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Sugar feeding *via* trehalose haemolymph concentration affects sex pheromone production in mated *Heliothis virescens* moths

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SUMMARY

Long-distance, female-produced sex pheromones are widespread among moths. Larval feeding provides most of the nutrients for development of these insects but is not thought to influence the *de novo* production of the fatty-acid derived compounds used as pheromones by most species. Feeding on plant nectar (sugar) by adult moths is important for increasing female fitness and also for the pollination of many plant species. In this paper, I show that feeding on sucrose solution, as opposed to water, increases sex pheromone titre in mated, but not virgin, female *Heliothis virescens*. Mating caused a rapid decrease in haemolymph trehalose concentration, which was restored to near-virgin levels by sugar ingestion. When isolated mated female abdomens were cultured with different concentrations of trehalose, pheromone titre increased with increasing trehalose concentration. This effect was not observed when abdomens were cultured on saline containing the sugar rhamnose, which insects cannot metabolise to glucose. Virgins injected with the juvenile hormone (JH) analogue, methoprene, showed the same effects as mated females with respect to pheromone titre and haemolymph trehalose concentration. Thus, following mating increases in JH titre increase demand for, and lowering of, blood sugar to develop oocytes, which can be compensated for by sugar ingestion. Haemolymph trehalose concentration probably influences glycolysis in gland cells and, consequently, levels of cytosolic citrate and acetyl-CoA for pheromone biosynthesis. This increase in pheromone titre in sugar-fed, mated females may facilitate further mating and increased fecundity. Thus, exogenous sugar feeding is behaviourally and physiologically integrated with endogenous JH titre to maximise female fitness.

Key words: juvenile hormone, methoprene, nectar feeding, pheromone biosynthesis.

INTRODUCTION

Many animals rely on pheromonal communication for critical behaviours involved in reproduction and survival (Wyatt, 2003). As most animals have a varied or unreliable diet, it is generally thought that diet has little direct effect on pheromone production, although there are exceptions known to this in which animals feed exclusively on a reliable food or in which the pheromone is not critical for completion of the behaviour (Bradbury and Vehrencamp, 1998). For instance, some bark beetles produce their aggregation pheromone components, verbenol and verbenone, from α-pinene from host trees during colonisation (Renwick et al., 1976; Hunt and Borden, 1989), although most bark beetle pheromone components are probably biosynthesised *de novo* (Seybold and Tittiger, 2003). Also, males of a number of arctiid moth species are known to produce their 'aphrodisiac pheromone' components, such as hydroxydanaidal, from pyrrolizidine alkaloids sequestered during larval feeding (Edgar et al., 2007; Schulz et al., 1993). Such aphrodisiac pheromones may enhance, but are not thought to be critical for, mating success (Bezzerides et al., 2005).

Long-distance, female-produced sex pheromones are widespread among moths and are critical for reproductive success (Tamaki, 1985). Most female moths produce their sex pheromone components *de novo* in a specialised gland *via* synthesis of saturated fatty acids and modification by a limited number of gland-specific enzymatic steps (Jurenka, 2003). Biosynthesis is controlled by the release of the pheromone biosynthesis-activated neuropeptide (PBAN) (Rafaeli and Jurenka, 2003) from the corpora cardiaca, which interacts with a G protein-coupled receptor on the membrane of pheromone gland cells (Choi et al., 2003). Feeding by larvae provides most of the nutrients for the development and reproductive activities of these holometabolous insects (O'Brien et al., 2004) but is not thought to influence the production of the sex pheromone components (Jurenka, 2003; Miller et al., 1976). Adult moths either do not feed or are restricted to feeding on liquids, which, for most species, constitute sucrose-rich plant nectar (O'Brien et al., 2004; Wheeler, 1996). This feeding is important for increasing female fitness (Wheeler, 1996; Ramaswamy et al., 1997), and also in the pollination of many species of nectar-producing plants (Pellmyr et al., 1996). All animals readily metabolise sugars to acetyl CoA, the building block of most moth pheromones (Jurenka, 2003), yet the effect of adult sugar feeding on pheromone production has been ignored. In this paper, I show that feeding on sucrose by the tobacco budworm moth, *Heliothis virescens* (Fabricius) (Lepidoptera; Noctuidae), causes a quantitative increase in pheromone production in mated, but not virgin, females through an increase in haemolymph trehalose concentration. Furthermore, this effect is mediated indirectly by juvenile hormone (JH), which causes increased egg maturation in females and consequently trehalose demand.

