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Linking larval nutrition to adult reproductive traits in the European corn borer *Ostrinia nubilalis*

RACHAEL E. BONOAN, NOORIA AL-WATHIQUI and SARA LEWIS

Department of Biology, Tufts University, Medford, Massachusetts, U.S.A.

Abstract. Throughout an organism's lifetime, resources are strategically allocated to many different functions, including reproduction. Reproduction can be costly for both sexes; females produce nutrient-rich eggs, whereas males of many species produce large and complex ejaculates. In capital breeding insects, nutrients are mainly acquired during the larval period, yet allocation decisions impact the reproductive fitness of adults. The present study examines the effect of larval dietary nitrogen on both male and female reproductive traits in the European corn borer moth *Ostrinia nubilalis* Hübner, whose adults do not feed and whose males transfer a large, nitrogen-rich spermatophore. One day post-eclosion, *O. nubilalis* adults reared on one of three different diets (3.0%, 1.6%, or 1.1% nitrogen) are mated and two experiments are undertaken: one to measure nitrogen and carbon content of male ejaculates, and the other to determine female fecundity and fertility. Although male larval diet does not alter the percentage nitrogen content of adult somatic tissue, males reared on the higher nitrogen diet (3.0%) produce spermatophores with increased nitrogen relative to somatic nitrogen. Furthermore, females raised on the 3.0% nitrogen diet receive spermatophores with lower carbon : nitrogen ratios and thus more nitrogen. Overall, females lay more eggs as their larval dietary nitrogen increases, although they lay fewer eggs when their mates are raised on the higher (3.0%) nitrogen diet. This suggests that *O. nubilalis* females may use male-derived nitrogen not to supplement egg production, but rather for somatic maintenance. Overall, the present study furthers our understanding of how larval diet can affect adult fitness in Lepidoptera.

Key words. Ejaculate tailoring, Lepidoptera, mating tactics, nuptial gift, sexual selection, spermatophore.

Introduction

An individual's reproductive success ultimately depends on the availability and allocation of its nutritional resources. In organisms that experience discrete life stages, juvenile nutrition is a key factor in determining adult fitness (Boggs, 2009; Raubenheimer *et al.*, 2009; Morehouse *et al.*, 2010; Tigreros, 2013). This is especially true for insects that feed only during the larval stage (capital breeders) because they must rely on larval-derived nutrients for somatic maintenance and reproduction throughout their adult lifespan (Wheeler, 1996; Jönsson, 1997). As such, larval resources should be strategically stored and allocated to maximize fitness (Jervis *et al.*, 2005; Boggs, 2009). Thus, larval nutrition is predicted to have far-reaching latent effects on adult survival and reproduction (Pechenik *et al.*, 1998).

Latent effects are likely to be particularly important for herbivorous insects such as lepidopterans; these insects are often nitrogen-limited as a result of the low nitrogen content of host plants (Slansky & Feeny, 1977). In the cabbage butterfly *Pieris rapae*, larval dietary nitrogen is shown to alter adult wing colouration, wing size and mating success (Tigreros, 2013). During mating, many insect males transfer an additional source of nutrition: a spermatophore that contains limiting resources, such as nitrogen and carbohydrates, in addition to sperm (Thornhill, 1976; Boggs, 1995; Lewis *et al.*, 2011). In the Lepidoptera, spermatophores appear costly to produce; they can weigh up to 15% of male body mass and contain up to 20% nitrogen (Svard & Wiklund, 1988).

Correspondence: Rachael E. Bonoan, Department of Biology, Tufts University, 163 Packard Avenue, Medford, Massachusetts 02155, U.S.A. Tel.: +1 617 627 2202; e-mail: rachael.bonoan@tufts.edu

In several species, female insects use these male-derived nutrients to support egg production (Boggs & Gilbert, 1979; Bowen *et al.*, 1984). Furthermore, a meta-analysis indicates that, across diverse insect taxa, females receiving larger, or more, spermatophores have a greater lifetime fecundity (Lewis & South, 2012).

