Address correspondence to: Dr. Stephen Foster
North Dakota State University
Entomology Department
NDSU Dept 7650
PO Box 6050
Fargo, ND 58108-6050
U.S.A
Ph. 1-701-231-6444
Fax 1-701-231-8557
Email: stephen.foster@ndsu.edu

Increased allocation of adult-acquired carbohydrate to egg production results in its decreased allocation to sex pheromone production in mated females of the moth *Heliothis virescens*

Stephen P. Foster, Karin G. Anderson and J.P. Harmon

Entomology Department, North Dakota State University,
PO Box 6050, Fargo, North Dakota 58108-6050, U.S.A

Running title: Egg production costs sex pheromone

SUMMARY

Females of most species of moths produce a volatile sex pheromone that attracts conspecific males over distance. In females of the polyandrous moth *Heliothis virescens*, feeding on carbohydrate (e.g., nectar) supplies precursor, via hemolymph trehalose, for both sex pheromone and egg production. With limited carbohydrate acquisition these two reproductive physiologies might compete for hemolymph trehalose, resulting in an allocation deficit to one. Using virgin and mated females, which have low and high egg maturation rates, respectively, we fed females a limited diet of ^{13}C-labeled glucose daily and, using mass isotopomer distribution analysis, determined allocations of adult-acquired carbohydrate (AAC) to newly synthesized pheromone and ovarian and egg fats, our proxies for allocation to egg production. With increased number of feeds, AAC enrichment of hemolymph trehalose increased, as expected. This led to mated females increasing their proportional allocation of AAC to ovarian and egg fats, but decreasing their proportional allocation of AAC to pheromone production. By contrast, virgins increased their proportional allocation of AAC to pheromone production with increased feeds, consistent with increasing AAC enrichment of hemolymph trehalose. These results show that with limited AAC intake, enhanced egg maturation in mated females results in reduced AAC allocation to pheromone production; this does not occur in virgins because of their lower egg maturation rate. This physiological competition for AAC corresponded with decreased pheromone production in mated moths to levels unlikely to attract mates. Therefore, the availability/allocation of AAC may be a proximate mechanism underlying the incidence of polyandry in this and other species of moths.

Keywords: hemolymph trehalose, pheromone biosynthesis, tradeoff, fatty acid, tracer-tracee, precursor enrichment, polyandry

INTRODUCTION

Moths, which account for approximately 90% of species of Lepidoptera, typically use volatile sex pheromones to locate mates over distance. These volatile, female-produced sex pheromones are a prerequisite for successful mating in most species in which males must locate females in relatively low population densities (Tamaki, 1985). The female moth typically produces and/or releases sex pheromone, a species-specific single component or blend of components, usually from a gland located between the 8th and 9th abdominal segments (Ma and Ramaswamy, 2003). Most moth sex pheromone components (so-called 'Type I'; Ando et al., 2004) are biosynthesized de novo, from acetate, via fatty acid synthesis followed by several steps peculiar to the pheromone gland, including desaturation, limited chain-shortening, reduction and acetylation and/or oxidation (Blomquist et al., 2011). In contrast to the voluminous work on the steps that modify the fatty acid chain of a pheromone component (Jurenka, 2003) and the regulation of biosynthesis by the pheromone biosynthesis activating neuropeptide (PBAN; Blomquist et al., 2011), the physiological pools that provide precursor for fatty acid and pheromone biosynthesis have received relatively little attention. Recent work (Foster, 2009; Foster and Johnson, 2010; Foster and Anderson, 2011) on the polyandrous moth *Heliothis virescens* Fabricius (Noctuidae) demonstrated that adult-acquired carbohydrate (AAC), via incorporation into hemolymph trehalose and subsequent glycolysis, is a major source of precursor (acetyl CoA) for sex pheromone production.

Carbohydrate acquired through adult feeding, usually from plant nectar, is also important in female Lepidoptera for providing metabolites for other physiologies, including flight (O'Brien, 1999), used for foraging for hosts for progeny to develop on and for additional adult carbohydrate and, most notably, for egg production (Boggs and Ross, 1993; Wheeler, 1996). AAC is especially important for females of longer-lived species, which mature eggs throughout the adult life. In such cases, AAC can have profound effects on female fecundity (Boggs and Ross, 1993; Wheeler, 1996), with its use increasing over a female's lifetime for the production of egg metabolites, including carbohydrates, fats (Telfer, 2009) and amino acids (O'Brien et al., 2000; O'Brien et al., 2002; O'Brien et al., 2004; O'Brien et al., 2005).

Given that polyandrous moths, such as *H. virescens*, will often mature eggs and produce and release sex pheromone for attracting mates at the same time, reiteratively throughout their life (e.g.,Ramaswamy, 1990), the common use of hemolymph trehalose suggested to us that there might be competition for this resource between these physiologies, especially if AAC were limited, as is likely in nature, where plant nectar is highly variable and widely utilized (Boggs and Ross, 1993; Wäckers et al., 2007). When AAC is limited, its allocations to egg production, pheromone production, or to both, might decrease. Although it has been postulated that production of sex pheromone might "cost" female fitness (Johansson and Jones, 2007; Harari et al., 2011), the relatively small quantities of fatty acid-derived sex pheromone produced by females suggest that a direct metabolic cost on egg production is unlikely (Greenfield, 1981; Cardé and Baker, 1984; Svensson, 1996). By contrast, the large amounts of metabolites required for egg production could impact the availability of precursor for pheromone production; i.e., high usage of AAC for egg production might result in its decreased use for pheromone production. As far as we know, all moths probably have sufficient larval resources prior to adult feeding to produce sex pheromone as virgins. However, as larval-acquired nutrients are depleted, pheromone production is likely to become increasingly dependent upon AAC which, if limited, would impact pheromone production (Foster, 2009; Foster and Johnson, 2010). This competition for AAC between the two reproductive physiologies is likely to impact mated (of polyandrous species) females more so than virgins, as mated females generally use hemolymph trehalose and other resources to mature eggs at greater rates (Ramaswamy et al., 1997).

In this paper, we test whether there is physiological competition for allocation of AAC between pheromone and egg production in female *H. virescens* through feeding ^{13}C-labeled glucose to both mated and virgin adult females, thus allowing us to quantify directly the allocations of AAC to pheromone and egg fat production. We demonstrate that, under a limited AAC scenario, allocation of AAC to egg production in mated, but not virgin, females results in its decreased proportional allocation to pheromone production.

METHODS AND MATERIALS

Insects. *Heliothis virescens* were from a colony maintained at the Department of Entomology, NDSU for nine years, with the original insects obtained from a colony at USDA-ARS-BRL, Fargo, ND. Larvae were reared individually in small plastic cups (Solo Cup Co., Lake Forest, IL) containing a wheat germ-casein diet, at 25°C under a 16:8 (L:D) photoperiod. Insects were sexed at the pupal stage, with females and males placed in separate containers. Adults were collected daily, and maintained under the same temperature and light conditions as larvae, until used in experiments.

Females were maintained individually in 140 ml plastic containers that precluded flight throughout an experiment. This ensured that females were relatively inactive and that respiratory differences between virgin and mated females were minimized. At the end of the scotophase on the day following eclosion (i.e., when females were 24–48 h old, hereafter referred to as 1 d old), each female was given a 37.5 μl drop (20.1 μmoles) of 10% (w/w) U-^{13}C-glucose (99%; Cambridge Isotope Laboratories, Andover, MA) on a watch glass to feed upon; 37.5 μl was the maximum amount of liquid that females consumed reliably. Only females that consumed the entire drop were used in experiments. Each subsequent day, at the end of the scotophase, females were given another 37.5 μl drop of labeled glucose, until they were analyzed for pheromone or fat (see below). Females were not given access to any liquids (water, sugar solutions, etc.) between feeds. Eggs were not collected from virgins, because the number laid by females was low and erratic (some days, virgins did not lay any eggs).

