ÂNGELA ALVES DE ALMEIDA

COMPORTAMENTO REPRODUTIVO E IDENTIFICAÇÃO DE FEROMÔNIO SEXUAL DE *Pseudaletia sequax* (LEPIDOPTERA: NOCTUIDAE)

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Doctor Scientiae.*

VIÇOSA
MINAS GERAIS – BRASIL
2007

ÂNGELA ALVES DE ALMEIDA

COMPORTAMENTO REPRODUTIVO E IDENTIFICAÇÃO DE FEROMÔNIO SEXUAL DE *Pseudaletia sequax* (LEPIDOPTERA: NOCTUIDAE)

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Doctor Scientiae*.

APROVADA: 31 de julho de 2007.

Prof. Evaldo Ferreira Vilela
(Co-orientador)

Prof. João Carlos Cardoso Galvão

Prof^{a}. Flávia Maria da Silva Carmo

Prof. Paulo Henrique Gorgatti Zarbin

Prof. Eraldo Rodrigues de Lima
(Orientador)

AGRADECIMENTOS

À natureza suprema, conhecida por muitos pelo nome de DEUS.

Ao Prof. Eraldo Rodrigues de Lima (DBA), por me orientar durante todos estes anos. Pelos conselhos, sugestões, e a grande amizade a mim dispensada. E ainda, pelos puxões de orelha que também me fizeram refletir, jamais o esquecerei.

Aos meus pais de coração, Salvador José Tenório e Maria da Penha, e aos meus irmãos que me ajudaram a continuar neste caminho.

Ao meu companheiro Cesar Nascimento Francischetti, por todo o seu amor, carinho e compreensão.

Ao querido amigo, Prof. João Carlos Cardoso Galvão (DFT), por me orientar durante o mestrado, pelos conselhos e incentivo que ajudaram a controlar os meus demônios pessoais que tanto me atormentaram durante o curso.

Ao Prof. Arne Janssen, pelos conselhos e amizade que tanto me ajudaram a evoluir.

Aos Co-Orientadores: Evaldo Ferreira Vilela e Ronaldo Reis Júnior, pela grande ajuda e aconselhamentos.

Aos Professores da Pós-Graduação em Entomologia, pela boa vontade com que sempre me atenderam.

Ao Prof. Paulo H. G. Zarbin pela grande ajuda intelectual e pelo empenho em identificar o feromônio sexual da *Pseudaletia sequax.* Sem a sua ajuda a maior parte deste trabalho não seria concretizada.

Ao Dr. José Roberto Salvadori pela ajuda.

À Universidade Federal de Viçosa por me aceitar e acolher desde a graduação, ao Programa de Pós-graduação em Entomologia pela oportunidade de realização deste trabalho, o meu maior orgulho é poder concorrer ao seu mais nobre Título, o de Doutorado.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pelo auxilio financeiro concedido em forma de bolsa.

Aos meus eternos amigos e companheiros de Jornada: Wanderlei, Dora, Ivaneide e Paulo César que tanto alegram a minha vida.

Ao meu gato, Alfredo César de Almeida Francischetti pelos momentos de carinho, amizade e descontração que sempre nos proporciona.

Aos amigos que fiz em Viçosa, Carina Mews, Carla Ribas, Clara Pimentel, Nina, Claudinei Lima, Eduarda Ribas, Marco Pimentel, Rogério Ribas, Teresa Telles, aos amigos que vieram do Rio de Janeiro estudar aqui na UFV, Simone Freitas (do Crato), Wagner e Silvia pelos ótimos momentos que passamos juntos.

A minha querida amiga e afilhada inesperada, Ana Raquel Souza, pelos incontáveis incentivos e elogios que alimentaram minha auto-estima.

Aos colegas e amigos de laboratório da velha guarda: Luciano Bellini, Tate Lana, Adriano Elias, Marcy Fonseca, Cristiane, Denise Euzébio, Ailton Lobo e Tito Bacca que sempre demonstraram grande amizade, companheirismo e consideração.

Aos amigos Bianca (Bibi) e Leandro (Lelê) pelo apoio, amizade e senso de humor que sempre me ajudaram durante todo este tempo.

Aos novos colegas e amigos de Laboratório: Wendel T. Pontes, Farah C. Gama, Dioenis Mattielo, Daniel Albeny, Silvana Souza, Luciane, Sandra e Aline, pela grande ajuda e amizade no final deste curso.

Aos meus inesquecíveis colaboradores e co-orientandos: Adolfo Reis e Fernanda Freitas (florzinha) pela ajuda, amizade e incentivo que possibilitaram a viabilidade deste trabalho.

Aos colegas da Pós-Graduação que sempre me incentivaram durante esses anos de convivência.

A Sra. Paula e Sra. Mirian, pela boa vontade com que sempre atenderam às minhas solicitações.

Ao Sr. Manuel, funcionário do insetário da UFV, pela boa vontade, amizade e grande ajuda durante o curso.

Por fim, aos colegas de curso que não conseguiram me compreender e optaram por me criticar o meu muito obrigada, suas críticas transformaram meu trabalho melhor, alegre, agradável e motivador. E ainda, que na maioria das vezes as críticas fossem destrutivas, eu consegui transformá-las em incentivos construtivos e dar continuidade ao meu trabalho.

SUMÁRIO

RESUMO

ALMEIDA, Ângela Alves de D.Sc., Universidade Federal de Viçosa, Julho de 2007. **Comportamento reprodutivo e identificação de feromônio sexual de *Pseudaletia sequax* (Lepidoptera: Noctuidae)**. Orientador: Eraldo Rodrigues de Lima. Co-Orientadores: Evaldo Ferreira Vilela e Ronaldo Reis Júnior.

Os efeitos da duração do período pupal e da idade de chamamento de 90 fêmeas virgens de *Pseudaletia sequax* foram determinados em laboratório durante seis dias consecutivos de chamamento. As lagartas foram criadas em dieta artificial até a pupação. O comportamento de chamamento foi avaliado de 10 em 10 minutos em sala de criação com fotoperíodo invertido. Fêmeas foram consideradas chamando quando assumiram a posição característica: asas e abdômen elevados com o ovipositor exposto, neste momento elas estavam emitindo feromônio sexual. Foi observado que o comportamento de chamamento é descontínuo. Fêmeas apresentaram grandes variações de idade de emergência, as emergiram com cinco dias de pupação levaram seis dias para iniciar o chamamento, e aquelas que emergiram com onze dias de pupação levaram apenas dois dias para iniciar o chamamento. O tempo para iniciar o chamamento em cada dia de observação diminuiu significativamente com a idade de chamamento. O chamamento foi influenciado positivamente pelo período pupal longo. Já o tempo total diário de chamamento diminuiu significativamente neste grupo de fêmeas. O número de bouts aumentou com a idade de chamamento e não foi influenciado pela duração do período pupal. Em seguida foram feitas análises dos extratos das glândulas de feromônio de *P. sequax* e a composição do feromônio sexual foi avaliada por cromatografia gasosa acoplada ao detector eletroantenográfico, a identidade dos compostos foi determinada por cromatografia gasosa e de espectro de massas. As posições das duplas ligações dos compostos foram determinadas por reações de DMDS. Estas análises identificaram três compostos ativos na mistura: (Z)-11-Hexadecenal (Z11-16:Al), (*Z*)-11-Hexadecenil acetato (Z11-16:OAc) e o (Z)-11-Hexadecen-1-ol (Z11-16:OH). A porcentagem de cada composto na mistura foi de 85.2% *Z*11-16:Al, 12.8% *Z*11-16:OAc e 2% *Z*11-16:OH. Estes compostos são candidatos a feromônio sexual de *P. sequax*. Em seguida foram realizados testes comportamentais em túnel de vento (3x1x1) de área útil. A velocidade do vento dentro do túnel foi calibrada para 0.40 m/s. As condições experimentais foram de 25±5°C e 70% de umidade relativa. Machos virgens foram testados diariamente durante a quinta e sexta hora de escuro o que corresponde ao período em que as fêmeas desta espécie estão liberando feromônio sexual. Os machos foram individualizados em tubos de vidro transparente e colocados para aclimatização nas condições experimentais. Após duas horas, os tubos de vidro com os machos foram levados para o interior do túnel de vento e colocados sobre uma plataforma em formato de V com 50 cm de altura durante dois minutos para a aclimatização. Os machos foram utilizados uma única vez e descartados em seguida. Os liberadores de borracha estavam a um 1.94m de distantes da plataforma e posicionados na mesma altura do tubo de vidro contendo o macho. Os tratamentos foram (blend) liberador de borracha com 100 µg/µl da mistura completa (85.2 % Z11-16:Al, 12. 8% *Z*11-16:OAc, 2% *Z*11-16:OH), álcool-free, liberador de borracha com 100 µg/µl (Z11-16:Al + *Z*11-16:OAc), acetato-free, liberador de borracha com 100 µg/µl (Z11-16:Al + *Z*11-16:OH) e aldeído: liberador de borracha com 100 µg/µl (Z11-16:Al). As análises do comportamento de vôo dos machos indicam que *Z*11-16:Al e Z11-16:OAc são essenciais para induzir vôos direcionados, pousos na fonte e tentativa de cópula e *Z*11-16:OH quando adicionado a mistura diminuiu significativamente o tempo levado para encontrar a fonte de feromônio. Entretanto na avaliação dos outros comportamentos o componente *Z*11-16: OH demonstrou ser desnecessário, pois não influenciou significativamente as respostas. Os resultados confirmaram que *Z*11-16: Al, *Z*11-16:OAc e *Z*11-16:OH são componentes ativos da mistura feromonal de *P. sequax,* pelo alto grau de atratividade que os machos tiveram pelos mesmos.

ABSTRACT

ALMEIDA, Ângela Alves de. D.Sc., Universidade Federal de Viçosa, July, 2007. **Reproductive behavior and sex pheromone identification of *Pseudaletia sequax* (Lepidoptera: Noctuidae)**. Adviser: Eraldo Rodrigues de Lima. Co-Advisers: Evaldo Ferreira Vilela and Ronaldo Reis Júnior.

The effects of pupal period and age on calling behavior of virgin females of *Pseudaletia sequax* were determined. Larvae were reared on artificial diet. Calling behavior of groups of females of similar age of pupation was observed every 10 minutes for six days at reversed photoperiod. Females were considered calling when they had assumed a characteristic position: wings and abdomen elevated, displaying the ovipositor, and presumably releasing pheromone. Calling behavior was discontinuous. Females that had emerged on the fifth day took more time to start calling those females with a longer pupal period. On the first day of calling, females took more time to initiate calling, on average during the seventh hour of the scotophase. On the second calling day, most females initiated calling during the fifth hour of the scotophase. On subsequent calling days, the average time to initiate calling changed to the fourth hour of the scotophase. Calling age had a significant effect on the onset of calling and mean time of calling per day. The first calling is influenced positively by pupal period, the calling length is influenced negatively mostly in groups of females the pupal period larger and mean number of calling bouts increased with calling age. Gas chromatographic and mass spectral analyses were conducted on pheromone gland extracts, volatiles collected from excised pheromone glands from females of *P. sequax* calling. Coupled Gas Chromatographic-electroantennographic Detection (CG-EAD) analysis of the female gland extract showed the presence of three EAD-active peaks, which were identified by CG-mass spectrometric (MS) analyses. The pheromone blend it is:(Z)-11-Hexadecenal (Z11-16: Al), (*Z*)-11-Hexadecenyl acetate (*Z*-11-16: OAc) and (*Z*)-11-Hexadecen-1-ol

(Z11-16: OH).The mean percentage of compounds identified from volatiles collected from calling females was 85.2% (*Z*)-11-Hexadecenal, 12.8% (*Z*)-11-Hexadecenyl acetate and 2% (*Z*)-11-Hexadecen-1-ol. The evaluation of antennal response of males to these pheromone components it was compared in laboratory by electroantennographic detection (CG-EAD) and the electroantennogram showed depolarization of antennae when in contact with these three compounds. Theses compounds is possible sex pheromone candidates of *P. sequax.* The behavioral response of *P. sequax* to synthetic sex pheromone was studied. Behavioral tests were carried out within a wind tunnel (3x1x1m) calibrated to 0.40m/s of wind speed. Virgin males were tested once during fifth and eighth hour of scotophase corresponding the female calling period. The males were placed at transparent glass cage and placed inside of tunnel on a wooden platform in v format (50 cm of height) during 2 minutes for acclimatization. A male was scored only once and then discarded. The rubber septa of pheromone it was located in (1,94m) the distance of platform of male. The treatments were: (blend) rubber septa of 100 μg/μl of the complete mixture (85.2 % Z11-16:Al, 12. 8% Z11-16:OAc, 2% *Z*11-16:OH), alcohol-free rubber septa 100 μg/μl (Z11-16:Al + *Z*11-16:OAc), acetate-free rubber septa 100 μg/μl (Z11-16Al + Z11-16:OH) and aldehyde alone rubber septa of 100 μg/μl (*Z*11-16:Al). Analyses of flight course indicted that *Z* 11-16:Al and *Z*11-16:OAc is an essential for inducing the upwind flight, landing and flight close and the minor component Z11-16:OH when added Z11-16:Al, Z11-16:OAc significantly increase flight close response, although in all others behavioral responses this compound revealed to be unnecessary. Ours results confirmed that *Z*11-16: Al, *Z*11-16:OAc and *Z*11-16:OH are sex pheromone component of the *P. sequax* because of the high male attractiveness.

INTRODUÇÃO GERAL

Pseudaletia sequax é uma importante praga de gramíneas no sul do Brasil e seus prejuízos mais evidentes são associados às culturas do trigo (*Triticum aestivum*) e do sorgo (*Sorghum bicolor*) (Gassen 2007). Em recente estudo Grego *et al.* (2006) reportaram a presença de danos provocados por *P. sequax* na cultura do triticale (*Triticosecale rimpaui* Wittm) sob plantio direto, na região de Campinas-SP, onde o nível de controle que melhor se ajustou foi o de 10 lagartas por metro quadrado. Os resultados também indicaram que as lagartas de *P. sequax* podem ocorrer de maneira agregada nesta cultura. Até o momento a única forma de controle disponível é o uso de inseticidas (Gallo *et al.* 2002). Não existem informações sobre o comportamento reprodutivo e nem sobre as substâncias químicas envolvidas na comunicação intra- específica deste inseto. Estas informações são essenciais para que estratégias de controle usando o comportamento sexual sejam desenvolvidas.