Nutrient limitation is expected to influence reproductive tactics in both sexes, particularly when males provide nuptial gifts such as spermatophores. In lepidopterans, limited dietary nitrogen may reduce male fitness by restricting spermatophore production. Accordingly, males are predicted to adjust their mating tactics by becoming choosier and strategically allocating their ejaculate (Dewsbury, 1982; Rutowski, 1982; Gwynne, 2008).

In the present study, *Ostrinia nubilalis* Hübner (the European corn borer) is used to investigate further how larval dietary nitrogen affects male and female reproductive strategies. *O. nubilalis* is a useful study species for such questions; they are readily reared on semi-synthetic diet and, at each successful mating, *O. nubilalis* males transfer a large spermatophore that contains up to 12% nitrogen (N. Al-Wathiqui, unpublished data). Additionally, both sexes mate multiple times (Royer & McNeil, 1993; Fadamiro & Baker, 1999), which is expected to select for strategic reproductive allocation in both sexes.

Specifically, the present study describes how nitrogen content of the male spermatophore, female lifetime fecundity, and fertility are altered when nitrogen content of larval diet is decreased. Regarding male mating tactics, males raised on reduced nitrogen diet are predicted to produce spermatophores containing less nitrogen relative to somatic nitrogen, as well as to provide more nitrogen-rich spermatophores when mated with females reared on the control diet. Regarding female tactics, female lifetime fecundity and fertility are predicted to increase when both sexes have been raised on control diets.

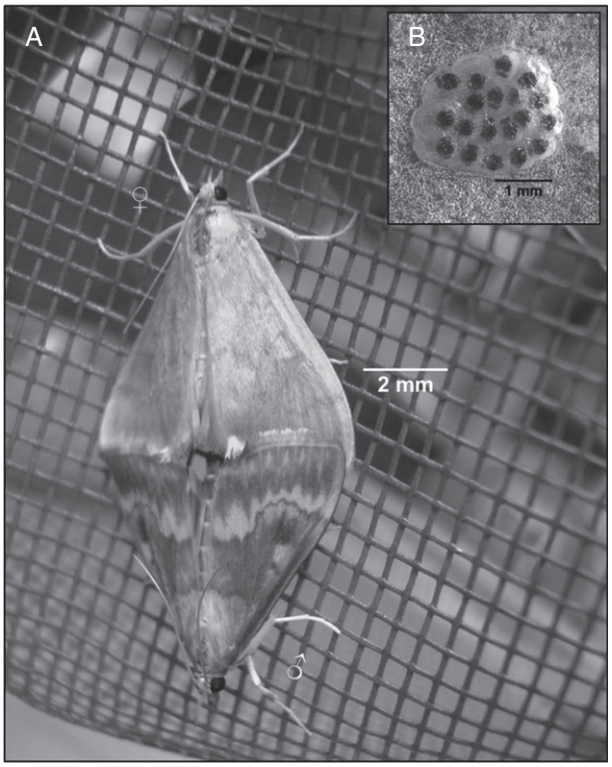

Fig. 1. *Ostrinia nubilalis* adults and eggs. (A) Mating pair. Spermatophore transfer was confirmed after mating by dissection of the female (Experiment 1) or observation of fertilized eggs 2–3 days post-laying (Experiment 2). (B) Fertilized eggs, 2–3 days post-laying, with small black head capsules visible.

Materials and methods

Dietary nitrogen manipulation

Three diets were created by manipulating nitrogen levels. This was achieved by using cellulose to replace casein, the main protein source in the *O. nubilalis* semi-synthetic larval diet (prepared by Bio-Serv, Flemington, New Jersey). The standard *O. nubilalis* diet contains 3.0% nitrogen by weight, and this is designated as the 'control' diet in the present study. Additionally, this resembles a high-quality natural diet; at the highest concentrations, the above-ground tissue of most healthy plants contains 3.0–7.0% nitrogen (Mattson, 1980). Two experimental low nitrogen treatments were used, consisting of 1.6% and 1.1% nitrogen. Each of the diets also differed slightly in energy content, with the control diet being 271 kcal L^{-1}, the 1.6% nitrogen diet being 218 kcal L^{-1} and the 1.1% nitrogen diet being 201 kcal L^{-1}.