Females were mated by placing 1-d-old virgins with 1-d-old virgin males, just prior to the start of scotophase. Pairs in copula were removed and left to finish copulation, after which the female was removed and placed in a small plastic container. At the end of scotophase, a female was given a 37.5 μl drop of 10% (w/w) U-^{13}C-glucose on which to feed. Feeding was repeated each day until females were analyzed. Each day following mating, either at the time of feeding or analysis, the number of eggs a female laid was counted. On the day a female was analyzed, the eggs she laid in the prior 20 hours were collected and analyzed for isotopic enrichment of fats (see below).

Determination of enrichment. Our aim was to determine how allocation of AAC to both mating and egg production changed with increased number of feeds (i.e., effectively over time). We gave adult females a daily feed (see above) of highly ^{13}C-enriched (99%) glucose and calculated precursor enrichment (see below) in the moieties that we analyzed. For pheromone production, we analyzed both the major pheromone component, (*Z*)-11-hexadecenal (Z11-16:Ald), and the stored pheromone precursor acids, (*Z*)-11-hexadecenoate (Z11-16:Acyl) and hexadecanoate (16:Acyl; Choi et al., 2005; Foster, 2005). We only analyzed the major pheromone component (and not the other pheromone components) because it constitutes over 90% of the mass of total pheromone produced and released by females (Teal et al., 1986), and because determination of enrichment in abundant compounds is inherently easier than determination of enrichment in trace compounds (i.e., the other components). Regardless, because the other pheromone components are biosynthesized from the same acetate precursor (Choi et al., 2005), precursor enrichments are likely to be similar to that of the major component. We analyzed the pheromone precursor acids because surplus (i.e., not converted to pheromone) quantities of these are stored in glandular glycerolipids (Foster, 2005). While the net biosynthetic rates of both stored acids (especially 16:Acyl) are considerably lower than that of the pheromone component (Foster and Anderson, 2011; Foster and Anderson, 2012), we wished to check that precursor enrichment patterns in these acids were consistent with that of the pheromone component.

In Lepidoptera, AAC is allocated to amino acid, carbohydrate and lipid in eggs (O'Brien et al., 2002; Telfer, 2009). We used allocation to de novo-synthesized fatty acids in eggs (and ovaries) as a proxy for AAC allocation to eggs because: (1) fats are a major component of *H. virescens* eggs (Cohen and Patana, 1985) and therefore constitute a major allocation; (2) fats and pheromone are biosynthesized by similar routes, with common precursors (Blomquist et al., 2011); (3) the allocation patterns for AAC to amino acids in eggs have been determined previously for a number of species of Lepidoptera (O'Brien et al., 2004; O'Brien et al., 2005; O'Brien et al., 2002), allowing comparison with our patterns for egg fat. In the first experiment, we measured enrichment in ovarian, as well as egg fats, because of the latency between when eggs were laid and when pheromone and hemolymph were sampled; the eggs sampled could

have been laid ca. 24 hours prior to sampling of the other parameters, whereas ovaries were sampled for enrichment at the same time as hemolymph and pheromone. For allocation to ovarian and egg fats, we measured precursor enrichment of 16:Acyl, because this is, by far, the most abundant de novo-biosynthesized acid in *H. virescens* eggs (Cohen and Patana, 1985); lineolate and linolenate are also highly abundant, but these are from larval dietary sources, as Lepidoptera cannot biosynthesize them (Blomquist et al., 1991). Other de novo-biosynthesized acids, such as octadecanoate and oleate, are incorporated into insect eggs, but these are likely synthesized from the same precursor pool and thus their precursor enrichments will be the same as that of 16:Acyl (Wolfe and Chinkes, 2005).

We determined precursor enrichment in fats and pheromone by mass isotopomer distribution analysis (MIDA; Hellerstein and Neese, 1992; Chinkes et al., 1996). MIDA is a combinatorial solution for determining the enrichment of a monomeric precursor pool by measuring the distribution of mass isotopomers in the resultant polymeric product(s). Assuming that the polymers are randomly assembled, then a given precursor enrichment will give a specific pattern of isotopomers in a polymer. If the isotopomer pattern is measured, then precursor enrichment can be determined. In practice, only two labeled mass isotopomers (usually those with one and two labeled precursor units are the most intense and, therefore, the easiest to quantify) need be determined (Wolfe and Chinkes, 2005). Precursor enrichment is the proportion of labeled (tracer) precursor of the true precursor pool (including labeled and unlabeled) in a polymeric product and its calculation is not affected by isotopic fractionation effects (Hellerstein and Neese, 1999). However, in our study, the true precursor pool is acetyl CoA, with labeled precursor derived from the labeled glucose fed to adults. Hence isotopic fractionation may occur during glycolysis and subsequent pyruvate decarboxylation; i.e., unlabeled glucose may be converted to acetyl CoA faster than labeled glucose, and thus may comprise proportionally more of the true precursor pool. Regardless, our aim was not to determine absolute amounts of AAC converted to pheromone or fatty acids but, rather, to compare how AAC allocation to the various moieties changed with increased feeding.

In MIDA, tracer (labeled) to tracee (unlabeled) ratios (TTRs) are calculated for singly and doubly labeled isotopomers of the various polymers. When using stable

isotopes, such as ^{13}C, the amount of label in an isotopomer has to be corrected for the amount of naturally occurring isotope and the overlap of the respective spectra of the singly and doubly labeled products (Chinkes et al., 1996). Thus:

$TTR(M+1) = (M+1 \div M+0)_{post} - (M+1 \div M+0)_{pre}$

$TTR(M+2) = (M+2 \div M+0)_{post} - (M+2 \div M+0)_{pre} - dT1 \times TTR(M+1)$

The $_{pre}$ and $_{post}$ terms refer to the measured isotopomer values before and after feeding on labeled glucose and correct for naturally occurring stable isotopes. We calculated the $_{pre}$ terms theoretically, based on known isotopic abundances, because previous studies have found these differ little from experimentally determined (females fed on unlabeled glucose) values (Foster and Anderson, 2011; Foster and Anderson, 2012). The term dT_1 corrects for the overlapping spectra of singly and doubly labeled isotopomers, and is the proportion of singly labeled tracer with two natural ^{13}C atoms (i.e., other than from a doubly labeled acetate from labeled glucose). Since our labeled acetate precursor contained two ^{13}Cs, these corrections were relatively minor.

Precursor enrichment (p) can then be calculated by:

$p = 2R/[(n-1)+2R]$, where $R = TTR(M+2)/TTR(M+1)$, and n = the number of precursor molecules in the product (eight for 16:Acyl and Z11-16:Ald).

Extraction and derivatization of pheromone, fats, and hemolymph trehalose. For sex pheromone, the pheromone gland of a female was dissected under a binocular microscope at the middle of the scotophase, and allowed to extract in 5 µl of *n*-heptane for at least three hours. For pheromone titers, 50 ng of (*Z*)-11-tetradecenal (Z11-14:Ald) was added as an internal standard. For pheromone gland fats, the dissected gland was placed in 2:1 dichloromethane:methanol and left at -15°C for at least 18 hours. After this, the solvent was decanted from the tissue and evaporated under a gentle stream of nitrogen. The residue was reacted with 0.5 M methanolic KOH for 1 hour before aqueous 1.0 M HCl was added (Bjostad et al., 1987). The resultant fatty acid methyl esters (FAMEs) were extracted with *n*-heptane after vigorous shaking.

Ovaries were dissected in physiological saline under a binocular microscope, just after dissection of the pheromone gland. After patting dry on absorbent paper, ovaries were placed in a 1.5 ml Eppendorf tube, along with ca 500 µl of 2:1 dichloromethane:

methanol, and ground with a disposable plastic pestle before centrifugation at 2000 x g for 5 min. The supernatant was decanted from the pellet and the solvent evaporated under a stream of nitrogen. Fatty acids from eggs were extracted by the same approach. FAMEs were generated from the extract residues as for the pheromone gland extract.