MATERIALS AND METHODS

Insects

Heliothis virescens larvae were reared on a wheat germ–casein diet at 25±0.5°C under a 16 h:8 h (light:dark) photoperiod. Males and females were separated after pupation and kept under the same conditions as larvae. Emerged adults were collected each day and placed in a container with either distilled water or a 10% (w/w)

sucrose solution, absorbed on cotton wool, to feed on. The next day, males and females were combined and copulating pairs of moths removed; after copulation, males were discarded. Females were generally placed back in containers with the food source (water or sucrose) that they had fed on previously, until used in experiments. Virgin females were maintained similarly. In one experiment, testing the effect of pre- and post-mating feeding on pheromone titre, newly-emerged females were placed in containers with either sugar or water, mated the next day and then placed on either sugar or water for 1–2 days further before pheromone analysis, yielding four treatments: sugar/sugar, sugar/water, water/sugar, water/water (pre-mating/post-mating).

Pheromone analysis

At mid-scotophase, when pheromone titre of female *H. virescens* is maximal (Roelofs et al., 1974), the pheromone gland of an individual female was extruded, excised and placed in ~15 ml of *n*-heptane, along with (*E*)-11-tetradecenal as internal standard. Glands were allowed to extract for at least 3 h at ambient temperature before analysis by gas chromatography–mass spectrometry (GC–MS), using a Hewlett-Packard 5890 gas chromatograph (GC) (Palo Alto, CA, USA) coupled with a 5972 mass selective detector (MSD) (Agilent Technologies, Palo Alto, CA, USA). The GC was fitted with a 30 m×0.25 mm i.d. (internal diameter) ZB-Wax column (Phenomenex, Torrance, CA, USA), temperature programmed from 80–180°C at 15°C min^{-1}, following an initial delay of 1 min. and then to 200°C at 3°C min^{-1}. Helium at a linear flow velocity of 30 cm s^{-1} was the carrier gas. The MSD was operated in the selected-ion monitoring mode with *m/z* of 192 (for the internal standard) and 220 [for (*Z*)-11-hexadecenal, the major pheromone component of *H. virescens* (Roelofs et al., 1974)] recorded. Quantity of pheromone was determined following integration of peak areas and compensation for relative *m/z* intensities using standard solutions of the two compounds. Pheromone quantity in the text and figures refers to the amount of (*Z*)-11-hexadecenal.

Sugar analysis

One day after mating (or 2 days after eclosion for virgins), a small hole was made dorsally between the 5th and 6th abdominal segments, gentle pressure was applied to the posterior abdominal segments and the resulting haemolymph droplet (~1–10 µl) was collected using a calibrated glass capillary. Fifty µg of sorbitol (in distilled water) was added to the haemolymph as an internal standard and the water removed from the sample by vacuum distillation. Sugars were acetylated (Wyatt and Kalf, 1957) by reaction with acetic anhydride in pyridine and analysed by GC–MS in the scanning mode using a 30 m×0.25 mm i.d., ZB-5 column (Phenomenex) and a temperature programme of 200–280°C at 10°C, following an initial delay of 1 min. As the sugars were not reduced prior to analysis, only trehalose octaacetate and sorbitol hexaacetate peaks were analysed. However, it should be noted that peaks for glucose pentaacetate anomers, and indeed those of other sugars, were always small (less than 5% of the trehalose peak) and so constituted a relatively insignificant fraction of the haemolymph sugars. Generally, sucrose octaacetate was not detected, indicating that the method for collecting haemolymph did not cause leakage from the gut. The few samples in which sucrose was detected were discarded. For samples from insects injected with oil (see below), haemolymph was collected more carefully, with only samples containing less than ~5% of oil in the total volume being analysed.

