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**EFFECTS OF DIET AND ABIOTIC CONDITIONS ON MALE SEX
PHERMONE CONTENT, AND THE IMPORTANCE OF MALE
PHEROMONES IN MALE MATING SUCCESS IN THE TRUE
ARMYWORM, PSEUDALETIA UNIPUNCTA**

Qi Xiu (Gordon) Chen

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EFFECTS OF DIET AND ABIOTIC CONDITIONS ON MALE SEX
PHERMONE CONTENT, AND THE IMPORTANCE OF MALE
PHEROMONES IN MALE MATING SUCCESS IN THE TRUE
ARMYWORM, *PSEUDALETIA UNIPUNCTA*

(Spine title: DIET, ABIOTIC CONDITIONS, AND PHEROMONE OF
THE TRUE ARMYWORM)

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By

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Graduate Program in Biology

2

Submitted in partial fulfilment of the
requirement for the degree of
Master of Science

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Abstract

Males of the true armyworm, *Pseudaletia unipuncta*, release sex pheromones from bilateral hairpencils, so fluctuating asymmetry (FA) of the pheromone source and concentration may be important in female choice. A survey of the field caught true armyworm males showed high inter-male variability in both concentration and FA from July 12 to August 5. I also quantified pheromones of males fed on artificial diet or corn leaves during larval development when reared under controlled laboratory and fluctuating field conditions. Again I found that both larval diet and abiotic conditions could influence both pheromone concentration and FA. Newly-emerged males reared under field condition had more pheromones and were more symmetrical than ones reared under controlled laboratory conditions on both larval diets. However, these differences were not significant by day six. I also tested the hypothesis that male pheromone was important for male mating success in a series of experiments, where the quantity/quality of larval or adult food resources varied. Female preferred males with higher pheromone content and lower FA when there had been some level of dietary stress, but not when individuals had been provided ample diet as larvae and adults.

Key Words: pheromones, female choice, fluctuating asymmetry (FA), true armyworm, *Pseudaletia unipuncta*, diet, temperature, abiotic conditions, field population

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1.1 Introduction

Darwin (1859) proposed the theory of natural selection, a cornerstone of modern biology, whereby favorable heritable traits become more common in successive generations of reproducing organisms while unfavorable ones become less common and may be eliminated from the population. However, as this theory could not explain bizarre, seemingly maladaptive traits, such as the flamboyant tail of the male peacock, he subsequently proposed the theory of sexual selection as the evolutionary process explaining such traits. He reasoned that the positive effects of such traits in both intra and inter-sexual interactions, relative to reproductive success, outweighed the negative effects on male survivorship (Darwin, 1871). Fisher (1958) built upon this theory, pointing out that for this to happen there must be a reproductive advantage for females mating with males possessing certain traits.

Our understandings of animal mating behaviors has dramatically improved as we refine our knowledge of sexual selection, including the degree of relative parental investment which controls the intensity of both intra-sexual competition and mate choice (Andersson, 1994; Trivers, 1972). It is clear that intra-sexual selection is an important force for the evolution of behavioral and morphological traits in males (Bradbury and Davies, 1987; Magurran and Garcia, 2000), such as penile morphology (Harcourt and Gardiner, 1992) and copulatory and mate-guarding behaviors (Møller and Birkhead, 1989; Zharikov and Nol, 2000). Furthermore, the degree of sexual dimorphism in body and canine size is much more pronounced in polygynous than monogamous species (Harcourt et al., 1981;

Kappeler, 1997), which correlates positively with the intensity of intra-sexual competition. However, intra-sexual competition is not limited to males, as females may compete for resources that directly affect their own, as well as their offspring's, survival (Palombit et al., 2000), resulting in traits such as reproductive suppression (Abbott, 1987; Woodroffe and MacDonald, 1995), maternal aggression (Maestripieri, 1992), territoriality (Agrell et al., 1998, and references therein), and infanticide (Wolff, 1997). Recent work on some mammals, where females are primary care-givers, suggest that they compete more frequently and/or intensively with each other, than they do with males (Bennett and Faulkes, 2000; Isaac, 2005). However, the extent to which females compete for limited resources and/or mates remains largely unresolved (Breiehagen and Slagsvold, 1988).

Inter-sexual selection, on the other hand, has been much more thoroughly studied (Searcy, 1982) and it is generally accepted that females are normally the choosy sex, given that they usually invest more in offspring than do males (Triver, 1972). Thus, a female's reproductive success is limited by resources she acquires while males are limited by the number of matings obtained. Consequently, males will compete for access to females, while females discriminate among males using visual, auditory and/or olfactory cues (Costanzo and Monteiro, 2007). The presence of non-random mating patterns does not automatically imply female choice, as intra-sexual competition may result in similar patterns (Searcy, 1982). However, experimental manipulations of specific male traits (Liu et al., 2006; Milinski and Bakker, 1990; Siitari and Huhta, 2002), the study of female preference (Coleman et al., 2004; Jennions and Petrie, 1997), and observations of

courtship behaviors of many species (Searcy, 1982) all lend support to the idea that the females are the choosy sex. However, sex role reversal can occur, where males become the choosy sex, when males have similar or higher parental investment than females (Barlow, 2005; Berglund et al., 2005; Kokko and Jennions, 2008; Simmons, 1992).

There are three hypotheses concerning the evolution of female choice: (1) the good gene hypothesis, (2) runaway sexual selection, and (3) sensory exploitation (Kirkpatrick, 1987). The good gene hypothesis assumes the gene(s) in question has evolved through direct natural selection, and female preference evolves indirectly as a correlated response to the male's superior heritable trait. A number of studies have shown that various fitness components are positively correlated with the expression of secondary sexual traits (Hasselquist et al., 1996; Møller, 1994; Petrie, 1994) and a recent meta-analysis on a large number of organisms also showed positive correlation between offspring survivorship and expression of male secondary sexual traits (Møller and Alatalo, 1999). The runaway sexual selection hypothesis proposes that female preference is genetically correlated with male display trait and consequently exerts a direct selection pressure on the male trait because males with selected trait enjoyed higher mating success (Hall et al., 2000; Pomiankowski and Iwasa, 1998). The third, the sensory exploitation hypothesis, proposes that females may be biased towards certain stimuli due to pre-existing preferences and consequently males evolve traits to exploit those pre-existing preferences (Ryan, 1990, 1998).

As the result of sexual selection, males of many species have very striking morphologies and behaviors (Ryan, 1998). However, males and females often have conflicting interests, so males are expected to manipulate receivers to maximize their own fitness while females must be able to appropriately interpret the signals received. The study of animal ornaments, displays, and songs (Keyser and Hill, 1999; Møller, 1992; Spencer et al., 2003) support the idea that signals are honest and costly, as do mathematical models of biological signaling (Enquist, 1985; Godfray, 1991; Grafen, 1990). There are three perspectives on honest signals: (1) The “Zahavi handicap” states that biological signals are honest with a direct viability cost, (2) the “revealing handicap” states that expression of certain traits directly reveals a male’s quality, while (3) the “condition-dependent handicap” proposes that the degree of trait expression correlates with male quality (Pomiankowski, 1988; Smith, 1987). However, dishonest signaling or cheating may occur, provided that the incidence is very low (Johnstone and Grafen, 1992).