Three replicates of each diet formulation were prepared in accordance with the manufacturer's instructions and poured into plastic containers ($30.5 \times 13\,cm^2$). After the diet solidified, 0.7 ± 0.05 g of fertilized *O. nubilalis* eggs (approximately 150 eggs from multiple laboratory-reared mothers) was placed on each diet. *O. nubilalis* consists of two strains distinguished by different female pheromone blends (Klun *et al.*, 1975; Kochansky *et al.*, 1975); Z-strain *O. nubilalis* moths from a laboratory-reared colony maintained at Tufts University were used for this study. Eggs and larvae were maintained in an incubator under a LD 16 : 8 h photocycle at 26 °C and 70% relative humidity. After 2 weeks, an unbleached paper towel was added to serve as a pupation substrate.

Fifth-instar larvae, pupae and 2-day post-eclosion adults were collected from each diet treatment and analyzed for percentage somatic nitrogen content to determine whether diet manipulation altered nitrogen stores at each life-history stage. Tissues from each individual were lyophilized, weighed and then packed into tin foil capsules for elemental micro-analysis using the CN mode on a vario MICRO cube microanalyzer (5mgChem90s method; Elementar, Mt Laurel, New Jersey).

All statistical analyses were performed using R, version 3.0.2 (R Core Team, 2013). Linear regressions were used to determine whether larval diet altered the percentage of nitrogen allocated to the somatic tissue of adults ($n = 8$), pupae ($n = 55$) and larvae ($n = 27$). The linear model (lm) used (percentage somatic nitrogen = larval diet × life-history stage) did not contain any random effects because the variance between replicates was negligible. All data satisfied test assumptions.