O estudo de substâncias químicas envolvidas na comunicação entre os insetos, tendo como escopo o desenvolvimento de técnicas viáveis para inclusão no manejo integrado de pragas, data de várias décadas (Cardé & Minks 1995). Tais substâncias são denominadas semioquímicos, terminologia proposta inicialmente por Nordlund & Lewis, (1976a). Dentro deste grupo, os feromônios sexuais são os mais estudados. São definidos como substâncias sintetizadas e secretadas por um indivíduo e quando percebidas por outro indivíduo do sexo oposto e da mesma espécie, provocam respostas comportamentais que podem levar a cópula (Karlson & Luscher, 1959).

Em Lepidoptera, geralmente os feromônios sexuais são produzidos e liberado pelas fêmeas via "comportamento de chamamento". Tal comportamento inclui eventos em série: exposição do ovipositor continuamente ou descontinuamente, vibração de asas, vôos à curta distancia ou simplesmente a adoção de uma posição estacionária. Estes comportamentos otimizam a dispersão do feromônio sexual no ambiente e aumentam a probabilidade de acasalamento (Cardé & Baker 1984).

A produção, emissão e percepção de feromônios sexuais em Lepidoptera são governadas por fatores extrínsecos e intrínsecos. Dentre estes fatores os mais estudados são: (I) Idade fisiológica, tanto das fêmeas quanto dos machos, pois normalmente fêmeas recém emergidas não estão fisiologicamente preparadas para produção ativa de feromônio sexual, em contrapartida, os machos também não estão preparados para percebê-los. (II) Presença de feromônio sexual de coespecíficos que no mesmo ambiente, pode determinar o adiantamento ou retardamento da produção e emissão de feromônio por fêmeas da mesma espécie (McNeil 1991). E ainda, machos de uma mesma espécie podem produzir substâncias inibidoras do comportamento de cópula em situações que haja alta competição por fêmeas (Hirai *et al.* 1978). (III) Presença das plantas hospedeiras que podem influenciar diminuindo ou aumentando o tempo necessário para o início da produção de feromônio. Por exemplo, em duas espécies de *Heliothis* as fêmeas não produzem feromônios na ausência dos voláteis da planta hospedeira (Raina 1988). (IV) Fatores ambientais, como temperatura, comprimento do dia, intensidade luminosa, que afetam o início da produção de feromônio por fêmeas (Nordlund & Brady 1974b, Turgeon & McNeil 1983, Howlader & Gerber 1986, Gerber & Howlader 1987, Han & Gatehouse 1991).

Até recentemente, estudos iniciais sobre a composição química dos feromônios sexuais eram relativamente simplificados, mostrando apenas uma molécula envolvida (Roelofs & Cardé 1977). Entretanto, a grande maioria dos lepidópteros possui em seus feromônios a característica multicomponente, com moléculas relativamente simples e com muitas similaridades estruturais (Inscoe 1982, Linn & Roelofs 1989). Muitos destes feromônios são misturas de acetatos, ésteres, álcoois e aldeídos que podem variar quanto ao comprimento da cadeia carbônica, posição da dupla ligação, configuração e grupo funcional. Estas variações conferem a especificidade da mistura e são responsáveis por desencadear a seqüência padrão de atração e comportamento pré-copulatório nos machos (Linn & Roelofs 1989), que inclui a movimentação das antenas, caminhamento, vibração de asas, levantar vôo, voar em zigue-zague, localizar a pluma de feromônio, voar contra o vento ("upwind flight") seguindo a pluma, localizar a fonte, pousar na fonte ("landing behavior") ou em áreas adjacentes, caminhar e bater asas até encontrar a fêmea.

Normalmente a cadeia de eventos acontece à longa distância. Já o encontro com a fêmea pode desencadear outra seqüência a curta distância e que eventualmente leva a cópula. Estes eventos são dependentes da composição química e estrutural do feromônio sexual (Mafra-Neto 1993, Mafra-Neto & Cardé 1996). As respostas mediante a presença de feromônios sexuais são moduladas pela qualidade e quantidade destes compostos que o macho percebe (Linn *et al*. 1987).

O comportamento dos machos perante o feromônio das fêmeas está atrelado ao conceito de especificidade do sinal químico ou canal químico de comunicação (Roelofs & Brown 1982). Em 1978, Roelofs propôs a hipótese do limiar de percepção dos componentes químicos. De acordo com esta hipótese, a especificidade de resposta do macho é controlada por dois efeitos principais que atuam no comportamento de vôo e que estão diretamente relacionados com a percepção da proporção dos componentes e da taxa de liberação do feromônio sexual. O papel dos componentes secundários na mistura feromonal em Lepidoptera levanta duas hipóteses: a dos componentes e a da mistura.

Linn *et al*. (1987), Linn & Roelofs (1989) demonstraram em várias espécies de insetos, que a composição química do feromônio sintético deve ser a mesma que a produzida pela fêmea, para que a seqüência de comportamentos seja produzida na sua plenitude. A maioria das espécies parece precisar da combinação completa de feromônio, que reproduza exatamente a composição natural emitida pelas fêmeas para que a resposta seja maximizada, ou seja, hipótese da mistura.

Posteriormente, Mafra-Neto (1993) demonstrou que em alguns sistemas o componente principal ou majoritário era o responsável pelo comportamento à longa distância e os componentes secundários responsáveis pelo comportamento à curta distância, ou seja, a hipótese dos componentes.

Estas hipóteses não são necessariamente excludentes. Entretanto, a existência de diferentes maneiras de percepção e resposta dos machos perante o feromônio sexual na natureza é um forte indício de que em Lepidoptera a pressão de seleção natural para percepção dos componentes da mistura levou o surgimento de mais de uma estratégia de comunicação química (Cardé, 1990).

Thornhill & Alcock (1983) propuseram que a seleção para percepção dos machos diante da mistura feromonal pode ocorrer em nível intraespecífico e interespecífico favorecendo o aumento da sensibilidade dos mesmos à mistura completa produzida pelas fêmeas. Esta seleção pode ser provocada pela competição entre os machos em busca de fêmeas viáveis para acasalamentos. Machos com alta sensibilidade à mistura feromonal podem ser favorecidos pela alta capacidade de encontrar fêmeas em um curto espaço de tempo. Esta alta especificidade pode prevenir acasalamentos entre espécies filogeneticamente próximas que sejam simpátricas e sincrônicas.

Um dos exemplos que corroboram com esta teoria é o estudo relacionando os noctuídeos *Trichoplusia ni* e *Pseudoplusia includens* (Walker), que são espécies simpátricas e sincrônicas e possuem em comum em suas misturas feromonais o Z7-12:OAc como componente principal e dois outros componentes minoritários Z7-12:OAc e Z11-12:OAc. *P. includens* produz adicionalmente os compostos Z7-12: propionato e Z7-12: butanoato. Estes dois compostos são responsáveis por isolar reprodutivamente estas duas espécies. Machos de *P. includens* na presença da mistura feromonal de *T. ni* respondem com alto nível de vôos a favor do vento. Este comportamento indica que *T. ni* pode ter em sua mistura algum composto com propriedade antagonista (Linn & Roelofs 1988). Grant *et al.* (1988), comprovaram que *P. includens* possui um grande número de receptores específicos para reconhecimento e resposta ao composto Z5-12: OAc, componente da mistura de *T. ni.* A adição deste composto em armadilhas para captura de *P. includens* diminui consideravelmente a captura de machos. Em outros estudos com as espécies simpátricas e sincrônicas *Helicoverpa zea* e *Heliothis virescens* componentes com propriedades antagonistas também foram identificados (Fadamiro & Baker 1997, Quero & Backer 1999).

Em contrapartida, fêmeas de alguns lepidópteros também desenvolveram ao longo do processo evolutivo a capacidade de perceber feromônios produzidos por coespecíficos. Este processo recentemente comprovado foi denominado auto-detecção. Este controverso fenômeno é observado em muitas famílias da ordem Lepidoptera e poucas são as explicações que justificam sua existência. Dentre elas, a mais aceita é que o aumento do número de fêmeas liberando feromônio sexual, em alta densidade populacional pode aumentar a probabilidade de atraírem machos ou induzir a dispersão das mesmas quando o nível populacional é alto o bastante para que haja competição por machos ou fontes de alimento.

Sendo assim, o reconhecimento do próprio feromônio é vantajoso na redução da competição por machos e por fontes de alimento ou aumentar a probabilidade de encontrar parceiros (Palanaswamy & Seabrook 1985). Exemplos de fêmeas que detectam o feromônio de coespecíficas estão documentados em muitos insetos da família Noctuidae e recentemente em Arctiidae (Palaniswamy & Seabrook 1978, Ochieng *et al*. 1995, Malo *et al*. 2004, Ansebo *et al*. 2005, Stelinski *et al*. 2006, Lim *et al*. 2007).

Diante do exposto, os feromônios sexuais são vitais na comunicação entre insetos da mesma espécie. Quando identificados, estudados e, sua função compreendida corretamente, permite sua manipulação de maneira eficaz transformando-os em potentes agentes reguladores de comportamento que podem ser disponibilizados e incluídos no manejo integrado de pragas.

O objetivo geral deste trabalho foi obter informações básicas sobre o comportamento reprodutivo de *P. sequax,* identificação da mistura feromonal e, posterior avaliação do comportamento dos insetos diante desta mistura em túnel de vento. Os objetivos específicos correspondem a cada capítulo da tese. (I) estudar o comportamento de chamamento de fêmeas de *P. sequax* e determinar a influência da duração do período pupal no início do período de chamamento, determinar em escotofase a melhor hora para extração de glândulas, ou seja, o pico máximo da produção e liberação de feromônio. (II) Identificar a mistura feromonal de *P. sequax* e testar a despolarização das antenas dos machos por meio de Cromatografia Gasosa acoplada ao Detector Eletroantenográfico (CG-EAG). (III) Estudar o comportamento de vôo dos machos em túnel de vento, testar diferentes combinações da mistura feromonal e determinar a importância de cada componente na atratividade dos machos.

As formatações dos capítulos estão segundo as normas das revistas as quais serão submetidos. O capítulo I foi aceito para publicação na revista Ethology, Capítulo II foi escrito seguindo as normas do Chemical Ecology Journal e o Capítulo III, seguindo as normas da revista Neotropical Entomology.

REFERÊNCIAS

ANSEBO, L.; IGNELL, R.; LÖFQVIST, J.; HANSON, B. S. Responses to sex pheromone and plant odours by olfactory receptor neurons housed in sensilla auricillica of the codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae). *Journal of Insect Physiology*, 51, 1066-1074, 2005

CARDÉ, R.T. Principles of mating disruption. In *Behavior-Modifying Chemicals for Insect Management* (eds). R.L. Ridgway, R.M. Silverstein and M. Inscoe, pp. 47-71. Marcel Dekker, New York, 1990.

CARDÉ, R.T.; MINKS, K.A. Control of moths pests by matting disruption: successes and Contraints. *Annual Review Entomology*, 40:559-585,1995.

CARDÉ, R.T.; BAKER, T.C. Sexual communication with pheromones, p.356-383.In Bell, W.J. & R.T. Cardé (Eds.), *Chemical ecology of insects*. London, Chapman and Hall, 524p, 1984.

FADAMIRO, Y.H.; BAKER, T.C. *Helicoverpa zea* males (Lepidoptera: Noctuidae) respond to intermittent fine structure of their sex pheromone plume and antagonist in a flight tunnel. *Physiological Entomology*. 22:316-324, 1997.

GALLO, D.; NAKANO, O.; SILVEIRANETO,S.; CARVALHO,R.P.L.; BATISTA, G.C.; BERTIFILHO, E.; PARRA, J.R.P.; ZUCHI, R.A.; ALVES, S.B.; VENDRAMIN, J.D.; MARCHINI, L.C.; LOPES, J.R.S; OMOTO, C. Entomologia agrícola. Piracicaba: FEALQ, 2002. 920p.

GASSEN, D.N. Principais pragas da cultura de trigo, cevada e aveia. Available in: WWW.cotrisoja.com..br/artigos/art-2005-0505.html.Acessed at 2007.

GREGO, R.L.; VIEIRA, S.R.; LOURENÇÃO, L.A. Spatial distribution of Pseudaletia sequax Franclemont in triticale under no-till management. *Scientia Agricola,* 63:321-327, 2006.

GERBER, H.G.; HOWLADER, A.M. The effects of photoperiod and temperature on calling behaviour and egg development of the bertha armyworm, *Mamestra configurata* (Lepidoptera: Noctuidae). *Journal of Insect Physiology*, 33: 429-436, 1987.

GRANT, A.J.; O'CONNELL, R.J.; HAMMOND, M.A comparative study of pheromone perception in two species of noctuid moths. *Journal of Insect Behavior*, 1: 75-96, 1988.

HAN, E.N.; GATEHOUSE, A.G. Effect of temperature and photoperiod on the calling behavior of a migratory insect, the oriental Armyworm, *Mythimna separata. Physiological Entomology*, 16: 419-427, 1991.

HIRAI, K.; SHOREY, H.H.; GASTON, L.K. Competition among courting male moths: male-to-male inhibitory pheromone. *Science,* 202: 644-645, 1978.

HOWLADER, A.M.; GERBER, H.G. Calling behavior of bertha armyworm, *Mamestra configurata* (Lepidoptera: Noctuidae). *The Canadian Entomologist,* 118: 735-743, 1986.

INSCOE, M.N. Insect attractants, attractant pheromones and related compounds, In: A.F. Kydoneus and M. Beroza (eds). *Insect suppression with controlled release pheromone systems*. CRC Press, Boca Raton, Florida, pp.201-295, 1982.

KARLSON, P.; LUSCHER, M. Pheromones, a new term for class of biologically active substances. *Nature,* 183: 55-56, 1959.

