



# Sex pheromone in the moth *Heliothis virescens* is produced as a mixture of two pools: de novo and via precursor storage in glycerolipids

Stephen Foster, Karin Anderson, Jérôme Casas

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1 Address correspondence to: Dr. Stephen Foster  
2 North Dakota State University  
3 Entomology Department  
4 NDSU Dept 7650  
5 PO Box 6050  
6 Fargo, ND 58108-6050  
7 U.S.A  
8 Ph. 1-701-231-6444  
9 Fax 1-701-231-8557  
10 Email: [stephen.foster@ndsu.edu](mailto:stephen.foster@ndsu.edu)  
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20 Sex pheromone in the moth *Heliothis virescens* is produced as a mixture of two pools: de  
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25 Stephen P. Foster<sup>1</sup>, Karin G. Anderson<sup>1</sup> and Jérôme Casas<sup>2</sup>  
26

27 <sup>1</sup>Entomology Department, North Dakota State University,  
28 PO Box 6050, Fargo, North Dakota 58108-6050, U.S.A  
29 and

30 <sup>2</sup>Université de Tours, Institut de Recherche sur la Biologie de l’Insecte,  
31 UMR CNRS 7261, 37200 Tours, FRANCE  
32  
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## Abstract

Most species of moths use a female-produced volatile sex pheromone, typically produced via de novo fatty acid synthesis in a specialized gland, for communication among mates. While de novo biosynthesis of pheromone (DNP) is rapid, suggesting transient precursor acids, substantial amounts of pheromone precursor (and other) acids are stored, predominantly in triacylglycerols in the pheromone gland. Whether these stored acids are converted to pheromone later or not has been the subject of some debate. Using a tracer/tracee approach, in which we fed female *Heliothis virescens* U-<sup>13</sup>C-glucose, we were able to distinguish two pools of pheromone, in which precursors were temporally separated (after and before feeding on labeled glucose): DNP synthesized from a mixed tracer/tracee acetyl CoA pool after feeding, and pheromone made from precursor acids primarily synthesized before feeding, which we call recycled precursor fat pheromone (RPP). DNP titer varied from high (during scotophase) to low (photophase) and with presence/absence of pheromone biosynthesis activating neuropeptide (PBAN), in accord with native pheromone titer previously observed. By contrast, RPP was constant throughout the photoperiod and did not change with PBAN presence/absence. The amount of RPP (6.3-10.3 ng/female) was typically much lower than that of DNP, especially during the scotophase (peak DNP, 105 ng/female). We propose an integral role for stored fats in pheromone biosynthesis, in which they are hydrolyzed and re-esterified throughout the photoperiod, with a small proportion of liberated precursor acyl CoAs being converted to pheromone. During the sexually active period, release of PBAN results in increased flux of glucose (from trehalose) and hydrolyzed acids entering the

61 mitochondria, producing acetyl CoA precursor for de novo fat and pheromone

62 biosynthesis.

63

64

65 **Keywords:** Chemical communication; stable isotope; tracer/tracee; mass isotopomer

66 distribution analysis; Noctuidae; Lepidoptera.

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## 1. Introduction

The use of volatile sex pheromones to bring mates together for copulation is prevalent among the group of insects known as moths (order: Lepidoptera) (Allison and Cardé, 2016). Female moths produce and/or release a sex pheromone, typically a blend of closely related chemicals, from a specialized gland usually located on the intersegmental membrane between the 8<sup>th</sup> and 9<sup>th</sup> abdominal segments (Ma and Ramaswamy, 2003). Elevated production and release of pheromone typically occur during a defined temporal period of the day, when a species is sexually active (Groot, 2014). In many species of moths, the period of elevated production is governed by the release of the pheromone biosynthesis-activating neuropeptide (PBAN) from the corpora cardiaca into the hemolymph (Blomquist et al., 2011; Rafaeli and Jurenka, 2003).

Species of moths that biosynthesize so-called “Type 1” sex pheromone components do so by a route involving rapid de novo fatty acid synthesis from acetyl CoA, followed by desaturation and/or cytosolic  $\beta$ -oxidation of the alkyl chain and modification of the carboxyl group to an alcohol, aldehyde or acetate ester (Ando et al., 2004; Blomquist et al., 2011; Foster, 2016). De novo biosynthesis of pheromone requires a supply of acetyl CoA precursor, which is provided by glycolysis/pyruvate oxidation of hemolymph trehalose, and mitochondrial  $\beta$ -oxidation of fatty acids from glandular glycerolipids (Foster and Anderson, 2015). In addition to providing fatty acids for  $\beta$ -oxidation, glandular glycerolipids also contain relatively substantial amounts of pheromone precursor acids, which may or may not subsequently be converted to pheromone (Bjostad et al., 1987; Fang et al., 1995; Foster, 2005b; Matsumoto, 2010). For instance, in the

silkworm moth, *Bombyx mori*, which uses a single component (bombykol) as its sex pheromone, triacylglycerol stores of pheromone precursor acids accumulate throughout the non-sexually active period. These are then hydrolyzed and reduced to pheromone following release of PBAN during the sexually active period (Matsumoto, 2010). By contrast, species such as the redbanded leafroller, *Argyrotaenia velutinana*, and the European corn borer, *Ostrinia nubilalis*, which use highly specific ratios of geometric isomers as pheromone components, may have a preponderance of the precursor acid of the minor pheromone component over that of the major component stored in glycerolipids (Bjostad et al., 1981; Foster, 2004). This suggests that there is little release and conversion of these acids directly to pheromone. In these cases, fatty acids may still be hydrolyzed from glycerolipids by lipases for  $\beta$ -oxidation, but the much greater abundances of other acids will likely result in a relatively low release of pheromone precursor acids with an insignificant effect on the ratio of pheromone components produced.

Sex pheromone biosynthesis and glandular glycerolipids have been extensively studied in the moth *Heliothis virescens* (Fabricius) (family: Noctuidae) (Choi et al., 2005; Foster and Anderson, 2011; Foster, 2005a, b; Foster and Anderson, 2012; Groot et al., 2016; Hagström et al., 2013), which uses a blend of (Z)-11-hexadecenal (Z11-16:Ald) and (Z)-9-tetradecenal as its sex pheromone (Roelofs et al., 1974). Recently, using the stable isotope tracer-tracee method of mass isotopomer distribution analysis (MIDA; Hellerstein and Neese, 1999), we established that females use a roughly 2:1 ratio of carbohydrate (from hemolymph trehalose) to stored fats as nutrients for production of acetyl CoA

during de novo pheromone biosynthesis (Foster and Anderson, 2015). However, we noticed that a significant portion of the unlabeled pheromone could not have been produced from the tracer/tracee pool of acetyl CoA used for de novo-produced pheromone (DNP). We concluded that this unlabeled pheromone must arise from a distinct precursor pool, containing no labeled acetyl CoA, most likely from pheromone precursor acids, specifically (*Z*)-11-hexadecenoate (Z11-16:Acyl), hexadecanoate (16:Acyl), and octadecanoate (18:Acyl), synthesized and stored before introduction of the tracer. These stored acids may be converted directly, without mitochondrial  $\beta$ -oxidation, to pheromone (Choi et al., 2005).

