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

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Short Title: Dietary nitrogen and reproductive traits

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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, while 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 reproductive fitness of adults. This study examines the effect of larval dietary nitrogen on both male and female reproductive traits in the European corn borer moth, *Ostrinia nubilalis*, whose adults do not feed and whose males transfer a large, nitrogen-rich spermatophore. Larvae were reared on three different diets (3.0%, 1.6%, or 1.1% nitrogen). Adults were mated and two experiments were done: one to measure nitrogen and carbon content of male ejaculates, and the other to determine female fecundity and fertility. Although male larval diet did not alter percent nitrogen content of adult somatic tissue, males reared on the higher nitrogen diet (3.0%) produced spermatophores with increased nitrogen relative to somatic nitrogen. Furthermore, females raised on the 3.0% nitrogen diet received spermatophores with lower C:N ratios and thus, more nitrogen. Overall, females laid more eggs as their larval dietary nitrogen increased, although they laid fewer eggs when their mates had been raised on the higher (3.0%) nitrogen diet. This suggests that *O. nubilalis* females may not use male-derived nitrogen to supplement egg production, but rather for somatic maintenance. Overall, this 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, 1998).

Latent effects are likely to be particularly important for herbivorous insects such as lepidopterans; these insects are often nitrogen-limited due to the low nitrogen content of host plants (Slansky-Feeny, 1977). In the cabbage butterfly *Pieris rapae*, larval dietary nitrogen was shown to alter adult wing coloration, 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, 1989). In several species, female insects have been shown to 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 that receive larger, or more, spermatophores have greater lifetime fecundity (South & Lewis, 2012).

Nutrient limitation is expected to influence reproductive tactics in both sexes, particularly when males are providing 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 this study, *Ostrinia nubilalis* (the European corn borer) was used to further investigate 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 (Al-Wathiqui, unpublished data). Additionally, both sexes mate multiply (Royer & McNeil, 1993; Fadamiro & Baker, 1999), which is expected to select for strategic reproductive allocation in both sexes.

Specifically, this study describes how nitrogen content of the male spermatophore, female lifetime fecundity, and fertility are altered when nitrogen content of larval diet was decreased. Regarding male mating tactics, males raised on reduced nitrogen diet are predicted to produce spermatophores containing less nitrogen relative to somatic nitrogen, and 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.

Materials and methods

Dietary nitrogen manipulation

Three diets were created by manipulating nitrogen levels. This was done by using cellulose to replace casein, the main protein source in *O. nubilalis* semi-synthetic larval diet (prepared by Bio-Serv, Flemington, NJ, USA). As standard *O. nubilalis* diet contains 3.0% nitrogen by weight, we designated this as the "control" diet. Additionally, this resembles a high quality natural diet; at highest concentrations, 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. The diets also differed slightly in caloric content, with the control diet being 271 kcal/L, the 1.6% nitrogen diet being 218 kcal/L, and the 1.1% nitrogen diet being 201 kcal/L.

Three replicates of each diet formulation were prepared following manufacturer instructions and poured into plastic containers (30.5 cm x 13 cm). After the diet solidified, 0.7 ± 0.05 grams of fertilized *O. nubilalis* eggs (approximately 150 eggs from multiple lab-reared mothers) were placed on each diet. *O. nubilalis* consists of two strains distinguished by different female pheromone blends (Klun *et al.*, 1973; Kochansky *et al.*, 1975); Z-strain *O. nubilalis* moths from a lab-reared colony maintained at Tufts University were used for this study. Eggs and larvae were maintained in an incubator at 26°C (RH 70%) on a LD 16:8 h cycle. After two weeks, 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 percent somatic nitrogen content to determine if 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 (5mgChem90s method, Elementar, Mt. Laurel, NJ).

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

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 the following 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 (11 cm diameter x 5.5 cm height) 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, Mt. Laurel NJ), and male nitrogen content was analyzed in the same way as 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 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 2x magnification at 1 day post-laying to determine female fecundity, and re-counted at 2-3 days post-laying to determine what proportion of eggs had been fertilized (n = 19 528 eggs), indicated by visible head capsules of the developing embryos (Fig. 1b).

To determine if 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 if 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 percent nitrogen in their somatic tissue (linear regression, F = 3.39, df = 1,25, P = 0.08) when compared with the control (3.0% nitrogen) diet (Fig. 3). In contrast, larval diet had no effect on the somatic nitrogen content of either pupae (linear regression, F = 0.83, df=1,53, P = 0.37) or adults (linear regression, F = 1.36, df = 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, $F = 3.88$, df = 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, $F = 0.08$, df = 1,67, $P = 0.77$). However, females that had been raised on a control diet received spermatophores with significantly lower C:N, and therefore a higher proportion of nitrogen (linear regression, $F = 3.36$, df = 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, χ^2=1179752, df=1, p<<0.001) and female diet (Poisson regression, $\chi^2 = 100164$, df = 1, $P << 0.001$). Lifetime fecundity increased with an increase in nitrogen content of female larval diet (Fig. 6a) but it 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, $\chi^2 = 11635$, df=1, $P << 0.001$).

