studies have found that some mature eggs [15] and sperm [22] are already present at the time of adult emergence in *S. litura*. Adult moths eclose at dusk, and no matings occur during the night of eclosion. Approximately 70 % of mating occurs on the subsequent night after emergence, and those unmated will mate on the third night [15, 43]. The present study showed that the highest reproductive performance was achieved when both sexes were 1 d old relative to older insects. If mating disruption were to delay female mating until 3 d of age, and if we assume that she mates with a male of the same age, her expected fecundity would decrease from 1840 ± 359 eggs to 1584 ± 207 eggs, a reduction of 16.16 %; a delay until 6 d of age would yield 1099 ± 146 eggs, a 40.27 % reduction. These results suggest that a delay of more than 6 d after emergence is necessary to achieve control. In addition, mating disruption potentially increases moth activities associated with mate searching and may thus increase the probability of attack by natural enemies [55]. The present study also suggests that the release of younger sterile males for control may yield the highest control efficacy, as young males had higher mating success than old males.

5. ACKNOWLEDGEMENTS

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Figure Captions

Fig. 1 Effect of age at mating on fecundity in *S. litura*. $y = \exp(7.5361 - 0.1604x_f + 0.2103x_m - 0.0590\beta_{11}x^2_m + 0.0345x_f\,x_m)$.

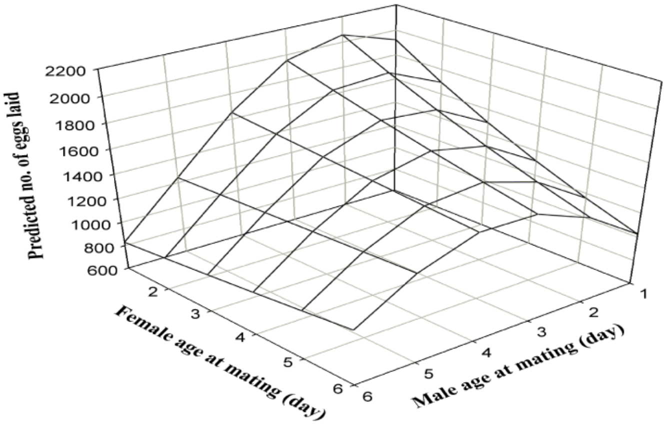

Fig. 2 Effect of age at mating on fertility rate in *S. litura*. $y = \exp(-0.1240 - 0.0158\ x_f)$.

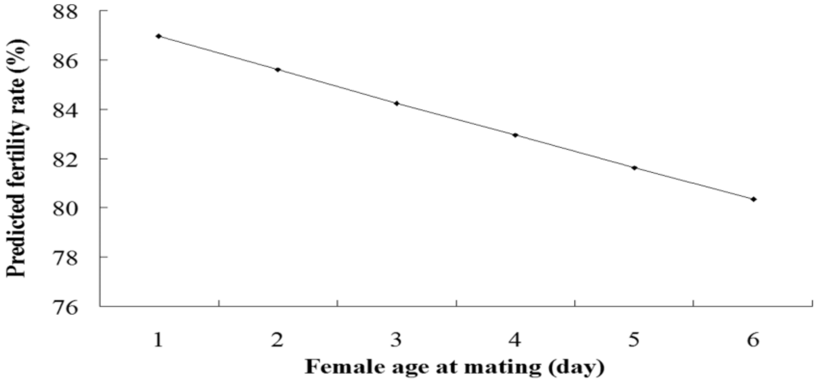

Fig. 3 Effect of age at mating on female longevity in *S. litura*. $y = \exp(2.4306 + 0.0161x_f + 0.0225x_f\,x_m)$.

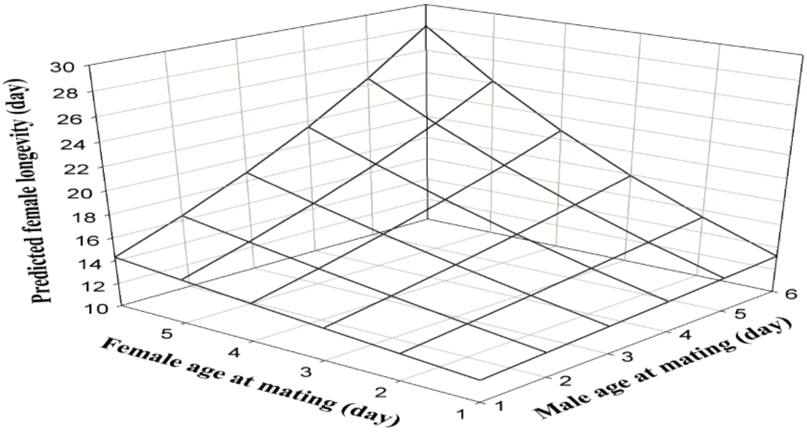

Fig. 4 Effect of age at mating on male longevity in *S. litura*. $y = \exp(2.7148 - 0.007x_f - 0.1898x_m + 0.0267\beta_{11}x^2_m + 0.0109x_f\,x_m)$.