This result suggested that recycling of stored precursor fats converted directly to pheromone (“recycled precursor fat pheromone”; RPP) might be an important contributor to pheromone production in *H. virescens*, particularly at different times of the photoperiod or with increased age. For example, most of the pheromone produced early in the scotophase, and released first by females, could be produced via this route before DNP production contributed more. Since we are developing a quantitative model to describe pheromone production and release in *H. virescens*, we sought to (i) characterize the overall production pathway structure, (ii) estimate some of its parameters, in particular the relative contributions of DNP and RPP, (iii) determine whether their contributions were time dependent with respect to photoperiod and age, and (iv) determine whether PBAN influenced the production of RPP and DNP, as it does for native pheromone (Eltahlawy et al., 2007; Groot et al., 2005).

## 2. Methods and Materials

### 2.1. Insects

*Heliothis virescens* were from a colony maintained at NDSU, Fargo, but originating from a colony previously established at USDA-ARS BRL, Fargo, and recently supplemented with insects supplied by Dr. F.A. Gould (North Carolina State University, Raleigh, NC). Larvae were reared at 25°C under a 16:8 L:D photoperiod and fed on a wheatgerm-casein diet until they pupated, after which they were sexed and the two sexes maintained separately under the same environmental conditions as larvae.

Adults were collected daily and categorized as 1-d-old the day after eclosion, 2-d-old, two days after eclosion, etc. Prior to the start of an experiment, adults were starved and denied access to any liquids. The stable isotope tracer was introduced by allowing an adult to feed on 25 µl of a 10% (w/v) aqueous solution of U-<sup>13</sup>C-glucose (99%; Cambridge Isotope Laboratories, Cambridge, MA) as a drop on a watch glass; only females that consumed the full amount were used in experiments. The labeled glucose, absorbed rapidly into hemolymph trehalose, undergoes glycolysis and oxidation to generate <sup>13</sup>C<sub>1,2</sub>-acetyl-CoA tracer, which is incorporated into pheromone and gland fats (Foster and Anderson, 2011; Foster and Anderson, 2012).

### 2.2. Extraction, Derivatization and Chemical Analysis

The pheromone gland of a female was extruded by applying gentle force to the abdomen and then excised with fine forceps. For pheromone extraction, the gland was placed in 5



161  $\mu\text{l}$  of *n*-heptane containing 25 ng of (*Z*)-11-tetradecenal (Z11-14:Ald) as an internal  
162 standard and allowed to extract for at least 1 h at ambient temperature before analysis.  
163 For fatty acid extraction, the gland was placed in 50  $\mu\text{l}$  of a 2:1 mixture of  $\text{CH}_2\text{Cl}_2$ :  
164 MeOH along with 250 ng of tripentadecanoin (Sigma-Aldrich, St Louis, MO) as an  
165 internal standard, and allowed to extract at  $-15^\circ\text{C}$  overnight.  
166  
167 The pheromone extract was injected into an Agilent 7890/5978A gas  
168 chromatograph/mass spectrometer (GC/MS), whereas the fat extract was subjected to  
169 base methanolysis to generate fatty acid methyl esters (FAMES). Briefly, after extraction,  
170 the solvent was decanted and removed by a gentle stream of nitrogen. Then, 50  $\mu\text{l}$  of 0.5  
171 M methanolic KOH was added and allowed to react for 1 h at ambient temperature before  
172 50  $\mu\text{l}$  of 1 M HCl (aq) was added along with 25  $\mu\text{l}$  of heptane. The solution was subjected  
173 to rapid vortexing for 30 sec., before the heptane layer (top) was decanted and injected in  
174 the GC/MS for analysis.  
175  
176 The GC used helium at a constant flow of  $1.5 \text{ ml} \cdot \text{min}^{-1}$  as carrier gas, and splitless  
177 injection. The column was a 30 m x 0.25 mm i.d x 250  $\mu\text{m}$  film thickness ZBWax  
178 (Phenomenex, Torrance, CA) and the oven temperature was programmed from  $80^\circ\text{C}$   
179 (delay of 1 min) to 180 at  $15^\circ\text{C} \cdot \text{min}^{-1}$ , then to 190 at  $5^\circ\text{C} \cdot \text{min}^{-1}$ , and finally to 220 at  
180  $20^\circ\text{C} \cdot \text{min}^{-1}$ . The MS was operated with electron impact ionization at 70 eV and used in  
181 the single ion monitoring mode. The MS source and quadrupole were set at  $230^\circ\text{C}$  and  
182  $150^\circ\text{C}$ , respectively.  
183

For the pheromone analyses, the following  $m/z$  were monitored: 192 for the internal standard (Z11-14:Ald), 220, 222, and 224, for Z11-16:Ald (we only analyzed the major pheromone component, as it comprises >90% of the mass of the pheromone and is biosynthesized similarly to the minor component Z9-14:Ald; Choi et al., 2005; Teal et al., 1986). The  $m/z$  192 and 220 were monitored because they are ions  $[(M-H_2O)^+]$  of intact (unlabeled) carbon skeletons of Z11-14:Ald and Z11-16:Ald, respectively, and carry more ion current than their respective parent ions. The  $m/z$  222 and 224 are the M+1 (+one  $^{13}C_2$  unit) and M+2 (+two  $^{13}C_2$  unit) isotopomers of Z11-16:Ald.

The FAMES of Z11-16:Acyl and 16:Acyl were analyzed similarly by monitoring their molecular ions, namely  $m/z$  268 (M+0 for Z11-16:Acyl), 270, (M+1 for Z11-16:Acyl, M+0 for 16:Acyl), 272 (M+2 for Z11-16:Acyl, M+1 for 16:Acyl) and 274 (M+2 for 16:Acyl), along with  $m/z$  256 of the internal standard methyl pentadecanoate.

### 2.3. Mass Isotopomer Distribution Analysis

MIDA is a combinatorial approach to determine isotopic (precursor) enrichment in a monomeric pool used to synthesize a polymer, following the introduction of a stable isotope-labeled monomer (Hellerstein and Neese, 1992; Wolfe and Chinkes, 2005). It accomplishes this by measuring intensities of both unlabeled and labeled isotopomers of the polymer, while accounting for abundances of natural isotopes. The precursor enrichment, i.e., the proportion of labeled monomeric units in the resulting polymer, can be calculated using the pattern of isotopomers. An advantage of MIDA is that it is not subject to isotopic discrimination in the precursor pool (Hellerstein and Neese, 1999).