Most insects follow the general predictions of sexual selection, with females being the choosy sex and male signals being honest and costly. It has been shown that females of different Orders select males for (i) the quality of their nuptial gifts which are costly for the males to produce or acquire (Cumming, 1994; Fedorka and Mousseau, 2002; Voigt et al., 2005), (ii) their acoustic signals, which maybe energetically costly (Burk, 1988; Ritchie, 2000; Tauber et al., 2001), body size (Brown et al., 1996; Juliano, 1985), and/or (iii) the degree of fluctuating asymmetry of secondary sexual characters (Hunt and Simmons, 1997). Fluctuating asymmetry (FA) is the random deviation in the symmetry of a bilateral trait in a given individual (Parsons, 1992; Tsubaki and Matsumoto, 1998)

and it has been proposed that FA increases with increasing stress during development (Parsons, 1990, 1992). Thus, if there is a relationship between male quality and FA, whereby high quality individuals will have lower FA, then females would select more symmetrical males within context of mate choice.

Generally, chemical signals are of considerable importance in the reproductive biology of nocturnal insects such as moths. Females attract conspecific males using long range sex pheromones released at very low rates and may be selecting for males who are better “searchers” (Fitzpatrick and McNeil, 1988; Greenfield, 1981). Once in proximity of a calling female, males often emit a short range sex pheromone from specialized scent organs and it has been postulated that these serve as a signal of male quality which may influence male reproductive success (Eisner, 1980; Fitzpatrick and McNeil, 1988; Hillier and Vickers, 2004; Lloyd, 1981; Royer and McNeil, 1992). However, there has not been a great deal of experimental work examining the importance of hairpencil odours on male reproductive success. It is possible that the level of fluctuating asymmetry in the pheromone content of left and right hairpencils may be of importance, as a number of studies have suggested that FA of bilateral traits may influence female choice (Harvey and Walsh, 1993; Liggett et al., 1993; Møller and Thornhill, 1998; Shykoff and Møller, 1999). It has been proposed that the level of FA is the result of gene-environment interactions, with well adapted individuals showing lower FA than poorly adapted ones under similar ecological conditions (Parsons, 1990) so male pheromone FA could serve as an honest signal for females. Several entomological studies have suggested that males with lower FA in pheromones had greater reproductive success than conspecifics with

higher FA (Schlaepfer and McNeil, 2000; Thornhill and Sauer, 1992). However, in these studies the chemical composition of the pheromones involved were unknown, thus it was impossible to test whether FA in pheromone content played a role in male reproductive success.

The true armyworm, *Pseudaletia unipuncta* (HAW.) (Lepidoptera: Noctuidae), is an important agricultural pest that provides an excellent model system to test the hypothesis that FA in male hairpencil content affects male reproductive success. In this species females release long range sex pheromone to attract males (Turgeon and McNeil, 1983; Dumont and McNeil, 1992); and once in close proximity of a potential mate, males release a short range sex pheromone, from bilateral hairpencils located on ventral surface of abdomen. Fitzpatrick and McNeil (1988) showed that males with intact hairpencils were rejected less often and enjoyed a higher mating success than individuals whose hairpencils had been excised. Given the insects are nocturnal and the hairpencils are not readily visible, it is highly unlikely that visual cues would be used in female choice, thus they postulated that the pheromone was important in mate choice. The male pheromone is comprised of acetic acid and benzaldehyde (Farine, 1982; Fitzpatrick et al., 1985), and there is a great deal of inter-individual variability in total pheromone titer, the relative proportion of each component, and the total amounts found in the left and right hairpencils of laboratory raised individuals (Fitzpatrick and McNeil, 1988).

However, if this is an important cue, it is not known which parameters play a role in male mating success. If females choose males based on male sex pheromones, then sufficient

variability in pheromone must exist. However, no previous studies examined variation in the hairpencil content of field collected moths. Furthermore, most studies examining male pheromones have used insects that were reared under control temperature, photoperiod and relative humidity on artificial diet. Moreover, it has been well documented that many parameters may differ when insects are reared under constant versus fluctuating conditions (Kingsolver et al., 2009; Vishalakshi and Singh, 2007; Worner, 1992). Similarly, larval food sources may impact adult reproduction (Awmack and Leather, 2002), including male quality (Delisle and Bouchard, 1995; Delisle and Hardy, 1997). Consequently, I evaluated the variability in pheromone concentration and FA of field collected *P. unipuncta* males, as well as in individuals reared under control versus fluctuating conditions when provided with either corn or artificial diet as a larval food source, testing the hypothesis that pheromone content will be higher and FA lower in males reared on natural host plants under fluctuating conditions than those on artificial diet under controlled laboratory conditions.

In a separate series of experiments, I examined the importance of male pheromone concentration and FA on male mating success as a function of stress (no stress, high stress, and low stress), where two males were held with one receptive female. In this experiment, I tested the hypothesis that males with higher pheromone concentration and lower FA would be more successful than those with lower concentration and higher FA.

1.2 Materials and methods

Natural pheromone variability:

Males were collected daily from light traps on the University of Western Ontario experimental farm from July 12 to August 5 in 2008. The moths were inactivated by chilling at -10°C for 15 minutes (Fitzpatrick et al., 1985), decapitated and the left and right hairpencils removed using fine forceps. Each hairpencil was placed in individual 1-dram Teflon-capped amber vials containing 1 ml of dichloromethane with 50 μg of naphthalene as an internal standard, and stored at -20°C until the pheromone extracts were analyzed by gas chromatography using Varian CP8400. The column used was CPWax52 with helium as the carrier gas. Injections were done in split mode with a split ratio of 10:1 and temperature was increased from 100°C to 150°C at 10°C per minute after initial holding of 2 minutes. Under these conditions, acetic acid had a retention time of 5.2 minutes, benzaldehyde 6.2 minutes, and naphthalene 9 minutes (see Figure 1). To account for accuracy of the automatic injector system, a control sample solution containing 260 $\mu\text{g}/\text{ml}$ AA, 260 $\mu\text{g}/\text{ml}$ BA, and 50 $\mu\text{g}/\text{ml}$ naphthalene was tested every 25th injection. Control samples were analysed over the course of the experiment and the mean concentration for acetic acid and benzaldehyde are 266.76 ± 4.34 μg (minimum: 243.34, maximum: 295.58; one outlier: 325.18) and 252.39 ± 2.73 μg (minimum: 227.20, maximum: 272.31; one outlier: 317.62), respectively.

The data obtained allowed us to determine the concentrations of both the individual components and total pheromone, as well as the degree of symmetry between left and

right hairpencils, throughout the summer. To calculate pheromone FA, we used the formula $|(Pr-Pi)/(Pr+Pi)|$, where Pr and Pi were the concentrations of pheromones in right and left hairpencils.