LINN, C.E. JR.; CAMPBELL, M.G.; ROELOFS, W.L. Pheromone components and active spaces: what do male moths smell and where do they smell it? *Science*, 237:650-652, 1987.

LINN, C.E.JR.; HAMMOND, A.; DU, J.W.; ROELOFS, W.L. Specificity of male response to multicomponent pheromones in noctuid moths *Trichoplusia ni* and *Pseudoplusia includens*. *Journal of Chemical Ecology*, 14: 47-57, 1988.

LINN, C.E.JR.; ROELOFS, W.L. Response specificity of male moths to multicomponent pheromones. *Chemical Senses*, 14: 421-437, 1989.

LIM, H.; PARK, K.C.; BAKER, T.C, Greenfield, M.D. Perception of conspecific female pheromone stimulates female calling in an arctiid moth *Utetheisa ornatrix. Journal of Chemical Ecology*, 33: 1257-1271, . 2007.

MAFRA-NETO, A.; Effects of the structure and composition of pheromone plumes on the response of the male almond moth Cadra cautella. Ph.D thesis, University of Massachusetts, Amherst. 1993.

MAFRA-NETO, A.; CARDÉ, T. R. Dissection of the pheromone-modulated fligth of moths using single -pulse response as a template. *Experientia,* 52: 373-379, 1996.

MALO, A. E.; CASTREJÓN-GÓMEZ, R. V.; CRUZ-LÓPEZ, L.; ROJAS, C.J. Antennal sensilla and electrophysiological response of male and female *Spodoptera frugiperda* (Lepidoptera: Noctuidae) to conspecific sex pheromone and plant odors. *Annals of the Entomolological Society of America,* 97: 1273–1284, 2004.

MCNEIL, N.J. Behavioral ecology of pheromone-mediated communication in moths and its importance in the use of pheromone traps. *Annual Review Entomology*, 36: 407-430, 1991.

NORDLUND, D.A.; LEWIS, J.W. Terminology of chemical-releasing stimuli in intraspecific interactions. *Journal of Chemical Ecology*, 2: 211-220. 1976 a.

NORDLUND, D.A.; BRADY, E.U. Calling behavior of female *Plodia interpunctella* (Hubner) (Lepidoptera: Pyralidae) under 2 light regimes. *Environmental Entomology,* 3: 793-796, 1974.

OCHIENG, A.S., ANDERSON, P.; HANSSON, B.S. Antennal lobe projection patterns of olfactory receptor neurons involved in sex pheromone detection in *Spodoptera littoralis* (Lepidoptera: Noctuidae). *Tissue & Cell,* 27: 221-232, 1995.

PALANISWAMY, P.; SEABROOK, D.W. Behavioral responses of female eastern spruce budworm *Choristoneura fumiferana* (Lepidoptera: Tortricidae) to sex pheromone of her own species. *Journal of Chemical Ecology*, 4: 649-655, 1978.

PALANISWAMY, P.; SEABROOK, D.W. The alteration of calling behaviour by female *Choristoneura fumiferana* when exposed to synthetic sex pheromone. *Entomologia Experimentalis et Applicata,* 37: 13-16, 1985.

QUERO, C.; BACKER, C.T. Antagonistic effect of (*Z*)-11-hexadecen-1-ol on the pheromone-mediated flight of *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae). *Journal of Insect Behavior,* 12: 701-710, 1999.

RAINA, A.K. Host plant, hormone interaction and sex pheromone production and release in *Heliothis* species. In Endocrinological Frontiers in Physiological Insect Ecology, ed. F. Sehnal, A. Zabza. D.L. Denlinger, pp. 33-36. Wroclaw: Wroclaw Tech. Univ. Press. 1988.

ROELOFS, W.L.; CARDÉ, R.T. Responses of Lepidoptera to synthetic sex pheromone chemicals and their analogues. Annual Review Entomology, 22: 377-405, 1977.

ROELOFS, W.L. Threshold hypothesis for pheromone perception. *Journal of Chemical Ecology,* 4: 685-699, 1978.

ROELOFS, W.L.; BROWN, R.L. Pheromones and evolutionary relationships of Tortricidae. *Annual. Review Ecologic Systems,* 13: 395-422, 1982.

STELINSKI, L.L.; IL'ICHEV, L.A.; GUT, J.L. Antennal and behavioral responses of virgin and mated oriental fruit moth (Lepidoptera: Tortricidae) females to their sex pheromone. *Annals of the Entomological Society of America*, 99: 898–904, 2006.

THORNHILL, R.; ALCOCK, J. The Evolution of Insect Mating Systems. Harvard University Press, Cambridge, MA, p. 53, 1983.

TURGEON, J.J.; MCNEIL, J.N. Modifications in the calling behavior of *Pseudaletia unipuncta* (Lepidoptera: Noctuidae) induced by temperature conditions during pupal and adult development. *The Canadian Entomology,* 115: 1015-1022, 1983.

CHAPTER I

Pupal Period Affects Calling Behavior of the Wheat Moth, *Pseudaletia sequax* (Lepidoptera: Noctuidae)

Ângela A. de Almeida, Eraldo R. Lima and Ronaldo Reis-Júnior.

Departamento de Biologia Animal, Setor Entomologia, Laboratório de Semioquímicos, Universidade Federal de Viçosa, Minas Gerais, Brazil.

Correspondence Author:

Ângela Alves de Almeida, Laboratório de Semioquímicos, Departamento de Biologia Animal, Universidade Federal de Viçosa, Avenida Ph. Rolfs s/n, 36570-000, Viçosa, Minas Gerais, Brazil.
E-mail: aaamoth@gmail.com

Abstract
The effects of pupal period and age on calling behavior of virgin females of *Pseudaletia sequax* were determined. Calling behavior of groups of females of similar age of pupation was observed every 10 minutes for six calling days. Females were considered calling when they assumed a characteristic position: wings and abdomen elevated, displaying the ovipositor, and presumably releasing pheromone. Calling behavior was discontinuous. Females with a shorter pupal period took more time to start calling than females with a longer pupal period and called for a longer period. On the first day of calling, females took more time to initiate calling, on average during the seventh hour of the scotophase, whereas on the second day of calling, most females initiated calling during the fifth hour of the scotophase. On subsequent days of calling, the average time to initiate calling changed to the fourth hour of the scotophase. The mean time of calling per day decreased significantly with the number of days since first calling, and the mean number of calling bouts increased with days since first calling.

Key Words: Sex pheromone, Wheat moth, Lepidoptera.

Introduction

Mating in moths generally depends on the expression of a series of behavioral patterns. In females, these behaviors include the emission of volatile sex pheromones (calling behavior), that leads to attraction of potential mates, and receptivity to (acceptance of) males that attempt mating. Pheromones are chemicals produced in specialized glands or specialized cells, they are released by individuals and induce responses such as orientation, pre-copulatory behavior and mating in the other gender of the same species (Cardé & Baker 1984; Kingan et al. 1993).

Calling is the release of the sex pheromone by female moths, which begins at the attainment of sexual maturity. In Noctuidae calling is conspicuous: females extrude their ovipositors intermittently or continuously, and fan their wings. Females assume a characteristic position optimizing the dispersion of volatile sex pheromones in to the environment, which leads to the attraction of males and enhances the probability of mating (Han & Gatehouse 1991a).

Pseudaletia sequax is an important pest, attacking wheat and forage crops in South America and in southern Brazil. Until now, there have been no reports on the reproductive biology of this moth. We investigated the calling behavior of *P. sequax* to determine the age at which females start calling following emergence, the average time that females remain calling, the influence on the duration of the pupal period on the calling behavior and average number of calling bouts.

Methods

The study was carried out between February and March, 2006. Larvae of *P. sequax* were collected from wheat crops in southern Brazil and reared on an artificial diet

(Salvadori & Parra 1990) with 10-ml of soy oil added as a complement of protein, fat acid and sterols. The first generation emerging in the laboratory was used for the experiments described here. After pupation, 90 female pupae of the same age and size were separated and isolated. After emergence, newly emerged virgin females were placed in individual plastic numbered cages. These cages were covered with cloth to permit circulation of air. The moths were supplied with a sugar-water solution of 10%.

Bioassays

The experiment was done in a dark room at temperature of 25 ± 5° C under reversed photoperiod (12D:12L) and a relative humidity of 70±5 %. Ninety pupae were observed daily to verify adult emergence and calculation of the duration of pupal period. Subsequently, ninety adult females were observed every 10 min throughout the scotophase to determine at what age calling was initiated. Moths were considered calling if the ovipositor was at least partially extended, because the sex pheromone in *P. sequax* is produced in specialized glands or specialized cells, located in ovipositor region.

All emerged females were observed during the scotophase following eclosion, the day on which a female first displayed calling behavior was designated as calling day 1, and subsequent days were designated as calling day 2, 3,… 6 respectively. Ninety females were observed for six calling days or until female death. Thus the calling behavior of females calling for the first time could be compared, regardless of differences in age since emergence and similar comparisons were made for subsequent calling days. If the females were calling during two consecutive observations, they were considered to have been calling for 20 min, if females called only one of two consecutive observations the calling period was considered 10 min. We used this information to obtain the mean onset of calling, the total time spent calling and the number of calling bouts.

Statistical analyses

All statistical analyses was done in R statistical system (R Development Core Team, 2006), using generalized linear modeling mixed effects and Poisson errors with log link (Crawley 2002), to obtain: mean time for first calling, the amount of time the females called and calling bouts. We use mixed models to remove the effect of temporal pseudo replication. The pupal period was calculated using linear modeling, followed by residual analyses to verify error distribution and suitability of the models employed, including checks for over-dispersion. Full models were built by including all variables and their interactions according to term complexity, starting from simplest one. All model simplification was achieved by extracting non-significant terms ($p>0.05$) from model according to their respective complexity, starting from the most complex one. When two non-significant terms presented the same complexity, the one explaining less deviance was extracted first. Each term deletion was followed by an ANOVA with F test, in order to recalculate the deviance explained by remaining terms.

Results

Calling females were easily recognized, as they raised their wings slightly above the abdomen, fully extruded their ovipositor and fluttered their wings. In general, they oriented vertically (head highest) during calling. When calling, females constantly vibrated their wings, and extruded their ovipositors intermittently and in a discontinuous pattern.

There was considerable variation in emergence of pupae of the same age emerged on different days; females emerging on the same day were grouped. The number of insects that emerged each day after pupation was: 9, 9, 14, 24, 15, 10 and 9 on the fifth to the eleventh day, respectively.

Since not all females initiated calling at the same chronological age we propose individual comparisons by "days since first calling". Thus the calling behavior of females calling for first time was compared, regardless of differences in age since emergence and similar comparisons were made for subsequent calling days.

Females that had emerged on the fifth day after pupation took more time to initiate calling than females that spent more time in the pupal stage. Hence, the day of first calling was influenced by the pupal period (Fig. 1).

We observed that the time until the start of calling was longer on the first calling day, whereas it decreased on subsequent calling days (Fig. 2). On the first calling day, females on average initiated calling during the seventh hour of the scotophase, whereas they started calling during the fifth hour of the scotophase on the second calling day (2). On subsequent calling days they initiated calling on average during the fourth hour of the scotophase. The average time that the females remained calling increased with calling days, hence, with physiological age. The daily calling period differed significantly in subsequent calling days. The total calling time was negatively affected by pupal period (Fig.3). The mean number of bouts increased with days since first calling (Fig. 4).

Discussion

Although all females pupated at the same time, females of *P. sequax* that had emerged earlier took more time to initiate calling compared with females that emerged later (Fig. 1). We also observed that the calling pattern was discontinuous, intermittent and females did not adopt a stationary posture. Intermittent calling has been associated with higher concentrations of pheromone produced (Turgeon & McNeil 1983). We also found that the time spent calling was shorter with increasing age since first calling (Fig. 2) and that the number of calling bouts increased with days since first calling (Fig. 4).

One explanation for the precocious calling by females of *P. sequax* that emerged later after pupation is that pheromone biosynthesis and chemical communication is under endocrine control and dependent of time since pupation. Possibly, females emerge physiologically more mature after a longer pupal period than after a shorter period and therefore start calling earlier (Fig. 1). Several studies showed that the regulation and activation of the biosynthesis of sex pheromones in moths are under control of hormonal systems (Raina & Klun 1984; Cusson & McNeil 1989; Han & Gatehouse 1991b Cusson et al. 1993; Picimbon et al. 1995; Rafaeli & Bober 2005).

We observed that, as moths aged, the calling length and the mean number of calling bouts was higher (Fig. 3, Fig. 4). Similar results were obtained with *Pseudaletia unipucta* (Turgeon & McNeil 1982; Turgeon & McNeil 1983; Hou & Sheng 2000). Generally, calling and courtship behaviors depend on ovarian development and age of the female, and are usually regulated by circadian rhythms under endocrine control, which are generally influenced by exogenous factors such as photoperiod and temperature (Turgeon & McNeil 1983). Many studies have shown an effect of photoperiod, ovarian development, age and temperature on calling behavior in insects (Nordlund & Brady 1974; Swier et al. 1977; Howlader & Gerber 1986; Gerber & Howlader 1987; Cusson et al. 1994a; Cusson et al. 1994a; Delisle & Simard 2003; Da Silva et al. 2006).

Our results demonstrate that *P. sequax* spends about 11 days as pupae, and females might be capable of compensating a longer pupal period by calling earlier than females with a shorted pupal period. Similar studies with *P. unipuncta*, a species in North America and Azores which consists of migratory and non-migratory populations, showed that migratory and non-migratory individuals have adopted different behavioral and physiological responses to temperature and photoperiod (McNeil & Tobe 2001). Calling behavior and rates of biosynthesis of juvenile hormone were initiated significantly earlier in adults of the non-migratory Azorean population (McNeil et al. 2000). Recent studies report the presence of populations of *P. sequax* in other Brazilian regions, indicating the migratory behavior of this insect (Reis et al. unpublished). Possibly, females of *P. sequax* that emerge earlier and call later may show a larger propensity to migrate to other regions in search of favorable conditions. Females that emerge later and call earlier may show propensity to non-migrant behavior. To this end, the calling behaviour and pupal period of resident and migratory populations of *P. sequax* should be compared. From an ecological perspective, it seems unlikely that the physiological processes involved in pheromone production will be identical in migratory and resident populations of *P. sequax*. In conclusion, this study shows that the calling behavior and pheromone release of *P. sequax* is affected by length of pupal period and days since first calling.