207

208 We used MIDA to calculate precursor enrichment following introduction of the tracer  
209 ( $^{13}\text{C}_2$ -acetyl CoA) formed after insects had fed on U- $^{13}\text{C}$ -glucose. We analyzed females  
210 at least 16 h after they had fed, so that the precursor pool and pheromone or pheromone  
211 precursor acids were in isotopic equilibrium (Foster and Anderson, 2011; Foster and  
212 Anderson, 2012). To calculate precursor enrichment, tracer/tracee ratios (TTRs) of singly  
213 (M+1) and doubly (M+2) labeled (i.e., with one and two  $^{13}\text{C}_2$  units, respectively)  
214 isotopomers were calculated for the acetyl CoA octomers Z11-16:Ald, Z11-16:Acyl and  
215 16:Acyl as follows:

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

217 (2)  $\text{TTR}(\text{M}+2) = (\text{M}+2/\text{M}+0)_{\text{post}} - (\text{M}+2/\text{M}+0)_{\text{pre}} - dT_1 \times \text{TTR}(\text{M}+1)$

218 Where ‘pre’ and ‘post’ subscripts, respectively, refer to the intensities of isotopomers  
219 before and after tracer is introduced. We used theoretically calculated values of the pre  
220 intensities (using known natural isotopic abundances), rather than experimentally  
221 determined ones, as previously (Foster and Anderson, 2011; Foster and Anderson, 2012)  
222 we found little difference between the two. The term  $dT_1$  is the contribution of the M+1  
223 isotopomer spectrum to the M+2 isotopomer. Then, precursor enrichment (“ $p$ ”, in molar  
224 percent excess) of an octomer can be calculated by:

225 (3)  $p = 2 \times [\text{TTR}(\text{M}+2)/\text{TTR}(\text{M}+1)] \div [7 + \text{TTR}(\text{M}+2)/\text{TTR}(\text{M}+1)]$

226

227 In order to calculate the amount of unlabeled pheromone not produced by de novo  
228 biosynthesis (i.e., RPP) after addition of the tracer, we used precursor enrichment to  
229 predict the entire isotopomer pattern (for all 9 isotopomers, M+0–8). Then, by using the

observed intensity of the M+2 isotopomer (minus any natural isotopic contributions from the M+0 and M+1 isotopomers), we calculated the expected intensity of the M+0 isotopomer produced via de novo synthesis from the labeled/unlabeled acetyl CoA pool. This was subtracted from the intensity of the observed M+0 isotopomer to allow the amount (relative to the internal standard) of RPP to be determined. Similarly, using the sum of all (nine) isotopomers expected for a given  $p$ , we calculated the amount of DNP. De novo-produced (i.e., labeled, including expected M+0 isotopomer, after U- $^{13}\text{C}$ -glucose was ingested) and previously synthesized (unlabeled, before feeding) stored Z11-16:Acyl and 16:Acyl were calculated similarly.

#### *2.4. Isotopic fractionation*

To test whether quantification of both DNP and RPP pools was affected by significant isotopic fractionation from use of the  $^{13}\text{C}$ -tracer, we fed 1 d females either unlabeled (>99% pure and natural isotopic composition; Sigma-Aldrich, St Louis, MO) or  $^{13}\text{C}$ -labeled glucose at the end of the scotophase. The following day, at the beginning of the scotophase (i.e., 16 h later), we quantified the amount of unlabeled pheromone in females fed unlabeled glucose, and the amounts of DNP and RPP in females fed labeled glucose. The total amounts (i.e., DNP + RPP for females fed U- $^{13}\text{C}$ -glucose vs total pheromone in females fed unlabeled glucose) for each treatment were compared.

#### *2.5. Effect of age and time of photoperiod*

We determined the amount of DNP and RPP in females of different ages and at different times during the scotophase, in order to test whether the respective amounts in the gland

varied through time. For this, we fed females of different ages (0, 1, 2, and 3 d) U-<sup>13</sup>C-glucose at the end of the scotophase and left them for at least 18 h before analyzing pheromone at hour 2 of the scotophase (hereafter, hours of the photoperiod are referred to as S2, P16, etc., with the letter indicating scotophase or photophase and the number the hour of the respective period). Females fed at 1 d were analyzed more extensively, starting at P14 of the subsequent photophase and then every 2 h throughout the subsequent scotophase (i.e., when 2 d old). In addition, 1 d females were fed 6 h into their first complete photophase (i.e., the photophase preceding the scotophase in which they were analyzed every 2 h) and analyzed 24 h later (at P6). From 5–13 females were analyzed for each time point.

## *2.6. Effect of decapitation*

To test whether the amounts of DNP and RPP are influenced by the absence of PBAN, we fed 1 d females U-<sup>13</sup>C-glucose at the end of the scotophase and decapitated them 18 h later (at Scot2). Decapitation stops PBAN reaching the pheromone gland and consequently results in a rapid decrease in pheromone titer (Rafaeli and Jurenka, 2003). We sampled females at 0 (immediately prior to decapitation), 0.25, 0.5, 1.0, 1.5, 2, 4, 6 and 24 h later. Five to eight females were analyzed at each time.

In the second part, 1 d females were fed U-<sup>13</sup>C-glucose and then, in the following scotophase (i.e., at least 16 h later) analyzed for total labeled (i.e., carbon chain synthesized after introduction of the labeled glucose) and excess unlabeled (i.e., carbon chain synthesized before introduction of the labeled glucose or from a pool of precursor

that was not derived from labeled glucose) FAMES of Z11-16:Acyl and 16:Acyl. Three groups of females were analyzed: (i) intact females at S0, (ii) intact females at S6, and (iii) females decapitated at S0 and analyzed at S6.

### *2.7. Effect of PBAN*

We conducted the complementary experiment to the previous one by injecting PBAN into decapitated females and determining the effect on DNP and RPP. One-day-old females were fed at the end of the scotophase and, immediately upon ingestion of the U-<sup>13</sup>C-glucose, decapitated. The decapitated females were then left for 18 h, at which time they were either injected with 5 pmole of PBAN (HeZ; Bachem, Torrance, CA) in saline (2.5 µl) or the same volume of saline (NaCl, 187.5 mmol.l<sup>-1</sup>, KCl, 4.83 mmol.l<sup>-1</sup>, CaCl<sub>2</sub>, 2.61 mmol.l<sup>-1</sup>, Hepes, 10 mmol.l<sup>-1</sup>, pH=6.8). They were analyzed for DNP and RPP at 0 (only saline injected), 0.25, 0.5, 1.0, 1.5 and 4.0 h later.

A similar experimental approach was adopted for analysis of total labeled and excess unlabeled FAMES of Z11-16:Acyl and 16:Acyl, except females were analyzed at 0 (no PBAN or saline injected), 0.5 and 4.0 h (for both saline- and PBAN-injected females).

### *2.8. Statistical analyses*

For the effect of photoperiod and age, we used ANCOVA with RPP, DNP or precursor enrichment as dependent variables, age as a covariate and time of photoperiod as a categorical independent variable, after first checking normality and heteroscedasity of the data. For all other data, we used ANOVA to test for differences, again after checking the

normality and heteroscedasity of the data, and tested differences among means by post-hoc Tukey-Kramer HSD tests with  $\alpha$  set at  $P = 0.05$ . In the decapitation experiment, we made two temporal comparisons: the first was of RPP and DNP titers over the first 6 h of decapitation, and the second was a binary comparison for both RPP and DNP titers just prior to and 24 h after, decapitation.