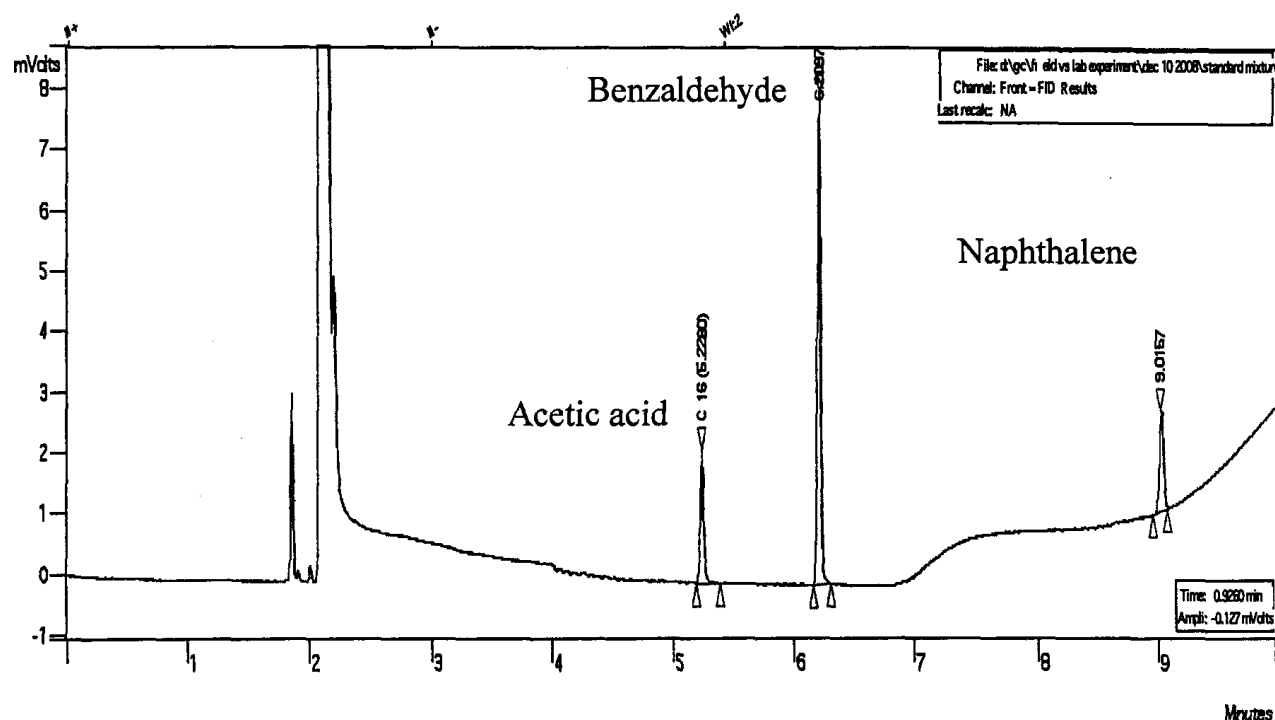


Figure 1. Chromatogram of a known concentration of acetic acid, benzaldehyde, the two pheromone components found in the hairpencils of *Pseudaletia unipuncta* males and internal standard (naphthalene) in split mode with split ratio of 10:1 with temperature 100°C to 150°C using Varian CP8400.

Effect of diet and abiotic conditions on male sex pheromone

Females collected in the light traps were placed in cages and allowed to oviposit on strips of wax paper. The newly-emerged larvae were reared either under constant laboratory conditions (25°C, 16L:8D, 70±5% RH) or under natural field conditions. Furthermore, under each rearing condition half of the larvae were reared on an artificial pinto bean diet (modified from Shorey and Hale, 1965), while the rest were fed fresh corn leaves. In all cases larvae were reared individually in solo cups, with a minimum of 100 larvae per diet per rearing condition. The resulting males in each combination were randomly assigned

to two groups and sacrificed at 0 or 6 days following emergence, as it has previously been shown that males contain little pheromone at emergence but reach a maximum about 5 days after emergence (Fitzpatrick et al., 1989) . The hairpencils were extracted and the content of the individual hairpencils were analysed using the techniques described above. Three-way ANOVA was used to compare mean pheromone content and FA with diet, age and temperature treated as categorical variables. Given that all male pheromones are produced prior to adult emergence, the possibility of correlations between pupal mass and total pheromone concentration and FA in six-day old virgin males was investigated. However, there were no significant correlations between pupal mass and pheromone concentration and FA in any rearing condition ($p > 0.08$, $R^2 < 0.3$; data not shown) so body mass was not considered in subsequent analyses.

Male reproductive success:

Three different experiments were carried out at 25°C, 16L:8D using the similar experimental design, in that, trials were set up with one calling female (the night after she first expressed the behavior associated with pheromone emission (Turgeon and McNeil, 1982)) and two randomly chosen 5-8 day old virgin males. As soon as mating was initiated the unsuccessful male was removed and held until the pair *in copula* had terminated. At this time the individual hairpencils of both successful and unsuccessful males were removed and prepared for analysis as outlined above.

In experiment 1, all individuals of both sexes were reared under standard conditions (25°C, 16L:8D) on excess artificial diet (no stress). In experiment 2, individuals came from a rearing where larvae developed on artificial diet that had inadvertently become infected with fungus, which represented a significant stress during larval development. In the third experiment adults were obtained from standard colony, as in experiment 1, but the virgin adult females were provided only water for the 48h after they initiated calling (low stress), before being placed in a cage with two, well fed, 5-8 day old virgin males that had been randomly chosen from the colony. We predicted that the nutrient-deprived females, be it during larval or adult development, would be more selective with respect to mate choice than their respective controls as males provide accessory gland secretions in their ejaculate which affect egg production (Marshall and McNeil, 1989; Svärd and McNeil, 1994).

Pheromone contents and FA were compared, using simple ANOVA, between successful and unsuccessful males as a function of rearing condition. Contingency table (χ^2) techniques were also used to test if pheromone concentration and FA influenced which male successfully mated. There was less than 15% variability in the repeated measures of the standard solution, however in order to be conservative in our analyses of both pheromone concentration and FA, we only considered the successful and unsuccessful males to be significantly different if the differences in pheromone concentration and/or FA was greater than 30%. This would even account for the one outlier seen for both acetic acid and benzaldehyde in the standard solution measurements.

1. 3 Results

Pheromone natural variability:

Sixty-two field-collected armyworm males had an average sex pheromone content (combined left and right hairpencils) of 31.19 ± 2.84 ug, with 17.62 ± 1.51 ug of acetic acid and 18.21 ± 1.53 ug benzaldehyde. There was considerable inter-individual variability in pheromone content of the field collected males, as previously reported for those from a laboratory colony (Fitzpatrick et al., 1985). Of the 62 males analyzed, 15 had no sex pheromones at all, while the others had at least one of the two pheromone components in the hairpencils (Table I). There were no significant differences in total pheromone content (Fig. 2), or the two components (acetic acid $R^2=0.018$, benzaldehyde $R^2=0.026$; data not shown), throughout the collecting period. The amount of acetic acid a male had was significantly correlated with benzaldehyde ($p=0.007$; Fig. 3) and total pheromone concentrations ($p<0.001$; Fig. 4).

Only males containing pheromone ($n=47$) were included in the analyses looking at the degree of male sex pheromone symmetry. There was a considerable inter-male variability in the degree of FA for total pheromone content but did not change significantly with time (Fig. 5). Similar trends were also observed for acetic acid ($R^2=0.014$) and benzaldehyde ($R^2=0.038$) (data not shown). The degree of symmetry of acetic acid was significantly correlated with that of benzaldehyde ($p=.007$, Fig. 6) and total pheromones ($p<.001$, Fig. 7).

Table I: The variability in the sex pheromone in the bilateral hairpencils of male true armyworm moths, *Pseudaletia unipuncta*, caught in light traps from July 12 to August 5 in 2008 at the University of Western Ontario experimental farm.

	Number of males with or without pheromones	
	With	Without
AA or BA	47	15
AA	44	18
BA	46	16
AA + BA	43	19

Note: Acetic acid (AA), Benzaldehyde (BA).