Acknowledgments

The authors thank Dr. Arne Janssen and Prof^{a} Flávia M.S.Carmo for valuable comments and corrections of the manuscript. Helpful comments on an earlier draft of this manuscript were made by two anonymous reviewers. Funding was provided by CAPES Foundation scholarship to A.A.Almeida, and CNPQ for the scholarship to E.R.Lima.

References

Cardé, R. T., Baker, T. C. 1984: Sexual communication with pheromones. In Chemical ecology of insects. (Bell, W.J. & Cardé, R.T., eds.). Chapman and Hall, New York, USA, 356-383.

Crawley, M. J. 2002: Statistical Computing: An introduction to data analysis using S-plus. Wiley, Oxford. 761pp.

Cusson, M. & McNeil, J. N. 1989: Involvement of juvenile hormone in the regulation of pheromone release activities in a moth. Science 243, 210-212.

Cusson, M., Yagi, K. J., Tobe, S. S. & McNeil, J. N. 1993: Identification of release products of corpora allata of male and female armyworm moths, *Pseudaletia unipuncta*. Journal of Insect Physiology 39, 775–783.

Cusson, M., Tobe, S. S. & McNeil, J. N. 1994: Juvenile hormones: their role in the regulation of the pheromone communication system of the armyworm moth, *Pseudaletia unipuncta*. Archives of Insect Biochemistry and Physiology 25, 329-345.

Cusson, M., Yu, G. C., Carruthers, K., Wyatt, R. G., Tobe, S. S. & McNeil. J. N. 1994: Regulation of vitellogenin production in armyworm moths, *Pseudaletia unipuncta.* Journal of Insect Physiology 40, 129–136.

Da Silva, E. L., de Carvalho, C. M., do Nascimento, R. R., Mendonça, A. L., da Silva, C. E., Gonçalves, G. B., de Freitas, M. D. T. & Sant'Ana, A. E. G. 2006: Reproductive behaviour of the Annona fruit borer, *Cerconota anonella.* Ethology 112, 971–976.

Delisle, J. & Simard, J. 2003: Age-related changes in the competency of the pheromone gland and the pheromonotropic activity of the brain of both virgin and mated females of two *Choristoneura* species. Journal of Insect Physiology 49, 91–97.

Gerber, H. G. & Howlader, A. M. 1987: The effects of photoperiod and temperature on calling behaviour and egg development of the bertha armyworm, *Mamestra configurata* (Lepidoptera: Noctuidae). Journal of Insect Physiology 33, 429-436.

Han, E. N. & Gatehouse, A. G. 1991a: Genetics of precalling period in the oriental armyworm, *Mythimna separata* (Walker) (Lepidoptera: Noctuidae), and implications for migration. Evolution 45, 1502-1510a.

Han, E. N. & Gatehouse, A. G. 1991b: Effect of temperature and photoperiod on the calling behavior of a migratory insect, the oriental Armyworm *Mythimna separata*. Physiological Entomology 16, 419-427b.

Hou, L. M. &. Sheng, F. C. 2000: Calling behaviour of adult female *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) of overwintering generation and effects of mating. Journal Applied Entomology 124, 71-75.

Howlader, A. M. & Gerber, H. G. 1986: Calling behavior of bertha armyworm, *Mamestra configurata* (Lepidoptera: Noctuidae). The Canadian Entomologist 118, 735-743.

Kingan, T. G., Thomas-Laemont, P. A. & Raina, A. K. 1993: Male accessory gland factors elicit change from virgin to mated behavior in the female corn earworm moth *Helicoverpa zea*. Journal of Experimental Biology 183, 61–76.

McNeil. J. N., Miller, D., Laforge, M. & Cusson, M. 2000. The biosynthesis of juvenile hormone its degradation and titres in females of the true armyworm: a comparison of migratory and non-migratory populations. Physiological Entomology 25, 103-111.

McNeil. J. N. & Tobe, S. S. 2001: Flights of fancy: possible roles of allatostatin and allatotropin in migration and reproductive success of *Pseudaletia unipuncta*. Peptides 22, 271-277.

Nordlund, D. A. & Brady, U. E. 1974: Calling behavior of female *Plodia interpunctella* (Hubner) (Lepidoptera: Pyralidae) under 2 light regimes. Enviromental Entomology 3: 793–796.

Picimbon, J., J. M. Becard, L., Sreng, J. L. Clement & Gardenne, C. 1995: Juvenile Hormone stimulates Pheromonotropic brain factor release in the female black cutworm, *Agrotis ipsilon*. Journal of Insect Physiology 41, 377– 382.

R Development Core Team. 2006. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org.

Rafaeli, A. & Bober, R. 2005: The effect of the juvenile hormone analog, fenoxycarb on the PBAN-receptor and pheromone production in adults of the moth *Helicoverpa armigera*: an aging hormone in adult females? Journal of Insect Physiology 51, 401–410.

Raina, A. K. & Klun, J. A. 1984: Brain factor control of sex pheromone production in the female corn earworm moth. Science 225, 531-533.

Salvadori, R. J. & Parra, P. R. J. 1990: Seleção de dietas artificiais para *Pseudaletia sequax* (Lepidoptera: Noctuidae). Pesquisa Agropecuária. Brasileira 25, 1701-1713.

Swier, S. R., Rings, W. R. & Musick, J. G. 1977: Age-related calling behavior of black cutworm, *Agrotis ipsilon*. Annals of the Entomological Society of America 70, 919–924.

Turgeon, J. J. & McNeil, J. N. 1982: Calling behaviour of the armyworm, *Pseudaletia unipuncta*. Entomologia Experimentalis et Applicata 31, 402-408

Turgeon, J. J. & McNeil, J. N. 1983: Modifications in the calling behavior of *Pseudaletia unipuncta* (Lepidoptera: Noctuidae) induced by temperature conditions during pupal and adult development. The Canadian Entomology 115, 1015-1022.

Figures and Captions

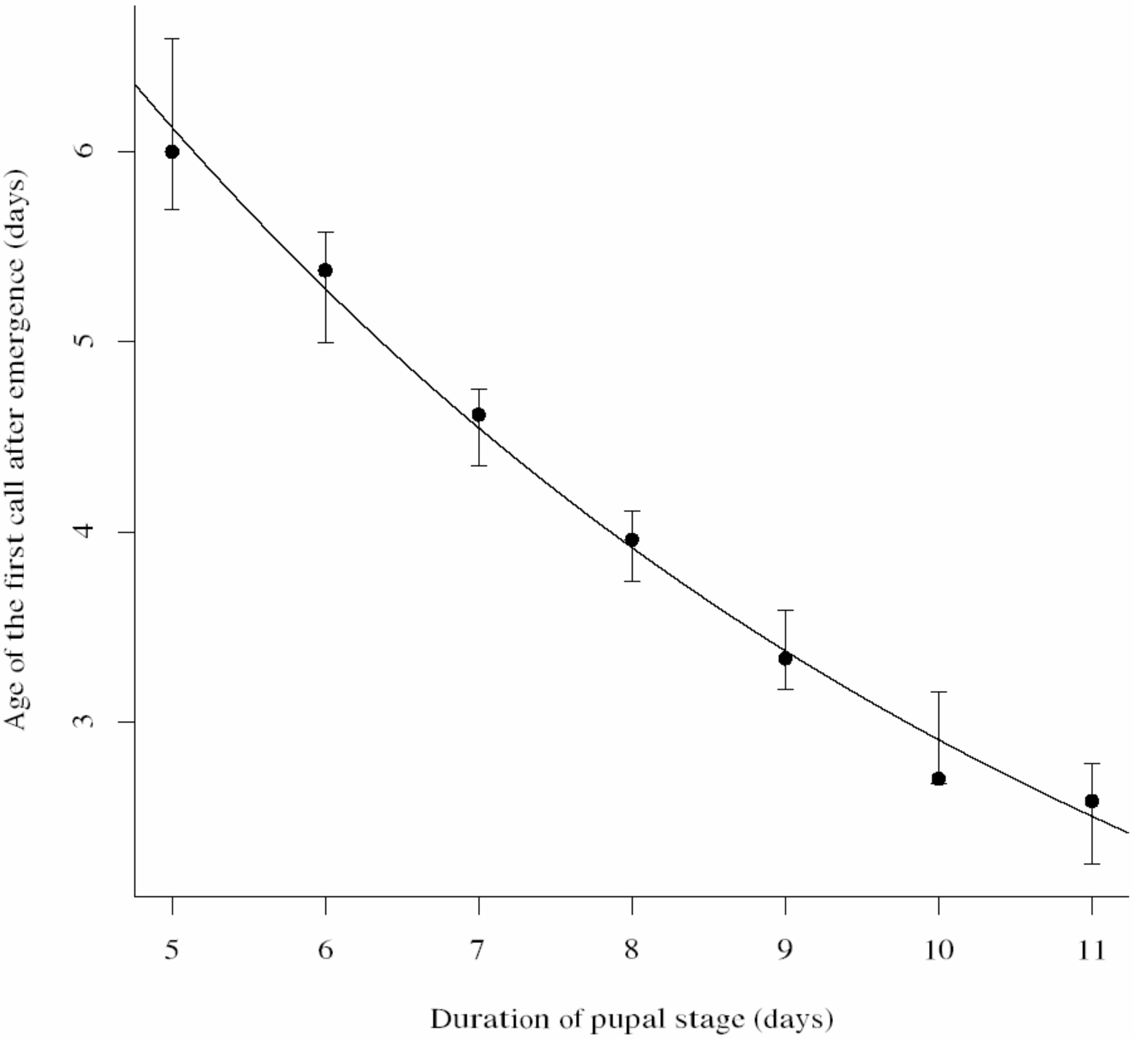

Figure 1: Relationship between duration of pupal stage and age of first calling after emergence of virgin females of *Pseudaletia sequax*. In 25 ± 5 C°; 12D:12L and 70% RH (n = 90), F-value =133.17, p-value $=2.2^{-16}$

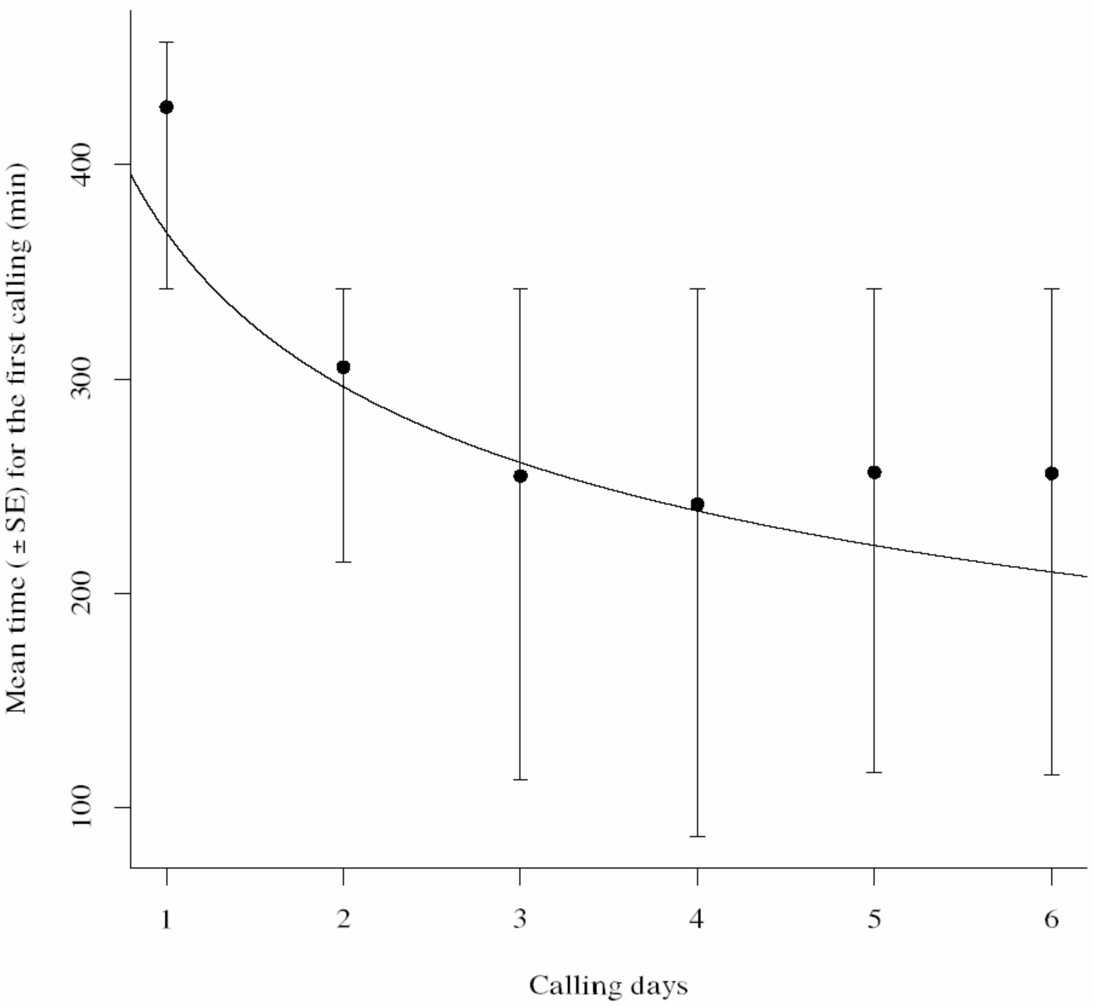

Figure 2: Relationship between days since first calling (calling days) and the onset time of calling from virgin females of *Pseudaletia sequax* in the scotophase at a temperature of 25 ± 5 C ° under a photoperiod of 12D: 12L and of 70% RH, (n=90), F-value 71.31, p-value =0.0001.