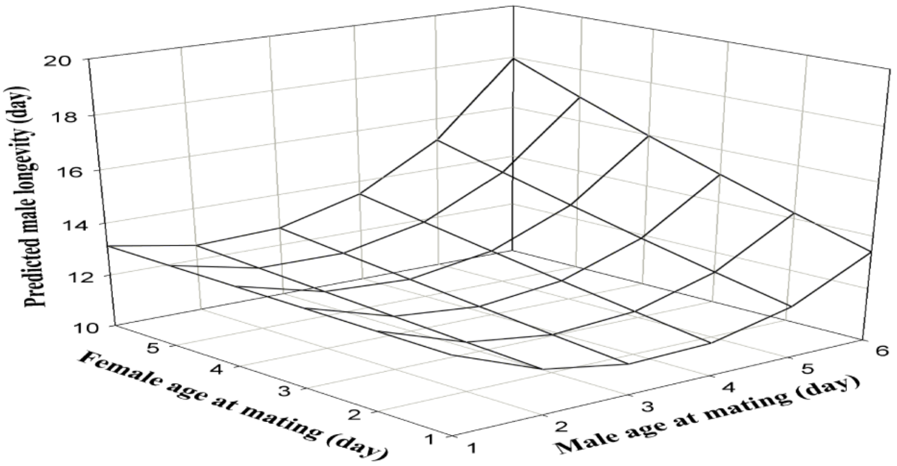

Fig. 5 Effect of age at mating on mating frequency (as evidenced by the number of spermatophores in females) in *S. litura*. $y = \exp(0.2707 - 0.0958x_m)$.

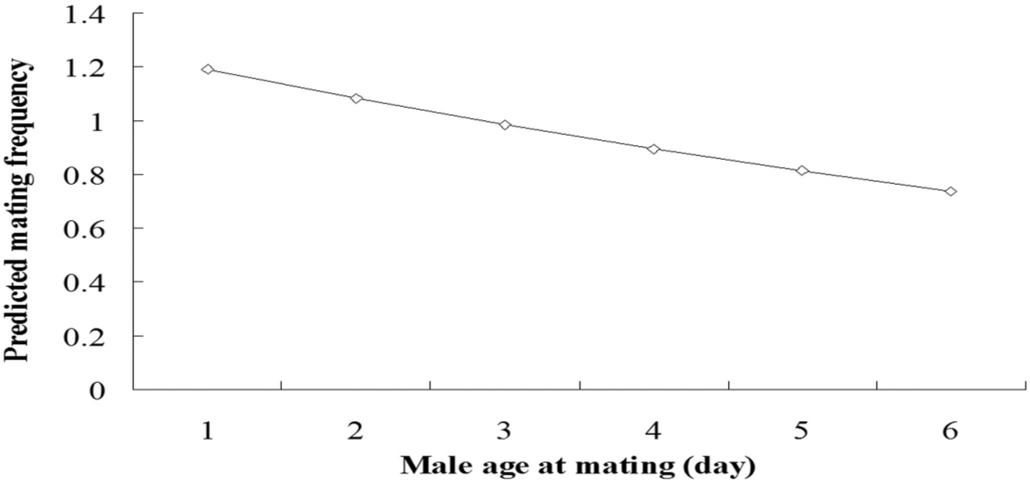

Fig. 6 Egg production, mating frequency and longevity between treatments 1 × 1 and 6 × 1 (female × male age (d)) in *S. litura*. For each parameter, bars with different letters indicate significant differences ($P < 0.05$).

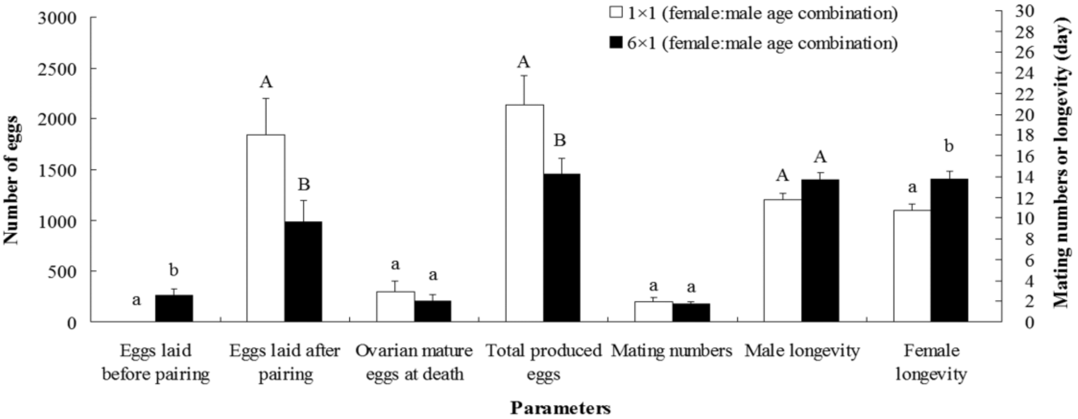

Table-1. Age combinations of pairs and associated sample sizes used to assess the effects of age at mating on reproductive fitness in *S. litura*.

Age (day) pair combinations		**No. of pairs**
Females	**Males**	
1	1	15
1	3	13
1	6	14
3	1	15
3	3	14
3	6	13
6	1	16
6	3	14
6	6	17