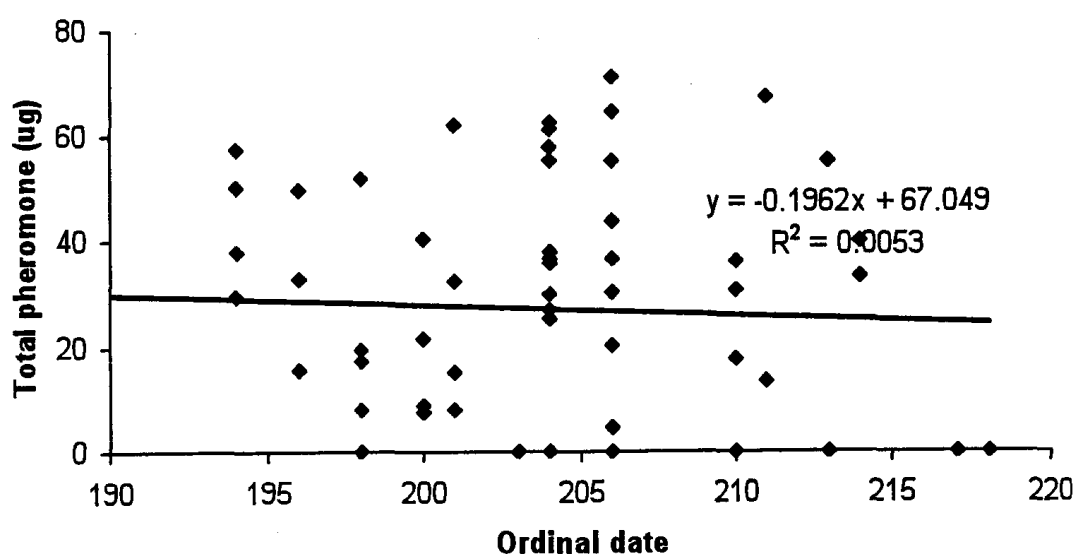


Figure 2: The total pheromone content of *Pseudaletia unipuncta* males caught in light traps from July 12 to August 5 in 2008 from University of Western Ontario experimental farm (n=62) as a function of ordinal date.

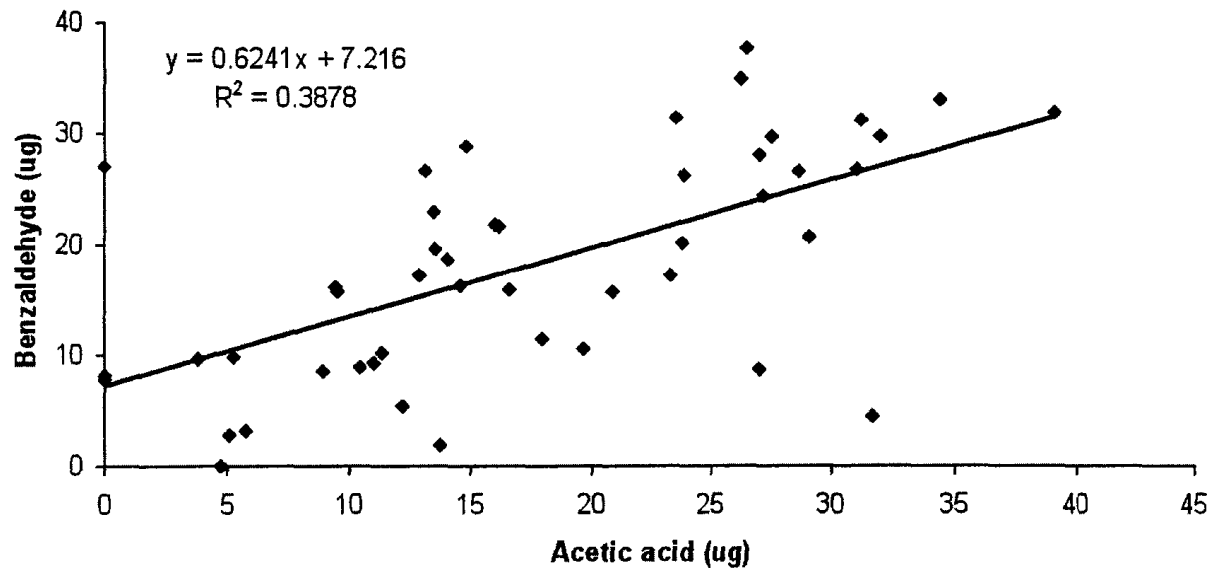


Figure 3: The correlation between total acetic acid and total benzaldehyde content in bilateral hairpencils of *Pseudaletia unipuncta* males (n=47; individuals with no pheromone were excluded) captured in light traps at the University of Western Ontario experimental farm from July 12 to August 5 in 2008.

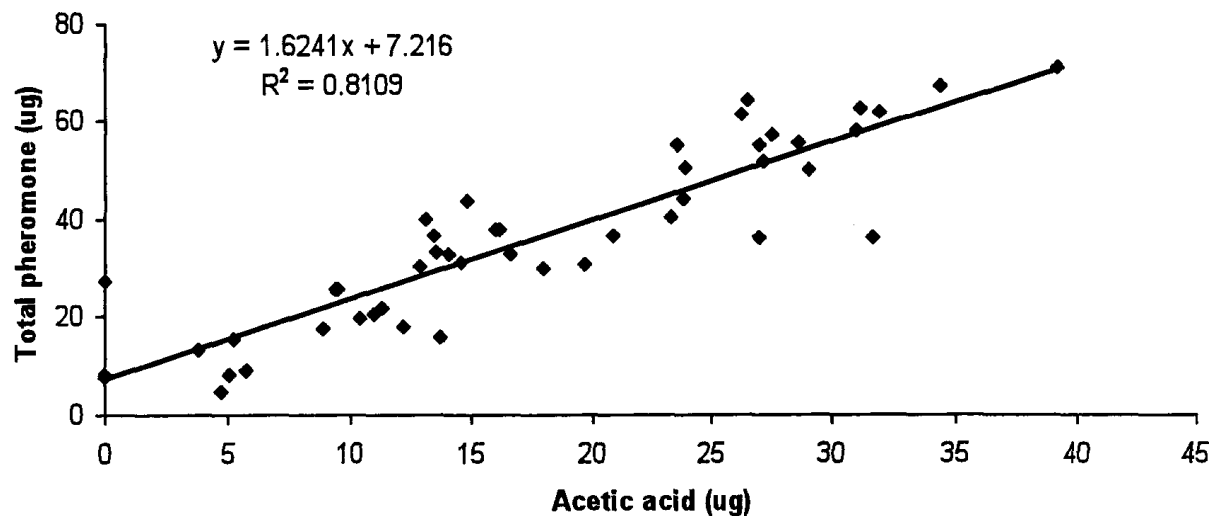


Figure 4: The correlation between total acetic acid and total pheromone content in bilateral hairpencils of male *Pseudaletia unipuncta* (n=47; individuals with no pheromone were excluded) captured in light traps at the University of Western Ontario experimental farm from July 12 to August 5 in 2008.