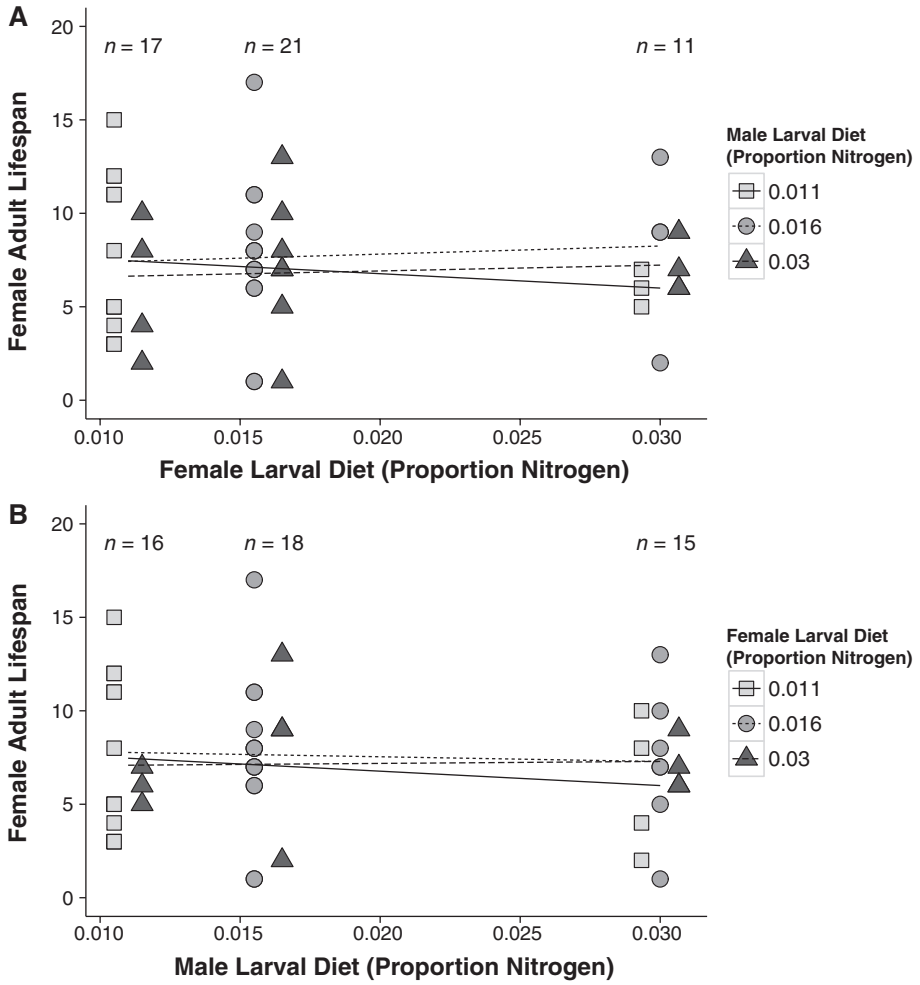

Fig. 2. Effects of (A) female and (B) male dietary nitrogen in *Ostrinia nubilalis* on adult lifespan of the female. The data presented were collected from a total of 49 successful matings.

Experimental matings

To determine the effects of dietary nitrogen on male and female reproductive tactics, 2-day post-eclosion adults were randomly assigned to create specific crosses (female–male larval diets): 3.0%–3.0%, 3.0%–1.6%, 1.6%–3.0%, 1.6%–1.6%, 3.0%–1.1%, 1.1%–3.0% and 1.1%–1.1% for a total of 145 experimental matings ($n = 96$ for Experiment 1; $n = 49$ for Experiment 2). All female–male pairings were conducted in paper cups (diameter 11 cm, height 5.5 cm) lined with wax paper (oviposition substrate). The mating cups were placed in an incubator and monitored every 10–15 min. Once the pair had mated (Fig. 1A), they were randomly assigned to one of the two experiments described below.

Experiment 1: Effects of dietary nitrogen on the male ejaculate. To investigate the effects of dietary nitrogen on *O. nubilalis* male ejaculate, spermatophores were collected from females immediately after copulation ended. Spermatophores were dissected from the female's bursa copulatrix, and the sperm-containing ampulla was removed. The remainder of the spermatophore ($n = 96$) and, when possible, the male ($n = 71$) that provided the spermatophore were lyophilized and weighed. Carbon and nitrogen content of spermatophores was determined by CHNOS analysis using a vario MICRO cube (2mgChem80s method; Elementar), and male nitrogen content was analyzed in the same way as for larvae, pupae and adults. For each male, relative spermatophore nitrogen was calculated by dividing the absolute nitrogen content (mg) of the spermatophore by the absolute nitrogen content (mg) of the male plus his spermatophore nitrogen content (mg). By calculating the relative nitrogen content, we can account for body size differences among males. CHNOS analysis also provided the carbon-to-nitrogen ratio (C : N) of these males and their spermatophores.

Relative nitrogen and C : N in male spermatophores were analyzed using a linear regression and saturated linear models (lm; $y =$ female larval diet × male larval diet). Random effects, such as replicate and individual, were not included in these

models; variances were near zero. All data fit the assumptions of this test.

Experiment 2: Effects of diet on fecundity and fertility. Mated females ($n = 49$) were supplied with water and kept in their mating cups to oviposit until death; female lifespan averaged 7.1 ± 3.8 days (Fig. 2). The oviposition substrate (wax paper) was replaced daily. Eggs from the 49 successful matings ($n = 29\,289$ eggs) were counted under $\times 2$ magnification at 1 day post-laying to determine female fecundity, and re-counted at 2–3 days post-laying to determine the proportion of eggs that had been fertilized ($n = 19\,528$ eggs), as indicated by visible head capsules of the developing embryos (Fig. 1B).

To determine whether larval diet altered female lifetime fecundity, a Poisson regression of a general linear model was run (glm; lifetime fecundity = female larval diet × male larval diet). The possible random effects of replicate and individual both had variances near zero and were not added to the model. Data were transformed (squared) to fit the assumptions. Mated females that did not lay any eggs throughout their lifetime were regarded as a failed mating and were not included in the analysis. The proportion of eggs fertilized was analyzed using a linear regression of a linear mixed effects model (number of eggs fertilized = female larval diet × male larval diet, random factor = female ID). Data for the proportion of eggs fertilized fit all assumptions. A Poisson regression was also run to determine whether male or female larval diet had an effect on lifespan of the laying female (glm; lifespan = female larval diet × male larval diet).