Hemolymph was sampled from females just prior to dissection of the pheromone gland. A small hole was made in the cuticle on the intersegmental membrane between the 6^{th} and 7^{th} abdominal segments, and the hemolymph droplet collected in a calibrated 5 µl glass capillary. After measurement of the volume (typically 2–5 µl), hemolymph was transferred to a glass vial, along with 50 µg of mannitol as internal standard, and water removed under vacuum. Trehalose was acetylated by reaction with acetic anhydride in pyridine at 100°C (Foster, 2009).

Analyses. Pheromone and FAMEs were analyzed by gas chromatography/mass spectrometry (GC/MS) on a Hewlett-Packard 5890/5972. The GC was fitted with a 30 m x 0.25 mm i.d. ZB-Wax column (Phenomenex, Torrance, CA, USA) and a splitless injector, and used helium as carrier gas at a constant flow of 1 $ml.min^{-1}$. The GC column oven temperature was programmed from 80–180°C at $15°C.min^{-1}$, following an initial delay of 1 min, and then to 220°C at $3°C.min^{-1}$.

For MIDA, we used the MS in the selected ion monitoring mode. For Z11-16:Ald, we recorded *m/z* 220 (M+0; unlabeled), 222 (M+1; one $^{13}C_2$-labeled acetate), and 224 (M+2; two $^{13}C_2$-labeled acetates). These ions correspond to the molecular ion with loss of a water molecule, a reasonably intense ion with an intact carbon skeleton. For methyl hexadecanoate (16:Me) and methyl (*Z*)-11-hexadecenoate (Z11-16:Me), we recorded, respectively, *m/z* 270, 272 and 274, and 268, 270 and 274. These were the molecular ions of the (M+0; unlabeled), (M+1; one labeled acetate), and (M+2; two labeled acetates) isotopomers of the FAMEs.

Pheromone of females fed only unlabeled glucose, for determination of titers, was quantified in SIM mode, monitoring *m/z* 220 (for Z11-16:Ald) and 192 (for the internal standard, Z11-14:Ald), after calibrating the MS's response with quantitative standards.

Trehalose octaacetate was quantified by GC, using a Varian 3700 equipped with a flame ionization detector, a splitless injector, and helium as carrier gas at a flow of 1

ml.min^{-1}. A 30 m x 0.25 mm i.d. ZB5 capillary column (Phenomenex), temperature programmed from 200–280^{o}C at 10^{0}C.min^{-1}, was used for the analysis. Hemolymph trehalose concentration (HTC) was calculated by dividing the amount of trehalose (determined relative to the internal standard, mannitol octaacetate) in a sample by volume. In one experiment (see below), we also determined the enrichment of trehalose by GC/MS, using an Agilent Technologies 6890/5973 and the same column and GC conditions as for the trehalose quantification. In electron impact ionization, trehalose octaacetate ions with an intact trehalose carbon skeleton are very weak. Consequently, we monitored the intense *m/z* 331 ($C_{14}H_{14}O_9^+$), a glucose tetraacetate fragment (M+0), and its $^{13}C_6$-labeled isotopomer (M+1). We did not correct for naturally occurring $^{13}C_6$-glucose, because of its extremely low abundance. Atom percent enrichment (APE) of trehalose was calculated (Wolfe and Chinkes, 2005) as:

APE = (M+1/M+0)/[1+(M+1/M+0)]

Experiments

We tested AAC allocations in both mated and virgin females, because they represent two different physiological demands for AAC: mated females mature eggs at a higher rate and, therefore, have a higher demand for AAC, while virgins mature eggs at a lower rate and have a lower demand for AAC (Ramaswamy et al., 1997; Foster, 2009). We tested a scenario in which AAC was limited: feeding was restricted to once a day and the concentration of carbohydrate was considerably less (up to one fifth) than that typically found in plant nectar (Perret et al., 2001). Moreover, this amount appeared sufficient to maintain HTC in virgins, but not in mated females (see Results). We tested this AAC-limited scenario, because we wanted to create physiological competition for AAC; a scenario with access to unlimited AAC would likely have produced a moot result, with mated females able to maintain AAC allocation to pheromone (e.g., Foster, 2009; Foster and Johnson, 2011), and therefore capable of producing enough pheromone for attracting males and remating, while maturing eggs. Separate experiments were required for analyses of pheromone and glandular fatty acids, as methanolic KOH (to produce FAMEs) reacts with aldehydes.

Experiment 1 – Pheromone, ovarian and egg fat enrichment in mated females.

We fed mated females U-^{13}C-glucose daily and analyzed individual females for each of HTC, and enrichment of trehalose, pheromone, ovarian (16:Acyl) and egg (16:Acyl) fats, for 0–9 days following mating. We analyzed four to eight different females for each day.

Experiment 2 – Enrichment of pheromone gland fats and eggs in mated females.

Mated females fed U-^{13}C-glucose daily were analyzed for precursor enrichment of pheromone gland (16:Acyl and Z11-16:Acyl) and egg fats (16:Acyl) for 1–4 days after mating. We analyzed four to five different females on each day.

Experiments 3 & 4 – Enrichment of pheromone and pheromone gland fats in virgin females.

Virgin females, fed U-^{13}C-glucose daily, were analyzed for precursor enrichment of pheromone (experiment 3) and pheromone gland fats (16:Acyl and Z11-16:Acyl; experiment 4) for 1–6 days and 1–4 days, respectively, after the initial feeding. For each enrichment measurement, four to six different females were analyzed on each day.

Pheromone titers

Pheromone titers of mated and virgin females were determined. Females were fed a drop of 37.5 μl of unlabeled 10% glucose daily, as in the enrichment experiments, until analyzed. Five to eight females of each type were analyzed for each day.

Statistical analyses

Changes in HTC and pheromone titers over time, for both mated and virgin females, were analyzed by linear regression, after first checking for normality and heteroscedasticity of data. Note, that the HTC data for mated females were from experiment 1, while new females (i.e., not used in experiments 3 or 4) were used for the virgin female data.

For experiment 1, we initially analyzed data by a structural equation modeling/path analysis approach (SAS Institute Inc., 2012), in order to investigate the relationships,

simultaneously, among days fed, enrichment of pheromone, ovaries and eggs, and total numbers of eggs laid. Multiple iterations of the model were investigated, but the core model included factors in which the number of days fed could influence all other variables, all enrichments were allowed to influence each other, and the enrichment of eggs and ovaries could relate with the number of total eggs. However, no insights were gained that weren't apparent with simpler and more common statistical analyses. Therefore, we used a general linear models (GLM) approach, with trehalose enrichment, days fed and total eggs laid as factors, and precursor enrichment of pheromone, ovaries (16:Acyl) and eggs (16:Acyl) as responses. Data for enrichment of trehalose and eggs were arcsine and log transformed, respectively, to ensure normality. We also tested linear correlations between pheromone enrichment and egg and ovarian fat enrichments to test whether any tradeoff in metabolite allocation was apparent at the individual level.

In experiment 2, we used linear models to test the relationships among days fed and eggs laid and precursor enrichments of pheromone gland fats (Z11-16:Acyl) and egg fat (16:Acyl), while in experiments 3 and 4, we used the same approach to test for the relationship between days fed and pheromone and between days fed and pheromone gland fats (Z11-16:Acyl and 16:Acyl), respectively. All GLM models were performed in JMP (2012).

RESULTS

Pheromone and HTC changes over time in mated and virgin females.