### 3. Results

#### 3.1. Isotopic fractionation effect

At the start of the scotophase, females fed unlabeled glucose the previous day had a mean pheromone titer of  $17.9 \pm 2.7$  ng/female ( $N=10$ ), whereas females fed  $U-^{13}C$ -glucose had a mean total RPP+DNP titer of  $22.2 \pm 3.5$  ng/female ( $N=14$ ); these means were not different (ANOVA,  $F_{1,22} = 0.86$ ,  $p = 0.36$ ). Thus, any isotopic effects that occurred in the biosynthesis of pheromone from  $U-^{13}C$ -glucose did not affect pheromone gland titer.

#### 3.2. Effect of photoperiod and age

At all times of the photoperiod and all ages tested, females had substantial and reasonably constant titers of RPP in the gland, varying from a mean of  $6.3 \pm 1.7$  to  $10.3 \pm 1.4$  ng/female (Fig. 1a). ANCOVA, with amount of RPP as the dependent variable, showed no effect of time of photoperiod ( $F_{5,65} = 0.64$ ,  $p = 0.67$ ), but an effect of age ( $F_{1,65} = 5.59$ ,  $p = 0.021$ ). Essentially, the amount of RPP declined slightly with increasing age of female, but the amount of RPP produced throughout the photoperiod did not differ.

The amount of DNP was generally much greater than that of RPP, especially during the scotophase (Fig. 1a). It also showed much greater change over time (both with photoperiod and age), varying from a mean of  $9.6 \pm 1.9$  to  $105.2 \pm 25.0$  ng/female. This difference was most noticeable between titers in the photophase and (especially middle of the) scotophase, in accord with previous studies on native pheromone titer in *H. virescens* and many other species of moths (e.g., Foster, 2005b; Groot, 2014; Heath et al., 1991; McNeil, 1991). ANCOVA, with amount of DNP as the dependent variable, revealed highly significant effects for both time of photoperiod ( $F_{5,65} = 5.96$ ,  $p < 0.001$ ) and age ( $F_{1,65} = 27.4$ ,  $p < 0.001$ ). There was a strong correlation ( $F_{1,70} = 40.2$ ,  $P < 0.001$ ;  $R^2 = 0.36$ ) between RPP and logDNP (Fig. 1b).

ANCOVA with precursor enrichment (Fig. 1c) as the dependent variable showed no effect of photoperiod ( $F_{5,65} = 1.89$ ,  $p = 0.11$ ), but an effect of age ( $F_{1,65} = 10.2$ ,  $p = 0.002$ ), consistent with decreasing levels of native hemolymph trehalose in older females (Foster et al., 2014).

### 3.3. Effect of decapitation

In the first 6 h after decapitation, DNP and RPP exhibited different patterns (Fig. 2a). DNP showed a rapid and significant decrease (ANOVA,  $F_{7,31} = 4.87$ ,  $p < 0.001$ ) from ca. 20 to 1.5 ng/female, consistent with the known effect of decapitation on total pheromone titer in decapitated females (Eltahlawy et al., 2007). The mean amounts of DNP 4 and 6 h after decapitation were lower (Tukey-Kramer HSD test) than the amounts prior to, or



0.25 h after, decapitation. By contrast, the mean amount of RPP did not change over the first 6 h of decapitation (ANOVA,  $F_{7,31} = 0.73$ ,  $p = 0.65$ ), ranging from 5.3–8.8 ng/female. Twenty four hours after decapitation, both DNP and RPP titers had declined to very low levels, both lower (ANOVA,  $F_{1,11} = 11.4$ ,  $P = 0.006$  and  $F_{1,11}=12.3$ ,  $P = 0.005$ , respectively) than their respective values before decapitation.

Precursor enrichment did not change over the first 6 h following decapitation (ANOVA,  $F_{7,31} = 0.75$ ,  $P = 0.63$ ; Fig. 2b). However, after 24 h of decapitation, precursor enrichment was slightly greater ( $F_{1,11}=10.6$ ,  $P = 0.008$ ) than that prior to decapitation (Fig. 2b).

With regard to fatty acyl stores, females decapitated for 6 h had a lower titer (ANOVA,  $F_{2,19} = 7.32$ ,  $P = 0.004$ ; Tukey-Kramer HSD test) of labeled Z11-16:Acyl than did intact females at the start of the scotophase or intact females 6 h into the scotophase (Fig. 2c). Titers of unlabeled Z11-16:Acyl in females decapitated 6 h were similar to those in intact females 6 h into the scotophase, but less than those in females at the start of the scotophase (ANOVA  $F_{2,19} = 6.15$ ,  $P = 0.004$ ; Tukey-Kramer HSD test; Fig. 2c). Of note is that 6 h of decapitation over this period yielded similar ratios of labeled to unlabeled Z11-16:Acyl as at the start of the scotophase (in intact females), whereas leaving females intact over this period (i.e., through the scotophase) resulted in a relative decline in the amount of unlabeled Z11-16:Acyl (compared to labeled Z11-16:Acyl) over the 6 h of the scotophase.

For both labeled (ANOVA,  $F_{2,19} = 0.56$ ,  $P = 0.58$ ) and unlabeled 16:Acyl (ANOVA,  $F_{2,19} = 0.56$ ,  $P = 0.58$ ), there were no differences in titer among any of the treatments (Fig. 2d). In contrast to the similar amounts of unlabeled and labeled Z11-16:Acyl, the amount of unlabeled 16:Acyl was much greater than that of labeled 16:Acyl, indicating a relatively slow turnover of this very large pool.

#### *3.4. Effect of PBAN*

Injection of PBAN into females decapitated 16 h earlier led to a rapid increase (ANOVA,  $F_{5,42} = 8.38$ ,  $P < 0.001$ ) in DNP (Fig. 3a), such that 1 h after injection the amount of DNP was greater (Tukey-Kramer HSD test) than that prior to injection (i.e., at  $t = 0$ ). DNP increased throughout the 4 h of the experiment, although the amount 4.0 h after PBAN injection was not different to that 1.5 h after injection (Tukey-Kramer HSD test).

Injection of PBAN also resulted in an initial small increase (ANOVA,  $F_{5,42} = 3.36$ ,  $P = 0.012$ ) in RPP (Fig. 3a), with the amount 1 h after injection being greater (Tukey-Kramer HSD test) than that prior to injection. After that, the level of RPP plateaued. The amount of RPP was always much less than that of DNP in females injected with PBAN.

Precursor enrichment (Fig. 3b) showed a small change over the experiment (ANOVA,  $F_{5,42} = 2.45$ ,  $P = 0.049$ ), with enrichment 4 h after injection of PBAN being greater than that 0.25 h after injection (Tukey-Kramer HSD test).

Injection of PBAN stimulated a large increase (ANOVA,  $F_{4,39} = 337.7$ ,  $P < 0.001$ ) in labeled Z11-16:Acyl (Fig. 3c); 4 h after injection, females had more (Tukey-Kramer HSD test) labeled Z11-16:Acyl than did females of all other treatments (which were similar).