Results

Effect of diet manipulation

Larvae raised on lower nitrogen diets tended to have a higher percentage nitrogen in their somatic tissue (linear regression, $F = 3.39$, d.f. = 1,25, $P = 0.08$) compared with the control (3.0% nitrogen) diet (Fig. 3). By contrast, larval diet had no effect on the somatic nitrogen content of either pupae (linear regression, $F = 0.83$, d.f. = 1,53, $P = 0.37$) or adults (linear regression, $F = 1.36$, d.f. = 1,6, $P = 0.29$) (Fig. 3).

Experiment 1: Effects of diet on male ejaculates. Male diet had a significant effect on relative spermatophore nitrogen (linear regression, $F = 3.88$, d.f. = 1,67, $P = 0.05$); males that were raised on control diet produced spermatophores with increased relative spermatophore nitrogen (Fig. 4). There was no effect of female larval diet on relative spermatophore nitrogen (linear regression, $F = 0.08$, d.f. = 1,67, $P = 0.77$). However, females that had been raised on a control diet received spermatophores with a significantly lower C : N, and therefore a higher proportion of nitrogen (linear regression, $F = 3.36$, d.f. = 1,92, $P = 0.05$) (Fig. 5).

Experiment 2: Effects of diet on fecundity and fertility. Female lifetime fecundity was significantly altered by both male (Poisson regression, $\chi^2 = 1\,179\,752$, d.f. = 1, $P \ll 0.001$) and female diet (Poisson regression, $\chi^2 = 100\,164$, d.f. = 1, $P \ll 0.001$). Lifetime fecundity increased with an increase in nitrogen content of female larval diet (Fig. 6A) but decreased with an increase in nitrogen content of male larval diet (Fig. 6B). There was also a significant interaction between male and female diet on lifetime fecundity (Poisson regression, $\chi^2 = 11\,635$, d.f. = 1, $P \ll 0.001$).

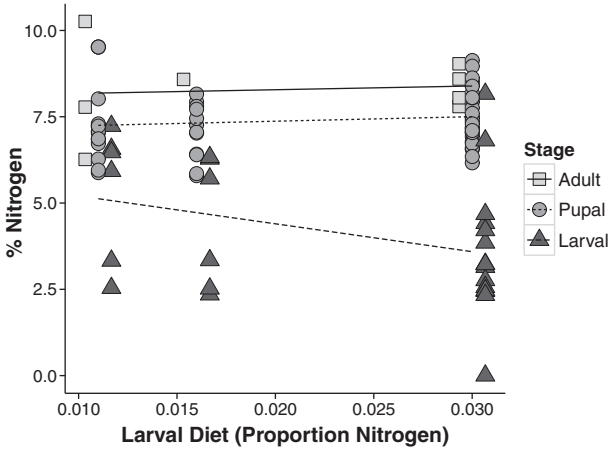

Fig. 3. Effect of dietary nitrogen on percent somatic nitrogen of *Ostrinia nubilalis* larvae ($n = 27$), pupae ($n = 55$) and adults ($n = 8$).

Despite these differences in fecundity, there was no effect of diet on the proportion ($63.7 \pm 0.03\%$) of eggs fertilized (linear regression for female diet, $F = 1.78$, d.f. = 1,45, $P = 0.90$; linear regression for male diet $F = 0.73$, d.f. = 1,45, $P = 0.40$) (Fig. 7). This may be a result of the low sample sizes per group and thus further investigation is warranted. Neither male, nor female diet had an effect on female lifespan (Poison regression for female diet, $\chi^2 = 0.002$, d.f. = 1, $P = 0.96$; Poisson regression for male diet $\chi^2 = 0.17$, d.f. = 1, $P = 0.68$) (Fig. 2).

