© 2015. Published by The Company of Biologists Ltd | The Journal of Experimental Biology (2015) 218, 1252-1258 doi:10.1242/jeb.119883

RESEARCH ARTICLE

Sex pheromones in mate assessment: analysis of nutrient cost of sex pheromone production by females of the moth *Heliothis virescens*

Stephen P. Foster* and Karin G. Anderson

ABSTRACT

It has been postulated that sex pheromones, in addition to their role in mate recognition and/or finding, may also serve a role in assessment of mate quality. For this, a sex pheromone must give honest information about a signaler's quality, with honesty ensured by a direct metabolic or indirect fitness cost to the signaler. Using a stable isotope tracer–tracee method, we characterized the nutrient pools that fuel sex pheromone production in females of the moth *Heliothis virescens*, as well as the relative importance of larval- and adult-acquired nutrients to this process. Females used three pools for *de novo* biosynthesis of sex pheromone, hemolymph trehalose, glycogen (via trehalose) and fat, and produced ca. 25% of pheromone directly from stored (previously synthesized) precursor fatty acids. Pheromone was produced roughly equally from carbohydrate and fat. Adult feeding was very important for pheromone biosynthesis, with a maximum of 65% of *de novo* biosynthesized pheromone produced from a single adult feed (carbohydrate). Although these nutrient pools are shared with other reproductive physiologies, notably oocyte production, it is unlikely that pheromone production imposes a significant metabolic cost on females, because (i) the amount of nutrients used for pheromone production is negligible compared with that available, (ii) the hemolymph trehalose pool is readily replaceable throughout the adult life, and (iii) in mated females, carbohydrate shortages result in reduced allocation to pheromone.

KEY WORDS: Lepidoptera, Nutrient acquisition, Sex pheromone biosynthesis, Mass isotopomer distribution analysis, Stable isotopes, Tracer–tracee

INTRODUCTION

Pheromones are used by animals for a range of behaviors and physiologies, of which perhaps the most widely distributed amongst taxa are those used for communication between mates (Wyatt, 2014). These sex pheromones are typically associated with finding and/or recognition of conspecific mates, although it has been suggested that they may also signal information about the 'quality' of a producer (Harari et al., 2011; Johansson and Jones, 2007); i.e. a responder derives information about whether mating with a sender should result in more or better quality offspring. The key for a sex pheromone to be effective in mate assessment is that it must honestly reflect a signaler's quality. To preclude widespread 'cheating' in a mate assessment signal, it is thought that the signal must be costly to produce, through either a direct metabolic or indirect cost to a signaler's fitness, and that the cost must be condition dependent, so that higher and lower quality signalers experience smaller and greater relative costs, respectively (Zahavi, 1977). In spite of numerous reports of pheromone-based mate assessment, few, if any, show direct evidence of any such costs (Kotiaho, 2001). In particular, there is a paucity of work on the direct metabolic costs of chemical mating signals (Johansson and Jones, 2007; Umbers et al., 2015).

The most well-studied sex pheromones are the volatile, female-produced sex pheromones of moths, notable for their ability to attract males over large distances (Ando et al., 2004). While their mate recognition function is established (Ando et al., 2004), a recent study on the moth *Lobesia botrana* (Harari et al., 2011) suggests that pheromone production might bear a 'cost', with smaller, but not larger, females exposed to conspecific pheromone laying fewer eggs than non-exposed females. Although this might indicate a mate assessment function for the sex pheromone, no mechanism for this 'cost' was investigated. In fact, little is known about the metabolic cost of sex pheromone production in moths, probably because it has been assumed that the relatively small quantities generally produced by females are not metabolically costly (Cardé and Baker, 1984; Harari and Steinitz, 2013; Johansson and Jones, 2007). However, this assumption may be flawed (Johansson and Jones, 2007), because it typically only considers pheromone amount at one time (usually maximal titer), fails to account for the amount of resources (nutrients) available for synthesizing pheromone and whether these nutrients are limited during the adult stage or not, and usually does not consider whether the nutrients may be shared, and therefore compete, with physiologies directly influencing fitness (e.g. egg production).

Most moth sex pheromones are biosynthesized *de novo* from acetyl CoA in a specialized gland via fatty acid synthesis and chain modification. While there have been numerous studies on the enzymes involved and its endocrine regulation, little is known about the nutrients used for biosynthesis (Blomquist et al., 2011). Adult Lepidoptera commonly feed on carbohydrate in plant nectar, which typically increases female fecundity (Wäckers et al., 2007). In the polyandrous moth *Heliothis virescens* Fabricius (Lepdioptera, Noctuidae), this adult-acquired carbohydrate (AAC) is also used for pheromone biosynthesis via uptake to hemolymph (as trehalose), followed by glycolysis and pyruvate oxidation to cytosolic acetyl CoA (Foster and Anderson, 2011; Foster, 2009; Foster and Johnson, 2010). Although this demonstrates that pheromone can be made from carbohydrate, we do not know whether this is the sole nutrient used or the relative importance of adult- versus larval-acquired nutrients. Females can produce pheromone solely from larval-acquired nutrients, although production is more limited than for feeding females (Foster, 2009; Foster and Johnson, 2010).

Entomology Department, North Dakota State University, PO Box 6050, Fargo, ND 58108-6050, USA.

*Author for correspondence (stephen.foster@ndsu.edu)

Received 22 January 2015; Accepted 19 February 2015

The Journal of Experimental Biology

In this paper, we used a stable-isotope tracer–tracee method to address, for the first time, whether pheromone production has a significant metabolic cost for a moth, by characterizing pools of nutrients used for pheromone production and quantifying the relative contributions of larval- and adult-acquired nutrients to this process.

RESULTS

Contribution of adult food to pheromone production – dose

Females fed increasing amounts (in 25 µl) of glucose had increased pheromone precursor enrichment (i.e. the proportion of acetate precursor derived from labeled compound, *p*; Table 1). The contribution of adult food (AAC) to pheromone production increased with the amount (i.e. percentage) of glucose fed before plateauing (Fig. 1A). An exponential 3P model (JMP 2015) gave an excellent fit (R^2=0.999), with an asymptote of 65.1% contribution of adult glucose to pheromone production.