Despite these differences in fecundity, there was no effect of diet on the proportion (63.7 ± 0.03%) of eggs fertilized (linear regression for female diet, $F = 1.78$, df = 1,45, $P = 0.90$; linear regression for male diet $F = 0.73$, df = 1,45, $P = 0.40$) (Fig. 7). This may be due to 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, $\chi^2 = 0.002$, df = 1, $P = 0.96$; Poisson regression for male diet $\chi^2 = 0.17$, df = 1, $P = 0.68$) (Fig. 2).

Discussion

Contrary to expectation, larval diet had a slight negative effect on the percent somatic nitrogen and no effect on adult or pupal somatic nitrogen (Fig. 3). These results suggest that larvae reared on low nitrogen diets compensated by consuming more food than those raised on the control diet. Compensatory feeding behavior has been previously reported in a number of lepidopteran larvae (e.g. Slansky & Scriber, 1985; Simpson & Simpson, 1990). The lower percent nitrogen in the larvae raised on the control diet may be due to our sampling method—larvae were sampled based on size however, if larvae raised on the lower nitrogen diets were eating more to compensate, they may have grown larger faster. Thus, larvae sampled for analysis may not have been exactly the same age.

In spite of likely compensatory eating, males raised on a control diet produced spermatophores with higher nitrogen relative to their total body nitrogen contentd (Fig. 4). Furthermore, females raised on the control diet received spermatophores containing more nitrogen (lower C:N) than females raised on lower nitrogen diets, regardless of male diet, but the effects of the diet treatments were not pronounced (Fig. 5). The C:N of male spermatophores may have been 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 has been observed in other insects, fish, and mammals, where female size and age dictate male ejaculate size (Wedell *et al.*, 2002). Although we did not measure female body size in experiment 2, females raised on the control diet may have been 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 have been observed in many insects (Honek, 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 increased, so did female lifetime fecundity (Fig. 6a). Interestingly, however, the total number of eggs females laid decreased as the nitrogen content in their mates' larval diet increased (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 2/3 of a female's eggs have not matured and could benefit from male-donated nutrients. However, out results indicate that females do not allocate male derived nitrogen to the production of more eggs. Although the sample size per group was relatively low, this result runs counter to the widely accepted assumption that spermatophore nitrogen, and thus protein content, primarily serves a nutritive function. A similar situation occurs in the Indian mealmoth (*Plodia interpunctella*), where males transfer a protein-rich spermatophore that does not augment female fecundity (Cook, 1999); females that received two spermatophores did not show increased fecundity compared to females who received one spermatophore. Thus, like the Indian mealmoth, 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 had 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 behavior. In fruit flies (*Drosophila melanogaster*), nearly 150 different seminal fluid proteins have been identified; these proteins initiate many physiological and behavioral changes within mated females (Avila *et al.*, 2011; Wolfner, 2009; Sirot *et al.*, 2009). *O. nubilalis* spermatophores include proteins that may similarly affect female reproductive processes (Al-Wathiqui *et al.*, unpubl.).

Interestingly, while both male and female larval diet affected female fecundity, fertility remained unchanged at about 63.7 ± 0.03% (Fig. 7). Thus, the observed reduction in fecundity when females mated with males reared on control diet remains puzzling, as it does not appear to be due to sperm limitation. It is plausible that males in better condition may strategically allocate their spermatophore protein and/or sperm, reserving some resources for future mating opportunities.

Our results indicate that larval nitrogen limitation influences the reproductive performance of both sexes in *O. nubilalis*. This study improves 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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Figure Legends

Fig. 1. *Ostrinia nubilalis* adults and eggs. (a) Mating pair. Following mating, spermatophore transfer was confirmed 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.

Fig. 2. Effects of *O. nubilalis* (a) female and (b) male dietary nitrogen on adult lifespan of the female. Data presented in both figures were collected from a total of 49 successful matings.

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

Fig. 4. Effect of male dietary nitrogen on relative nitrogen of spermatophores transferred by *O. 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.

Fig. 6. Effects of *O. nubilalis* (a) female and (b) male dietary nitrogen 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 hours until the female died.

Fig. 7. Effect of *O. 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.