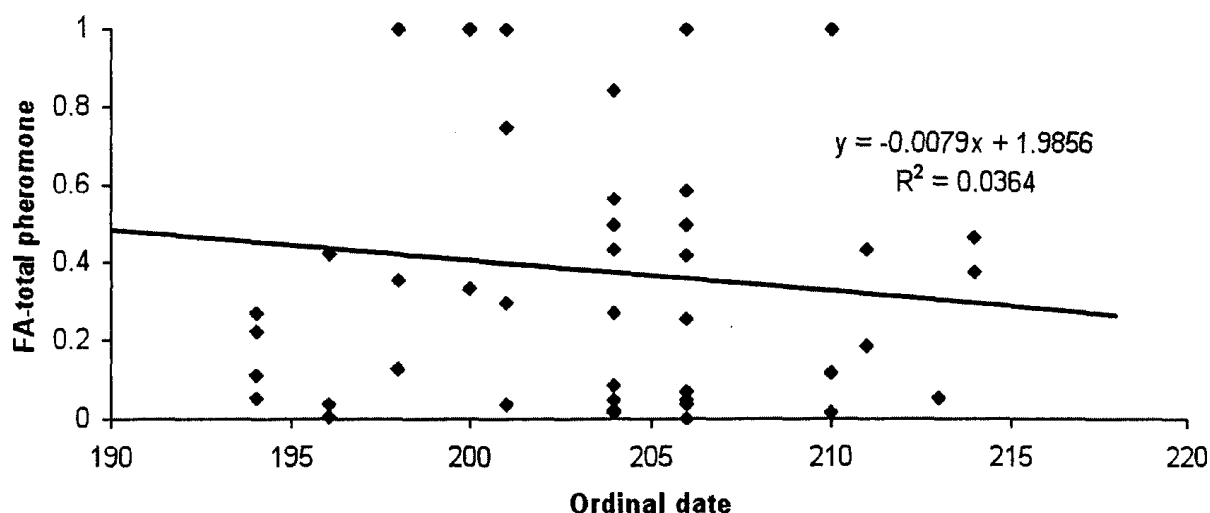


Figure 5: The degree of FA in total pheromone found in the bilateral hairpencils of male *Pseudaletia unipuncta* (n=47; individuals with no pheromone were excluded) captured in light traps at the University of Western Ontario experimental farm as a function of ordinal date in 2008. Value of 1 indicates perfect asymmetry, whereas 0 is perfect symmetry.

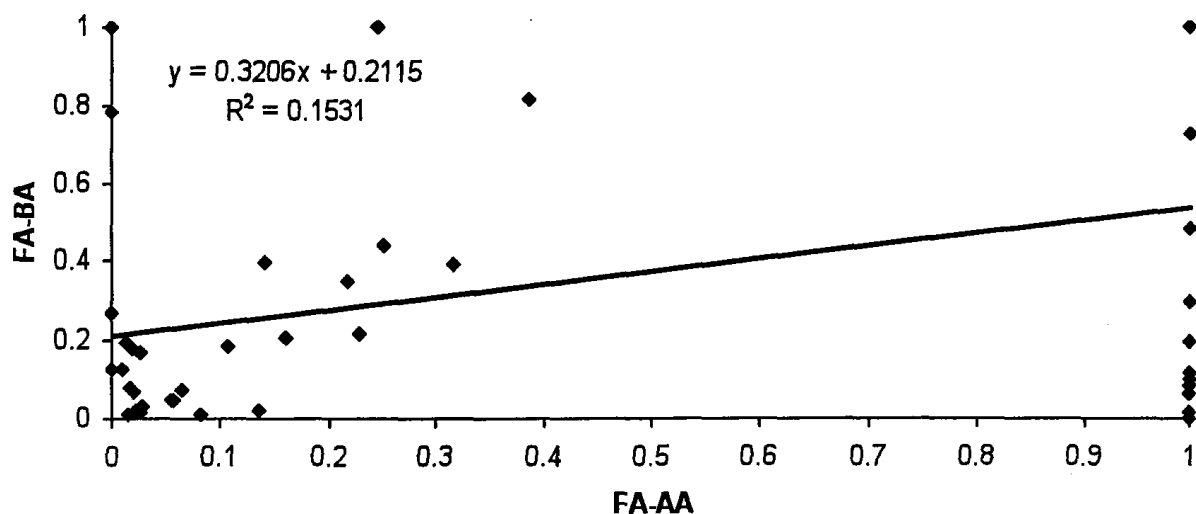


Figure 6: The correlation between the degree of acetic acid FA and benzaldehyde FA in bilateral hairpencils of male *Pseudaletia unipuncta* (n=47; individuals with no pheromone were excluded) captured in light traps at the University of Western Ontario experimental from July 12 to August 5 in 2008. Value of 1 indicates perfect asymmetry, whereas 0 is perfect symmetry.

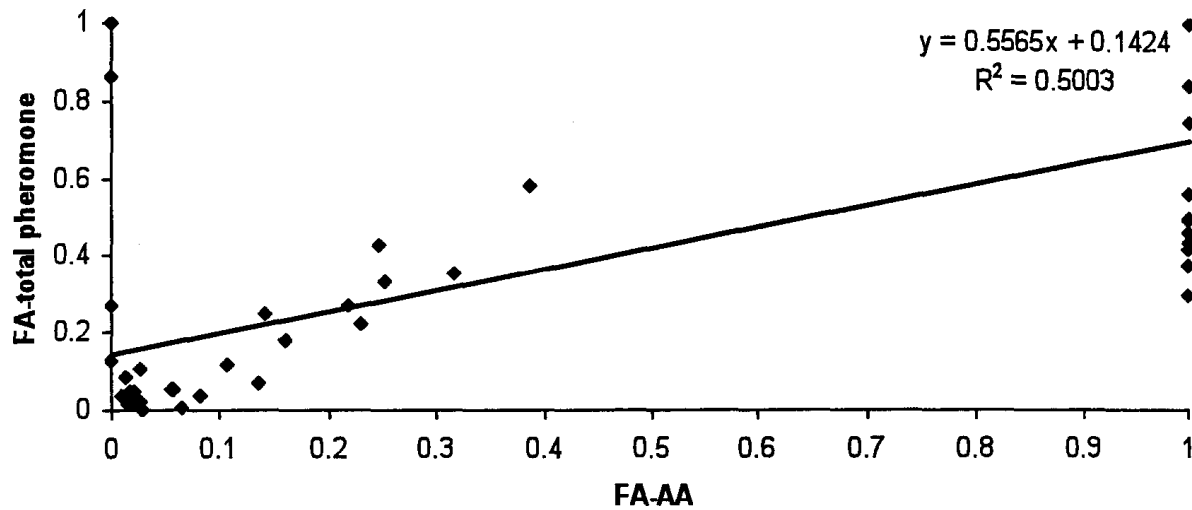


Figure 7: The correlation between the degree of acetic acid FA and total pheromone FA in bilateral hairpencils of male *Pseudaletia unipuncta* (n=47; individuals with no pheromone were excluded) captured in light traps at the University of Western Ontario experimental farm from July 12 to August 5 in 2008. Value of 1 indicates perfect asymmetry, whereas 0 is perfect symmetry.

Effects of diet and abiotic conditions on male pheromone:

A significantly higher proportion of field-reared males had pheromone in their hairpencils on the day of emergence than those reared at constant laboratory conditions, whether larvae were fed on artificial diet or corn (see Table II). However, for any given rearing condition there was no significant effect of diet. By day six, there were no significant differences in the proportion of males with pheromone as a function of either abiotic conditions or larval diet (see Table III).