Contribution of adult food to pheromone production – time, multiple feeds and mating status

Females fed once on 10% glucose had declining *p* (Table 1) and percentage contribution of adult diet to pheromone values with time; after 2 days, only 5% of pheromone was derived from AAC, compared with ca. 27% 4 h after feeding (Fig. 1B). Four hours after a second feed given 24 h later, both *p* and percentage pheromone from adult food were restored to levels comparable to those 4 h after the first feed. However, 24 h later, *p* and percentage pheromone derived from AAC had again more than halved, similar to the result after one feed. Mated females had higher levels of *p* and percentage pheromone from adult food 4 h after feeding than did virgins (Table 1, Fig. 1B).

Contribution of adult food to pheromone production – photoperiod

Females fed glucose, either in the scotophase or photophase, and analyzed 4 or 28 h later showed effects of treatment on *p* (ANOVA, $F_{3,36}$=28.77, P<0.0001) and excess unlabeled pheromone (EUP; ANOVA, $F_{3,36}$=22.53, P<0.0001). EUP (see Materials and methods) is a measure of the relative amount of unlabeled pheromone not explained by *de novo* biosynthesis after the tracer ([U-^{13}C]glucose) has been introduced (note that some unlabeled pheromone is produced *de novo* from the tracer/tracee pool), which must have been biosynthesized either *de novo* before introduction of the tracer or from some other (unlabeled) precursor. Females fed glucose in the scotophase and analyzed 4 h later had a greater (P<0.05, Student's *t*-test) pheromone *p* than all other treatments (Fig. 2). All four treatments had substantial (at least 0.3× the total amount of pheromone synthesized from the acetate pool) levels of EUP, with females fed during the photophase and analyzed 4 h later having greater (P<0.05, Student's *t*-test) levels than the other three treatments.

Change in enrichment of pheromone and trehalose over time.

Four hours after feeding, females fed 30% glucose showed a linear increase in enrichment of both pheromone and trehalose with increases in [U-^{13}C]glucose fed (Fig. 3). For each [U-^{13}C]glucose enrichment, trehalose enrichment was slightly greater than pheromone *p*, consistent with slight isotopic fractionation through the pathway. As for other experiments, enrichment of both pheromone and trehalose in females fed 25:75 [U-^{13}C]:[U-^{12}C] glucose was much lower 28 h after feeding compared with that at 4 h. In fact, pheromone and trehalose enrichments were reduced by roughly similar proportions after 28 h. This is evident when these values are placed on the respective lines derived from analysis 4 h after feeding different glucose enrichments (Fig. 3).

Contribution of fatty acids to pheromone production

Labeled pheromone was produced rapidly following injection of [U-^{13}C]-(*Z*)-9-hexadecenoic acid ([U-^{13}C]Z9-16:COOH). Precursor enrichment peaked at 0.11 at 20 min following injection, before declining slowly over 2 h (Fig. 4A). This *p* was converted to 0.389 units of singly labeled pheromone by Eqn 8 (see Materials and methods) and the rate of increase of singly labeled pheromone calculated as 0.149 h^{-1} (Fig. 4A), yielding a fractional synthetic rate (FSR; Eqn 9, Materials and methods) of 38.3% h^{-1}, less than half that determined previously with [U-^{13}C]glucose as a tracer (Foster and Anderson, 2011).

[16,16,16-D_3]-Hexadecenoic acid (D_3-16:COOH) injected into females was rapidly converted to pheromone (Fig. 4B). Over the experiment, pheromone enrichment was constant.

Females fed unlabeled glucose and injected with [U-^{13}C]Z9-16:COOH had lower (ANOVA, $F_{1,26}$=11.50, P=0.022) pheromone *p* than females fed water (Fig. 4C). When females were injected with DMSO or (*Z*)-9-octadecenoic acid (Z9-18:COOH) in DMSO, or not injected (control), and then fed [U-^{13}C]glucose, there was an effect ($F_{2,26}$=3.45, P=0.044) of treatment on pheromone *p*, due to a small reduction in *p* following both DMSO and Z9-18:COOH injections relative to the control (Fig. 4C). Pheromone *p* values in experiments with either glucose or fatty acid as tracer were similar.

Table 1. Precursor enrichment (*p*) for various treatments determining the contribution of adult food to pheromone production in female *Heliothis virescens*

Treatment	N_1	Glucose enrichment 1	p_1	N_2	Glucose enrichment 2	p_2
5% glucose	9	0.5	0.102±0.010	10	0.25	0.057±0.009
10% glucose	10	0.5	0.145±0.008	9	0.25	0.069±0.003
20% glucose	10	0.5	0.201±0.006	10	0.25	0.092±0.005
50% glucose	10	0.5	0.271±0.013	12	0.25	0.117±0.007
75% glucose	9	0.25	0.142±0.004	8	0.125	0.061±0.002
1×10% glucose+4 h	8	1.0	0.309±0.018	8	0.5	0.133±0.011
1×10% glucose+28 h	12	1.0	0.112±0.018	10	0.5	0.058±0.007
1×10% glucose+44 h	10	1.0	0.091±0.011	12	0.5	0.070±0.006
2×10% glucose+4 h	9	1.0	0.359±0.025	10	0.5	0.191±0.009
2×10% glucose+28 h	8	1.0	0.174±0.026	7	0.5	0.095±0.006
M1×10% glucose+4 h	9	1.0	0.270±0.024	9	0.5	0.172±0.013

Two enrichments of glucose solution were fed to different females for each treatment. *N* and s.e.m. are also given. All females were 1 day old virgins, except for the treatment designated as M, in which females were 2 days old and mated. In the lower part of the table, the number of feeds that females received (1×, 2×) is indicated.

