**HERNANE DIAS ARAÚJO**

# LABORATORY AND FIELD STUDIES ON INFOCHEMICALS OF BRAZILIAN NOCTUIDS

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.*

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APROVADA: 23 de janeiro de 2017.

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Eraldo Rodrigues de Lima
(Orientador)

A meus pais, Nilsa e David,
dedico.

“There are many reasons for using pheromones.
One is that they are elegant”
Heinrich Arn

“You can observe a lot just by watching”
Yogi Berra

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# Contents

# ABSTRACT

ARAÚJO, Hernane Dias, D.Sc., Universidade Federal de Viçosa, January, 2017. **Laboratory and field studies on infochemicals of Brazilian noctuids**. Adviser: Eraldo Rodrigues de Lima. Co-advisers: Angelo Pallini Filho and Og Francisco Fonseca de Souza.

Chemical compounds are mediators of various intra and interspecific interactions between insects, such as reproduction, pollination, food and host finding, predation and parasitism. Through the decoding of these channels of chemical communication over decades of research, it was possible to develop pest control techniques using behavior-modifying chemicals - the semiochemicals. In this thesis, we evaluated an initial step in the research with semiochemicals and the practical application in the field of a product already developed through research focused on the control of noctuid moths. In the first study, we evaluated the calling behavior and sex pheromone production pattern of the invasive moth *Helicoverpa armigera*. It has been present in Brazil for a few years and caused great losses in different cultures. We observed that the production and emission of sex pheromone by females of a Brazilian population occurs generally at the end of the scotophase, different from that found in other populations and rarely seen in other moths. *Helicoverpa armigera* has a closely related species, the native moth *Helicoverpa zea*, and they have the same sex pheromone components. Therefore, the pattern found may help to elucidate a possible interaction between both species, since they can occur in the same place and at the same time. In the second study, we evaluated the efficiency of the plant-based attractant NOCTOVI® as an attract-and-kill method when mixed with 2% of the insecticide methomyl. The product proved to be efficient against *Spodoptera frugiperda* in corn crops, evidenced by the finding of dead moths in the areas treated with the attractant plus the insecticide. In addition, the density of males trapped in sex pheromone traps in the treated area was lower than in the area with no application. NOCTOVI® proved to be a promising product for the control of noctuid pest moths.

# RESUMO

ARAÚJO, Hernane Dias, D.Sc., Universidade Federal de Viçosa, janeiro de 2017. **Estudos laboratoriais e de campo em infoquímicos de noctuídeos brasileiros**. Orientador: Eraldo Rodrigues de Lima. Coorientadores: Angelo Pallini Filho e Og Francisco Fonseca de Souza.

Compostos químicos são mediadores de várias interações intra e interespecificas entre insetos, como a reprodução, polinização, encontro de alimento e hospedeiro, predação e parasitismo. Através da decodificação destes canais de comunicação química ao longo de décadas de pesquisa, foi possível o desenvolvimento de técnicas de controle de pragas utilizando-se compostos químicos modificadores de comportamento - os semioquímicos. Nesta tese, nós avaliamos um passo inicial na pesquisa com semioquímicos e a aplicação prática em campo de um produto já desenvolvido através de pesquisas voltado para o controle de noctuídeos. No primeiro estudo, avaliamos o comportamento de chamamento e produção do feromônio sexual da mariposa invasora *Helicoverpa armigera*, presente no Brasil há poucos anos e causadora de grandes perdas em diferentes culturas. Observamos que a produção e emissão de feromônio sexual pelas fêmeas de uma população brasileira ocorre de forma geral no final da escotofase, diferente do encontrado em outras populações e pouco visto em outras mariposas. *Helicoverpa armigera* é uma espécie próxima da mariposa nativa *Helicoverpa zea* e ambas apresentam os mesmos componentes em seu feromônio sexual. Logo, o padrão encontrado pode ajudar a elucidar uma possível interação entre ambas as espécies, uma vez que elas podem ocorrer no mesmo local e ao mesmo tempo. No segundo estudo, avaliamos a eficiência do atraente baseado em plantas NOCTOVI® como um método atrai-e-mata quando misturado com 2% do inseticida metomil. O produto se mostrou eficiente contra *Spodoptera frugiperda* na cultura do milho, evidenciado pelo encontro de mariposas mortas nas áreas tratadas com o atraente mais o inseticida. Além disso, a densidade de machos capturados em armadilhas de feromônio sexual na área tratada foi menor que na área sem aplicação. NOCTOVI® se mostrou um produto promissor para o controle de mariposas praga da família Noctuidae.