Mated females fed unlabeled glucose daily showed a decrease in pheromone titer over the 6 days of sampling (Fig. 1a; $F_{1,33}$= 17.70, P = 0.0002). This was paralleled by a similar decline ($F_{1,56}$= 10.06, P = 0.0025) in HTC (Fig. 1a). Virgins under the same feeding regime also showed a decline in pheromone titer over the course of the sampling period (Fig. 1b; $F_{1,40}$= 26.29, P < 0.0001). Considering the data qualitatively across the two different experiments, titers of virgins were typically greater than those of mated females at the same sampling time, as observed in other studies (Raina, 1989; Foster, 2009). In contrast to the mated females, HTC of virgins did not change over the 6 days of sampling (Fig. 1b; $F_{1,36}$= 1.05, P = 0.312).

Experiment 1 – Pheromone, ovarian and egg enrichment in mated females.

As expected, hemolymph trehalose enrichment in mated females increased over time (Fig. 2a). Even after a single feed, trehalose enrichment was very high (ca. 0.80), demonstrating that most of the hemolymph trehalose at this time (and subsequent) was derived from AAC, consistent with the high turnover of hemolymph trehalose by mated females. Our two-factor model ($F_{2,47}$ = 9.782, P= 0.0003) showed an effect of the number of feeds (F = 15.178, P = 0.0003), but not of total eggs laid (F = 0.0137, P = 0.907), on enrichment of hemolymph trehalose.

In our three-factor model, days fed had an effect on all three measures (pheromone, ovarian and egg fat) of enrichment (Table 1). Total eggs laid had near-significant effects on pheromone and ovarian fat enrichments, but less so on egg fat enrichment, while trehalose enrichment had no effect on any of the three enrichments (Table 1), probably because its effects were largely accounted for in days fed. Of particular note was that while precursor enrichment of ovarian and egg fats increased with increased feeding, consistent with the increasing proportion of hemolymph trehalose derived from AAC, precursor enrichment of pheromone decreased (Fig. 2a). That is, the proportion of pheromone synthesized from AAC decreased with increased feeding, even though hemolymph trehalose was increasingly derived from AAC.

It was not clear to us what the significance of the effect of total eggs laid on precursor enrichment in ovarian (and to a lesser extent egg) fat meant in terms of an actual physiological effect, especially as the three factors in our model were all temporally related and consequently highly correlated. Therefore, we calculated the residuals from the linear relationship between the total eggs laid and the number of days fed. This gave us a sense of how many more or fewer eggs were laid by an individual female that had been fed for a certain period of time compared to the overall average across all individuals in the experiment. We then determined the relationships between these residuals and ovarian and egg fat enrichments. Both relationships were significant ($F_{1,58}$ = 5.107, P = 0.028 for ovarian fat and $F_{1,49}$ = 5.993, P = 0.022 for egg fat). Thus, by a given day, females that laid fewer eggs than average tended to use proportionally more AAC to synthesize new fats for eggs than did females that laid more eggs than average, consistent with the former females retaining greater amounts of AAC. Typically, across

all the days, precursor enrichments of ovarian and egg fats were similar, with both enrichments lower than that of the pheromone component.

We then tested whether the populational effects observed in our analyses held up at the individual level, by testing linear correlations between pheromone enrichment and egg and ovarian fats; i.e., do individuals show the same relationship between enrichments that we see when looking across all the individuals in the population? There was no correlation across individuals between pheromone enrichment and egg enrichment ($F_{1,32}$ = 0.014, P = 0.91; data not shown), but a slight trend of a negative correlation ($F_{1,38}$ = 1.24, P = 0.13) between pheromone enrichment and ovarian enrichment.

Experiment 2 – Enrichment of pheromone gland and egg fats in mated females.

As in experiment 1, precursor enrichment of 16:Acyl in eggs laid in the preceding 20 hours increased over time (Fig. 2b). Our model showed an effect of number of feeds (F = 5.74, P = 0.031), but not of number of eggs laid (F = 0.064, P = 0.864), on precursor enrichment of 16:Acyl in eggs. Precursor enrichment of the stored pheromone precursor acid, Z11-16:Acyl, did not change over time; consequently there were no effects of either number of feeds (F = 3.69, P = 0.075) or numbers of eggs laid (F = 1.53, P = 0.236). By contrast, precursor enrichment of the stored pheromone precursor acid, 16:Acyl, decreased over time, with an effect of number of feeds (F = 8.20, P = 0.012), but not of total eggs laid (F = 1.62, P = 0.223). Overall, precursor enrichment of 16:Acyl in the pheromone gland tended to be less than that of Z11-16:Acyl, but both were typically greater than precursor enrichment of egg fat. Although strictly not comparable, it was apparent that precursor enrichment of Z11-16:Acyl was substantially lower at the start of the experiment (i.e., after one or two feeds) than that of the pheromone component in experiment 1.

Experiments 3 & 4 – Enrichment of pheromone and pheromone gland fatty acids in virgins.

In experiment 3, precursor enrichment of pheromone produced by virgins fed U-^{13}C-glucose daily increased ($F_{1,29}$= 30.56, P < 0.0001) with increased number of feeds, such that by the end of the experiment (when females were 6 d old) pheromone precursor

enrichment had more than doubled. This demonstrates the proportional increase of AAC allocated to pheromone production in virgins, and is consistent with an increase in hemolymph trehalose derived from AAC, expected from repeated adult feeding on (labeled) glucose. Similarly, precursor enrichment of Z11-16:Acyl also increased ($F_{1,18}$= 6.97, P = 0.017) over time in Experiment 4, although precursor enrichment of 16:Acyl did not change ($F_{1,18}$ = 1.06, P = 0.317). As in experiment 2, precursor enrichment of 16:Acyl tended to be less than that of Z11-16:Acyl. If we again, make the (not strictly valid) cross-experimental comparison of precursor enrichment of pheromone and pheromone precursor acid, it is apparent that, in contrast to what we observed for mated females, precursor enrichments in pheromone and Z11-16:Acyl were similar at the start of the experiment.

DISCUSSION

With limited intake of AAC, female *H. virescens* could reduce its allocation to all reproductive physiologies or selectively reduce its allocation to one (or more) physiology. By feeding females successive, but limited, amounts of, ^{13}C-labeled glucose we tested how allocation of AAC to pheromone and egg production changed with successive adult feeds over time. In our experiments, females were housed individually in small containers that precluded flight; i.e., their activity, and respiratory, levels were relatively low, and, we assume, similar between virgin and mated females. Therefore, we could observe relative allocation differences to the two reproductive physiologies between virgin and mated females without complications from other metabolically demanding activities, such as flight (Candy et al., 1997, O'Brien, 1999). However, it is important to recognize that our results and interpretations could be modified in natural conditions by high demands for the same metabolite by other physiological activities, such as flight.

With successive feeds, the proportion of hemolymph trehalose derived from AAC increased, as expected. If allocation of this pool to the two reproductive physiologies were to follow the same pattern, then precursor enrichment of pheromone and egg fats should also increase over time. While this was the case for virgins, it was not so in mated females, for which ovarian and egg fat precursor enrichments increased, but pheromone enrichment decreased. The most likely explanation for this effect is that increased egg

maturation rate in mated females, mediated by higher juvenile hormone titers controlling ovarial patency (Ramaswamy et al., 1997), causes increased demand for hemolymph trehalose (Foster, 2009; O'Brien et al., 2000), resulting in a deficit of this metabolite for pheromone production. By contrast, virgins maintained or increased their proportional allocation of AAC to pheromone because of their lower egg maturation rate and its consequent lower demand for hemolymph trehalose (Foster, 2009; Ramaswamy et al., 1997). That demand for AAC was lower in virgins than in mated females was apparent by HTC being maintained over the duration of the experiment in the former, while decreasing substantially in the latter, under the same feeding regimen. Thus, for mated females, in particular, sufficient AAC is important for maintaining allocation of hemolymph trehalose to both reproductive physiologies, but when AAC is limited, egg production limits allocation of this resource to pheromone production.