The Journal of Experimental Biology (2015) 218, 1252-1258 doi:10.1242/jeb.119883

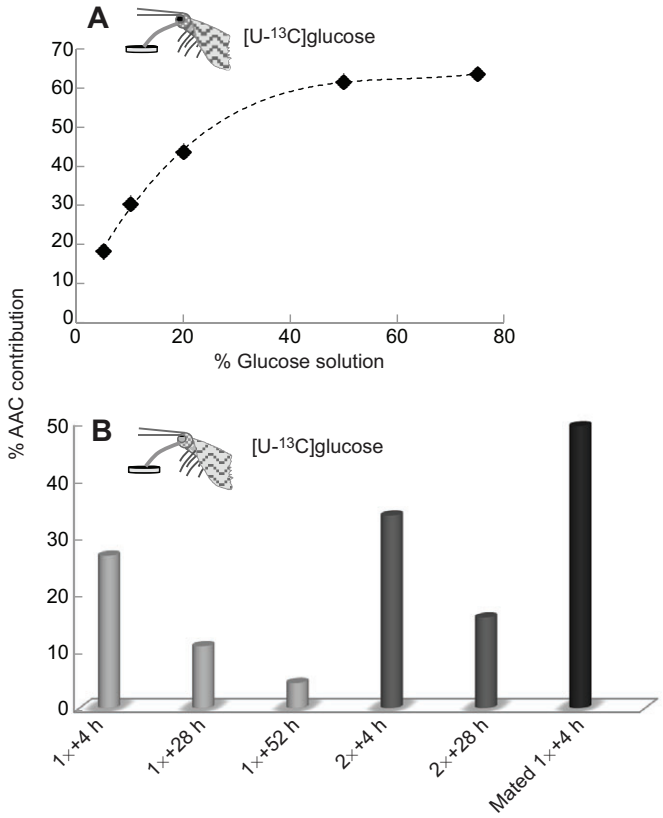

Fig. 1. Percentage contribution of adult food to pheromone production for female *Heliothis virescens* fed glucose. (A) Adult-acquired carbohydrate (AAC) contribution for virgins fed different concentrations of glucose. (B) Data for virgins fed once (1×) and analyzed 4, 28 or 52 h later; virgins fed twice (2×) at 1 day old and then 24 h later, and analyzed 4 or 28 h later; mated females fed (when 2 days old) 24 h after mating and analyzed 4 h later. Different females from each treatment were fed two different enrichments of [U-^{13}C]:[U-^{12}C] glucose. For details, see Materials and methods and Table 1.

DISCUSSION

Female *H. virescens* use both carbohydrate and fat for *de novo* biosynthesis of sex pheromone. Both are initially (at eclosion) derived from larval feeding, but become differentially enriched with AAC after feeding. Enrichment by AAC is more significant for the carbohydrate pool than for the fat pool, because AAC is directly and rapidly taken up into this pool (as trehalose), while the *de novo* biosynthesis of fats from carbohydrates in adult females is relatively much slower (Foster and Anderson, 2012; Foster et al., 2014). Both increased feeding frequency and mating caused increases in the contribution of AAC to pheromone production, presumably because of increased AAC enrichment of the carbohydrate pool and perhaps, to a lesser extent, the fat pool (see below). Although mated females were only fed once, as were comparable virgins, their greater pheromone enrichment by AAC was likely due to greater usage of nutrients, especially trehalose (Foster, 2009; Ramaswamy et al., 1997). Regardless, AAC is very important for pheromone production, with a single feed of carbohydrate capable of supplying a significant amount of precursor for pheromone produced during the same scotophase: a maximum of ca. 65% from our dose–response experiment.

But what of the contributions of the various nutrient pools (irrespective of whether they were of larval or adult source)? The trehalose/pheromone enrichment experiment showed that increasing the amount of labeled glucose ingested (in a fixed dose of total glucose) increases trehalose AAC enrichment, which, in turn, increases pheromone AAC enrichment. A similar result of increasing pheromone enrichment was obtained with the dose–response experiment. If one considers that the amount of glucose (18.8 µg) in the 75% feed was roughly 10 times that in hemolymph trehalose before feeding [ca. 2 µg, assuming 50 µl volume, as for the similar size *Helicoverpa zea* (Bushman et al., 2002), at ca. 20 mmol l^{-1} (Foster, 2009)], then this suggests that the contribution of AAC to pheromone production is largely replacing the contribution of larval-acquired trehalose. If so, then the maximum contribution of AAC to pheromone production is likely close to the maximum carbohydrate contribution to pheromone production. Whether this maximum is actually the norm (i.e. 65% of pheromone is usually produced from carbohydrate, regardless of source) is not known, although we note that in spite of the increase in hemolymph trehalose concentration resulting from AAC, pheromone titer does not actually increase in young virgins (Foster and Johnson, 2010), suggesting that this carbohydrate contribution may be the norm in females with a 'normal' [>5 mmol l^{-1} (Foster and Johnson, 2010)] hemolymph trehalose concentration.

While trehalose is the carbohydrate pool used directly for *de novo* biosynthesis of pheromone, our data indicate that this pool is slowly supplemented by glucose from glycogen stores. We first observed that the percentage of pheromone (or *p*) derived from AAC declined from 4 to 28 h after feeding, and demonstrated that this was caused by a comparable decline in trehalose enrichment. In unfed virgins, 1–2 days following eclosion, hemolymph trehalose concentration changes relatively little, before declining slowly (Foster and Johnson, 2010), whereas in mated females, hemolymph trehalose concentration drops significantly 24 h after mating, indicating that glucose from glycogen largely meets the demands of carbohydrate

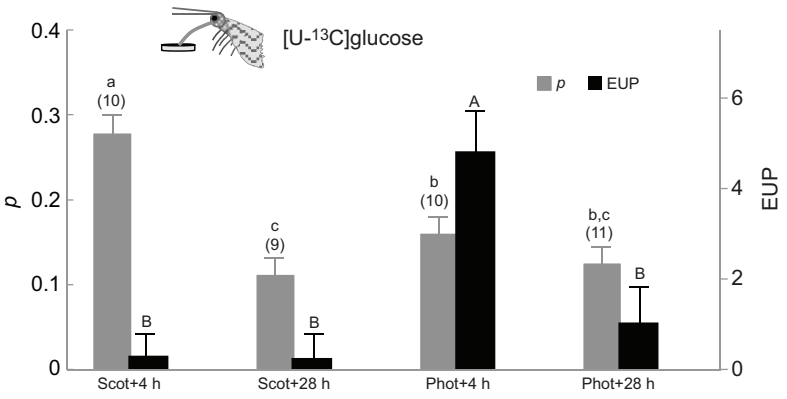

Fig. 2. Mean precursor enrichment (*p*) and excess unlabeled pheromone (EUP) proportion of virgin female *H. virescens*. Females were fed 30% 50:50 [U-^{13}C]:[U-^{12}C]glucose during either scotophase (Scot) or photophase (Phot) and analyzed 4 or 28 h later. EUP is a measure of the relative amount of unlabeled pheromone not explained by *de novo* biosynthesis. Different letters of the same case above bars indicate significant (P<0.05) differences; N is given in parentheses.