Assays

For the PBAN assay, females that fed on either water or sucrose were mated and one day later decapitated. These females were left for 6 h [when pheromone titre had declined to a very low level, <0.5 ng (Eltahlawy et al., 2007)], before being injected with 5 pmol of synthetic PBAN in saline (NaCl, 187.5 mmol l^{-1}; KCl, 4.83 mmol l^{-1}; $CaCl_2$, 2.61 mmol l^{-1}; Hepes, 10 mmol l^{-1}; glucose, 14 mmol l^{-1}; adjusted to pH=6.8). After 1.5 h, pheromone glands were dissected and analysed for pheromone. To test the effect of trehalose on pheromone titre, water-fed females that had been mated for one day were decapitated and left for a further day. After this, the terminal six abdominal segments were excised and placed on a 10 µl drop of saline similar to the one used in the PBAN assay but lacking glucose and containing different concentrations of trehalose. After 15 min equilibration time, 5 pmol of PBAN (in 5 µl saline lacking glucose) was added to the abdomen saline and the assay left for 1.5 h after which the pheromone gland was excised and analysed. A similar experiment was performed comparing iso-osmotic salines with either 200 mmol l^{-1} trehalose or 200 mmol l^{-1} rhamnose, the latter a deoxy hexose that is not converted to glucose (or trehalose) by insects. Data are reported for initial concentration of sugar in the saline; however, actual concentrations experienced by the gland were likely to be half as much because of dilution by the PBAN saline and the insect haemolymph.

For the methoprene assay, 5 µl of methoprene in vegetable oil (1 µg $µl^{-1}$) or vegetable oil (control) was injected into 1-day-old virgin females that had been fed on either water or sucrose and left for a further day before pheromone was analysed. For the analysis of trehalose levels in oil- and methoprene-injected females, only water-fed females were used. For females injected with the higher dosage of methoprene, a more concentrated solution (5 µg $µl^{-1}$) was used.

Data analysis

Data were analysed using the program JMP-IN (SAS Institute, Cary, NC, USA). Prior to analysis by parametric methods, data were checked for normality and homogeneity of variance. Data are presented as means ± standard error of mean (±s.e.m.). For a one-factor comparison, differences among means were tested by analysis of variance (ANOVA) or Student's *t*-test (for multiple mean comparisons). Two-way ANOVA, with or without an interaction term, was used for two-factor comparisons.

Chemicals

(*E*)-11-tetradecenal was purchased from Pherobank (Wageningen, The Netherlands), synthetic *Helicoverpa zea* PBAN from Bachem (Torrance, CA, USA), D-sorbitol from Sigma (St Louis, MO, USA), L-(+)-rhamnose from Aldrich (Milwaukee, WI, USA), trehalose from USB (Cleveland, OH, USA), acetic anhydride from Alfa Aesar (Ward Hill, MA, USA) and methoprene (98% pure, racemic mixture of *R* and *S* enantiomers) from Chem Service, Inc. (West Chester, PA, USA).

RESULTS

When allowed to feed *ad libitum* on 10% sucrose solution, mated, but not virgin, female *H. virescens* had significantly greater titres of sex pheromone than corresponding females that fed *ad libitum* on water (Fig. 1). This difference was apparent for at least three days (the maximum time tested) following mating. When synthetic PBAN was injected into sugar- or water-fed mated females that had been decapitated, sugar-fed females had significantly greater titres of pheromone than water-fed ones (179.2±22.6, *N*=9 *versus*