The amount of unlabeled Z11-16:Acyl increased slightly with PBAN injection (ANOVA,  $F_{4,39} = 3.41$ ,  $P = 0.018$ ), with the amount 4 h after PBAN injection being greater (Tukey-Kramer HSD test) than that prior to injection (i.e., at  $t = 0$ ). Neither labeled ( $F_{4,39} = 2.24$ ,  $P = 0.083$ ) nor unlabeled ( $F_{4,39} = 1.77$ ,  $P = 0.15$ ) 16:Acyl titers changed with PBAN injection (Fig. 3d).

## 4. Discussion

### 4.1. Identification of two pathways

Through feeding  $^{13}\text{C}$ -labeled glucose to female *H. virescens*, we demonstrated that females produce two pools of pheromone: DNP, which incorporates  $^{13}\text{C}$ -tracer, and RPP, which does not. At most times, especially during the scotophase, the titer of DNP was much larger than that of RPP and, importantly, their respective titer profiles differed with regard to photoperiod and presence/absence of PBAN. The photoperiodic pattern of DNP titer closely resembled that of native titer in *H. virescens* (Foster, 2005b; Heath et al., 1991) and other moths (Groot, 2014; McNeil, 1991), with a substantial difference between the sexually inactive (photophase, titer low) and active (scotophase, titer high) periods. By contrast, RPP titer was constant throughout the photoperiod. For DNP (and native pheromone) this photoperiodic variation is explained by the presence/absence of PBAN acting on the gland (Eltahlawy et al., 2007; Groot et al., 2005; Rafaeli and Jurenka, 2003). RPP titer was largely independent of the presence/absence of PBAN, although females decapitated for ca. 16–24 h showed a small decline in RPP, which could be rectified by injection of PBAN. This, together with the concomitant changes in

precursor enrichment, showing a small decline in fat usage for DNP, suggest that extended absence of PBAN, or perhaps another effect of decapitation, may result in other (than fat and pheromone synthesis) minor effects on fat metabolism in the gland. The small effect of age on both DNP and RPP is consistent with a senescent decline in biosynthetic capability in the gland (Foster, 2005b; Raina et al., 1986)

The incorporation of  $^{13}\text{C}$ -label into DNP and its lack of incorporation into RPP, show the two pools of pheromone must be biosynthesized by distinct, but related, routes (Fig. 4). In our experiments, Z11-16:Ald DNP was biosynthesized de novo, after females fed on labeled glucose. This route involves a cytosolic pool of labeled/unlabeled acetyl CoA, formed from glycolysis/pyruvate oxidation and  $\beta$ -oxidation of stored fatty acids (Foster and Anderson, 2015), and synthesis of transient 16:Acyl and Z11-16:Acyl precursors (Fig. 4) (Choi et al., 2005). Since label from glucose is incorporated into pheromone very rapidly after feeding (Foster and Anderson, 2011), the lack of label in RPP, at least 16 h after feeding, shows that it cannot be produced from the same cytosolic acetyl CoA pool. Instead, it must be derived largely from precursors in the female before adult feeding (i.e., from nutrients acquired during larval feeding).

As suggested previously (Foster and Anderson, 2015), the most likely candidates for a precursor of RPP are glandular glycerolipid stores of the pheromone precursor(s), Z11-16:Acyl, 16:Acyl, and perhaps 18:Acyl. These acids are stored predominantly in triacylglycerols in the gland, mostly on *sn*-1 and *sn*-3 positions of the glycerol backbone (Foster, 2005b). Following hydrolysis by glandular lipases, these precursor acids can be

converted directly to pheromone (Choi et al., 2005; see also Fig. 4). Therefore, assuming pheromone gland lipases (Du et al., 2012) are typical triacylglycerol lipases, with little selectivity toward acyl groups of similar chain length (Watt and Steinberg, 2008), then the most probable precursor for production of RPP in our experiments, by virtue of its much greater abundance (Foster, 2005b; see also, Figs. 2b, 3b), is unlabeled 16:Acyl, although unlabeled 18:Acyl may also contribute. Stored Z11-16:Acyl probably contributes little to RPP measured in our experiments, since its isotopic enrichment was similar to that of pheromone (see also Foster et al., 2014). Hence, hydrolysis of stores of this moiety and conversion to pheromone, while strictly speaking also forming “recycled precursor fat” pheromone, will yield apparent increases in DNP from the isotopomer patterns. The much larger amount of stored 16:Acyl relative to Z11-16:Acyl, combined with the small production of RPP from 16:Acyl, suggests that only a small amount of DNP measured in our experiments was produced from stored Z11-16:Acyl. This may account for the small amount of “apparent” DNP observed throughout the photophase in our experiments, although it is possible that this is produced by a low level of de novo production in the absence of PBAN.

#### 4.2. PBAN control

The small amount of RPP compared to the large amount of 16:Acyl (and to a lesser extent, Z11-16:Acyl) available in the gland of female *H. virescens* suggests limited but constant lipolysis that is largely independent of PBAN, in contrast to the situation in *B. mori* (Du et al., 2012; Matsumoto, 2010). However, during PBAN stimulation in *H. virescens*, acyl CoAs are also used for  $\beta$ -oxidation, producing acetyl CoA precursor for

DNP (Foster and Anderson, 2015), and perhaps for energy for cell function. Since fats are used to produce up to one third of precursor for DNP (Foster and Anderson, 2015), it is likely that there is considerably more lipolysis than indicated by RPP production alone. When PBAN is absent, the likely fate of most acyl CoAs is re-esterification but when present, most or a significant portion of liberated acyl CoAs is  $\beta$ -oxidized. During PBAN stimulation, acyl CoAs may be compartmentalized, with de novo-synthesized acyl CoAs (16:Acyl) being primarily converted directly to pheromone, while hydrolyzed, stored acyl CoAs are  $\beta$ -oxidized, producing precursor for de novo synthesis. We do not suggest that PBAN directly controls the flux of fatty acids into the mitochondria. Rather, it likely controls this flux indirectly, as well as glycolytic flux, by controlling a downstream process, such as conversion of acetyl CoA to malonyl CoA by acetyl CoA carboxylase (Eltahlawy et al., 2007; Rafaeli and Jurenka, 2003)

Compartmentalization of different sources of acyl CoAs with different metabolic fates is well established. In mice, the enzymes glycerol-3-phosphate acyltransferase (GPAT) and carnitine palmitoyl transferase (CPT) compete for acyl CoAs at the outer mitochondrial membrane, with GPAT preferentially loading de novo-synthesized acyl CoAs for incorporation into glycerolipids, while CPT preferentially loads acyl CoAs from glycerolipids for carnitine esterification and transport across the mitochondrial membrane for  $\beta$ -oxidation (Cooper et al., 2015). This compartmentalization of newly synthesized and stored fats is supported by our FAME experiments. Six hours after decapitation, both labeled and unlabeled Z11-16:Acyl stores had declined by similar amounts; females continue to use stores but cannot replenish them since they are not synthesizing new fatty

acids. By contrast, only unlabeled Z11-16:Acyl stores declined in intact females over the same period; both labeled and unlabeled stores were utilized as in decapitated females, but labeled stores were replenished via the tracer/tracee pool. In females injected with PBAN, there was an expected increase in labeled Z11-16:Acyl stores, but also a small increase in unlabeled stores, but only to levels similar to those prior to decapitation.