The Journal of Experimental Biology (2015) 218, 1252-1258 doi:10.1242/jeb.119883

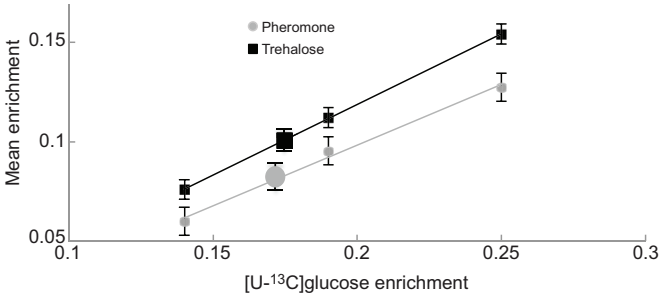

Fig. 3. Mean pheromone enrichment (*p*) and hemolymph trehalose (atom percentage excess, APE) from virgin female *H. virescens*. Females were fed 30% glucose of different enrichments (25:75, 19:81 or 14:86 [U-^{13}C]:[U-^{12}C]glucose) at the start of scotophase and analyzed 4 h later. Females fed 25:75 enrichment were also analyzed 28 h after feeding (indicated by larger symbols). Data are means±s.e.m.

usage in virgins but not the greater demands of mated females, which need to feed regularly to maintain hemolymph trehalose concentration and pheromone levels (Foster, 2009). In our single adult feed experiments, glucose from glycogen was all derived from larval feeding. However, well-fed adult Lepidoptera increase glycogen stores (Ziegler, 1991), meaning that with increased adult feeding, glycogen should become increasingly enriched with AAC, and hence any glucose mobilized from these stores will be too.

Given a maximum of ca. 65% of *de novo* biosynthesized pheromone is produced from carbohydrate, the balance (35%) must be the minimum produced from fat stores. Female *H. virescens* eclose with a large fat store that, even after adult feeding, remains largely larval derived, with the exception of the pheromone precursor acid (*Z*)-11-hexadecenoate (Foster and Anderson, 2012; Foster et al., 2014). We demonstrated that pheromone was produced *de novo* from fat through mitochondrial β-oxidation of [U-^{13}C]-Z9-16:COOH. The *p* of ca. 0.1 is substantial, but less than our expected ca. 0.35. This lower enrichment is probably due mainly to β-oxidation of other (non-labeled) endogenous fatty acids, and perhaps also to kinetic isotope effects on β-oxidation of the fatty acid to acetyl CoA, and methodological problems from delivering a bolus of fatty acid in DMSO. This last point was suggested by pre-injection of fatty acid or DMSO prior to feeding on [U-^{13}C]glucose yielding lower *p* values than the untreated control (no injection). Interestingly, in the reverse experiment, in which females were fed unlabeled glucose or water before injection of [U-^{13}C]-Z9-16:COOH, those fed glucose had a slightly lower *p* than those fed water, indicating that more pheromone was made from other sources, presumably carbohydrate. This suggests that the proportions of carbohydrate and fat used for *de novo* pheromone biosynthesis are not fixed but change according to endogenous conditions of the female, although probably not greatly. Using [U-^{13}C]-Z9-16:COOH as a tracer gave a FSR of ca. 38% h^{-1}, less than the ca.100% h^{-1} obtained previously with [U-^{13}C]glucose (Foster and Anderson, 2011). This lower FSR is possibly due to inhibitory effects of the bolus of acid in DMSO on biosynthetic enzymes (Foster, 2005a). Regardless, both tracers indicated rapid *de novo* biosynthesis during the scotophase.

In addition to *de novo* biosynthesis, sex pheromone can also be made from previously synthesized fatty acids stored in glycerolipids (Bjostad et al., 1987). Only certain acids though can be converted directly to pheromone components. Female *H. virescens* produce a multi-component blend (Teal et al., 1986) via the intermediate acids (*Z*)-11-hexadecenoic, (*Z*)-9-hexadecenoic, (*Z*)-9-tetradecenoic, hexadecenoic and octadecenoic acid (Choi et al., 2005). Although the