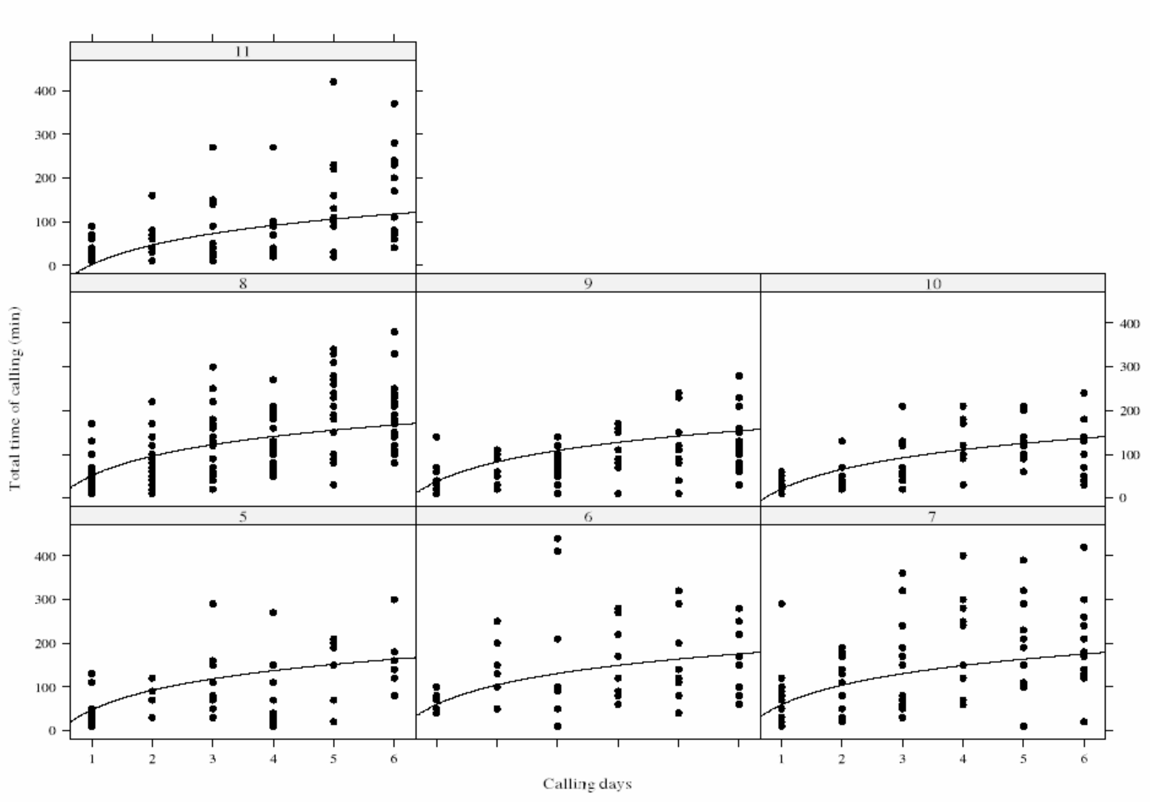

Figure 3: Relationship between calling day, F-value=151.64, p-value =0.0001, calling length and pupal period (5, 6,…, 11 respectively) F-value=13.69, p-value =0.0004 of virgin females of *Pseudaletia sequax* in the scotophase at a temperature of 25 ± 5 C ° and a photoperiod of 12D: 12L and of 70% RH,(n=90).

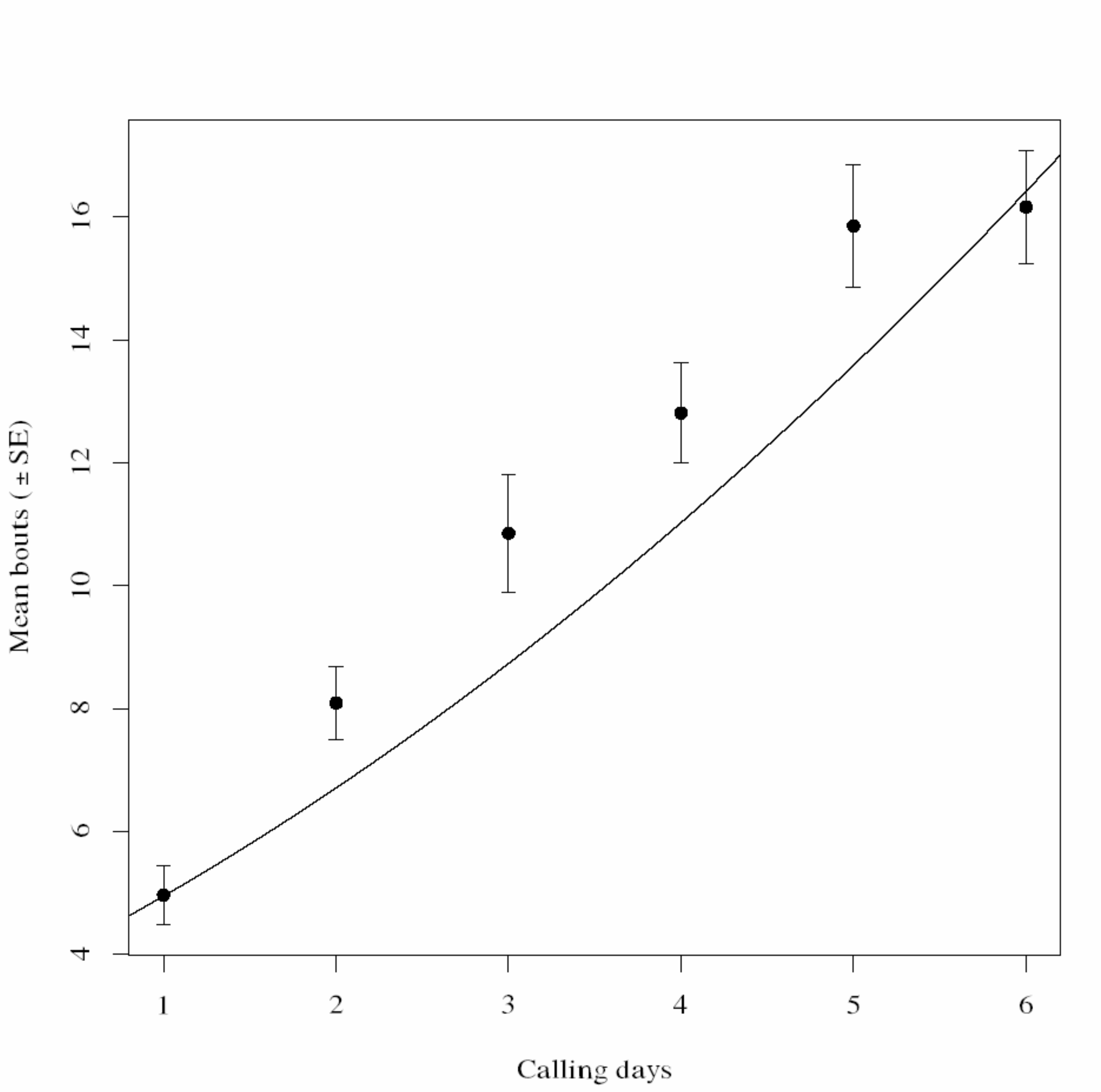

Figure 4: Relationship between days since first calling and mean number of calling bouts of virgin females of *Pseudaletia sequax* in the scotophase at a temperature of 25 ± 5 C ° and a photoperiod of 12D: 12L and 70%RH (n=90), F-value= 202.251, p- value 0.0001.

CHAPTER II

Identification of the sex pheromone candidates of *Pseudaletia sequax* (Franclemont) (Lepidoptera: Noctuidae: Hadeninae)

Ângela Alves de Almeida, Eraldo Rodrigues de Lima and Paulo H. G. Zarbin

Corresponding Author:

A. A. Almeida
Laboratório de Semioquímicos, Departamento de Biologia Animal, Universidade Federal de Viçosa, 36570-000, Viçosa-MG, Brazil.
E-mail: aaamoth@gmail.com; angela@insecta.ufv.br

E.R. Lima
Laboratório de Semioquímicos, Departamento de Biologia Animal, Universidade Federal de Viçosa, 36570-000, Viçosa-MG, Brazil

P.H.G. Zarbin
Departamento de Química, Laboratório de Semioquímicos, Universidade Federal do Paraná, C. Postal 19081,81531-990 Curitiba-PR, Brazil

Abstract

Gas chromatographic and mass spectral analyses were conducted on pheromone gland extracts, volatiles collected from excised pheromone glands from females of *Pseudaletia sequax* calling. Coupled Gas Chromatographic-electroantennographic Detection (CG-EAD) analysis of the female gland extract showed the presence of three EAD-active peaks, which were identified by CG-mass spectrometric (MS) analyses. The pheromone blend it is:(Z)-11-Hexadecenal (Z11-16: Al), (*Z*)-11-Hexadecenyl acetate (*Z*-11-16: OAc) and (*Z*)-11-Hexadecen-1-ol (Z11-16: OH).The mean percentage of compounds identified from volatiles collected from calling females was 85.2% *Z*-11-Hexadecenal, 12.8% *Z*-11-Hexadecenyl acetate and 2% Z-11-Hexadecen-1-ol. The evaluation of antennal response of males to these pheromone components it was compared in laboratory by electroantennographic detection (CG-EAD) and the electroantennogram showed depolarization of antennae when in contact with these three compounds. Theses compounds is possible sex pheromone candidates of *P. sequax.*

Key Words: Chemical ecology, wheat moth, (*Z*)-11-Hexadecenal, (*Z*)-11-Hexadecenyl acetate and (*Z*)-11-Hexadecen-1-ol.

Introduction

P. sequax belongs to noctuid subfamily Hadeninae that includes several agronomic pests species whose outbreaks must be controlled on pasture grass, forage crops, wheat sorghum and soybean (Franclemont and Todd 1983). In south of South America, including Brazil, Argentine and Uruguay, *P. sequax* is an important pest of wheat and forage crops due to destruction of leaves and spikes**.** However, so far there is no information about the pheromone composition of this insect.

Renou et al. (1988a) used a multivariate analysis of the correlation between Noctuidae subfamilies, the chemical structure of their sex pheromones and phylogeny to demonstrate that subfamilies Heliothinae, Plusiinae, Noctuinae and Hadeninae contribute with 94% of the 44 known pheromone and attractant molecules of Noctuidae. The molecules have one or two double bonds and an acetate, alcohol or aldehyde functional group. In all species studied in the subfamily Hadeninae the sex pheromone components Z11-16:Ac and Z11-16:OH are always present. The hexadecenals are associated with Heliothinae but are not specific of this subfamily. Z11-16:Al elicits strong electroantennogram responses in various neotropical Amphipyrinae and Hadeninae subspecies (Renou et al., 1988 a).

Based in the Renou et al. (1988a) study, our hypothesis is that *P. sequax* as a member of Hadeninae complex the sex pheromone should contains that compounds at a specific mixture. Our objective was to identify the compounds of the sex pheromone from *P. sequax.*

Methods and Materials

Insects Approximately 50 pupae of *P. sequax* were obtained from Curitiba region in South Brazil. The colony was established at Laboratory of Semiochemicals and Behavior of Insects at Federal University of Viçosa and maintained at 25°C and 70% relative humidity. After emergence newly emerged females and males were placed in cages for mating, and all moths were supplied with a 10% sugar-water solution. Kikuyu leaves (*Pennisetum clandestinum*) were used as substrate for oviposition in the cages. During the oviposition period, the eggs were removed daily and dated. After eclosion neonate larvae of *P. sequax* were reared on an artificial diet (Salvadori and Parra, 1990) with 10 ml of soy oil added as protein source. In the subsequent generation female pupae of the same cohort were separated after pupation until emergence. After emergence females were placed in individual numbered plastic cages that were covered with cloth to permit air circulation. The experiment was done in a dark room at a temperature of 25 ± 5°C under reversed photoperiod (12D: 12L) and relative humidity 70%.

Pheromone Gland Extraction Pheromone glands were removed from actively calling females five days after emergence during the fourth and sixth hour of the scotophase. Females of *P. sequax* begin calling 4-5 h after scotophase and may continue to call intermittently for 3-4 h (Chapter 1). Females were considered to be calling when their ovipositor was obviously protruded. Calling females were removed from the holding cage, and pressure was applied to the abdomen to cause the tip to protrude. The pheromone gland situated in a membrane between the eighth and ninth abdominal segments, was excised with a small scissors. Careful was taken to in order to remove as little as possible the abdominal tip with the glandular tissue. The extraction of the excised glands were done in 20-40µl of n-hexane for 30 min and then stored in a freezer (-18º C).

Gas chromatography-electroantennographic detection Gland extracts were analysed by gas chromatography-electroantennographic detection (GC-EAD) using a Shimadzu (Japan) 17A v. 3 series chromatograph equipped with a splitless injection system and a DB-5 capillary column (30 m × 0.32 mm i.d., 0.25 µm film thickness; Supelco. Inc., Bellefonte, PA, USA). Hidrogen at 100 kPa was used as carrier gas. The oven was programmed from 70 to 280 °C at 7° C per min 1 min after injection; injector and detector temperature was held at 250 and 280 °C, respectively. To prevent condensation a heating system surrounded the column arm that goes to the antennae at 280 °C. The antennal preparation that was connected to an amplifier via Ag-AgCl electrodes immersed in 0.1 M KCl. Electroantennographic detection responses were amplified (50 ×) with an AC/DC UN-6 amplifier in DC mode (Syntech Laboratories, Hilversum, The Netherlands).

Gas Chromatography- Mass Spectrometry We analyzed pheromone gland extracts (three batches of 23, 6, and 6 pheromone glands) by CGMS and compared the retention times and mass spectra of the EAD-active compounds detected in a gland extract (1µl, ca. 23-female equivalents). Gas Chromatotograph-Mass Spectrometry (CG-MS) analyses were performed with a Varian 3800-Saturn 2000 CG-MS-MS ion trap detector gas chromatograph, equipped with a DB-5 column (30 m x 0.25 mm x 25µm; Agilent Technologies, Santa Clara, CA, USA) and splitless injector was used in these analyses. The oven was programmed for 70°C (1min hold) to 250°C at 7°C/min and kept at this temperature for 5 min -Saturn 2000 CG-MS-MS ion trap detector, fitted with a DB-5 capillary column. Identifications were confirmed by comparison of retention times and mass spectral data with those of authentic samples. The double bond position of the compounds by CG-MS was determined using a pooled pheromone gland extract of 23 females by dimethyl disulfide (DMDS) derivatizations procedure described below.