Although this effect of egg production on pheromone production in mated females was apparent in our full model by the effect of days fed on the various enrichments, for individual moths there was no correlation between egg fat and pheromone enrichment and only a weak negative correlation (consistent with our trend from the full model) between ovarian fat and pheromone enrichment. Since time, in general, had a strong effect on enrichments, the correlation between ovarian and pheromone enrichments is likely to be more appropriate, as these enrichments were sampled at the same time, whereas egg fat enrichment was from eggs laid roughly 24 hours prior. The weaker correlation between ovarian fat and pheromone enrichment, in comparison to the significant trends over time in our full model, likely resulted from the fewer points available for the correlation compared to the full model. In particular, pheromone enrichment of older females (i.e., when both pheromone titer and enrichment were declining) became difficult to determine because of the smaller amounts of pheromone and incorporation of label into isotopomers.

This physiological competition for AAC between pheromone and egg production could be widespread across moths, especially given the biosynthetic similarity of many moth sex pheromone components (Blomquist et al., 2011), but is likely to be most significant for species, like *H. virescens*, that feed as adults, mature eggs following eclosion, and are polyandrous. However, even in species that do not fit these criteria, it is

503 conceivable that starvation during the larval stage could result in reduced carbohydrate
504 reserves available for pheromone production at eclosion. In such cases, lower production
505 of pheromone could make females less competitive for attracting mates (Johansson and
506 Jones, 2007), with adult feeding being crucial to compensate for this.
507 The decline in pheromone precursor enrichment over time in mated females means
508 that AAC (hemolymph trehalose) is used proportionally less for producing precursor for
509 synthesis of pheromone and, consequently, proportionally more larval-derived
510 (unlabeled) sources of precursor are used. The most likely pool for this is fat stored in
511 glycerolipids in the pheromone gland, with this relatively large pool (Foster, 2005)
512 providing acetate precursor for de novo biosynthesis, as well as, potentially, preformed
513 stored acids (especially 16:Acyl and Z11-16:Acyl) for pheromone biosynthesis.
514 However, this decrease in proportional allocation of AAC to pheromone biosynthesis was
515 accompanied by a very substantial decline in pheromone titer, suggesting that the
516 increased proportional use of larval carbon is unable to compensate fully for the
517 decreased availability of AAC. The low absolute amount of pheromone produced by
518 these mated females is unlikely to be sufficient to attract conspecific males over distance
519 (Foster and Johnson, 2011). Thus, with limited AAC intake, the reproductive cost of egg
520 production in mated females is no further mating and the direct and indirect benefits that
521 would accrue from such polyandry (Jennions and Petrie, 2000). With an adequate supply
522 of AAC throughout the lifetime, mated female *H. virescens* continue to produce sufficient
523 quantity of pheromone to attract males for further mating (Foster, 2009; Foster and
524 Johnson, 2011). Thus, availability of AAC is a proximate mechanism underlying, at least
525 in part, the frequency of mating in this and perhaps other polyandrous moth species.
526 Comparison of precursor enrichment in the pheromone component and its precursor
527 acid, Z11-16:Acyl, showed apparent differences between mated and virgin females,
528 especially at the start of experiments: mated females had substantially higher precursor
529 enrichment of the pheromone component than of the precursor acid, while virgins had
530 similar values for both moieties. Although this acid is the immediate pheromone
531 precursor, most of it is rapidly reduced and oxidized to pheromone; i.e., the Z11-16:Acyl
532 pool analyzed largely consists of acid stored in glycerolipids (Foster and Anderson,
533 2012). Exactly why stored Z11-16:Acyl in mated females is lower in AAC-derived

precursor than the pheromone component is unclear, although the fact that the two are similar in virgins suggests it is a function of the greater demand for hemolymph trehalose in mated females. The generally lower precursor enrichment of stored 16:Acyl, compared to both pheromone and Z11-16:Acyl, suggests that (at least a portion of) this pool of 16:Acyl may be compartmentally distinct from that of the other two moieties, especially given its ubiquity and the possibility of contamination from non-glandular tissue. Regardless, the rate of synthesis of this stored acid is slow and it consequently represents a relatively small allocation of AAC in the gland (Foster and Anderson, 2012).

The use of both hemolymph trehalose, increasingly derived from AAC, and stored fats, largely derived from larval-acquired nutrients, as two pools supplying precursor to pheromone production in *H. virescens* is similar to the situation for carbon allocation to egg production reported in other Lepidoptera (and indeed in other insects in which adults feed on sugars, e.g., Rivero et al., 2001; Min et al., 2006). In studies on various moth and butterfly species fed larval and adult diets with different carbon isotopic signatures, carbon allocation to eggs was found to fit a two-compartment (pool) model, with one pool composed principally of larval-acquired carbon and the other a mixture of larval and adult-acquired carbon (O'Brien et al., 2000; O'Brien et al., 2004). The increasing allocation of AAC, in the mixed pool, to ovarian and egg fats in our study was consistent with other studies on allocation of AAC to egg metabolites in other species of Lepidoptera (O'Brien et al., 2000; O'Brien et al., 2002; O'Brien et al., 2004; O'Brien et al., 2005), thereby supporting the validity of our fat proxy for the pattern of allocation of ACC to egg production.

In summary, AAC supplies a common pool, hemolymph trehalose, of precursor for pheromone and egg production in the moth *H. virescens*. When AAC is limited for mated females, physiological competition for this metabolite results in its allocation to egg production occurring at the expense of its allocation to pheromone production, thus limiting the ability of females to attract further mates. Thus, availability of AAC to adult females may be a proximate mechanism underlying the incidence of polyandry in this and other species of moths.

Acknowledgments

This work was funded by a Hatch grant from the North Dakota Agricultural Experiment Station.

REFERENCES

Ando, T., Inomata, S. and Yamamoto, M. (2004). Lepidopteran sex pheromones. In *The Chemistry of Pheromones and Other Semiochemicals I*, vol. 239 (ed. S. Schulz), pp. 51-96. Berlin / Heidelberg: Springer

Bjostad, L. B., Wolf, W. A. and Roelofs, W. L. (1987). Pheromone biosynthesis in lepidopterans: Desaturation and chain shortening. In *Pheromone Biochemistry*, eds. G. D. Prestwich and G. J. Blomquist), pp. 77-120. New York: Academic Press.

Blomquist, G. J., Borgeson, C. E. and Vundla, M. (1991). Polyunsaturated fatty acids and eicosanoids in insects. *Insect Biochemistry* **21**, 99-106.

Blomquist, G. J., Jurenka, R., Schal, C. and Tittiger, C. (2011). Pheromone production: biochemistry and molecular biology. In *Insect Endocrinology*, (ed. L. I. Gilbert), pp. 523-567. San Diego, CA: Academic Press.

Boggs, C. L. and Ross, C. L. (1993). The effect of adult food limitation on life history traits in *Speyeria mormonia* (Lepidoptera: Nymphalidae). *Ecology* **74**, 433-441.

Candy, D. J., Becker, A. and Wegener, G. (1997). Coordination and integration of metabolism in insect flight. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **117**, 497-512.

Cardé, R. T. and Baker, T. C. (1984). Sexual communication with pheromones. In *Chemical Ecology of Insects*, eds. W. J. Bell and R. T. Cardé), pp. 355-383. London: Chapman and Hall Ltd.

Chinkes, D. L., Aarsland, A., Rosenblatt, J. and Wolfe, R. R. (1996). Comparison of mass isotopomer dilution methods used to compute VLDL production in vivo. *American Journal of Physiology - Endocrinology and Metabolism* **271**, E373-383.