These trends were not apparent in 16:Acyl stores, perhaps because the amounts are much greater and less subject to apparent change, and also because our dissections of glands almost certainly contained other tissue in which 16:Acyl was abundant, masking any effects peculiar to the gland.

#### *4.3. Contribution of stored fat to pheromonal communication*

The fact that a small amount of RPP is constantly produced throughout the entire photoperiod, regardless of whether DNP is being synthesized or not, suggests that its production is largely a consequence of constant hydrolysis of fats from glycerolipids, for either  $\beta$ -oxidation or re-esterification, with only a small portion of free precursor acyl CoAs (predominantly 16:Acyl) reaching the endoplasmic reticulum for conversion to pheromone (Hagström et al., 2013). Furthermore, the amount of pheromone produced directly (RPP route) by female *H. virescens* from stored precursor fats is relatively small, especially in comparison to the amount of DNP produced during the sexually active period. Therefore, RPP is likely, at best, to make a small contribution to the pheromone released, especially for younger females attracting males for their first mating.

505 This dual pathway of pheromone production has been established, so far, only for *H.*  
506 *virescens*. Hence, we do not know whether or not RPP contributes more or less to  
507 pheromone production and release in other species of moths. Its contribution likely  
508 depends on the amounts of pheromone precursor acids stored, as well as glandular  
509 lipolytic activity. In the case of *B. mori*, females appear to produce all their pheromone  
510 from precursor acyl CoAs hydrolyzed from triacylglycerols. However, photoperiodic  
511 control of pheromone biosynthesis is somewhat unusual in this species, in that release of  
512 PBAN appears to control fatty acid reduction (Matsumoto, 2010), rather than fatty acid  
513 synthesis, as found for *H. virescens* (Eltahlawy et al., 2007) and other moths studied  
514 (Rafaeli and Jurenka, 2003). Thus, fats are synthesized and stored in the absence of  
515 PBAN, but not mobilized and reduced to pheromone until PBAN is released (Matsumoto,  
516 2010). By contrast, in *H. virescens*, new precursor acyl CoAs are synthesized only when  
517 DNP is also being produced, with only a small portion being stored in triacylglycerols;  
518 their primary fate being pheromone (Foster and Anderson, 2012). It is also worth noting  
519 that *H. virescens* adults feed (on nectar) and hence can replenish carbohydrate used  
520 throughout the adult life; more than two thirds of acetyl CoA precursor for DNP  
521 production in *H. virescens* is derived from hemolymph carbohydrate (trehalose) (Foster  
522 and Anderson, 2015). Many species of moths, including *B. mori*, do not feed as adults  
523 and hence may be more dependent upon stored fats for pheromone production, as both  
524 DNP and RPP. Studying nutrient use for pheromone production across a range of species,  
525 with different life history traits, is needed to determine whether recycling of stored  
526 precursor fats is more than a minor route for contributing to pheromone production in  
527 moths.



528

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**Captions**

**Figure 1. Mean titers ( $\pm$ SEM) of de novo-produced pheromone (DNP) and recycled precursor fat pheromone (RPP) in female *Heliothis virescens* of different age and times of the photoperiod.** a) DNP and RPP. (b) Relation between DNP (log scale) and RPP titers. (c) Pheromone precursor enrichment (in molar percent excess; MPE) of DNP. Age of female (in days) given below figures. Numbers above bars are numbers of replicates.

**Figure 2. Mean titers ( $\pm$ SEM) of various pheromone-related moieties in female *Heliothis virescens* fed U-<sup>13</sup>C-glucose the previous day and decapitated at the start of the subsequent scotophase.** A) De novo-produced pheromone (DNP) and recycled precursor fat pheromone (RPP). (b) Pheromone precursor enrichment (in molar percent excess; MPE) of DNP. Labeled and unlabeled (c) (Z)-11-hexadecenoate (Z11-16:Acyl) and (d) hexadecanoate (16:Acyl). Decap6 = decapitated (at S0) for 6 h. Different letters (of the same case) above bars indicate means that are different ( $P < 0.05$ , Tukey-Kramer test), while numbers in parentheses are the numbers of replicates.

**Figure 3. a) Mean titers ( $\pm$ SEM) of various pheromone-related moieties in decapitated female *Heliothis virescens* fed U-<sup>13</sup>C-glucose and analyzed following injection with pheromone biosynthesis-activating neuropeptide (PBAN).** a) De novo-produced pheromone (DNP) and recycled precursor fat pheromone (RPP). (b) Pheromone precursor enrichment (in molar percent excess; mpe) for DNP. Labeled and unlabeled (c) (Z)-11-hexadecenoate (Z11-16:Acyl) and (d) hexadecanoate (16:Acyl). Letters above

bars indicate means that are different ( $P < 0.05$ , Tukey-Kramer test), while numbers in parentheses are numbers of replicates.

**Figure 4. Biosynthesis of pheromone [(Z)-11-hexadecenal] in the gland of *Heliothis virescens* females by two distinct routes: de novo-produced pheromone (DNP) and recycled precursor fat pheromone (RPP).** Processes that occur throughout the day have grey arrows, while processes that occur primarily during the sexually active period (scotophase) have black arrows. Features are: (1) stored acyl CoAs are continuously hydrolyzed and re-esterified to glycerolipids, (2) DNP production is controlled by release of pheromone biosynthesis activating neuropeptide (PBAN) from the corpora cardiaca, which acts on a step in de novo synthesis of hexadecanoate (16:Acyl), and (3) this indirectly controls fluxes of  $\beta$ -oxidation of glandular fats and glycolysis/pyruvate oxidation of glucose (from hemolymph trehalose) to produce acetyl CoA precursor. Z11-16:Acyl is the pheromone precursor acid (Z)-11-hexadecenoate.