Dimethyldísulfide Derivatizations Biological samples in hexane extract were determined by reaction with Dimethyl disulfide (DMDS) derivatizations according to the method of Vincenti et al. (1987). A sample in hexane (23µl) was treated with 50-100 µl DMDS (Aldrich, gold label) and one drop of iodine solution (60 mg iodine in 1ml diethyl ether). Reaction mixtures were kept at 40°C oven for 24 hr, cooled and diluted with (ca. 200 µl). Iodine was removed by shaking with 5% aqueous $Na_2S_2O_3$ (ca 100 µl). The organic phase was removed and the aqueous phase extracted with 100 µl hexane. The combined hexane solution was dried over Na_2 SO_4 and concentrated to small volume (12-15 µl) and kept at 4°C. Subsequently the sample was injected in CGMS. Derivatizations of authentic standards confirmed the position and configuration of the double bond. All synthetic compounds were purchased from commercial sources and synthetic standards were prepared in our laboratory. The purity of the compounds was confirmed by gas chromatography.

Results and Discussion

Analysis of pheromone extracts glands by combined CG-EAD showed that male antennae consistently responded to three compounds (Fig.1). The retention times in CG-MS Mass spectra analysis of the major EAD-active peak gave identifying features of an aldehyde (22.6min.) followed by alcohol (23.5 min.) and acetate (25.3 min.), (Fig.2).

The chemical analysis CG-MS showed that the three peak present in extract (Fig.2). The peak A is one mass spectrum characteristic of hexadecenal. Peak B gave a mass spectrum characteristic of 11-hexadecen-1-ol, and peak C gave a mass spectrum characteristic of 11-hexadecenyl acetate. The flame ionization detector showed that the tree compounds are present in extract at percentage 85.2 (Al), 12.8 (OAc) and 2% (OH) (Fig.2). The minor constituent was presumed to be related alcohol. Double-bond positions in the three components of the pheromone extract were determined by reaction with DMDS followed by CG-MS analyses. The reaction indicated the presence of DMDS derivated for 11-16: Al, 11-16: OAc and 11-16: OH, both in Z configurations.

The components (Z-11:16 Ald, Z-11:16 OAc and Z-11:16 OH) presents in ours extracts corroborated with our hypothesis that sex pheromone of *P. sequax* is associated with moths of the sub family Hadeninae and that those compounds are common in insects of *Pseudaletia* genus. For example, studies of sex pheromone of the *Pseudaletia unipuncta* that occurs in North America demonstrate that the mixture is constituted by Z-11:16 OAc free and isomer *E*-11:16 OAc (<1%), also with the possible presence of Z-9:16 OAc and Z-11:16 OH (McDonough et al., 1980).

The compounds Z-11:16 Al, Z-11:16 OAc and Z-11:16 OH are part of the blend sex pheromone of several moths of the genus *Pseudaletia* (Arn et al., 2000 El-Sayed, 2007). Additionally, our results corroborate with of the correlation proposed by Renou et al. (1988 a, 1988 b) between Noctuidae subfamilies and the chemical structure of their sex pheromones.

Morse and Meighen (1986), in a revision about pheromone biosynthesis, role of functional groups and pheromone specificity in *Choristoneura fumiferana* described several biosynthetic pathways of particular value, since it can explain how distinctive pheromone signals utilizing different functional groups (ester, alcohol and aldehyde) and also may explain pheromone differences in species of other genera of Lepidoptera. The formation of aldehyde and alcohol pheromones is positively associated with the enzymes alcohol oxidase and acetate esterase in order. It is probable that the acetate esterase and alcohol oxidase are exterior to the gland cells in the cuticle since metabolic energy and cellular cofactors are not necessary for conversion of the acetate ester to aldehyde pheromone, only water and oxygen. Thus, specific communication systems composed of pheromones containing functional group blends would arisen without additional energy or metabolic factors being required from the cell (Morse and Meighen, 1986).

Additionally, Roelofs and Rooney (2003) in revision about molecular genetics and evolution of pheromone biosynthesis in Lepidoptera, showed the presence of desaturases used in the pheromone biosynthetic pathway in various moths species has revealed that one way to make a major shift in the pheromone blend is by activation of different desaturases from mRNA that already exists in the pheromone gland.

In general the reactions of conversion and synthesis in moths are controlled by hormonal systems, specifically 33-residue peptide, termed Pheromone Biosynthesis Activating Neuropeptide (PBAN), which originates from the subesophageal ganglion. However, the juvenile hormone (JH), secreted by corpora allata (CA) also demonstrated to be essential for pheromone biosynthesis (Raina and Klun, 1984; Cusson and McNeil, 1989; Cusson et al., 1993; Cusson et al., 1994; Picimbon et al., 1995; Rafaeli and Bober, 2005).

However, the synthesis and release of pheromones compounds with lower energy cost would be advantageous in many migratory moths because of the high energy cost required for migration. There is evidence of migratory behavior in *P. sequax* (not published data). Moreover it is possible that synthesis of the compounds of lower energy cost may be advantageous to this moth. This is corroborated by the presence of aldehyde, acetate, alcohol and in sex pheromone blend.

Currently, the sex pheromone identification in Lepidoptera is applicable for monitoring and possible mate disruption or control. Therefore the main semiochemical used in pest management programs are sex pheromones (McNeil, 1991; Wyatt, 1997). On the other hand, a new type of classification called chemotaxonomy is proposed in some studies using the chemical structure of sex pheromone and biosynthetic pathways as a new character for the classification for some families of Noctuidae: especially the subfamily Hadeninae and other families as Pyralidae and Bombycidae (Renou et al, 1988; Frerot et al, 1993; Honda et al, 1994; Moto et al, 2003).

Same studies of the distribution of *P. sequax* reported the common occurrence of this species in the French islands Guadeloupe and Martinique. In theses regions *P. sequax* is variable in size in accordance with xerophilous or hygrophilous habitat. The largest individuals are encountered in hygrophilous zone, whereas the smallest are found in xerophilous zone. The distribution of individuals considered small is also registered in Cuba, Jamaica, Mexico, Argentine, Brazil and Uruguay independently of the habitat characteristics (Franclemont, 1951).

The identification of the sex pheromone blend of *P. sequax* can have applicability in agronomic scoped activities as well as in studies of biology and characterization of this specie in different regions. For example in regions that *P. sequax* is variable in size.

Acknowledgments

The authors thank Dr. Simon Luke Elliot for comments and corrections of the manuscript. Funding was provided by Brazilian Ministry of Education through the CAPES Foundation, and CNPQ for the scholarship to A.A. Almeida and E.R. Lima.

References

ARN, H., TÓTH, M. and PRIESNER, E. 2000. The Pherolist: (http// www. stud. slu. se /~h7toblim/ oldpherolist).

CUSSON, M. and MCNEIL, J.N. 1989. Involvement of juvenile hormone in the regulation of pheromone release activities in a moth. *Sci*. 243: 210-212.

CUSSON, M., YAGI, K.J., TOBE, S.S. and MCNEIL, J.N. 1993. Identification of release products of corpora allata of male and female armyworm moths, *Pseudaletia unipuncta*. *J. Insect. Physiol.* 39: 775–783.

CUSSON, M., TOBE, S.S. and MCNEIL, J.N. 1994. Juvenile hormones: their role in the regulation of the pheromone communication system of the armyworm moth, *Pseudaletia unipuncta*. *Arch. Insect Biochem Physiol*. 25: 329-345.

EL-SAYED, A.M. 2007. The Pherobase: Database of Insect Pheromones and Semiochemicals. http://www.pherobase.net.

FRANCLEMONT, J.G. and TODD, E.L. 1983. Noctuidae, T.XXIV, pp.12-159, in R. W. Hodges (ed.). Check-list of the Lepidoptera of America North of Mexico. E. W. Classey Lim. And the Wedge Entomological Research Foundation, London.

FRANCLEMONT J.G., 1951. Proc. Entomol. Soc. Wash. 53: 57-74.

FREROT, B., DUGDALE, J.S. and FOSTER, S.P. 1993. Chemotaxonomy of some species of moths in the New- Zealand genus Graphania based on sex- pheromones. *N. Zeal. J. Zool.* 20: 71-80.

HONDA, H., HIMENO, K. and YOSHIYASU, Y.1994. Chemotaxonomy of the cotton leaf-roller (Lepidoptera: Pyralidae) in Japan with special reference to differences in sex pheromones. *App. Entomol. Zool.* 29:323-330.

MCDONOUGH, M. L., KAMM, A.J. and BIERL-LEONHARDT, A.B. 1980. Sex pheromone of the armyworm, *Pseudaletia unipuncta* (Haworth) (Lepidoptera: Noctuidae). *J. Chem. Ecol.* 6: 565-572.

MCNEIL, J.N. 1991. Behavioral ecology of pheromone-mediated communication in moths and its importance in the use of pheromone traps. *Ann. Rev. Entomol.* 36: 407-430.

MORSE, D. and MEIGHEN, E. 1986. Pheromone biosynthesis and role of functional groups in pheromone specificity. *J. Chem. Ecol.*12:335-351.

MOTO, K., YOSHIGA, T., YAMAMOTO, M., TAKAHASHI, S., OKANO, K., ANDO, T., NAKATA, T. and MATSUMOTO, S. 2003. Pheromone gland –specific fatty acyl reductase of the silkmoth, *Bombyx mori. PNAS.* 100:9156-9161.

PICIMBON, J., BECARD, M.J., SRENG, L., CLEMENT, L.J. and GARDENNE, C. 1995. Juvenile hormone stimulates pheromonotropic brain factor release in the female black cutworm, *Agrotis ipsilon*. J. *Insect Physiol.* 41:377-382.

RAFAELI, A. and BOBER, R. 2005. The effect of the juvenile hormone analog, fenoxycarb on the PBAN-receptor and pheromone production in adults of the moth *Helicoverpa armigera*: an “aging” hormone in adult females? *J. Insect Physiol.* 51:401-410.

RAINA, A.K. and KLUN, J.A. 1984. Brain factor control of sex pheromone production in the female corn earworm moth. *Sci.* 225: 531-533.

RENOU, M., LALANNE-CASSOU, B., MICHELOT, D., GORDON, G. and DORÉ, J-C. 1988a. Multivariate analysis of the correlation between Noctuidae subfamilies and the chemical structure of their sex pheromones or male attractants. *J. Chem. Ecol.* 14:1187-1215.

RENOU, M., LALANNE-CASSOU, B., DORÉ, J-C., MILAT, M-L. 1988b. Electroantennographic analysis of sex pheromone specificity in Neotropical Catocalinae (Lepidoptera: Noctuidae) a multivariate approach. *J. Chem. Ecol.* 34:481-488.

ROELOFS, L.W. and ROONEY, P.A. 2003. Molecular genetics and evolution of pheromone biosynthesis in Lepidoptera. Proc. Natl. Acad. Sci. USA. 100:9179-9184.

SALVADORI, R.J. and PARRA, P.R.J. 1990. Seleção de dietas artificiais para *Pseudaletia sequax* (Lepidoptera: Noctuidae). *Pesq. Agrop. Bras*. 25:1701-1713.

SCHNEIDER, D. 1963: Electrophysiological investigation of insect olfaction. In: Zotterman, I. Y. (ed.), Olfaction and taste. Pergamon Press, Oxford.

WYATT, T.D.1997. Putting pheromones to work, pp.445-459. In CARDÉ, T. R. and MINKS [eds.], Insect pheromone research: new directions. Chapman & Hall, New York.

VINCENTI, M., GUGLIELMETTI, G., CASSANI, G. and TONINNI, C. 1987. Determination of double-bond position in diunsaturated compounds by mass-spectrometry of dimethyl disulfide derivatives. Anal. Chem. 59:694–699.

Figures and Captions

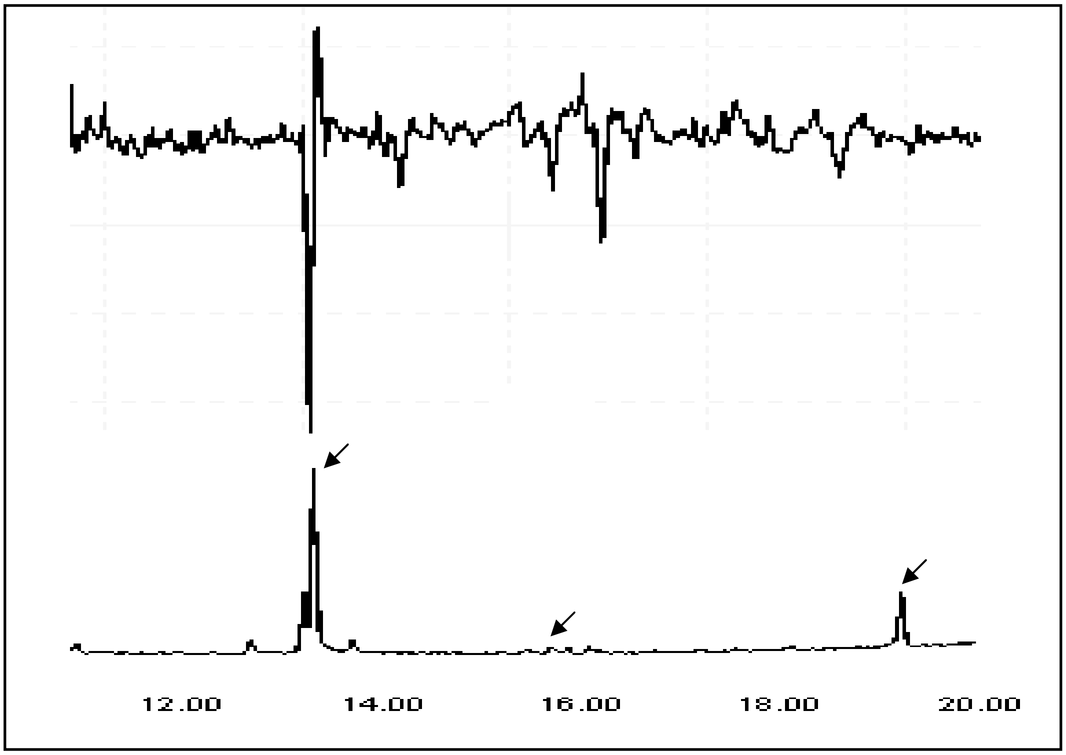

Fig.1 Coupled gas chromatograph electroantennogram detection (CG-EAD) analysis of female of the *Pseudaletia sequax* pheromone gland extract; the flame ionization detector response is bellow EAD-active peak. The EAD response of antennae of male is above.