Choi, M. Y., Groot, A. and Jurenka, R. A. (2005). Pheromone biosynthetic pathways in the moths *Heliothis subflexa* and *Heliothis virescens*. *Archives of Insect Biochemistry and Physiology* **59**, 53-58.

Cohen, A. C. and Patana, R. (1985). Chemical composition of tobacco budworm eggs during development. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry* **81**, 165-169.

Foster, S. and Anderson, K. (2011). The use of mass isotopomer distribution analysis to quantify synthetic rates of sex pheromone in the moth *Heliothis virescens*. *Journal of Chemical Ecology* **37**, 1208-1210.

Foster, S. and Johnson, C. (2011). Signal honesty through differential quantity in the female-produced sex pheromone of the moth *Heliothis virescens*. *Journal of Chemical Ecology* **37**, 717-723.

Foster, S. P. (2005). Lipid analysis of the sex pheromone gland of the moth *Heliothis virescens*. *Archives of Insect Biochemistry and Physiology* **59**, 80-90.

Foster, S. P. (2009). Sugar feeding via trehalose haemolymph concentration affects sex pheromone production in mated *Heliothis virescens* moths. *Journal of Experimental Biology* **212**, 2789-2794.

Foster, S. P. and Anderson, K. G. (2012). Synthetic rates of key stored fatty acids in the biosynthesis of sex pheromone in the moth *Heliothis virescens*. *Insect Biochemistry and Molecular Biology* **42**, 865-872.

Foster, S. P. and Johnson, C. P. (2010). Feeding and hemolymph trehalose concentration influence sex pheromone production in virgin *Heliothis virescens* moths. *Journal of Insect Physiology* **56**, 1617-23.

Greenfield, M. D. (1981). Moth sex pheromones: an evolutionary perspective. *Florida Entomologist* **64**, 4-17.

Harari, A. R., Zahavi, T. and Thiéry, D. (2011). Fitness cost of pheromone production in signaling female moths. *Evolution* **65**, 1572-1582.

Hellerstein, M. K. and Neese, R. A. (1992). Mass isotopomer distribution analysis: a technique for measuring biosynthesis and turnover of polymers. *American Journal of Physiology - Endocrinology And Metabolism* **263**, E988-1001.

Hellerstein, M. K. and Neese, R. A. (1999). Mass isotopomer distribution analysis at eight years: theoretical, analytic, and experimental considerations. *American Journal of Physiology - Endocrinology And Metabolism* **276**, E1146-1170.

Jennions, M. D. and Petrie, M. (2000). Why do females mate multiply? A review of the genetic benefits. *Biological Reviews* **75**, 21-64.

JMP. (2012). JMP Version 10. Cary, North Carolina: SAS Institute Inc.

Johansson, B. and Jones, T. (2007). The role of chemical communication in mate choice. *Biological Reviews of the Cambridge Philosophical Society* **82**, 265-289.

Jurenka, R. (2003). Biochemistry of female moth sex pheromones. In *Insect Pheromone Biochemistry and Molecular Biology*, eds. G. J. Blomquist and R. C. Vogt), pp. 54-80. Amsterdam: Elsevier.

Ma, P. W. K. and Ramaswamy, S. B. (2003). Biology and ultrastructure of sex pheromone-producing tissue. In *Insect pheromone biochemsitry and molecular biology.*, eds. G. J. Blomquist and R. C. Vogt), pp. 19-51. London: Elsevier Academic Press.

Min, K.-J., Hogan, M. F., Tatar, M. and O'Brien, D. M. (2006). Resource allocation to reproduction and soma in *Drosophila*: A stable isotope analysis of carbon from dietary sugar. *Journal of Insect Physiology* **52**, 763-770.

O'Brien, D. M. (1999). Fuel use in flight and its dependence on nectar feeding in the hawkmoth *Amphion floridensis*. *J Exp Biol* **202**, 441-451.

O'Brien, D. M., Boggs, C. L. and Fogel, M. L. (2004). Making eggs from nectar: the role of life history and dietary carbon turnover in butterfly reproductive resource allocation. *Oikos* **105**, 279-291.

O'Brien, D. M., Boggs, C. L. and Fogel, M. L. (2005). The amino acids used in reproduction by butterflies: A comparative study of dietary sources using compound-specific stable isotope analysis. *Physiological and Biochemical Zoology* **78**, 819-827.

O'Brien, D. M., Fogel, M. L. and Boggs, C. L. (2002). Renewable and nonrenewable resources: Amino acid turnover and allocation to reproduction in Lepidoptera. *Proceedings of the National Academy of Sciences* **99**, 4413-4418.

O'Brien, D. M., Schrag, D. P. and Martinez del Rio, C. (2000). Allocation to reproduction in a hawkmoth: a quantitative analysis using stable carbon isotopes. *Ecology* **81**, 2822-2831.

Perret, M., Chautems, A., Spichiger, R., Peixoto, M. and Savolainen, V. (2001). Nectar sugar composition in relation to pollination syndromes in Sinningieae (Gesneriaceae). *Annals of Botany* **87**, 267-273.

Raina, A. K. (1989). Male-induced termination of sex pheromone production and receptivity in mated females of *Heliothis zea*. *Journal of Insect Physiology* **35**, 821-826.

Ramaswamy, S. B. (1990). Periodicity of oviposition, feeding, and calling by mated female *Heliothis virescens* in a field cage. *Journal of Insect Behavior* **3**, 417-427.

Ramaswamy, S. B., Shu, S., Park, Y. I. and Zeng, F. (1997). Dynamics of juvenile hormone-mediated gonadotropism in the Lepidoptera. *Archives of Insect Biochemistry and Physiology* **35**, 539-558.

Rivero, A., Giron, D. and Casas, J. (2001). Lifetime allocation of juvenile and adult nutritional resources to egg production in a holometabolous insect. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **268**, 1231-1237.

SAS Institute Inc. (2012). SAS® Structural Equation Modeling 1.3 for JMP® . Cary, NC: SAS Institute Inc.

Svensson, M. (1996). Sexual selection in moths: the role of chemical communication. *Biological Reviews of the Cambridge Philosophical Society* **71**, 113-135.

Tamaki, Y. (1985). Sex pheromones. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 9 eds. G. A. Kerkut and L. I. Gilbert), pp. 145-191. New York: Pergamon Press.

Teal, P. E. A., Tumlinson, J. H. and Heath, R. R. (1986). Chemical and behavioral analyses of volatile sex pheromone components released by calling *Heliothis virescens* (F.) females (Lepidoptera: Noctuidae). *Journal of Chemical Ecology* **12**, 107-126.

Telfer, W. H. (2009). Egg formation in Lepidoptera. *Journal of Insect Science* **9**, 1-21.

Wäckers, F. L., Romeis, J. and van Rijn, P. (2007). Nectar and pollen feeding by insect herbivores and implications for multitrophic interactions. *Annual Review of Entomology* **52**, 301-323.

Wheeler, D. (1996). The role of nourishment in oogenesis. *Annual Review of Entomology* **41**, 407-431.

Wolfe, R. R. and Chinkes, D. L. (2005). Isotope Tracers in Metabolic Research, Second Edition: John Wiley & Sons, Inc.