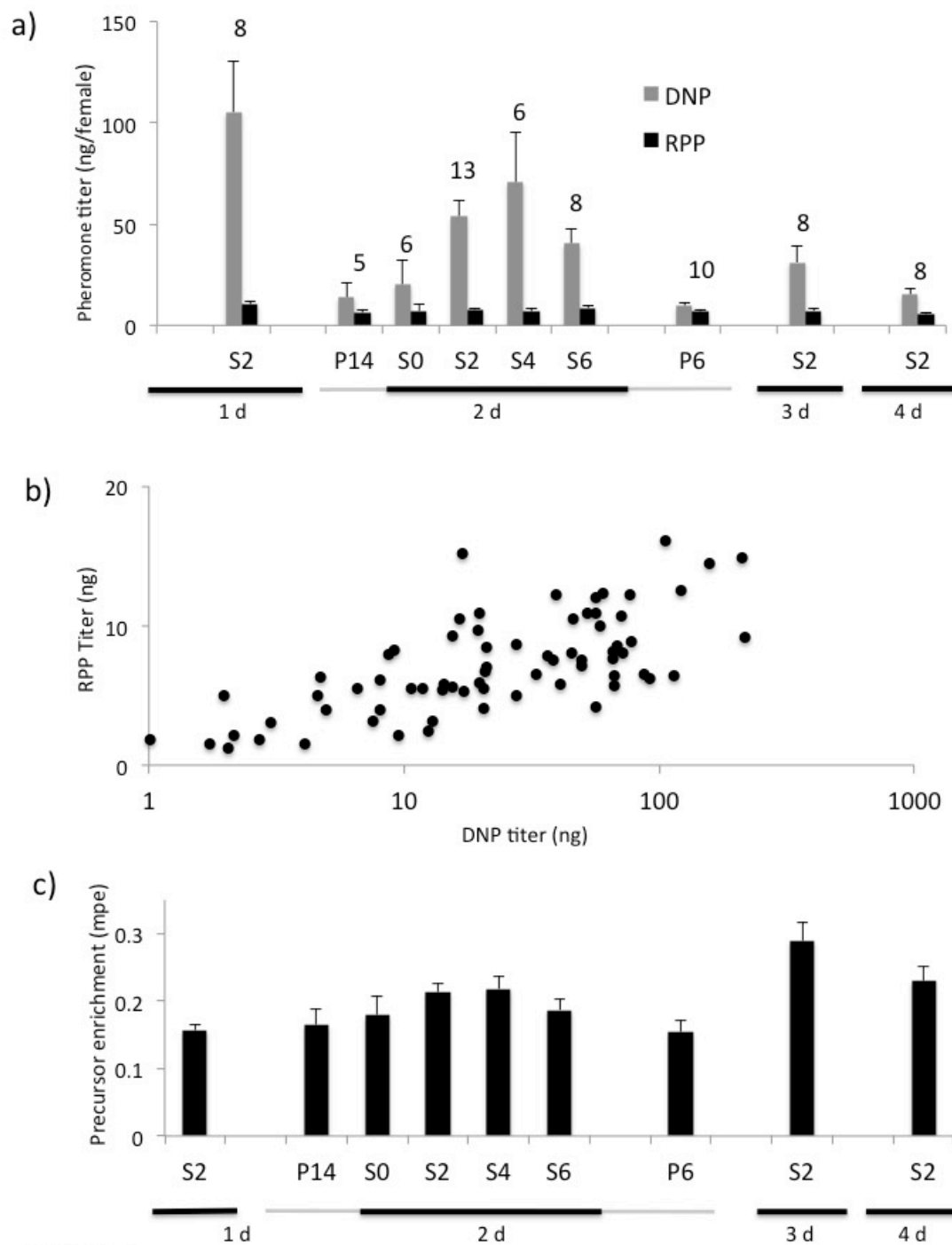


Figure 1

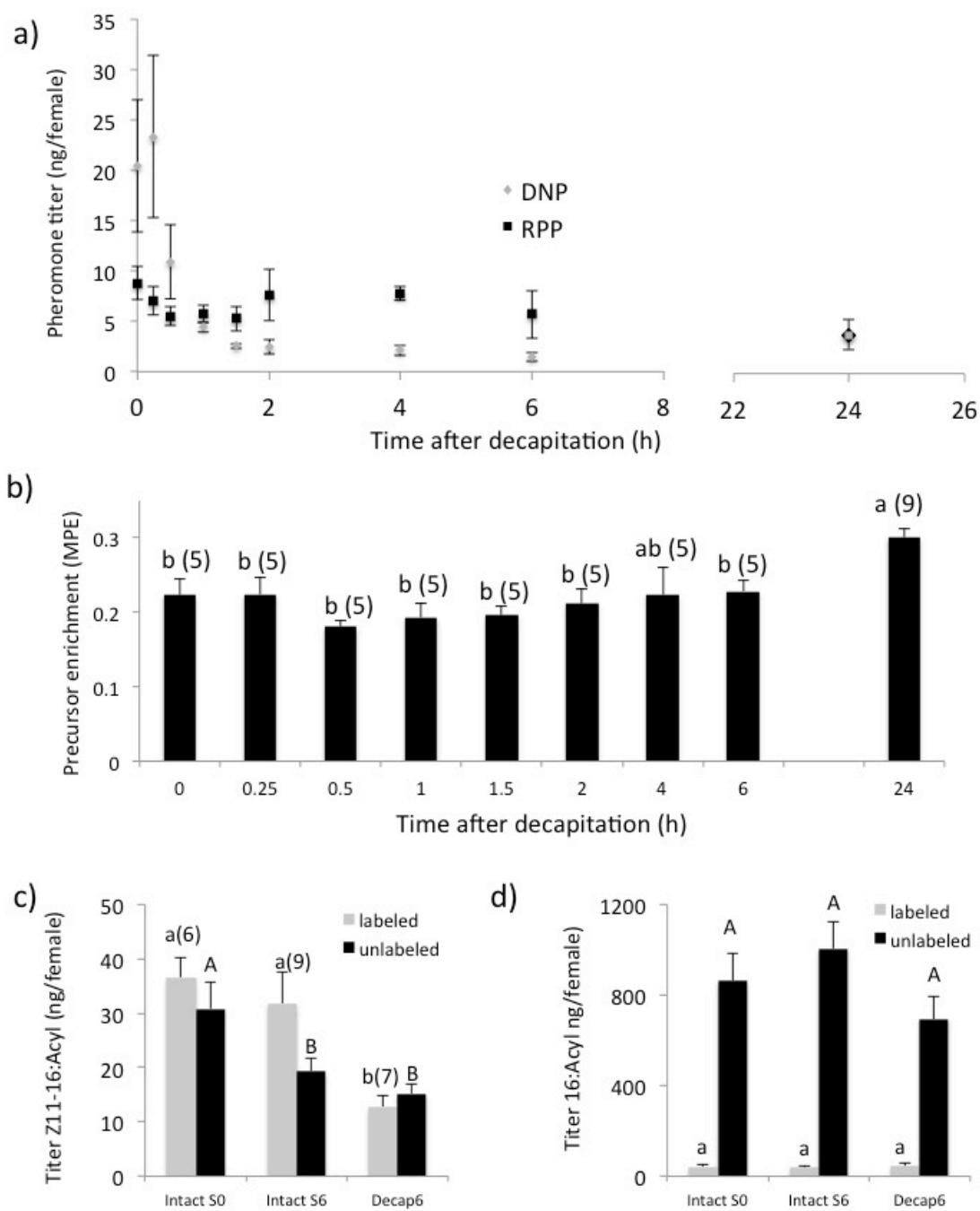


Figure 2

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