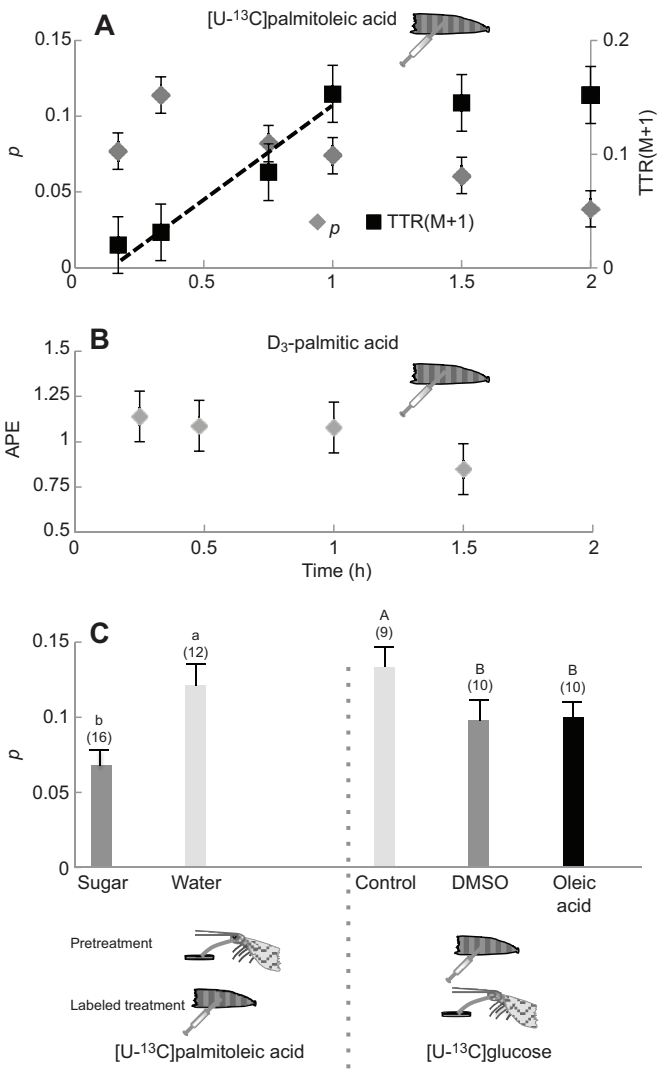

Fig. 4. Mean pheromone enrichment of virgin female *H. virescens* after various treatments. (A) Precursor enrichment (*p*) and tracer:tracee ratio for singly labeled pheromone [TTR(M+1)] after injection with [U-^{13}C]-(*Z*)-9-hexadecenoic (palmitoleic) acid. (B) APE after injection with [16,16,16-D$_3$]-hexadecenoic (palmitic) acid. (C) Precursor enrichment after pretreatment with unlabeled substrate and then treatment with labeled substrate. In the left panel, females were fed glucose (sugar) or water, and then injected with [U-^{13}C]-(*Z*)-9-hexadecenoic acid. In the right panel, females were injected with (*Z*)-9-octadecenoic (oleic) acid (in DMSO), DMSO or not injected (control), and then fed 10% [U-^{13}C]glucose. Different letters of the same case above bars indicate significant (P<0.05) differences; *N* is given in parentheses.

insect has large stores of other acids (Foster, 2005b), only stores of the above acids can be converted directly to pheromone components. Pheromone was produced directly from D$_3$-16:COOH, but our approach precluded quantification of the amount produced via this route, as we could not quantify unlabeled pheromone produced from other precursor acids, especially (*Z*)-11-hexadecenoate. However, our experiments with labeled glucose always showed substantial EUP, ca. 30% of the total labeled pheromone, over that predicted by mass isotopomer distribution analysis (MIDA). Given the rapid turnover of pheromone during the scotophase (Foster and Anderson, 2011), this EUP likely comes from stored precursor acids converted directly to pheromone. If so, then ca. 20–25% of pheromone must be produced this way, with the balance from *de novo* biosynthesis utilizing glucose and (other) fatty acids. Given its biosynthetic proximity to

The Journal of Experimental Biology (2015) 218, 1252-1258 doi:10.1242/jeb.119883

pheromone, stored (*Z*)-11-hexadecenoate is likely the major contributor to this, especially considering the low enrichment of pheromone we obtained from the more biosynthetically distal D_3-16:COOH. Disregarding whether stored fats were biosynthesized previously by the insect from carbohydrate or not, during the scotophase, pheromone must be biosynthesized roughly equally from carbohydrate and fat (via both routes) stores. The relatively large amount of EUP 4 h after feeding in the photophase must be taken into account with the relatively low amounts of pheromone produced during this period. It could result from either a relatively high (compared with *de novo* biosynthesis) production of pheromone from stored precursor acids or a slow turnover during this period of previously synthesized (unlabeled) pheromone (Foster and Anderson, 2011), especially as 24 h later EUP was much lower.

Fats in the pheromone gland are thought to play a role in pheromone biosynthesis in moths, acting as a reservoir for storing and/or releasing pheromone precursor acids (Bjostad et al., 1987; Foster, 2005a). In *H. virescens* and other moths, most glandular fat appears shortly before or after eclosion (Foster, 2005b). We had assumed that this pool was largely, if not exclusively, biosynthesized *de novo* in the gland. However, the relatively high production of pheromone from abdominal fatty acid injections, and the relatively slow rate of fat synthesis in the gland in *H. virescens* females (Foster and Anderson, 2012), suggests that most glandular fats may actually originate elsewhere, such as the fat body (Arrese and Soulages, 2010), and are transported to the gland by lipophorins (Schal et al., 1998) from the late pupal stage onwards.

The multiple pools of nutrients used by females for pheromone production are shared with other reproductive physiologies, notably oocyte production (Arrese and Soulages, 2010; O'Brien et al., 2002; Ramaswamy et al., 1997). Given this sharing, does pheromone production impose a significant metabolic cost on other reproductive activities? In virgins, the amount of precursor used for pheromone production over a day is equivalent to <1% of that potentially available in hemolymph trehalose (as only 50% of pheromone is made from carbohydrate, it is probably <0.5%) and inconsequential compared with that potentially available from fat in the body (Wood et al., 1969) or even the gland (Foster, 2005b). Not only are the amounts of nutrients used for pheromone production small compared with that potentially available, they are also small compared with that used for other physiologies. For example, the change in trehalose enrichment over 24 h indicated that ca. 30% of labeled trehalose was used for various physiologies over this period, much higher than that used for pheromone biosynthesis (see above). Especially given the relatively high contribution of adult food to pheromone production in *H. virescens* females, it seems highly unlikely that pheromone production can impose a significant metabolic cost to virgins. For mated females, the amount of nutrients used for pheromone production is similar to that for virgins; however, nutrient use for other metabolic activities, especially oocyte production, is much greater (Foster, 2009; O'Brien et al., 2002; Telfer, 2009). When mated females feed on abundant exogenous sugar, they maintain pheromone (Foster, 2009) and increase egg production (Ramaswamy et al., 1997). However, if they are unable to obtain sufficient sugar, then allocation of trehalose to pheromone production decreases, with a concomitant reduction in pheromone titer, but allocation to oocytes is maintained (Foster, 2009; Foster et al., 2014). Thus, in mated female *H. virescens*, pheromone production also appears to impose little or no significant metabolic cost. In terms of pheromone titer, *H. virescens* females produce relatively large amounts of pheromone compared with most moth species (www.pherobase.com). Hence, we might expect that the metabolic cost of pheromone production will also be insignificant across other species of moths. However, some species of Arctiidae and Bombycidae produce relatively large amounts of pheromone (micrograms), while numerous moth species do not feed as adults (Miller, 1996). In such cases, nutrients used for pheromone production could become limiting over time, leading to a more substantial metabolic cost of pheromone production on other reproductive physiologies. Further research on such species is required to test whether this occurs or not.