691 **Captions**

692

693 **Figure 1. Mean (±SEM) hemolymph trehalose concentrations and pheromone titers**
694 **of (a) mated (when 1 day old) and (b) virgin female *Heliothis virescens* at various**
695 **ages or days after mating. Females were fed daily on 10% glucose solution, starting**
696 **when 1 day old.**

697

698 **Figure 2. Mean (±SEM) ^{13}C-enrichments of various moieties, over time, of mated**
699 **female *Heliothis virescens* fed daily on U-^{13}C-glucose: (a) hemolymph trehalose (in**
700 **atom percent excess), (*Z*)-11-hexadecenal (pheromone), and hexadecanoate in**
701 **ovaries and eggs (all in mole percent excess) from experiment 1; (b) hexadecanaote**
702 **(16:Acyl) and (*Z*)-11-hexadecenoate (Z11-16:Acyl) in the female sex pheromone**
703 **gland (both in mole percent excess), and 16:Acyl from eggs, from experiment 2.**
704 Females were mated one day after eclosion and were fed initially after copulation. Linear
705 models (see text) were used to test for effects. In 1a, there was a positive effect of number
706 of feeds (i.e., days after mating) on enrichment of hemolymph trehalose ($F = 15.178$, $P =$
707 0.0003; data arcsine transformed), and on enrichment of pheromone, and 16:Acyl in eggs
708 and ovaries (see Table 1; egg data log transformed). In 1b, number of feeds had a positive
709 effect ($F = 5.74$, $P = 0.031$) on enrichment of 16:Acyl in eggs, no effect ($F = 3.69$, $P =$
710 0.075) on enrichment of glandular Z11-16:Acyl, and a negative effect ($F = 8.20$, $P =$
711 0.012) on glandular 16:Acyl.

712

713 **Figure 3. Mean (±SEM) ^{13}C-enrichments (mole percent excess) of various moieties,**
714 **over time, of virgin female *Heliothis virescens* fed daily on U-^{13}C-glucose: (a) (*Z*)-11-**
715 **hexadecenal (pheromone) in experiment 3; (b) hexadecanaote (16:Acyl) and (*Z*)-11-**
716 **hexadecenoate (Z11-16:Acyl) in the female sex pheromone gland in Experiment 4.**
717 Females were fed initially on the day after eclosion (i.e., when one day old). Linear

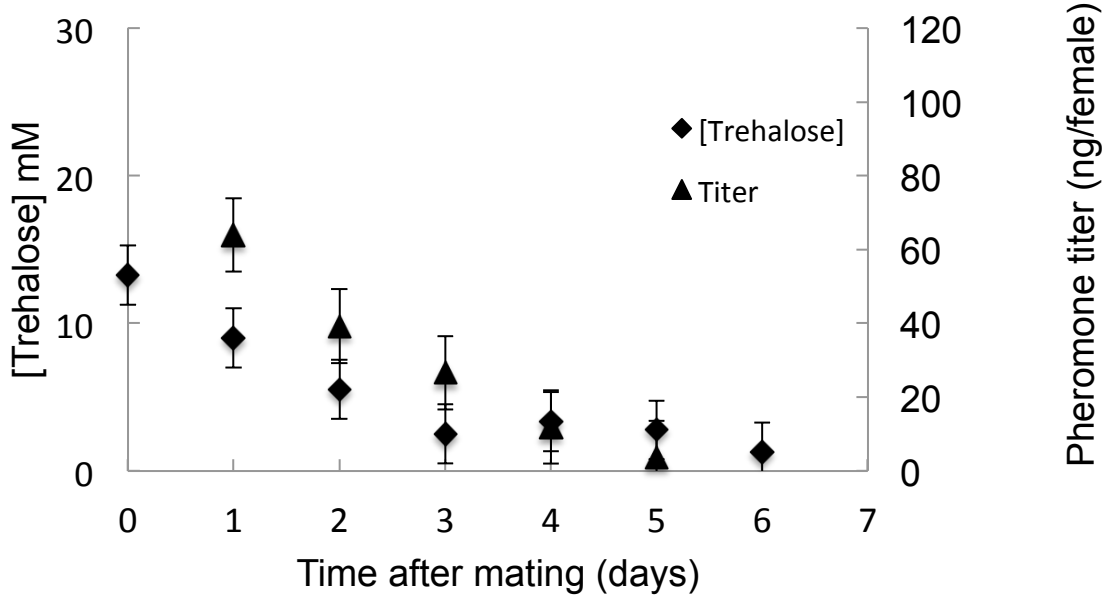
718 models (see text) were used to test for effects. In 3a, number of feeds (age) had a positive
719 effect ($F_{1,29}$= 30.56, P < 0.0001) on precursor enrichment of pheromone. In 1b, number of
720 feeds had a positive effect ($F_{1,18}$= 6.97, P = 0.017) on enrichment of glandular Z11-
721 16:Acyl, but not on enrichment of glandular 16:Acyl ($F_{1,18}$ = 1.06, P = 0.317).
722
723
724

725 Table 1. Statistical analyses of mated female *Heliothis virescens* in Experiment 1, testing
726 the effects (parameter estimates±SEM) of days fed, total eggs laid and trehalose
727 enrichment on the responses of pheromone, ovarian and egg fat enrichment.

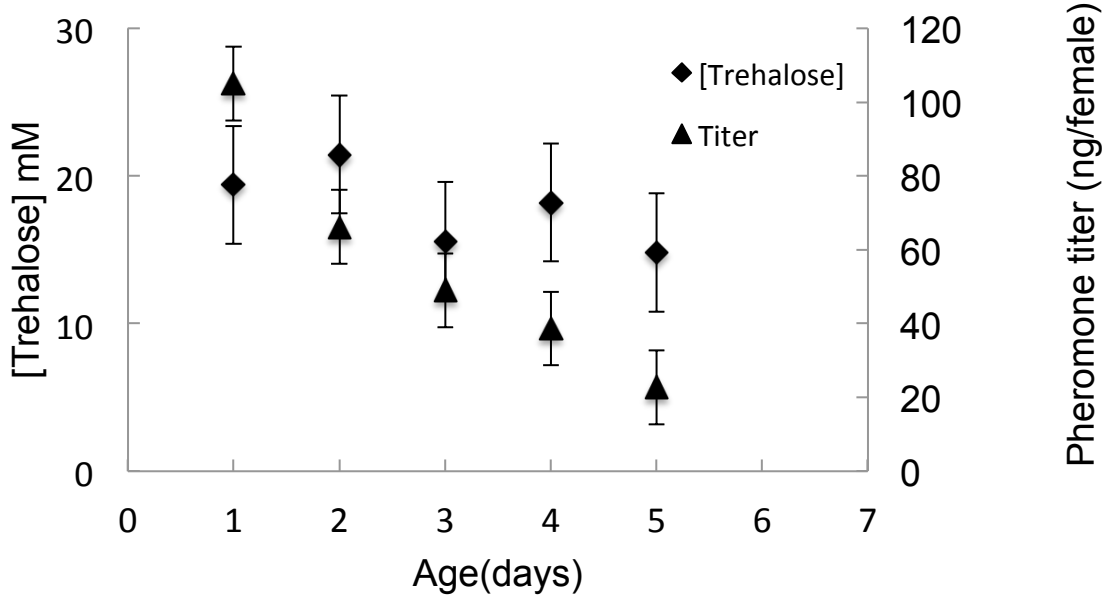
Response	Model	Days fed	Total eggs laid	Trehalose enrichment
Pheromone precursor enrichment	$F_{3,31} = 2.096$, P= 0.12	-0.035±0.017 t = -2.14, P = 0.040	0.0004±0.0002 t = 2.01, P = 0.054	-0.096±0.34 t = -0.22, P = 0.83
Ovaries precursor enrichment	$F_{3,43} = 3.919$, P= 0.015	0.0086±0.0041 t = 2.09, P = 0.042	-0.00008±0.00004 t = -1.98, P = 0.054	0.124±0.079 t = 1.57 P = 0.12
Eggs precursor enrichment	$F_{3,37} = 2.005$, P= 0.13	0.013±0.0057 t = 2.25, P = 0.031	-0.00008±0.00005 t = -1.68, P = 0.14	-0.062±0.099 t = -0.62, P = 0.54