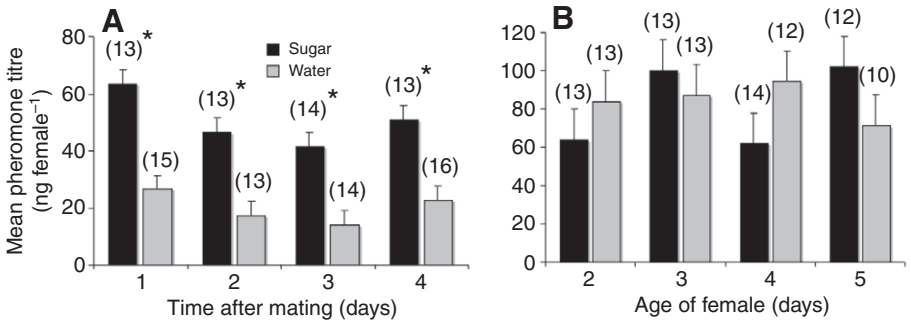

Fig. 1. Mean sex pheromone titre of adult female *Heliothis virescens* fed on 10% sucrose solution or distilled water (A) mated when 1-day-old and (B) virgin. *Indicates a significant difference (P<0.05, ANOVA) between a pair of treatments (time after mating or age of virgin) of sugar-fed and water-fed females. Error bars represent s.e.m.; N is in parentheses above each treatment.

108.6±24.0, N=8, ng female^{-1}, respectively; $F_{1,15}$=4.58, P=0.049, ANOVA), indicating that the observed effect in intact, mated females was related to sugar feeding directly and not to attenuation by neural or neurohemal factors originating from the head.

In a test of whether access to sugar solution before or after mating had a similar effect on pheromone titre or not, the four combinations of sugar/water feeding before and after feeding showed distinct differences (Fig. 2). Two-way ANOVA analysis of the data showed no significant effect (F=0.001, P=0.98) on pheromone titre of access to water or sugar before mating but a strong significant effect (F=6.7, P<=0.01) of access to water or sugar after mating. After mating, females (i.e. combined water- or sugar-fed females before mating) that had access to sugar had a mean titre of 65.5±8.0 (N=27) compared with 36.5±7.8 (N=28) for females that had access to water. Thus, access to (and presumably feeding on) sugar after mating had a much stronger effect on pheromone titre in mated females than access before mating.

As the most obvious effect of sucrose feeding should be to raise haemolymph sugar levels (Blatt and Roces, 2001), trehalose, the major blood sugar in most insects (Wyatt and Kalf, 1957; Thompson, 2003), concentrations were determined for both virgin and mated females that had been fed sugar or water (Fig. 3). Factorial analysis showed significant main effects of mating status (F=4.35, P=0.047) and feeding (F=23.5, P<0.001) but no significant interaction (F=0.005, P=0.95) between the two effects. Both virgin and mated females that fed on sugar had significantly higher (P<0.05, Fisher's t-test) trehalose concentrations than the respective females that fed on water.

Comparison of pheromone titre produced by excised abdomens of water-fed mated females, cultured on saline droplets with different concentrations of trehalose, showed that abdomens cultured on saline with an initial concentration of 200 mmol l^{-1} trehalose had a significantly higher (P<0.05, Fisher's t-test) pheromone titre than abdomens cultured on lower trehalose concentrations (Fig. 4). In a separate experiment, abdomens cultured on saline with an initial concentration of 200 mmol l^{-1} trehalose produced a significantly higher (ANOVA, $F_{1,23}$=7.51, P=0.01) pheromone titre (mean=82.9±9.6; N=12) than did abdomens cultured on iso-osmotic saline with 200 mmol l^{-1} rhamnose (mean=46.5±9.2; N=13).

The effects of methoprene on virgin female *H. virescens* that had fed on either water or sugar were tested. For females injected with methoprene, sugar-fed ones had a significantly (P<0.05, Fisher's t-test) higher pheromone titre than water-fed ones (Fig. 5A). By contrast, there was no difference between control females (injected with oil only) that had fed either on sugar or water. Factorial analysis of the data showed a barely significant (F=3.83, P=0.05) main effect of sugar feeding and no main effect of methoprene (F=2.01, P=0.16) but a strong interaction (F=6.75, P=0.01) of methoprene and sugar feeding; the interaction indicating that the effect of methoprene on pheromone titre was strongly dependent on whether a female had fed on sugar or water. Water-fed virgin females injected with methoprene at both a 5 and 25 mg dose had significantly lower haemolymph trehalose concentrations than control females injected with oil (Fig. 5B), indicating that methoprene treatment caused a reduction in haemolymph trehalose concentration.