# General Introduction

Chemical ecology is a multidisciplinary field of science that examines the role of chemical signals that mediate the interactions between plants, animals and other organisms, inserted in communities and ecosystems; as well as the evolutionary and behavioral consequences of these interactions. In short, it is the study of the communication between organisms through chemical substances generally called "semiochemicals". It began more properly as a branch of science after Butenandt *et al.* (1959) isolated for the first time the sexual attractant of an insect. In the same year, the term "pheromone" was coined by Karlson & Lüscher (1959), defined as a substance secreted externally by an individual and received by a second individual of the same species, where specific responses such as defined behaviors or physiological processes are elicited. When the substance is emitted and perceived by organisms of different species, it is called an "allelochemical". Chemical communication is involved in various processes of nature, such as reproduction, pollination, food and host finding, predation and parasitism.

From the exploration of these interactions between organisms, of the same species or not, researchers can develop sustainable strategies for pest insect management. After the work of Butenandt *et al.* (1959), several pheromones were identified, mainly between the 60s and 90s, and also several allelochemicals (see El-Sayed, 2016). It is estimated that the number of metabolites identified is greater than 200.000 (Cortesero *et al.*, 2016).

The use of pheromones in pest control was then developed based on the idea of specific manipulation of the insect communication system without affecting other organisms. There are several advantages of this method of control over the use of conventional insecticides. Pheromones are non-toxic and non-persistent; being species-specific, they elicit behavioral responses in extremely low doses. The application of pheromones focuses on two aspects: mating disruption and attraction to traps, used for the monitoring of the insect pest or its mass collection. The concept of mating disruption is to interfere or strongly block the transmission of signals between sexual partners. The monitoring of insects is useful in detecting or determining the premature incidence of pests, leading to a reduction in the use of conventional insecticides. Mass trapping is a control method that uses synthetic pheromone in many traps to selectively capture as many individuals as possible from the target pest insect to maintain its population below the economic injury level (Bento, 2001; Matthews & Matthews, 2010). There is also the method known as attract-and-kill, known for many years but little used in comparison with other techniques (Jones, 1998). It consists essentially of two components: a lure, which can be a pheromone, a visual or chemical attractant or both, and a killing or disabling agent (insecticide, growth regulator, sterilizer or even a pathogen) that will control the insect (Bento, 2007). Recently, plant-based lures that do not mimic any particular plant have been shown to be an efficient and cost-effective alternative to synthetic pheromone (Gregg *et al.*, 2016). The mating disruption has been used primarily against moths while the use of traps has had an effect against moths, beetles and flies (see Witzgall *et al.*, 2010).

To achieve the level of knowledge required to develop the most appropriate control technique, a wide range of basic science research are required. The first step is to observe the insect to determine if any behavior is mediated or not by some semiochemical. Making these observations in the field can be a difficult and unproductive task. In the laboratory, these observations can be made more easily, mainly through structures such as arenas, olfactometers and wind tunnels. Each of these methods is recommended according to the insect and behavior to be observed, but they are always used to measure the qualitative and quantitative response of the insect to a probable semiochemical (see Haynes & Millar, 2012b). A common observation in the laboratory when working with moth pheromones is the observation of the female calling behavior. This consists of the continuous or intermittent extrusion of its ovipositor, where the pheromone gland is located. The female can assume characteristic positions and fan the wings to optimize the dispersion of the pheromone (Almeida *et al.*, 2008). This technique is demonstrated in Chapter 1 of this thesis.

After the necessary observations, the probable semiochemical is collected, either by extracting the compound by immersing glands or parts of the organism in solvents or by collecting volatile chemicals from the organism (headspace odors). The latter occurs in an adsorbent filter that is subsequently washed with solvent or in an adsorbent fiber in the case of the solid phase microextraction technique (SPME), which does not require solvent. Subsequently, the compounds are separated by means of liquid phase (HPLC) or gas (GC) chromatographic techniques and their structures identified by means of mass spectrometry (MS), nuclear magnetic resonance (NMR) and others (see Haynes & Millar, 2012a).

The electroantennography (EAG) technique allows to determine which compounds are perceived by the insect (see Haynes & Millar, 2012a), although it is not able to determine its function.