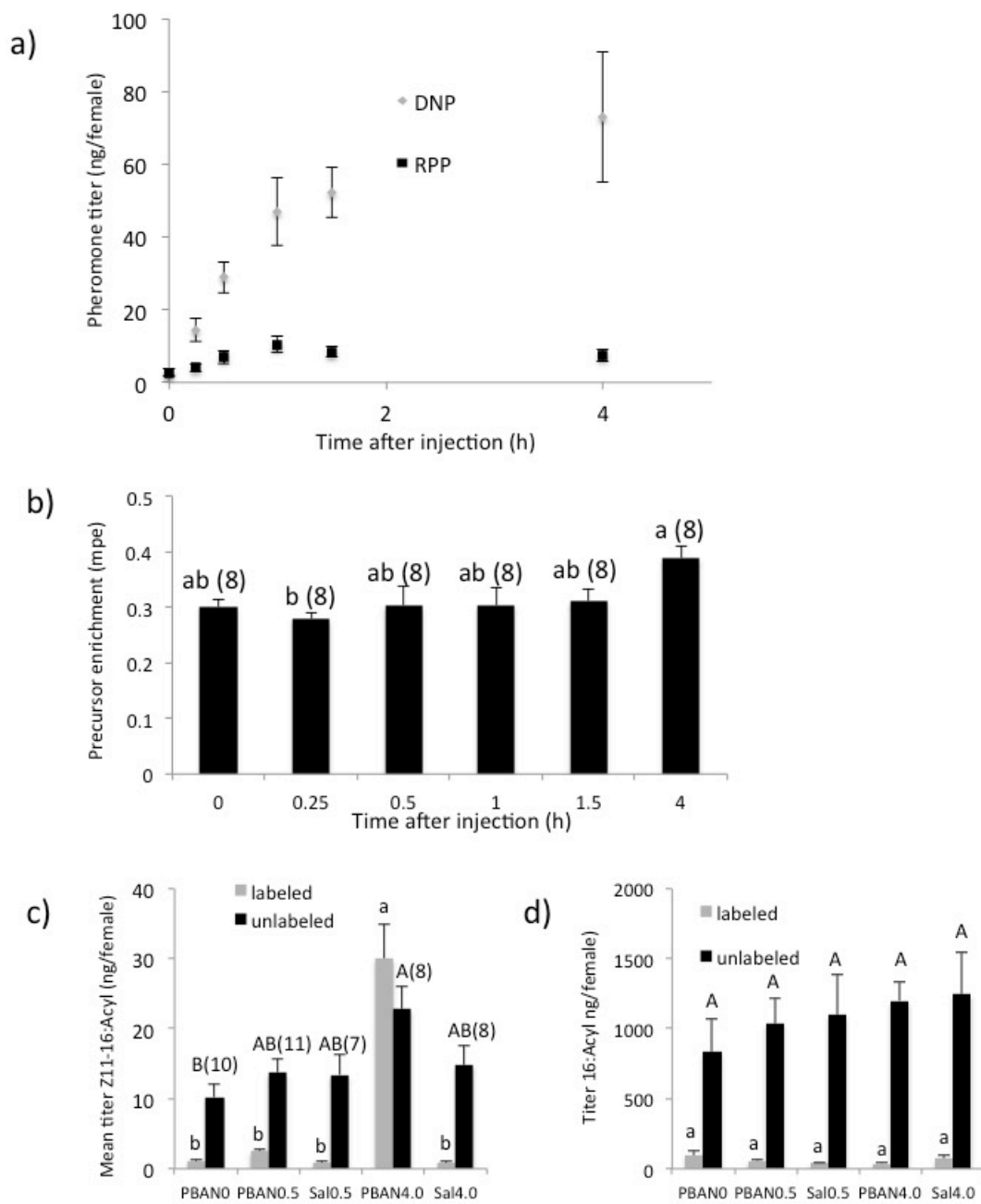


Figure 3

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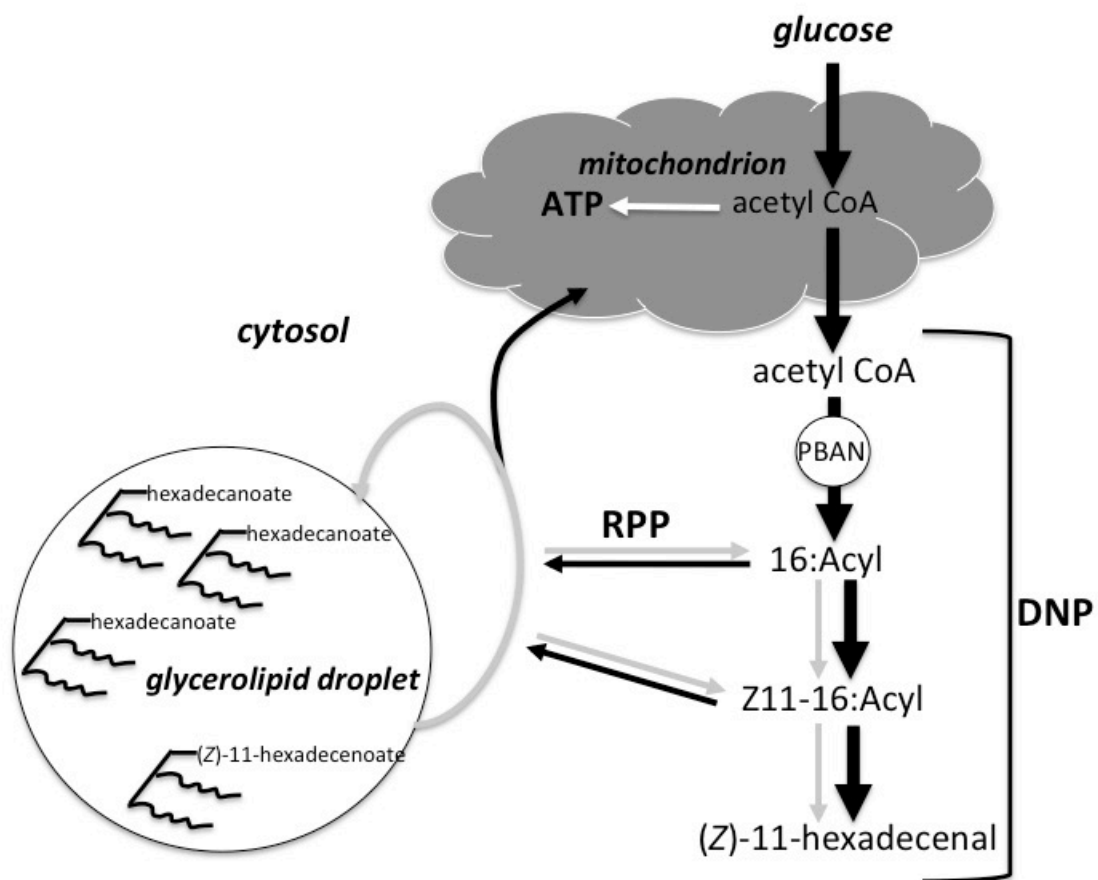


Figure 4