DISCUSSION

Mating typically causes permanent or reduced production of sex pheromone in female moths, depending upon whether females of the respective species are monandrous or polyandrous (Rafaeli and

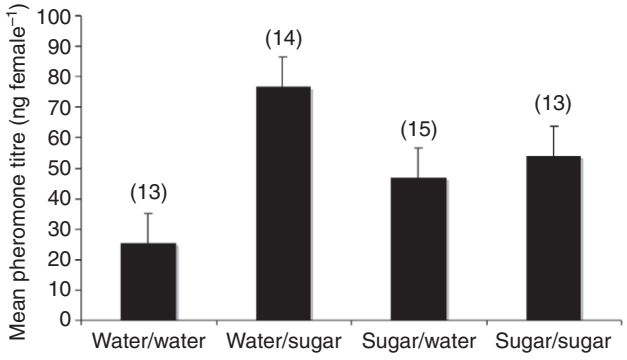

Fig. 2. Mean sex pheromone titre of adult female *Heliothis virescens* fed for 1 day on either 10% sucrose solution or distilled water, mated, then fed for a further 1–2 days on either 10% sucrose solution or distilled water. Treatments are given as pre-mating food/post-mating food. Different letters above bars indicate means (+s.e.m.) that are significantly different (P<0.05, Fisher's t-test); N is in parentheses above each treatment.

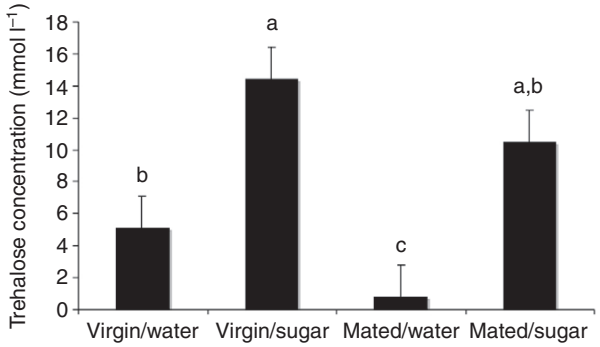

Fig. 3. Mean trehalose concentrations in haemolymph of virgin and mated adult female *Heliothis virescens* that had fed on either distilled water or 10% sucrose solution. Bars represent s.e.m. Different letters above bars indicate means that are significantly different (P<0.05, Fisher's t-test); N=7 for all treatments.

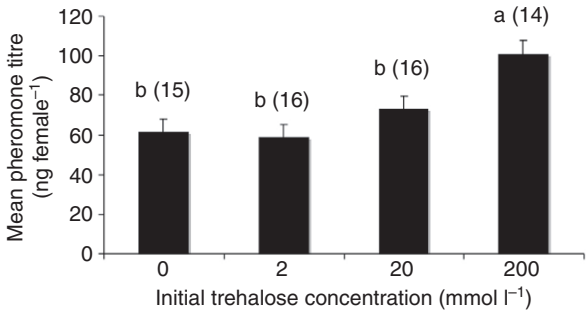

Fig. 4. Mean sex pheromone titre of mated female *Heliothis virescens* abdomens cultured in saline with different initial concentrations of trehalose. Females were water-fed and mated, left for one day, decapitated, and left for a further day, before the assay. Synthetic pheromone biosynthesis-activated neuropeptide (PBAN) (5 pmol) was added to stimulate pheromone biosynthesis. Bars represent s.e.m. Different letters above bars indicate means that are significantly different (P<0.05, Fisher's t-test); N is in parentheses above each bar.