If there is an insignificant direct metabolic cost of sex pheromone production for female *H. virescens*, then females must incur an indirect fitness cost for producing sex pheromone if it indeed acts as a mate assessment signal (Umbers et al., 2015). The most likely indirect fitness cost to female moths is interception of the chemicals by various natural enemies. However, across all moth species this appears to be rather uncommon (Zuk and Kolloru, 1998) and, indeed, any such costs may be difficult to ascertain or quantify. Thus, for *H. virescens*, and possibly most other species of moths, it seems unlikely that the female-produced sex pheromone acts as a mate assessment signal.

MATERIALS AND METHODS

Insects

Heliothis virescens were from a laboratory colony originally obtained from the United States Department of Agriculture-Agricultural Research Service (USDA-ARS), Fargo, ND, USA, and reared on a wheatgerm/casein diet. After pupation, males and females were placed in separate containers at 25°C under a 16 h:8 h light:dark photoperiod. Adult females were collected each day and either used in experiments or mated the next day (i.e. when 1 day old); mated females were used in experiments 1 day later (i.e. when 2 days old).

Chemicals

[U-^{13}C]glucose (99%) was purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA), [U-^{13}C]-Z9-16:COOH from Larodan Fine Chemicals (Malmo, Sweden), D_3-16:COOH from MSD Isotopes (Rahway, NJ, USA), and unlabeled glucose and Z9-18:COOH from Sigma-Aldrich (St Louis, MO, USA).

Analysis of pheromone and trehalose

The pheromone gland was dissected and placed in *n*-heptane for at least 3 h at ambient temperature before analysis by gas chromatography/mass spectrometry (GC/MS) using a Hewlett-Packard 5890/5972 (Palo Alto, CA, USA) with splitless injection and helium as the carrier gas, at a constant flow of 1 ml min^{-1}. The GC was fitted with a 30 m×0.25 mm i.d., 250 µm film thickness ZB-Wax column (Phenomenex, Torrance, CA, USA), programmed from 80 to 180°C at 15°C min^{-1}, with an initial delay of 1 min, and then to 220°C at 3°C min^{-1}. The mass spectrum was run in electron ionization mode at 70 eV. We analyzed only the major component, (*Z*)-11-hexadecenal (Z11-16:Ald), because it comprises >90% of the pheromone mass (Teal et al., 1986) and is biosynthesized from the same acetate precursor pool as the other components (Choi et al., 2005).

Trehalose was analyzed as trehalose octaacetate, generated by removing water from hemolymph under vacuum and then acetylating the sugar through reaction with acetic anhydride in pyridine at 100°C (Foster, 2009). Trehalose enrichment was determined by GC/MS, as above, except using a 30 m×0.25 mm i.d. ZB5 capillary column (Phenomenex), temperature programmed from 200 to 280°C at 10°C min^{-1}.

In experiments with [U-^{13}C]glucose and [U-^{13}C]-Z9-16:COOH, we recorded specific isotopomers of Z11-16:Ald: *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 are intact structures minus a water molecule, a more intense ion than the molecular ion. In the experiment with D_3-16:COOH, we recorded *m*/*z* 220 and 223, representing loss of water from the molecular ion

The Journal of Experimental Biology (2015) 218, 1252-1258 doi:10.1242/jeb.119883

of Z11-16:Ald of unlabeled and D_3-labeled isotopomers, respectively. For trehalose enrichment, we could not measure any ions of the intact C_{12} skeleton of trehalose octaacetate because of weak intensities. Therefore, we monitored *m/z* 331 [$C_{14}H_{14}O_9^+$;(M+0)], a glucose tetraacetate fragment (Foster et al., 2014) and its $^{13}C_6$-isototopomer [*m/z*=337; (M+1)].

Enrichment of pheromone and trehalose

In most experiments, we determined *p* of Z11-16:Ald, using the combinatorial approach of MIDA (Hellerstein and Neese, 1992; Wolfe and Chinkes, 2005). In experiments with D_3-16:COOH, or when analyzing trehalose, we calculated enrichments in APE.

MIDA assumes that a polymer is assembled randomly from a pool of monomeric precursor (i.e. for pheromone, from cytosolic acetyl CoA) and that a specific proportion (i.e. *p*) of tracer (labeled) to tracee (labeled) will give a specific distribution of isotopomers. Therefore, by measuring the isotopomer pattern by GC/MS, we can determine *p* of the pool of precursor from which the polymer is synthesized. Only two labeled isotopomers – (M+1) and (M+2) are usually the most intense (Wolfe and Chinkes, 2005) – need be measured to calculate *p*. From these, tracer to tracee ratios (TTRs) are calculated and corrected for naturally occurring isotopes and overlap of the spectra of singly and doubly labeled isotopomers (Wolfe and Chinkes, 2005). Thus:

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

$$\mathrm{TTR(M+2)} = (\mathrm{M+2}) \div (\mathrm{M+0})_{\mathrm{post}} - (\mathrm{M+2} \div \mathrm{M+0})_{\mathrm{pre}} - \mathrm{dT_1} \times \mathrm{TTR(M+1)}. \quad (2)$$

The ‘pre’ and ‘post’ terms are, respectively, the isotopomer values before and after introduction of the tracer and correct for naturally occurring ^{13}C and ^{2}H. We used theoretically calculated ‘pre’ terms, based on known isotopic abundances, because previous work found these to differ little from values determined experimentally (Foster and Anderson, 2011, 2012). The term dT_1 corrects for overlap of spectra of the singly and doubly labeled isotopomers. Precursor enrichment is calculated as:

$$p = 2R/[(n-1) + 2R], \quad (3)$$

where *R*=TTR(M+2)/TTR(M+1) and *n* is the number of precursor molecules in the product (eight for Z11-16:Ald).