Rearing condition (see Table IV) had an effect on pheromone content in day 0 males, generally being higher in individuals reared in the field, while differences due to diet were only seen for benzaldehyde in the field (Table V). In 6 day old males, rearing conditions affected male pheromones for individuals reared on corn but not artificial diet (see Table IV), while dietary affects were only observed under controlled laboratory conditions (see Table V). Thus, there were significant interactions between diet treatments and rearing conditions ($p < 0.001$; $F = 28.78$) on hairpencil pheromone content.

Analysis of pheromone FA was not carried out on day 0 as a large portion of males had no pheromones in their hairpencils. The degree of FA for individuals fed on corn was significantly lower when males were reared under fluctuating field than constant laboratory conditions, but not for those reared on artificial diet (Table VI). Larval diet affected the degree of FA in male pheromone under both rearing conditions; however,

higher FA occurred in artificial diet reared individuals in the field while the opposite was observed under controlled laboratory conditions (see Table VII).

Table II: The number of newly-emerged virgin *Pseudaletia unipuncta* males with pheromone in their hairpencils as a function of larval diet (artificial diet or corn leaves) and rearing conditions (25°C, 16L:8D or field conditions) in summer 2008.

	n	With pheromones	Without Pheromones	Diet treatments	P-value
Lab	19	4	15	Artificial diet	p=0.8
	17	2	15	Corn	
Field	10	6	4	Artificial diet	p=0.39
	10	8	2	Corn	
p=0.027 (Artificial diet); p<0.001 (Corn)					

Note: Acetic acid (AA), Benzaldehyde (BA). Degree of freedom is 1.

Table III: The number of six day old virgin *Pseudaletia unipuncta* males with pheromone in their hairpencils as a function of larval diet (artificial diet or corn leaves) and rearing conditions (25°C, 16L:8D or field conditions) in summer 2008.

	n	With pheromones	Without Pheromones	Diet treatments	P-value
Lab	12	10	2	Artificial diet	p=0.64
	11	8	3	Corn	
Field	10	8	2	Artificial diet	p=0.61
	17	15	2	Corn	
p=1 (artificial diet); p=0.35 (corn)					

Note: Acetic acid (AA), Benzaldehyde (BA). Degree of freedom is 1.

Table IV: The mean (\pm SEM) pheromone concentration in bilateral hairpencils of 0 and 6 day old virgin *Pseudaletia unipuncta* males as a function of rearing conditions (25°C, 16L:8D or fluctuating field conditions) when larvae were fed artificial diet or corn leaves in summer 2008.

	Day 0				Day 6			
	AA(ug)	BA(ug)	AA+BA(ug)	n	AA(ug)	BA(ug)	AA+BA(ug)	n
<u>Artificial diet</u>								
Field	32.16 \pm 5.62	2.03 \pm 0.62	34.19 \pm 5.68	10	51.96 \pm 7.75	28.80 \pm 6.37	80.76 \pm 11.93	10
16L8:25°C	5.63 \pm 2.77	2.34 \pm 1.26	7.98 \pm 4.00	19	42.78 \pm 7.75	42.48 \pm 7.61	85.37 \pm 15.21	12
P-value	p<0.001	p=0.86	p=0.001		p=0.43	p=0.21	p=0.83	
F-value	F=22.75	F=0.03	F=14.50		F=0.64	F=1.66	F=0.05	
<u>Corn diet</u>								
Field	37.50 \pm 4.00	7.16 \pm 1.76	44.65 \pm 5.29	10	52.55 \pm 5.82	44.77 \pm 5.65	97.32 \pm 10.78	17
16L8:25°C	5.12 \pm 2.03	1.11 \pm -.98	6.23 \pm 2.93	15	21.27 \pm 5.02	21.25 \pm 5.68	42.52 \pm 10.08	11
p-value	p<0.001	p=0.002	p<0.001		p=0.001	p=0.002	p=0.002	
F-value	F=62.86	F=10.51	F=47.13		F=14.17	F=7.86	F=12.18	

Note: Acetic acid (AA), Benzaldehyde (BA). Degree of freedom is 1. All individuals were used to calculate mean pheromone content.

Table V: The mean (\pm SEM) pheromone concentration in bilateral hairpencils of 0 and 6 day old virgin *Pseudaletia unipuncta* males as a function of larval diet (artificial diet or corn leaves) when reared at 25°C, 16L:8D or fluctuating field conditions in summer 2008.

Field condition	Day 0				Day 6			
	AA(ug)	BA(ug)	AA+BA(ug)	n	AA(ug)	BA(ug)	AA+BA(ug)	n
Artificial diet	32.16 \pm 5.62	2.03 \pm 0.62	34.19 \pm 5.68	10	51.96 \pm 7.75	27.80 \pm 6.37	80.76 \pm 11.93	10
Corn	37.49 \pm 4.00	7.16 \pm 1.76	44.65 \pm 5.29	10	52.55 \pm 5.82	44.77 \pm 5.65	97.32 \pm 10.78	17
P-value	p=0.45	p=0.013	p=0.194		p=0.95	p=0.10	p=0.362	
F-value	F=0.60	F=7.55	F=1.82		F=0.003	F=0.29	F=0.87	
<hr/>								
<u>16L8:25°C</u>								
Artificial diet	5.63 \pm 2.77	2.34 \pm 1.26	7.98 \pm 4.00	19	42.78 \pm 7.75	42.58 \pm 7.61	85.37 \pm 15.21	12
Corn	5.12 \pm 2.03	1.11 \pm 0.98	6.23 \pm 2.93	15	21.27 \pm 5.02	21.25 \pm 5.68	42.52 \pm 10.08	11
p-value	p=0.89	p=0.46	p=0.74		p=0.03	p=0.038	p=0.032	
F-value	F=0.02	F=0.55	F=0.74		F=5.22	F=4.90	F=5.30	

Note: Acetic acid (AA), Benzaldehyde (BA). Degree of freedom is 1. All individuals were used to calculate mean pheromone content.

Table VI: The mean (\pm SEM) pheromone FA in bilateral hairpencils of six day old virgin *Pseudaletia unipuncta* males as a function of rearing conditions (25°C, 16L:8D or fluctuating field conditions) when larvae were reared on artificial diet or corn leaves in summer 2008.

	Artificial diet				Corn			
	AA	BA	AA+BA	n	AA	BA	AA+BA	n
Field	0.33 \pm 0.15	0.23 \pm 0.13	0.26 \pm 0.12	10	0.15 \pm 0.06	0.05 \pm 0.01	0.09 \pm 0.03	17
16L8:25°C	0.15 \pm 0.10	0.08 \pm 0.04	0.11 \pm 0.06	12	0.58 \pm 0.12	0.48 \pm 0.13	0.50 \pm 0.12	10
p-value	p=0.295	p=0.238	p=0.224		p=0.002	p<0.001	p<0.001	
F-value	F=1.17	F=1.15	F=1.60		F=12.42	F=21.13	F=19.99	

Note: Acetic acid (AA), Benzaldehyde (BA), Fluctuating Asymmetry (FA). Degree of freedom is 1. Individuals with no pheromones were excluded.

Table VII: The mean (\pm SEM) pheromone FA in bilateral hairpencils of six day old virgin *Pseudaletia unipuncta* males as a function of diet (artificial diet or corn leaves) reared at 25°C, 16L:8D or fluctuating field conditions in summer 2008.