Discussion

Contrary to expectation, larval diet has a slight negative effect on percentage somatic nitrogen in developing *O. nubilalis*, but no effect on adult or pupal somatic nitrogen (Fig. 3). These results suggest that larvae reared on low nitrogen diets compensate by consuming more food than those raised on the control diet. Compensatory feeding behaviour is reported in a number of lepidopteran larvae (Slansky & Scriber, 1985; Simpson & Simpson, 1990). The lower percentage nitrogen in the larvae raised on the control diet in the present study may be a result of the sampling method used: larvae are sampled based on size; however, if larvae raised on the lower nitrogen diets eat more to compensate, they may be growing larger faster. Thus, larvae sampled for analysis may not be exactly the same age.

Despite probable compensatory eating, males raised on the control diet produce spermatophores with higher nitrogen relative to their total body nitrogen content (Fig. 4). Furthermore, females raised on the control diet receive spermatophores containing more nitrogen (lower C : N ratio) than females raised on

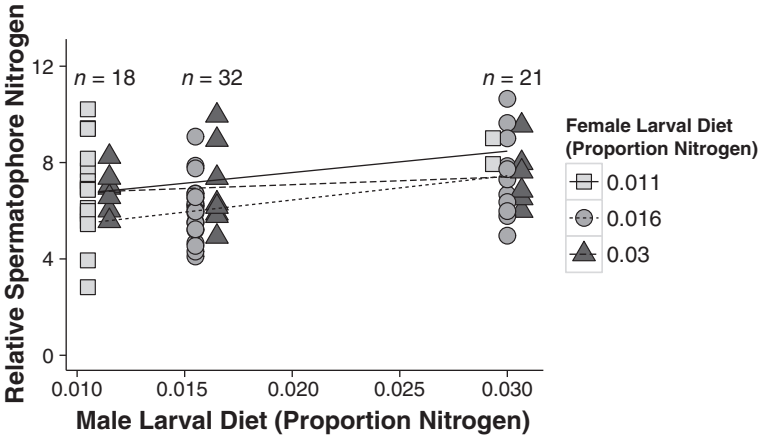

Fig. 4. Effect of male dietary nitrogen on relative nitrogen of spermatophores transferred by *Ostrinia nubilalis* males (absolute nitrogen of spermatophore/absolute total nitrogen).

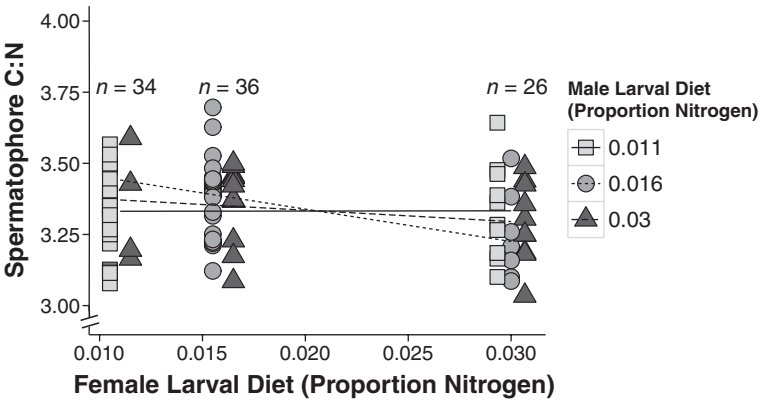

Fig. 5. Effect of female dietary nitrogen on the carbon to nitrogen ratio (C : N) of transferred spermatophores in *Ostrinia nubilalis*.

lower nitrogen diets, regardless of male diet, although the effects of the diet treatments are not pronounced (Fig. 5). The C : N ratio of male spermatophores may be affected by compensatory eating; however, compensatory eating would not change how males allocate their resources based on female quality.

These findings indicate that *O. nubilalis* males may tailor the composition of their ejaculate based on the perceived fecundity of their mate. Ejaculate tailoring based on female quality is observed in other insects, fish and mammals, where female size and age dictate male ejaculate size (Wedell *et al.*, 2002). Although female body size is not measured in Experiment 2, females raised on the control diet may be larger than those raised on the low nitrogen diets, which would allow males to use body size as an indicator of female fecundity. Direct correlations between female size and fecundity are observed in many insects (Honěk, 1993), including green stink bugs (Capone, 1995), curculionid beetles (Harari *et al.*, 1999) and winter moths (van Dongen *et al.*, 1997).