For the experiment using D_3-16:COOH, we determined the intensities of unlabeled (M+0) and D_3-labeled (M+1) isotopomers of Z11-16:Ald, allowing us to calculate TTR(M+1), as above. For trehalose, we calculated TTR(M+1), but did not allow for naturally occurring *m/z* 337, because of its negligible abundance. For both of these, we calculated enrichment as APE (Wolfe and Chinkes, 2005):

$$\mathrm{APE} = \mathrm{TTR(M+1)}/[\mathrm{TTR(M+1)}+1]. \quad (4)$$

Contribution of adult food to pheromone production

As production of acetyl CoA from glucose or fatty acid is subject to kinetic isotope effects, we could not use *p* directly as a measure of the contribution of adult food. We eliminated fractionation effects using the method of O’Brien et al. (2002), by feeding two isotopic enrichments of glucose to different females and calculating the respective *p* values, allowing calculation of the contribution of adult diet to pheromone production.

Briefly, *p* will result from the isotopic enrichments (Enrich) and proportional contributions (*A*) of adult and larval diets, with respective fractionation effects (Δ):

$$p = A(\mathrm{Enrich_{Adult}} + \Delta_{\mathrm{Adult}}) + (1 - A)(\mathrm{Enrich_{Larval}} + \Delta_{\mathrm{Larval}}). \quad (5)$$

Because the fractionation effects are characteristic of the biochemical route, feeding females two glucose enrichments and solving the two equations simultaneously allows the proportional contribution of the adult diet to pheromone production to be calculated:

$$A_{\mathrm{Adult}} = (p_1 - p_2)/(\mathrm{Enrich_{Adult1}} - \mathrm{Enrich_{Adult2}}). \quad (6)$$

Calculation of EUP

EUP was calculated for each female by using its *p* value and the measured M+2 isotopomer value to calculate the expected total amount of all nine isotopomers (TAI). We did not correct for overlap of spectra from the M+1 isotopomer or for the occurrence of natural isotopes, which are both relatively small:

$$\mathrm{TAI} = (\mathrm{M+2})/[28 \times p^2 \times (1-p)^6]. \quad (7)$$

Next, we calculated the expected area of the M+0 isotopomer, assuming it was all synthesized from the tracer/tracee pool [=$(1–p)^8$×TAI] and subtracted this from the actual observed area to give $\mathrm{Excess_{M+0}}$. EUP was calculated as $\mathrm{Excess_{M+0}}$/TIA. If all unlabeled pheromone was synthesized from the tracer/tracee pool, EUP=0.

Calculation of FSR

As calculation of FSR utilizes the rate of change of singly labeled product, and *p* is enrichment of acetate precursor, we first converted *p* to units of singly labeled product (*Ep*):

$$Ep = np(1-p)^{n-1}. \quad (8)$$

FSR was then calculated as:

$$\mathrm{FSR} = (Et_2 - Et_1) \div [(t_2 - t_1) \times Ep], \quad (9)$$

with the rate of change of singly labeled product calculated by linear regression before isotopic steady state was reached.

Experiments

Contribution of adult food to pheromone production

Dose

At the start of the scotophase, we fed 1 day virgins with 25 µl of 5%, 10%, 20%, 50% or 75% (w/v) ^{13}C-enriched glucose, and analyzed pheromone *p* after 4 h [when production is greatest and the acetyl CoA pool has reached isotopic equilibrium and all *de novo* biosynthesized pheromone is likely derived from the equilibrated tracer/tracee pool (Foster and Anderson, 2011)]. For each glucose concentration, two enrichments were fed to different females: 50:50 and 25:75 [U-^{13}C]:[U-^{12}C]glucose for 5%, 10%, 20% and 50% glucose, and 25:75 and 12.5:87.5 ([U-^{13}C]:[U^{12}C]glucose) for 75% glucose.

Time, multiple feeds and mating status

One day old virgins and 2 day old mated females were fed 25 µl of one of two enrichments (100:0 or 50:50 of [U-^{13}C]:[U-^{12}C]glucose) of 10% glucose at the start of the scotophase. Then: (i) virgins were analyzed for pheromone *p* 4, 28 or 52 h later; (ii) virgins were fed again 24 h later, and analyzed for pheromone *p* 4 or 28 h later; and (iii) mated females were analyzed for pheromone *p* 4 h later.

Photoperiod

We fed 1 day old virgins with 25 µl of 30% 50:50 [U-^{13}C]:[U-^{12}C]glucose, either at the beginning of the scotophase or in the 8th hour of the photophase, and determined pheromone *p* 4 and 28 h later. In the previous experiment, we noticed that females had more unlabeled pheromone than expected (by MIDA) if all the pheromone was biosynthesized from the mixed tracer/tracee pool. Therefore, we also calculated EUP.

Change in enrichment of pheromone and trehalose over time

In previous experiments, *p* was always lower at 24 h than at 4 h. As hemolymph trehalose concentration is constant in virgins over this period (Foster and Johnson, 2010), we determined trehalose enrichment. Females were fed 25 µl of 30% of one of 25:75, 19:81 or 14:86 [U-^{13}C]:[U-^{12}C] glucose at the start of the scotophase and analyzed for trehalose and pheromone enrichments 4 h later. A sub-group fed 25:75 [U-^{13}C]:[U-^{12}C] glucose was analyzed 24 h later. Hemolymph was sampled (Foster, 2009) prior to dissection of the pheromone gland.