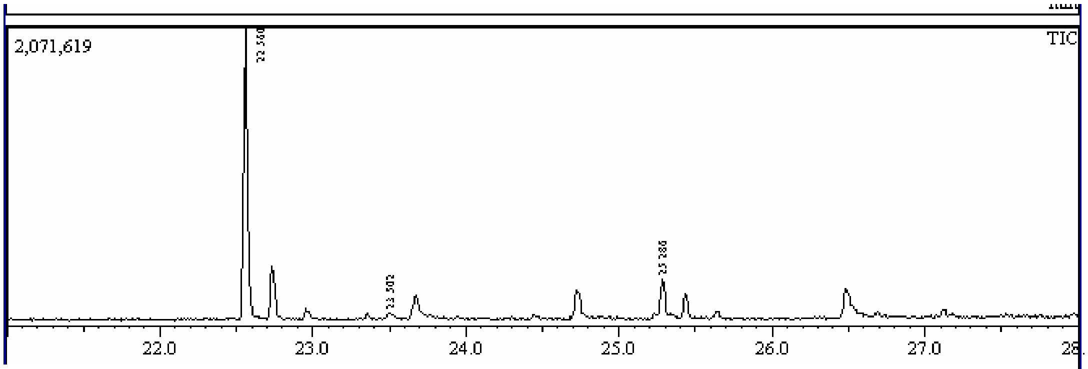

Fig.2 Retention times and fragmentation patterns of Z11-16:Al, Z11-16:OH and Z11-16:OAc respectively the three sex pheromone components are to *Pseudaletia sequax.*

CHAPTER III

Evaluation of sex pheromone candidates of *Pseudaletia sequax* (Lepidoptera: Noctuidae) in wind tunnel

Ângela Alves de Almeida and Eraldo Rodrigues de Lima

Corresponding Author:

A. A. Almeida
Laboratório de Semioquímicos, Departamento de Biologia Animal, Universidade Federal de Viçosa, 36570-000, Viçosa-MG, Brazil.
E-mail: aaamoth@gmail.com; angela@insecta.ufv.br,

E.R. Lima
Laboratório de Semioquímicos, Departamento de Biologia Animal, Universidade Federal de Viçosa, 36570-000, Viçosa-MG, Brazil.

Abstract

Behavioral response of the *P. sequax* to synthetic sex pheromone was studied. Behavioral tests were carried out within a wind tunnel (3x1x1m) calibrated to 0.40 m/speed. Virgin males were tested once during fifth and eighth hour of scotophase corresponding the female calling period. The males were placed at transparent glass cage and placed inside of tunnel on a wooden platform in v format (50 cm of height) during 2 minutes for acclimatization. A male was scored only once and then discarded. The rubber septa of pheromone were located in a 1,94m distance of a platform of male. The treatments were: (blend) rubber septa of 100µg/µl of the complete mixture (85.2 % Z11-16:Al, 12. 8% Z11-16:OAc, 2% Z11-16:OH), alcohol-free rubber septa 100 µg/µl (Z11-16:Al + Z11-16:OAc), acetate-free rubber septa 100µg/µl (Z11-16Al + Z11-16:OH) and aldehyde alone rubber septa of 100µg/µl (Z11-16:Al). Analyses of flight course indicted that Z11-16:Al and Z11-16:OAc is an essential for inducing the upwind flight, landing, flight close and the minor component Z11-16:OH when added Z11-16:Al, Z11-16:OAc significantly increase flight close response, although in all others behavioral responses this compound revealed to be unnecessary.

Key Words: Mating behavior, wind tunnel, upwind flight, Lepidoptera, wheat moth, sex pheromone.

Introduction

Successful mate location, courtship and copulation in insects are dependent on a chemical communication channel, blend composition and plume structure (Cardé & Baker 1984, Mafra-Neto & Cardé 1994). Studies on sex pheromones in Lepidoptera have revealed that many of them use multi-component systems in pheromonal communication between male and female (Linn & Roelofs 1989). Additionally, it has frequently been pointed out that for many lepidopterans a blend of two or more components of their sex pheromones is very important in attracting conspecific males that respond to the signal by flying upwind along the odor plume to the female. However, the role of individual pheromone components in communication between sexes still remains unknown except for a few species (Linn *et al*.1985, Roelofs *et al*. 2002).

In previous studies we identified a sex pheromone candidate of wheat moth, *Pseudaletia sequax* (Franclemont, 1951) (Lepidoptera: Noctuidae). This composed of (*Z*)-11-hexadecenal (*Z*)-11-hexadecenyl acetate and (*Z*)-11-hexadecen-1-ol in percentage 85.2, 12.8 and 2% respectively. In the present study we investigate the flight behavior of males of *P. sequax* to determinate the importance of each component of the sex pheromone in causing upwind flight towards a sex pheromone source in a wind tunnel.

Material and Methods

Insects. The study was carried out between September and December, 2006. Larvae of *P. sequax* were reared on an artificial diet (Salvadori & Parra 1990) with 10 ml of soya oil added as protein source. The colony was established at the Laboratory of Pheromones and Behavior of Insects at the Federal University of Viçosa and maintained at 25°C and 70% relative humidity. After pupation, pupae were sexed daily and male pupae of the same age were separated and isolated in a dark room at 25± 5 C° under reversed photoperiod (12D:12L) and 70% relative humidity for acclimatization. Newly emerged virgin males were placed in individual plastic cages and separated by age. Cages were covered with cloth to permit air circulation. The males were supplied daily with a 10% sugar-water solution. At 4 days post-emergence males were used for tests in the wind tunnel, since previous observations indicated that younger males did not show antennal responses when in contact with the sex pheromone. It has been a common observation that in moth communication systems. Males often do not respond optimally to female sex pheromone until 2-6 days after emergence (Werner 1977, Tóth 1979, Turgeon *et al.*1983, Gemeno & Haynes, 2000). In many such cases male response begins within a day of the age at which females begin calling (Werner 1977, Gemeno & Haynes, 2000).

Wind tunnel. Behavioral tests were carried out within a wind tunnel (3x1x1m) calibrated to 0.35m/s wind speed under reversed photoperiod (12D: 12L), at 25± 5 C° and 70% relative humidity. Groups of male moths were placed daily in transparent glass tubes (15x3 cm) and allowed to acclimatize to the tunnel conditions for at least two hours prior to testing. The virgin males were tested once during the fifth and eighth hours of scotophase corresponding to the female calling period. After acclimatization, males were placed inside the tunnel on a wooden platform in a V formation (50 cm height) during 2 minutes for acclimatization and liberated. Each male was used only once and then discarded. The rubber septa of pheromone were located at 1.94m from the male platform. A smoke plume of hydrochloric acid and ammonium was regularly used to visualize the structure of the pheromone plume, to assure that it passed over the platform containing the males and to determine the best wind speed (established at 0.40m/s).

The treatments were: (blend) rubber septa of 100µl of the complete mixture (*Z*11-16:Al, *Z*11-16:OAc and *Z*11-16:OH), alcohol-free rubber septa 100µg/µl (*Z*11-16:Al, *Z*11-16:OAc), acetate-free rubber septa 100 µg/µl (*Z*11-16:Al, *Z*11-16:OH) and aldehyde alone, rubber septa of 100 µg/µl (*Z*11-16:Al).

Observed Behaviors: antennal waving, upwind flight, landing, claspers extrusion and time in seconds to termination of flight or flight close.

The wind tunnel was cleaned with pure air after tests and males moths were substituted after each flight. Four treatments were tested daily for durations of approximately 3-4h.

Statistical analyses. All statistical analyses were done in R (R Development Core Team 2006), using binomial models (Crawley 2002) to obtain mean percentage of antennal waving, upwind flight, landing in the pheromone source, claspers extrusion, and time in seconds for flight close. Full models were built by including all variables and their interactions. Subsequent model simplification was achieved by extracting non-significant terms ($p>0.05$) from the models, starting with higher-order interactions (backward method). When two non-significant terms were of the same order, the one explaining least deviance was extracted first. Each term deletion was followed by an ANOVA with Chi square test, in order to recalculate the deviance explained by remaining terms. Analysis was made with the number of males exhibiting each behavior in sequence ($N=91$ for blend, 81 for alcohol free, 71 for aldehyde and 71 for acetate free). To obtain the mean time in seconds for flight close response we used an F-test with a normal distribution ($p<0.05$). Only males that flew the complete distance were used.

Results

Antennal waving. Males of *P. sequax* waved antennae in the presence of all treatments: blend, alcohol- free, acetate- free and aldehyde alone (98, 93.9, 77.5 and 76.6% respectively). However, the blend and alcohol-free significantly enhanced the waving of antennae of males (Fig.1).

Upwind flight. Males in the presence of blend and the alcohol-free mixture significantly respond with upwind flight and there was no significant difference between the blend and the alcohol-free mixture (71.4 and 70.3% respectively). We observed that before upwind flight many males performed the following behavioral sequence: wing-fanning activation response, leading to flight after the release cage, zigzagging in an upwind direction in the odor plume, upwind flight to the source. Obviously, these behaviors were evident only in males that exhibited flying in an upwind response (Fig. 1).

Landing. The subtraction of the *Z*11-16:OAc compound led to zero landing while aldehyde alone led to a landing response of 22%. Blend and alcohol-free also were best to enhanced landing behavior 58.2 and 58% (Fig.1).

Claspers extrusion. Both the blend and alcohol-free treatments were best for causing claspers extrusion in males (56.4 and 60% respectively). On the other hand, the acetate-free treatment showed a significantly enhanced mating behavioral response of 21% when compared with aldehyde alone at 4.2% (Fig.1).

Flight close. The mean time for males spent completing flight significantly increased in the aldehyde alone and acetate-free treatments because of the decrease in source localization resulting in more zigzagging and erratic flights. We observed that aldehyde and acetate-free treatments caused very few flight completions (N =10, 4, respectively).

However, males in the presence of blend and alcohol-free treatments diminished significantly the mean time for location of the pheromone source and the number of males exhibiting flight completion response was greater (n= 69, 57 respectively). Although a male in the presence of all compounds (blend treatment) exhibited significantly higher response levels (Fig.2).

Discussion

Significant differences in behavioral responses were recorded for male moths of *P. sequax* with different combinations of sex pheromone. The results obtained here show that the components *Z*11-16:Al and *Z*11-16:OAc are responsible together for the majority of the behavioral response of males in a wind tunnel (Fig. 1). Our results also demonstrated that in the presence of all sex pheromone combinations used males had initiated response sequence with waved antennae (Fig.1) indicating that the activation of behavioral response did not depend of the complete mixture. However, the mixture containing all pheromone components blend and alcohol-free increased significantly the initial response sequence and suggesting that males of *P. sequax* are able to detect the presence of different compounds in pheromone mixture. However, ours results corroborate with a fundamental paradigm in pheromone research, the concept of signal specificity, which states that sex pheromones function as species-specific mate recognition signals (Cardé & Baker 1984, Baker 1985).

Another explanation for signal specificity is that male competition for available females is a potential selective force and one could postulate that enhanced sensitivity to the pheromone blend will result in males being able to locate conspecific females more rapidly. On an interspecific level, sensitivity to a specific blend or ratio of components could enable males to avoid making mistakes by being attracted to a closely related female (Cardé & Baker 1984, Thornhill & Alcock 1997, Roelofs *et al*. 2002).

Presumably *P. sequax* is able to distinguish signals in an environment where there are other species using the same or similar compounds. Our studies demonstrate that blend and alcohol-free treatments enhanced the response levels of upwind flight to 71% and 70.3%, respectively. Furthermore, the major component *Z*11-16:Al alone was not responsible for activating these behavioral response. However *Z*11-16:Al added to *Z*11-16:OAc triggered upwind flight in a sustained wind tunnel. Maximal or peak levels of response dropped significantly with the removal the *Z*11-16:OAc. A similar level of response was significantly greater for landing behavior and mating. Landing and mating behavior were significantly inhibited by subtraction of *Z*11-16:OAc from the blend, but not by the subtraction of *Z*11-16:OH. These results suggest that the *Z*11-16:OAc is an indispensable component in pheromone mixture of conspecific females and the minor component Z11-16:OH is apparently unnecessary for attracting males (Fig.1). In addition, there is a possible discrepancy of less male attraction to synthetic pheromone when compared to the natural one, which is known to occur in wind tunnel with other moths (Vetter & Baker 1983, Sanders 1984, Baker *et al*. 1991, Coracini *et al*. 2003).

Linn & Roelofs (1989) proposed three explanations for the role of the minor components (i) the same minor components are produced and released as a by-product of the synthesis. The neutral impact of these components would not affect the importance of the active blend for male response; (ii) males are capable of detecting the entire minor component. This suggests the possibility that components might interact at common receptor sites, perhaps to amplify the signal in some manner; (iii) what appear to be neutral components may in fact have important roles as interspecific signals, specifically as behavioral antagonists between closely related species. Several studies demonstrate the antagonistic effects on the upwind flight pheromone response of male moths to certain compounds, when added to the pheromone blend of a given species, although little is known about the mechanism of antagonism in upwind flight and many of these compounds were interspecific pheromones in several closely species (Fadamiro & Backer 1997, Cossé *et al*. 1998, Quero & Baker 1999, Linn *et al*. 2007).