	Field conditions				16L8:25°C			
	AA	BA	AA+BA	n	AA	BA	AA+BA	n
Artificial diet	0.33 \pm 0.15	0.23 \pm 0.13	0.26 \pm 0.12	10	0.15 \pm 0.10	0.08 \pm 0.04	0.11 \pm 0.06	12
Corn	0.15 \pm 0.06	0.05 \pm 0.01	0.09 \pm 0.03	17	0.58 \pm 0.12	0.48 \pm 0.13	0.50 \pm 0.12	11
p-value	p=0.187	p=0.067	p=0.075		p=0.012	p=0.005	p=0.006	
F-value	F=1.86	F=3.75	F=3.51		F=7.96	F=10.89	F=10.23	

Note: Acetic acid (AA), Benzaldehyde (BA), Fluctuating Asymmetry (FA). Degree of freedom is 1. Individuals with no pheromones were excluded.

Mate reproductive success:

When the caterpillars were reared on standard artificial diet, the incidence of mortality was low, with >90% emerging as adults, and 33 of 35 trials had successful matings during last two hours of scotophase. There were no significant differences in mean concentrations or FA of major components or total pheromone between successful and unsuccessful males (see Table VIII), nor in the proportion of females that mated with more symmetrical individuals (see Table IX). The concentration of acetic acid was significantly correlated with concentration of both benzaldehyde and total pheromones in both successful and unsuccessful males, but not with respect to the levels of FA (see Table X).

When reared on a low quality larval diet, <50% of the individuals emerged as adults, and successful mating only occurred in 10 out of 50 trials. In this experiment successful males had significantly higher overall concentrations of acetic acid and marginally more total pheromone, but not benzaldehyde, than unsuccessful ones but there were no significant difference in FA values between two groups (Table VIII). However, females mated significantly more often with males having higher concentration and lower FA in both acetic acid and total pheromone in the choice assay (see Table IX). Again the concentration of acetic acid was significantly correlated with concentration of benzaldehyde and total pheromone for both successful and unsuccessful males, although acetic acid FA was only significantly correlated with the total pheromone FA in successful males (see Table X).

When reared on standard artificial diet during larval development and virgin adult females starved for 48h, 16 out of 17 resulted in successful matings. There were no differences in concentration for either the individual components or total pheromone or in FA, between successful and unsuccessful males (see Table VIII). However, when given a choice starved females preferred to mate with males of higher concentration and FA of acetic acid and (see Table IX). Again the concentration of acetic acid was significantly correlated with concentration of benzaldehyde and total pheromone for both successful and unsuccessful males but not with respect to levels of FA (see Table X).

Table VIII: The concentration and FA of total and individual components in the hairpencil of virgin *Pseudaletia unipuncta* males reared on artificial diet under controlled laboratory conditions (25°C, 16L:8D) at different levels and types of nutritional stress.

Exp. 1*	n	AA (ug)	BA (ug)	AA+BA (ug)	AA-FA	BA-FA	(AA+BA)-FA
Successful	33	24.41±4.43	31.59±4.18	56±8.08	0.45±0.11	0.35±0.08	0.40±0.07
Unsuccessful	33	25.11±4.50	37.43±4.18	62.54±7.98	0.45±0.10	0.19±0.05	0.23±0.05
p-value		p=0.912	p=0.327	p=0.566	p=0.97	p=0.10	p=0.07
F-value		F=0.12	F=0.98	F=0.33	F=0.08	F=2.78	F=3.42
Exp. 2**							
Successful	10	23.92±2.38	18.10±3.13	42.02±5.35	0.28±0.12	0.22±0.10	0.29±0.12
Unsuccessful	10	12.75±3.51	11.35±3.77	24.09±7.06	0.43±0.15	0.36±0.20	0.38±0.13
p-value		p=0.017	p=0.185	p=0.058	p=0.453	p=0.509	p=0.617
F-value		F=6.94	F=1.90	F=4.10	F=0.54	F=0.40	F=0.26
Exp. 3***							
Successful	16	42.92±5.56	35.54±4.08	78.46±8.15	0.32±0.11	0.24±0.08	0.17±0.04
Unsuccessful	16	29.52±5.88	28.17±4.32	57.69±9.62	0.49±0.13	0.32±0.10	0.34±0.09
p-value		p=0.108	p=0.225	p=0.110	p=0.33	p=0.59	p=0.11
F-value		F=2.74	F=1.54	F=2.71	F=0.99	F=0.30	F=2.81

Note: Acetic acid (AA), Benzaldehyde (BA), Fluctuating Asymmetry (FA). Degree of freedom is 1. Males with no pheromones were excluded in FA calculation.

* All individuals were reared on standard artificial diet during larval stage. All adults had unlimited supply of 8% sugar solution

** All individuals were reared on low quality artificial diet during larval stage. All adults had unlimited supply of 8% sugar solution

*** All individuals were reared on standard artificial diet during larval stage. Sexually mature female adults were starved for two days with water supplied.

Table IX: 2x2 contingency table was constructed to compare concentration and FA of total and individual components in the hairpencils of mated (successful) and no-mate (unsuccessful) *Pseudaletia unipuncta* males reared on artificial diet under controlled laboratory conditions (25°C, 16L:8D) at different levels and types of nutritional stress.

TABLE 2. Effect of AA and BA on the success of the treatment at different levels and types of nutritional stress.													
Exp. 1*		AA Conc. (ug)		BA Conc. (ug)		AA+BA Conc. (ug)		AA FA		BA FA		AA+BA FA	
	n	More	Less	More	Less	More	Less	Less	More	Less	More	Less	More
Successful	33	11	13	11	16	11	15	14	11	13	15	14	17
Unsuccessful	33	13	11	16	11	15	11	11	14	15	13	17	14
p-value		p=0.773		p=0.276		p=0.423		p=0.572		p=0.790		p=0.612	
Exp. 2**													
Successful	10	7	1	6	2	7	2	8	1	6	2	8	1
Unsuccessful	10	1	7	2	6	2	7	1	8	2	6	1	8
p-value		p=0.010		p=0.132		p=0.057		p=0.003		p=0.132		p=0.003	
Exp. 3***													
Successful	16	9	3	7	3	8	4	10	3	9	5	9	4
Unsuccessful	16	3	9	3	7	4	8	3	10	5	9	4	9
p-value		p=0.039		p=0.179		p=0.220		p=0.017		p=0.257		p=0.115	

Note: Acetic acid (AA), Benzaldehyde (BA), Fluctuating Asymmetry (FA). Degree of freedom is 1. Males with no pheromones were excluded in FA calculation. In cases where the difference in pheromone concentration and FA was less than 30% between successful and unsuccessful males, data were excluded.

* All individuals were reared on standard artificial diet during larval stage. All adults had unlimited supply of 8% sugar solution

** All individuals were reared on low quality artificial diet during larval stage. All adults had unlimited supply of 8% sugar solution

*** All individuals were reared on standard artificial diet during larval stage. Sexually mature female adults were starved for two days with water supplied.

Table X: Correlations between acetic acid and benzaldehyde or total pheromone, both concentration and FA, in the hairpencils of *Pseudaletia unipuncta* males reared on artificial diet under controlled laboratory conditions (25°C, 16L:8D) at different levels and types of nutritional stress.