Jurenka, 2003). Female *H. virescens* are polyandrous, with females mating on average 2.6 times during their lifetime (Raulston et al., 1975). The work reported herein shows that ingestion of sugar especially after mating allows females to produce a higher pheromone titre than mated females that have fed on water. Ingestion of sugar by mated females caused an increase in haemolymph trehalose concentration, which, in turn, caused increased pheromone production. This was shown by the changes in haemolymph trehalose concentration with feeding and the gland culture assay, in which an increased trehalose concentration in the saline (plus PBAN) gave an increased pheromone titre whereas iso-osmotic saline with the sugar rhamnose did not. That a higher than normal trehalose concentration was required to elicit a significant response (see Fig. 3) may be due to the assay design, with the open haemocoel and transected dorsal vessel hampering circulation of the trehalose to the pheromone gland. Moreover, we know little of the time required for changes in trehalose concentration to influence pheromone production, other than effects can be manifest within a day (see Fig. 1); the 1.5 h duration of the assay may have been too short for lower trehalose concentrations to exert their effect. It seems likely that the effect of haemolymph trehalose concentration on pheromone titre is to modulate the flux of glucose into gland cells, influencing glycolysis and consequently levels of cytosolic citrate and acetyl-CoA for pheromone biosynthesis (Jurenka, 2003). This effect was observed only for mated female *H. virescens*, probably because the greatest decline in haemolymph trehalose levels, observed in this study, occurred following mating. That water-fed virgins had substantially lower haemolymph trehalose concentrations than the corresponding sugar-fed virgins, but their pheromone titres were similar, suggests that there may be a threshold haemolymph trehalose concentration above which pheromone production is relatively constant and below which pheromone production decreases.

In *H. virescens* and other moths, the switchover from typical virgin physiology/behaviour to typical mated physiology/behaviour is strongly correlated with increases in JH titres in the haemolymph of females; injections of JHs or their analogues into virgin females can elicit many of these physiological changes, such as increased egg maturation (Ramaswamy et al., 1997). The JH mimic, methoprene, causes a decrease in haemolymph trehalose concentration in the fly *Delia radicum* (Young and Gordon, 1987), and similarly caused a decline in haemolymph trehalose concentration, and pheromone titre, when injected into virgin *H. virescens* females in this study. In mated *H. virescens* females, JH therefore appears to play an indirect role in modulating pheromone titres by lowering haemolymph trehalose concentration with a consequent decline in pheromone production. This JH-mediated decrease in haemolymph trehalose concentration is probably caused by a high demand for carbon, usually provided for by carbohydrates (O'Brien et al., 2004; O'Brien et al., 2000), for increased egg maturation in mated females (Ramaswamy et al., 1997). Previously, JH has been implicated in mediating development of competency of the pheromone gland in pharate moths (Rafaeli and Jurenka, 2003), down-regulation of pheromone production in older female moths (Rafaeli and Bober, 2005) or in controlling PBAN release until after a migratory flight (Cusson and McNeill, 1989). This mechanism of feeding influencing pheromone production in mated *H. virescens* females has some parallels with the effect of feeding on aggregation pheromone production in the bark beetle *Ips pini*. In *I. pini*, feeding by the adult male raises JH levels causing up-regulation of mevalonate pathway genes and a concomitant increase in flux of acetyl-CoA through this pathway (Seybold and Tittiger, 2003). In the bark beetle case, however, pheromone biosynthesis occurs in anterior midgut cells and the increase in acetyl-CoA flux could result either from increased sugar absorption from ingested phloem or from the JH-mediated degeneration of flight muscle tissue (Ivarsson et al., 1998).

Variation in sex pheromone production among female moths is well documented (Löfstedt, 1993). Pheromone titre is known to vary temporally, with age or time of photoperiod (Schal et al., 1987). Non-temporal variation is usually attributed to genetic differences among individuals (Allison and Cardé, 2006; Löfstedt, 1993; Symonds and Elgar, 2008). However, it is clear from the work