However, in *P. sequax* the component *Z*11-16:OAc is important for attraction of conspecific males and the minor component *Z*11-16:OH presumably is unnecessary for attraction or would have an antagonist effect in closely related species. Until now no information is available about behavioral antagonists between closely related species of the *P. sequax.*

In the current study the mean time males spent completing flights significantly increased with aldehyde alone and acetate-free treatments because of a decrease in source location by males resulting in increased zigzagging. Also, the blend and alcohol-free mixtures diminished significantly the mean time for locating pheromone source (Fig.2). Males are capable of sustaining upwind flight, locate pheromone source in less time in response to blend. The alcohol-free mixture also caused upwind flight in a short time. All the observed behaviors corroborate with the importance of the principal component *Z*11-16:Al added of *Z*11-16: OAc in causing maximal levels of response. However, the true role for *Z*11-16:OH still is less evident.

Moreover, several studies have demonstrated that sex pheromones are blends of a main compound and other compounds that are synergists. Females usually produce further compounds in the gland, which do not play an overt behavioral role (Arn *et al.*1992, 2000). Synergists can be substituted with other gland compounds to produce the same behavioral effect. This phenomenon has been termed pheromone redundancy (Linn *et al*. 1984, King *et al*. 1995, Mayer and Mitchell 1999). The component Z11-16:OH did not demonstrate an antagonist effect in intraspecific communication. Nevertheless, when Z11-16:OH is added in blend a significant increase in flight close response occurred, although in all other behavioral responses this compound was revealed to be unnecessary. Our results suggests that *Z*11-16:Al, *Z*11-16:OAc and Z11-16:OH were sex pheromone components of *P. sequax* because all compounds of same manner were important contributors to male attraction in a wind tunnel. Thus these compounds are essential pheromonal components and together elicit optimal behavioral reactions from the males. Future studies should investigate the role of the Z11-16:OH in communication with closely related species, for example, *Pseudaletia adultera,* which is sympatric and synchronic with *P. sequax* in South Brazil region. Furthermore, field bioassays are necessary to confirm the males' responsiveness using this pheromone mixture in traps.

Acknowledgments

The authors thank Dr. Simon Luke Elliot for comments and corrections of the manuscript. Funding was provided by Brazilian Ministry of Education through the CAPES Foundation, and CNPQ for the scholarship to A.A. Almeida and E.R. Lima.

References

Arn, H., Tóth, M. & Priesner, E. 1992. List of sex pheromones of Lepidoptera and Related Attractants. Montfavet: International Organization for Biological Control.

Arn, H., Tóth, M. & Priesner, E. 2000. The Pherolist. Internet edition. http://www.phero.net/pherolist.

Baker, T.C. 1985. Insect mating and courtship behavior. In Kerkut, G.A. & Gilbert, L.I. (eds), Comprehensive Insect Physiology Biochemistry and Phamacology. Pergamon Press, New York. 9: 621-672.

Baker, T.C. Francke, W., Millar, J.G., Löfstedt, C., Hansson, B.S. Du, J.-W., Phelan, P.L., Vetter, R.S., Yougman, R. & Todd, J.L. 1991. Identification and biossay of sex pheromone components of carob moth *Ectomyelois ceratoniae* (Zeller). J. Chem. Ecol. 17:1973-1987

Cardé, R.T. & Baker, T.C. 1994. Sexual communication in insects. In Bell,W. & Cardé, R.T.(eds), Chemical Ecology of Insects. Sinauer Assoc., Sunderlund, MA. 335-386.

Coracini, A.D.M., Bengtsson, M., Reckziegel, A., Eiras, E.A., Vilela, F.E., Anderson, P., Francke, W., Löfqvist, J. & Witzgall, P. 2003. Behavioural effects of minor sex pheromone components in Brazilian apple leafroller *Bonagota cranaodes* (Lep., Tortricidae). J. App. Ent.127:427-434.

Cossé, A.A., Todd, J.L. & Baker, C.T. 1998. Neurons discovered in male *Helicoverpa Zea* antennae that correlate with pheromone- mediated attraction and interspecific antagonism. J. Comp. Physiol. 182:585-594

Crawley, M. J. (2002) *Statistical Computing*: An introduction to data analysis using S-plus. Wiley, Oxford. 761pp.

Fadamiro, Y.H. & Baker, T.C. 1997. *Helicoverpa zea* males (Lepidoptera: Noctuidae) respond to intermittent fine structure of their sex pheromone plume and antagonist in a flight tunnel. Physiol. Entomol. 22:316-324

Gemeno, C. & Haynes, K.F. 2000. Periodical and age-related variation in chemical communication system of black cutworm moth Agrotis ipsilon. J. Chem. Ecol. 26:329-342.

Linn, C.E. Jr., Bjostad, L.B., Du, J.W. & Roelofs, W.L. 1984. Redundancy in a chemical signal: behavioral response of male *Trichoplusia ni* to a 6 component sex pheromone blend. J. Chem. Ecol. 10:1635-1658.

Linn, C.E. Jr., Campbel, M.G. & Roelofs, W.L.1985. Male moth sensitivity to multicomponent pheromones: the critical role of the female-released blend in determining the functional role of components and the active space of the pheromone. J. Chem. Ecol. 12: 659-668.

Linn, C.E. Jr., & Roelofs, W.L. 1989. Response specificity of male moths to multicomponent pheromones. Chem. Senses. 14: 421-437.

Linn, C.E. Jr., Musto, J.C. & Roelofs, W.L. 2007. More rare males in *Ostrinia*: response of Asian corn borer moths to the sex pheromone of the European corn borer. J. Chem Ecol. 33:199-212.

Quero, C. & Backer, T.C. 1999. Antagonistic effect of (*Z*)-11-hexadecen-1-ol on the pheromone-mediated flight of *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae). J. Insect Behav. 12:701-710.

R Development Core Team. (2006) R. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org.

Roelofs, W.L., Liu, W., Hao, G., Jiao, H., Rooney, P.A., & Linn, C.E. Jr. 2002. Evolution of moth sex pheromones via ancestral genes. Proc. Natl. Acad. Sci. USA. 99:13621-13626.

Salvadori, R.J. & Parra, P.R.J. 1990. Seleção de dietas artificiais para *Pseudaletia sequax* (Lepidoptera: Noctuidae). *Pesq. Agrop. Bras*. 25: 1701-1713.

King, G.G.S., Gries, R., Gries, G. & Slessor, K.N. 1995. Optical isomers of 3,13-dimethylheptadecane: sex pheromone components of western false hemlock looper, *Neptytia freemani* (Lepidoptera: Geometridae). J. Chem. Ecol. 21:2027-2045.

Mafra-Neto, A. & Cardé, R.T. 1994. Fine-scale structure of pheromone plumes modulates upwind orientation of flying moths. Nat. 369:142-144.

Mayer, M.S., Mitchell, E.R. 1999. Subtleties in perception and discrimination by cabbage looper moths, *Trichoplusia ni*, of the two 14-carbon acetate sex pheromone components. J. Chem. Ecol. 25:455-469.

Sanders, C.J. 1984. sex pheromone of the spruce budworm (Lepidoptera: Tortricidae): evidence for missing component. Can. Entomol. 116:93-100.

Tóth, M., 1979. Pheromone related behavior of *Mamestra suasa* (Schiff): daily rhythm and age dependence. Acta Phyt. Acad. Sci. Hung. 14:189-194.

Turgeon, J.J., McNeil, J.N. & Roelofs, W.L. 1983. Responsiveness of *Pseudaletia unipuncta* males to the female sex pheromone. Physiol. Entomol. 8:339-344.

Thornhill, R. & Alcock, J. 1983. The evolution of insects mating systems. Harvard University Press, Cambridge, MA, p.53

Vetter, R.S. & Baker, T.C., 1983. Behavioral responses of the male *Heliothis virescens* in a sustained flight tunnel to combinations of seven compounds identified from female sex pheromone glands. J. Chem. Ecol. 9:747-759.

Werner, R.A. 1977. Behavioral responses of spear marked black moth, *Rheumaptera hastata* (Lepidoptera: Geometridae) to a female produced sex pheromone. Ann. Entomol. Soc. Am. 70:84-86.

Figures and Captions

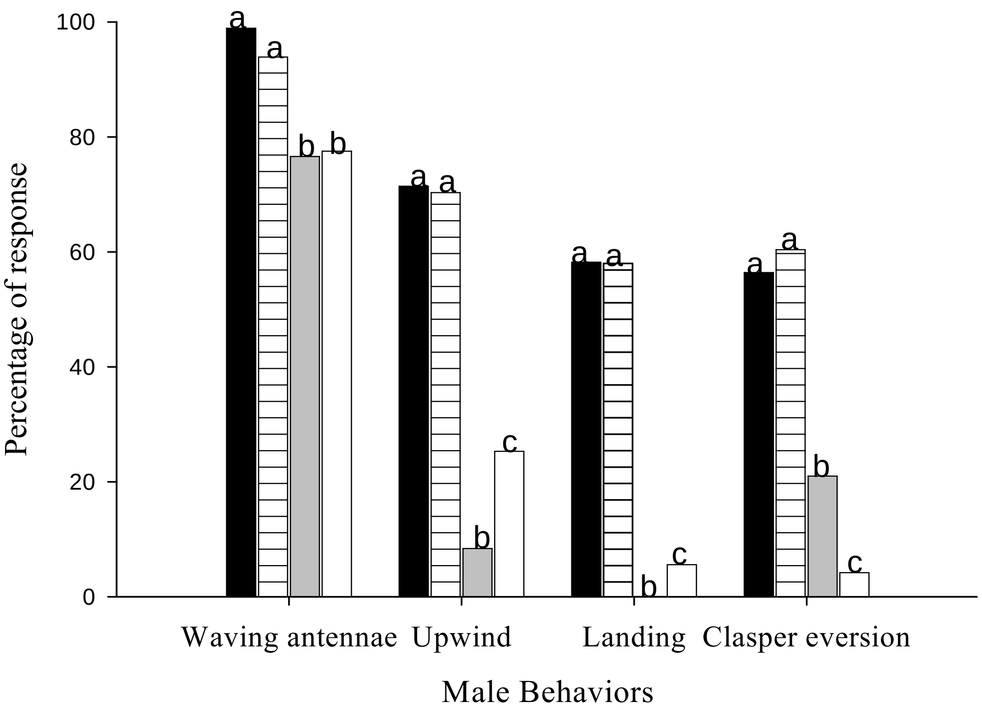

Figure 1: Percentage of male of the *Pseudaletia sequax* exhibiting behaviors in wind tunnel in the presence of four pheromone combinations. Solid bars blend; horizontal lines, alcohol-free; grey bars, acetate-free; clears bar, aldehyde alone. Bars with identical letters did not differ statistically (p>0.05) in comparisons of individual behaviors.

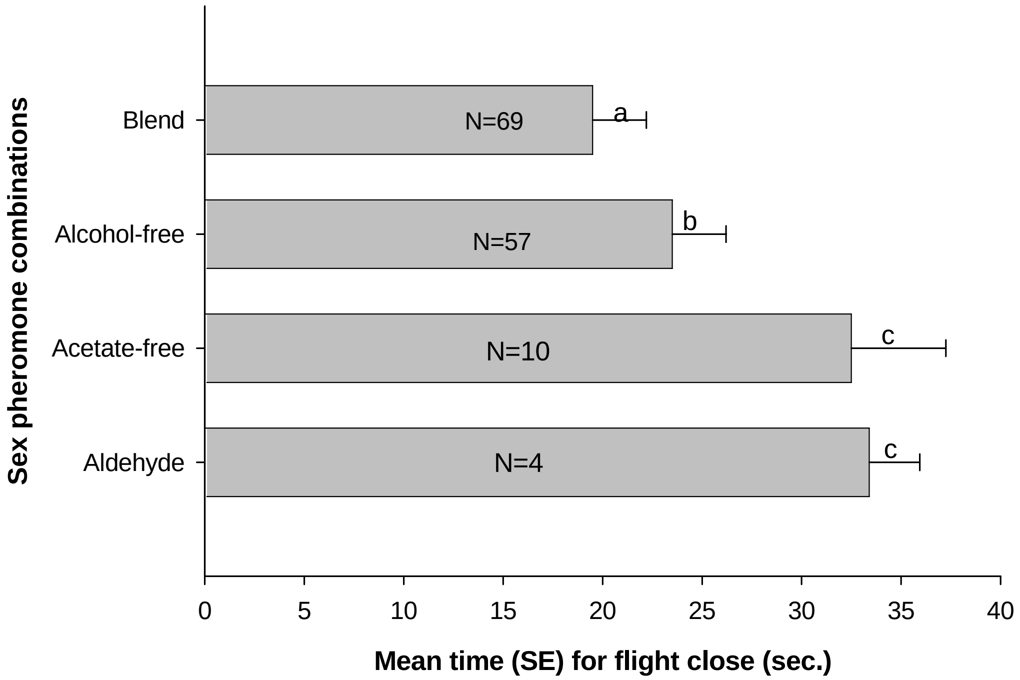

Figure 2: Mean time in seconds for flight completion male response of the *Pseudaletia sequax* in contact with sex pheromone combinations. Bars of the identical letters did not statistically differ ($p>0.05$).

CONCLUSÃO GERAL

- Fêmeas de *P. sequax* em mesmas condições de temperatura e umidade relativa podem ter diferentes tempos de emergência.
- Fêmeas que passaram 11 dias em estágio de pupa compensaram o longo período pupal produzindo chamando mais cedo em comparação com as que emergiram no quinto dia de período pupal.
- O comportamento de chamamento de fêmeas de *P. sequax* é descontínuo.
- O Comprimento de chamamento, em minutos foi afetado negativamente pelo período pupal.
- A mistura feromonal de *P. sequax* tem a característica multicomponente. E é composta de um componente majoritário Z11-16:Al (85.2%), Z11-16:OAc (12.8%) e Z11-16:OH (2%) respectivamente.
- Os testes comportamentais em túnel de vento mostraram que os componentes Z11-16:Al e Z11-16:OAc são os mais relevantes para que a seqüência de comportamentos pré copula seja exibida.
- A adição de o Z11-16:OH à mistura diminuiu significativamente o tempo gasto pelos machos para chegar até a fonte de feromônio.
- A avaliação individual do Z11-16:OH é necessária para que sua função seja totalmente elucidada.
- A mistura completa Z11-16:Al, Z11-16:OAc e Z11-16:OH demonstrou ser a mais eficiente para atração de machos de *P. sequax*.