728
729
730

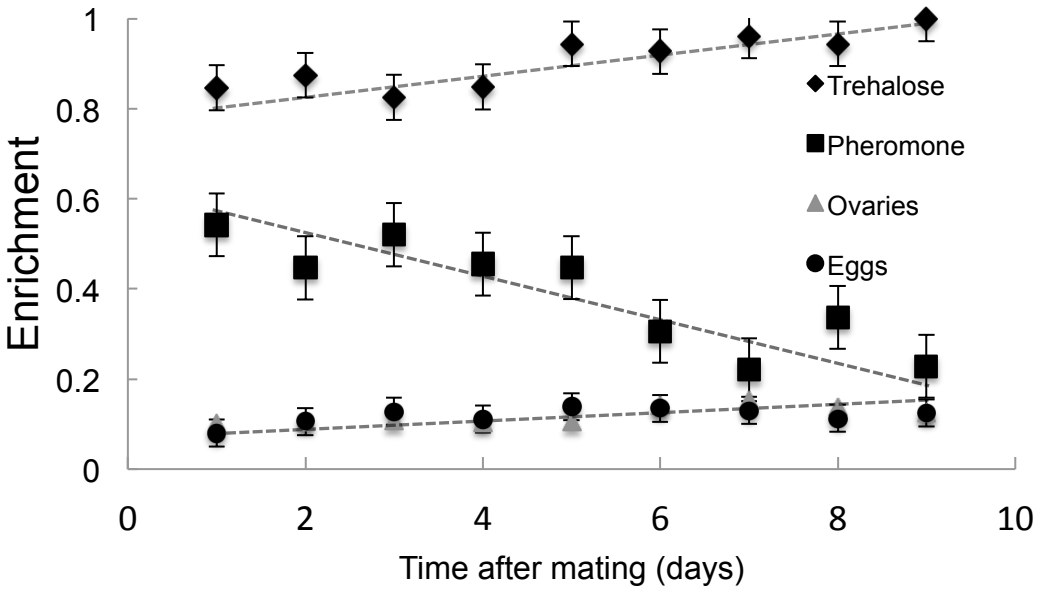
a)



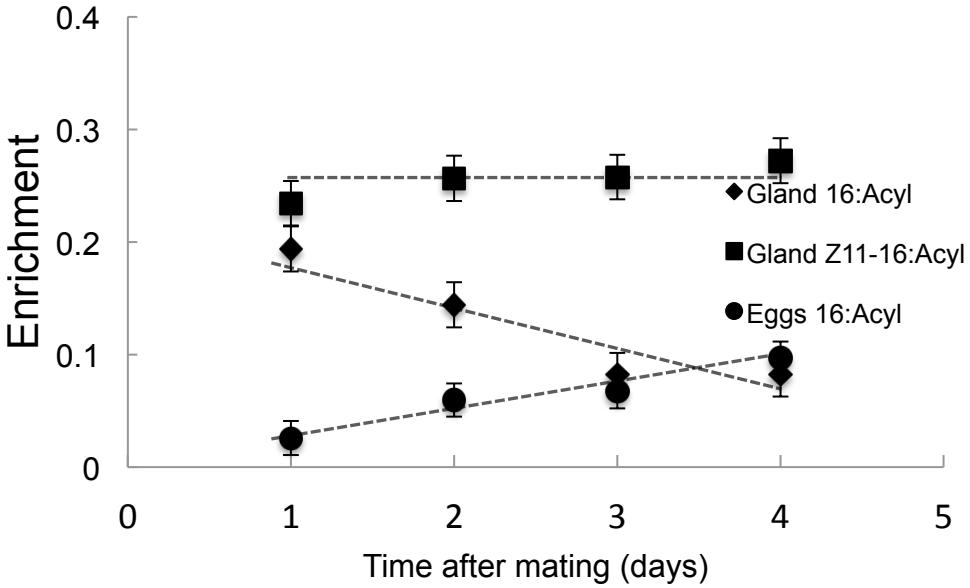
b)



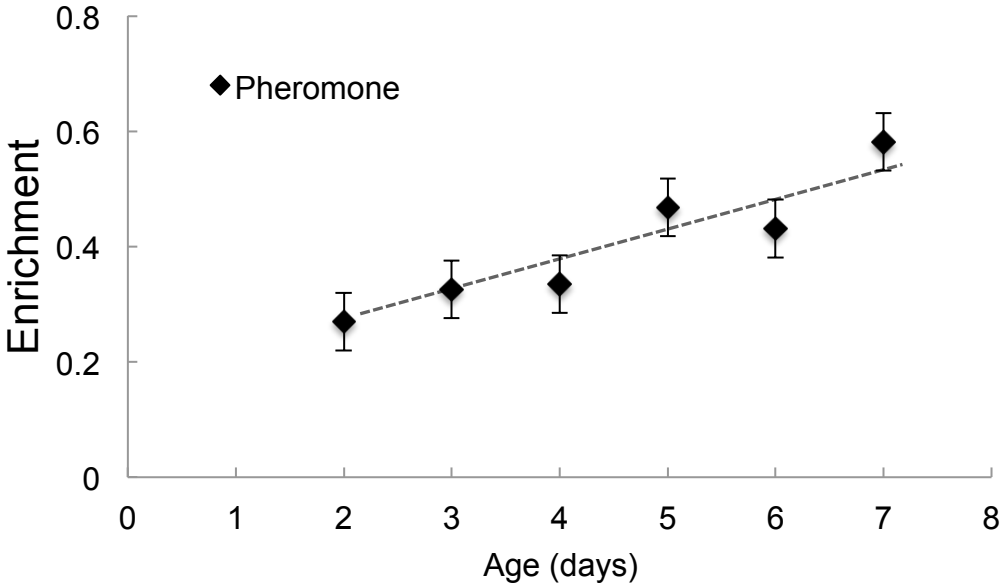
a)


Enrichment
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
Time after mating (days)
Trehalose
Pheromone
Ovaries
Eggs

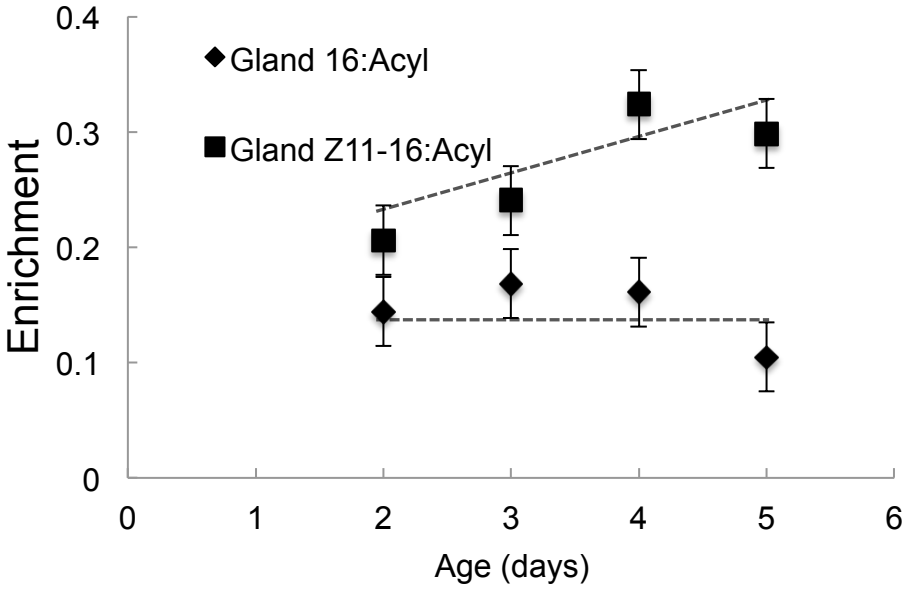
b)


Enrichment
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
Time after mating (days)
Gland 16:Acyl
Gland Z11-16:Acyl
Eggs 16:Acyl

a)


Pheromone
Enrichment
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
7
8
Age (days)

b)


Gland 16:Acyl
Gland Z11-16:Acyl
Enrichment
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
6
Age (days)