Exp. 1 *	Successful males		Unsuccessful males			Successful males		Unsuccessful males	
AA (ug) as a predictor of BA (ug)	R ²	p-value	R ²	p-value	AA FA as a predictor of BA FA	R ²	p-value	R ²	p-value
AA+BA(ug)	0.579	<0.001	0.472	<0.001	AA+BA FA	0.022	0.524	0.035	0.427
	0.887	<0.001	0.855	<0.001		0.002	0.836	0.003	0.811
Exp. 2**									
BA (ug)	0.782	<0.001	0.778	<0.001	BA FA	0.022	0.702	0.081	0.586
AA+BA(ug)	0.926	<0.001	0.937	<0.001	AA+BA FA	0.694	<0.001	0.086	0.574
Exp. 3***									
BA (ug)	0.174	0.108	0.598	<0.001	BA FA	0.015	0.668	0.031	0.582
AA+BA(ug)	0.793	<0.001	0.919	<0.001	AA+BA FA	0.468	0.005	0.049	0.490

Note: Acetic acid (AA), Benzaldehyde (BA), Fluctuating Asymmetry (FA), mated (successful), no-mate (unsuccessful). Males with no pheromones were excluded in FA calculation.

* All individuals were reared on standard artificial diet during larval stage. All adults had unlimited supply of 8% sugar solution

** All individuals were reared on low quality artificial diet during larval stage. All adults had unlimited supply of 8% sugar solution

*** All individuals were reared on standard artificial diet during larval stage. Sexually mature female adults were starved for two days with water supplied.

1.4 Discussion

It is clear that considerable inter-male variability exists in the concentrations of total and individual pheromone components, as well as the degree of fluctuating asymmetry, in the hairpencils of field-collected *P. unipuncta*. These findings are similar to those previously reported from males reared under controlled laboratory conditions (Fitzpatrick et al., 1985; this study). As nothing is known about the history of males captured in light traps, there are a number of possible, non-exclusive, explanations for this variation. The pheromone content in male hairpencils may be determined genetically, as demonstrated in selection studies on female pheromones (Sheck et al., 2006, and references therein), and modified by a variety of biotic factors. For example, there are significant effects of age (our data; Fitzpatrick et al., 1989), diet (our data), and previous mating history, as pheromone is lost during courtship (Fitzpatrick and McNeil, 1988). The differences observed between our laboratory and field rearings suggest that climatic conditions during larval and pupal development may also modify the various pheromone parameters measured.

Twenty-five percent of field-collected males had no pheromone or were missing one component. Furthermore, even when both components were present, there was a high degree of FA. This pattern was constant during the second generation flight period, so if as proposed by Fitzpatrick and McNeil (1988) that hairpencil content is a reliable cue for female choice in this species, then there would be sufficient variability for females to evaluate male quality during courtship.

The hypothesis that females would select males with higher concentration and more symmetrical pheromones when in the presence of two potential mates, was clearly not supported when all larvae were reared on unlimited amount of high quality artificial diet and adults were provided an *ad lib.* sugar source. This suggests that under ideal conditions, neither the concentration nor the degree of FA is a major cue for mate choice. However, when individuals had been reared on poor larval diet, even though adults were provided with ample food source, males with higher concentration and lower FA had a higher mating success than their competitors. Similarly, when all individuals were provided high quality larval diet but females had limited adult food, a similar pattern was observed. Thus, it would appear that male pheromone concentration and/or FA only play a role in mate choice under stressful conditions, such as when larval and/or adult nutrition is suboptimal, even though there was no correlation between pupal mass and pheromone qualities under controlled laboratory conditions. I am confident that the results showing greater mating success of individuals with higher concentrations and lower FA in pheromone content is real and not an artifact of sampling. Firstly, I took a conservative approach by only considering there was a real difference in any of the pheromone parameters if there was > 30% difference between the two males. Furthermore, males lose about 9% to 13% during courtship (Fitzpatrick and McNeil, 1988), so the values observed for successful males would be an underestimate of their actual pheromone content at the start of the assay.

It has been well documented that males of many insect species provide nutrients in their ejaculates, and that these may be used in egg production and/or increased survivorship

(Bissoondath and Wiklund, 1996; Pivnick and McNeil, 1988; Svärd and McNeil, 1994).

Our results suggest that the relative importance of these male-derived nutrients in the true armyworm, and thus the degree of mate choice by females, will vary within a context of nutritional ecology. In most years, true armyworm larval densities are low and under these endemic conditions larval food sources would not be limiting, similar to individuals reared with excess, high quality artificial diet in the laboratory. Consequently, the absence of female choice based on male sex pheromones is not entirely surprising especially as this species is polyandrous in the field (Svärd and McNeil, 1994) and the donation of the first male may not be that critical for well fed females. However, in epidemic years, intraspecific larval competition for food is intense, a situation replicated in the experiment where both sexes were reared on poor quality larval diet. Under such conditions females would benefit from choosing males that provided the best nuptial gift (Marshall and McNeil, 1989; Pivnick and McNeil, 1988; Svärd and McNeil, 1994), and would explain why the successful males had higher pheromone titers and lower FA than unsuccessful ones. The same argument could be made in the experiment where adult females that had been starved for 48h. These individuals would have depleted reserves for body maintenance and under such conditions there would be a definite advantage for females to select mates with high pheromone content and lower FA, if these were reliable indicators of male quality and the nuptial gift.

Thus, based on our findings females could be utilizing pheromone concentrations and/or the level of FA as cues when choosing a mate, although the concentration and FA of acetic acid would appear to be more important than BA. In fact only acetic acid was

significantly different between successful and unsuccessful males in experiment 3, where adult females were subjected to a low level of stress. Future research should include experiments artificially manipulating the concentration of the different pheromone components and observe mating of males whose hairpencils had been excised at emergence. Lopez et al. (unpublished) working on the Azorian populations of the armyworm reported that there was a significant correlation between the levels of fluctuation in the FA of benzaldehyde and acetic acid, although I did not find such a consistent relationship in North American armyworms. However, in most cases where there was evidence of female choice in our experiments there were significant correlations between both the concentrations and FA of acetic acid and total pheromone. This could be due to differences in geographic races, as these are known to differ with respect to female pheromone emission (McNeil et al., 1996; McNeil et al., 2000) and future research should examine this possibility.

The findings reported in this study support the idea of context-dependent mate choice, where the relative importance of pheromone concentration and FA as a cue to assess male quality will vary, being low in an "ideal" world but of importance in a "less than ideal" one. Environmental conditions are almost never static in nature and recent work has suggested mate choice can be plastic with respect to population density (Welch, 2003), environmental factors such as predation risk (Johnson and Basolo, 2003; Reynolds et al., 1993), social composition (Royle et al., 2008), and communications that signal prospective parental care (Warner et al., 1995). A review of some entomological studies looking at the correlation between FA and reproductive success found five reported a

significant relationship between the two parameters (Allen and Simmons, 1996; Liggitt et al., 1993; Thornhill, 1992; Thornhill and Sauer, 1992; Tsubaki and Matsumoto, 1998) while three others did not (Bjorksten et al., 2000; Blanckenhorn et al., 1998; Eggertt and Sakuluk, 1994). Interestingly, only the studies conducted under field conditions reported significant correlations supporting the idea that any study on the role of FA in mate choice must be considered within a realistic ecological context and not only examine using insects that have been reared on high quality diets under favorable abiotic conditions at moderate densities.

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