As the nitrogen content of their larval diet increases, so does female lifetime fecundity (Fig. 6A). Interestingly, however, the total number of eggs that females lay decreases as the nitrogen content in their mates' larval diet increases (Fig. 6B). *O. nubilalis* adult females eclose with approximately 90 mature oocytes (Miller, 1988) but lay an average of 250 eggs during their lifetime (Dopman *et al.*, 2010). This means that almost two-thirds of a female's eggs are not matured and could benefit from male-donated nutrients. However, the present results indicate that females do not allocate male derived nitrogen to the production of more eggs. Although the sample size per group is relatively low in the present study, this result runs counter to the widely accepted assumption that spermatophore nitrogen, and thus protein content, primarily provides a nutritive function. A similar situation occurs in the Indian meal moth (*Plodia interpunctella*), where males transfer a protein-rich spermatophore that does not augment female fecundity (Cook, 1999); females that receive two spermatophores do not show increased fecundity compared with females who receive one spermatophore. Thus, similar to the Indian meal moth, male-derived nutrients in *O. nubilalis* may not contribute directly to female egg production.

One possible explanation for the inverse relationship between male larval diet and female fecundity may be that females use male-derived protein mainly for somatic maintenance; females raised on a diet low in nitrogen have the same lifespan as those raised on the control diet (Fig. 2A). Another possibility is that *O. nubilalis* spermatophores may contain male seminal proteins that, rather than serving a nutritive function, directly affect female post-mating physiology and behaviour. In fruit flies (*Drosophila melanogaster*), almost 150 different seminal fluid proteins are reported to be identified; these proteins initiate many physiological and behavioural changes within mated females (Wolfner, 2002; Sirot *et al.*, 2009; Avila *et al.*, 2011). *O. nubilalis* spermatophores include proteins that may similarly