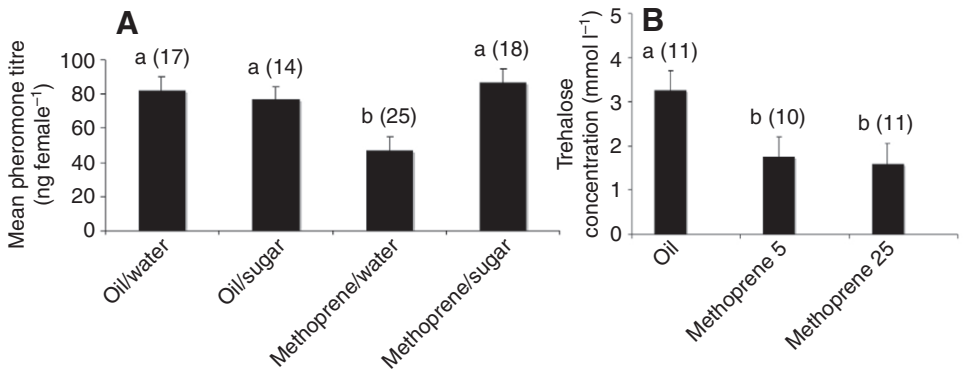

Fig. 5. (A) Mean sex pheromone titre of virgin female *Heliothis virescens* that had fed on either distilled water or 10% sucrose before being injected with either vegetable oil or 5 mg methoprene in vegetable oil. (B) Mean concentration of trehalose in the haemolymph of virgin female *H. virescens* that had fed on water before injection with vegetable oil, 5 mg or 25 mg of methoprene (in vegetable oil). Bars are s.e.m. Different letters above bars indicate means that are significantly different (P<0.05, Fisher's t-test); N is in parentheses above each bar.

presented here that pheromone titre, in mated *H. virescens* females at least, is phenotypically plastic, varying with haemolymph trehalose concentration. Although only mated females exhibited this effect in this study, it seems reasonable to assume that other environmental or physiological effects that cause drastic lowering of haemolymph trehalose concentration (e.g. below 1–5 mmol l^{-1}) (see Fig. 3) could lead to phenotypic variation in pheromone titre in virgin (as well as mated) females. For example, senescence (Wyatt and Kalf, 1957) and prolonged flight (Rockstein and Srivastava, 1967) have a strong effect on insect haemolymph trehalose concentration. Pheromone titre also decreases with increasing age in virgins of many species of moths (Jurenka, 2003) while, as mentioned previously, some species of moths delay pheromone production until after a migratory flight (Cusson and McNeill, 1989; Rafaeli and Jurenka, 2003), which might be expected to lower carbohydrate and lipid reserves substantially. Moreover, because most studies on sex pheromone variation in moths have not controlled for adult feeding, it is conceivable that not all observed variation in quantity (e.g. Haynes and Baker, 1988) is attributable to genetic differences but may be caused by variation in adult feeding or haemolymph trehalose concentration determined from prior nutrition (Hahn, 2005). As the likely effect of haemolymph trehalose concentration on pheromone production is to alter acetyl-CoA concentrations, the common building block of all fatty acid-derived pheromone components (Jurenka, 2003), it follows that changes in trehalose concentration will affect production of all components of the pheromone [e.g. of *H. virescens* (Klun et al., 1980)] equally. Therefore, it should probably affect pheromone blend quantity and not blend quality (i.e. ratios of components) in most cases.

Nectar or sugar feeding by female Lepidoptera has a strong effect on fecundity (Wheeler, 1996). The mechanisms for this increase, particularly given the relatively low composition of amino acids in nectars (Wheeler, 1996), are not well understood, although in some species an increasing amount of carbon from ingested sugar is allocated to later egg production (O'Brien et al., 2004). Sugar feeding is also thought to contribute in some way to increases in JH titre in females, which causes increased fecundity through increased egg maturation (Ramaswamy et al., 1997). The increase in pheromone titre in sugar-fed, relative to water-fed or starved, mated females probably results in an increased release rate of pheromone (Casimero et al., 2001). This should allow sugar-fed mated females to attract males over greater distances (Baker and Roelofs, 1981), thereby increasing the likelihood of further mating. Although sugar-fed mated females produced slightly less pheromone than either sugar- or water-fed virgins, the high level of polyandry observed in *H. virescens* (Lamunyon, 2000; Raulston et al., 1975) suggests that virgins are readily mated and that competition for additional mates among mated females may be important. Therefore, sugar feeding by mated females of polyandrous moth species may be important for increasing fecundity directly (Ramaswamy et al., 1997), as well as indirectly, by increasing the likelihood of further matings, which in turn causes increased fecundity (Torres-Vila et al., 2004). Such enhanced effects on fecundity should be accounted for in studies on female fitness and benefits of nectar feeding by night-flying pollinating moths.

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