Contribution of fatty acids to pheromone production

We determined whether fatty acids contributed to pheromone production via mitochondrial β-oxidation and *de novo* biosynthesis or through biosynthesis directly from a precursor acid. For the former, we injected 0.2 mg (in 2 µl DMSO) of [U-^{13}C]-Z9-16:COOH into a 1 day old female and determined pheromone *p* by MIDA. Preliminary experiments indicated 0.2 mg was the minimum dose for detectable levels of the (M+2) isotopomer. Two hours into the scotophase, we injected the acid into the abdomen and analyzed pheromone in different females 10, 20, 45, 60, 90 or 120 min later. We also calculated FSR (Wolfe and Chinkes, 2005).

For biosynthesis from a precursor acid, we injected 0.2 mg (in 2 µl of DMSO) of D_3-16:COOH into females 2 h into the scotophase, and determined APE of pheromone 15, 30, 60 and 90 min later.

To test whether levels of one nutrient influenced biosynthesis from the other, we pretreated females with unlabeled fat or carbohydrate, introduced a labeled form of the other, and then analyzed pheromone *p* 30 min later: (i) females were fed either 25 µl of water or 10% glucose (unlabeled) in the scotophase and, within 5 min, injected with 0.2 mg of [U-^{13}C]-Z9-16:COOH; and (ii) females were injected with either 2 µl DMSO or 0.2 mg of unlabeled Z9-18:COOH (in DMSO) and, within 5 min, fed 25 µl of 10% [U-^{13}C]glucose. For the latter, we also analyzed untreated females (not injected) fed 25 µl of 10% [U-^{13}C]glucose. The amounts of glucose fed or fatty acid injected had roughly the same potential amount of acetate precursor: 2.5 µmol glucose=5 µmol acetate; 0.74 µmol [U-^{13}C]-Z9-16:COOH and 0.71 µmol of Z9-18:COOH=5.9 and 6.4 µmol of acetate, respectively.

Statistical analyses

Data were analyzed by ANOVA, checking first for normality and heteroscedasity, and means were compared by Student's *t*-tests. Differences among means are reported at $P<0.05$.

Acknowledgements

The authors are grateful to Mark Spanier for help with some of the calculations.

Competing interests

The authors declare no competing or financial interests.

Author contributions

S.P.F. designed and performed the research, analyzed the data and wrote the manuscript. K.G.A. performed the research.

Funding

This work was funded, in part, by a United States Department of Agriculture – National Institute of Food and Agriculture Hatch Grant ND02367 to S.P.F. Deposited in PMC for release after 12 months.

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.

Arrese, E. L. and Soulages, J. L. (2010). Insect fat body: energy, metabolism, and regulation. *Annu. Rev. Entomol.* **55**, 207-225.

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

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.

Bushman, D. W., McGinley, S. M. G., Einsmann, J. E. and Robel, D. K. (2002). The role of carbohydrate metabolism in the regulation of postfeeding diuresis in adult *Helicoverpa zea* (Lepidoptera: Noctuidae). *Ann. Entomol. Soc. Am.* **95**, 492-497.

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

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

Foster, S. P. (2005a). The fate of topically applied fatty acids in the sex pheromone gland of the moth *Heliothis virescens*. *Insect Biochem. Mol. Biol.* **35**, 1021-1031.

Foster, S. P. (2005b). Lipid analysis of the sex pheromone gland of the moth *Heliothis virescens*. *Arch. Insect Biochem. Physiol.* **59**, 80-90.

Foster, S. (2009). Sugar feeding via trehalose haemolymph concentration affects sex pheromone production in mated *Heliothis virescens* moths. *J. Exp. Biol.* **212**, 2789-2794.

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

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 Biochem. Mol. Biol.* **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. *J. Insect Physiol.* **56**, 1617-1623.

Foster, S. P., Anderson, K. G. and Harmon, J. P. (2014). 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*. *J. Exp. Biol.* **217**, 499-506.

Harari, A. R. and Steinitz, H. (2013). The evolution of female sex pheromones. *Curr. Zool.* **59**, 569-578.

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. *Am. J. Physiol. Endocrinol. Metab.* **263**, E988-1001.

Johansson, B. G. and Jones, T. M. (2007). The role of chemical communication in mate choice. *Biol. Rev. Camb. Philos. Soc.* **82**, 265-289.

Kotiaho, J. S. (2001). Costs of sexual traits: a mismatch between theoretical considerations and empirical evidence. *Biol. Rev.* **76**, 365-376.

Miller, W. E. (1996). Population behavior and adult feeding capability in Lepidoptera. *Environ. Entomol.* **25**, 213-226.

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. *Proc. Natl. Acad. Sci. USA* **99**, 4413-4418.

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

Schal, C., Sevala, V. and Cardé, R. T. (1998). Novel and highly specific transport of a volatile sex pheromone by hemolymph lipophorin in moths. *Naturwissenschaften* **85**, 339-342.

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). *J. Chem. Ecol.* **12**, 107-126.

Telfer, W. H. (2009). Egg formation in Lepidoptera. *J. Insect Sci.* **9**, 1-21.

Umbers, K. D. L., Symonds, M. R. E. and Kokko, H. (2015). The mathematics of female pheromone signaling: strategies for aging virgins. *Am. Nat.* **185**, 417-432.

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

Wolfe, R. R. and Chinkes, D. L. (2005). *Isotope Tracers in Metabolic Research*, 2nd edn. Hoboken, NJ: John Wiley & Sons, Inc.

Wood, R., Harlow, R. D. and Lambremont, E. N. (1969). GLC analysis of *Heliothis virescens* triglycerides at various metamorphic stages. *Lipids* **4**, 159-162.

Wyatt, T. D. (2014). *Pheromones and Animal Behaviour: Chemical Signals and Signatures*. New York: Cambridge University Press.

Zahavi, A. (1977). The cost of honesty: further remarks on the handicap principle. *J. Theor. Biol.* **67**, 603-605.

Ziegler, R. (1991). Changes in lipid and carbohydrate metabolism during starvation in adult *Manduca sexta*. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **161**, 125-131.

Zuk, M. and Kolluru, G. R. (1998). Exploitation of sexual signals by predators and parasitoids. *Q. Rev. Biol.* **73**, 415-438.