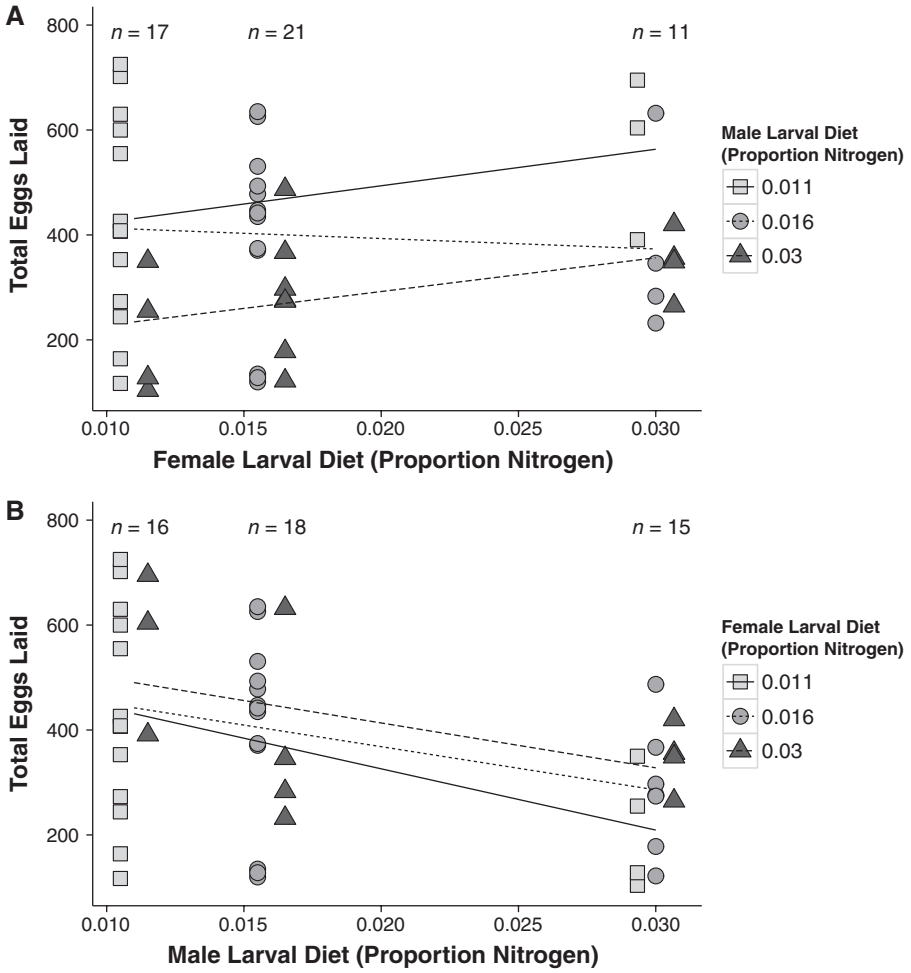

Fig. 6. Effects of (A) female and (B) male dietary nitrogen in *Ostrinia nubilalis* on females' lifetime fecundity. Both figures are based on the total number of eggs ($n = 29\,289$ eggs) produced from 49 matings. After each mating, eggs were collected every 24 h until the female died.

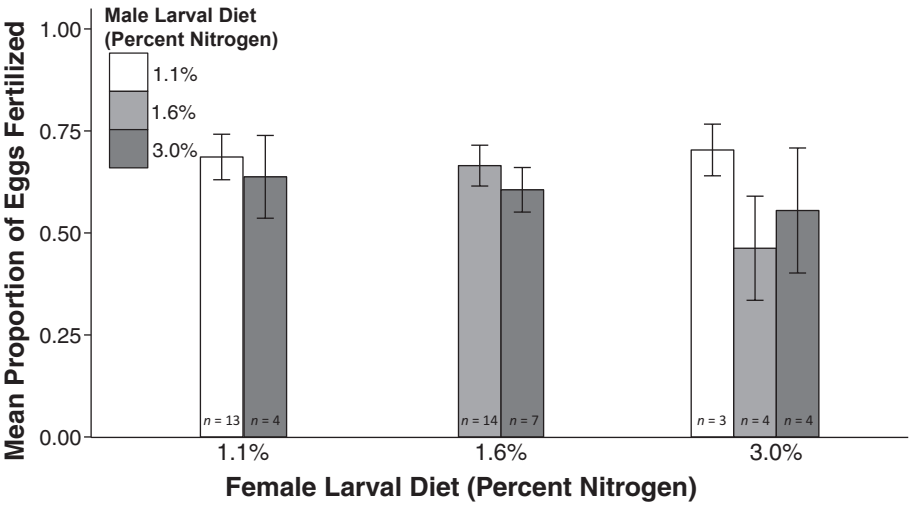

Fig. 7. Effect of *Ostrinia nubilalis* male and female dietary nitrogen on the proportion of eggs fertilized throughout a female's life span. Eggs were collected and counted daily, and fertilized eggs (see Fig. 1B) were assessed 2–3 days post-laying.

affect female reproductive processes (N. Al-Wathiqui, S.M. Lewis, E.B. Dopman, unpublished observations).

Interestingly, although both male and female larval diet affects female fecundity, fertility remains unchanged at approximately $63.7 \pm 0.03\%$ (Fig. 7). Thus, the observed reduction in fecundity when females mate with males reared on a control diet remains puzzling because it does not appear to be a result of sperm limitation. It is plausible that males in better condition may allocate their spermatophore protein and/or sperm strategically, reserving some resources for future mating opportunities.

The results of the present study indicate that larval nitrogen limitation influences the reproductive performance of both sexes in *O. nubilalis*. The findings improve our understanding of the link between larval nutrition and adult reproductive tactics in capital-breeding insects that do not feed as adults. Further studies are needed to investigate the possibility of compensatory feeding behavior, as well as the development times for each life-history stage. There is also merit in running this same experiment but with larger sample sizes for each treatment group.

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