To do this, the behavior of the insect in the presence of the identified chemical compounds is again observed. Now is the time to test whether the semiochemical is suitable for use as a pest control or monitoring method, which also brings new challenges. What kind of trap and releaser to use? What is the most efficient method? Is the synthetic semiochemical durable in the field? Climatic variables such as temperature, humidity and wind should be analyzed in the implementation of a pest management program with the use of semiochemicals. In addition, physical aspects of the traps should also be considered, such as color, shape, position and installation height (see Howse *et al.*, 1998).

As you can see, there are many steps between laboratory work and practical field application. Lately, cutting-edge insect semiochemical research has focused on identifying and determining the role of proteins and receptors that bind to odorants and genes involved in the production, reception, and interpretation of chemical signals. Still, basic research will always be necessary for the chemical communication of insects to be understood and used for the sustainable management of pests. The big challenge is to fit the small parts developed in the laboratory into the big puzzle that is the multitrophic interactions.

## Overview of Chapter 1

Inadequate knowledge of insect behavior or inadequate formulation of the sex pheromone may be some of the causes of failure of control methods using semiochemicals. Chapter 1 of this thesis deals with the evaluation of the calling behavior and sex pheromone production of a Brazilian population of the invasive moth *Helicoverpa armigera*. Such assessment is important since the process of adaptation to a new habitat can affect the moth reproductive behavior. As main results, it was shown that the components of the sex pheromone were the same found in other populations and in similar proportions. However, an unusual calling behavior was observed. It occurred only in the last quarter of the 10h scotophase, with the highest frequency reached in its last hour. Females of 1 and 3 days old produced the highest amounts of (Z)-11-hexadecenal, the major pheromone component, at the last 4 hours of the scotophase, while 5 days old females had the pheromone highest amounts in the last 2 hours. The calling behavior was synchronized with the pheromone production, as expected. This so late calling pattern and pheromone production does not occur very often in other moths and is reported for the first time in *H. armigera*. *Helicoverpa armigera* is close related with the native moth *Helicoverpa zea* and they have the same sex pheromone. Therefore, the study of *H. armigera* pheromone production and emission pattern can help to elucidate a possible interaction between both species.

## Overview of Chapter 2

In Chapter 2, we entered another point of studies in chemical ecology: the practical application of the acquired knowledge for use in the sustainable control of insect pests. Here, we assessed the efficacy of the plant-based attractant NOCTOVI® as an attract-and-kill method against *Spodoptera frugiperda* and other noctuid corn pests when used together with 2% of the insecticide methomyl. Moths from the families Noctuidae and Erebidae were found dead on the ground in corn crop areas treated with the attractant plus the insecticide, but not in areas containing only the attractant or without application. Also, we observed no mortality of non-target organisms. Moreover, the mean amount of *S. frugiperda* males collected in sex pheromone traps was higher in areas without application than in areas with NOCTOVI® and with the NOCTOVI® + methomyl mixture. So, the product has proved to be efficient to attract *S. frugiperda* in corn crops, evidenced by fewer moths being caught in the latter treatment.

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# Chapter 1

# An unusual pattern of sex pheromone production and calling behavior in *Helicoverpa armigera* (Lepidoptera: Noctuidae)

# An unusual pattern of sex pheromone production and calling behavior in *Helicoverpa armigera* (Lepidoptera: Noctuidae)

Hernane Dias Araújo[1], Janice de Souza Lopes[2] and Eraldo Lima[3]

**Abstract**

*Helicoverpa armigera* is one of the most important pests of the Old World. Recently, it has been documented in Brazil and is already distributed all over the country. The process of adaptation to this new habitat can affect the moth reproductive behavior. Here, we assessed the rhythm of the calling behavior and of the sex pheromone production in a Brazilian *H. armigera* population from Luís Eduardo Magalhães, Bahia State. The mean onset time of calling declined and the mean time spent calling increased after the first day of calling, but remained constant thereafter. The mean number of calling bouts was not affected by the calling age of females. The calling behavior occurred only in the last quarter of the 10h scotophase, with the highest frequency reached in its last hour. Females of 1 and 3 days old produced the highest amounts of (Z)-11-hexadecenal, the major pheromone component, at the last 4 hours of the scotophase, while 5 days old females had the pheromone highest amounts in the last 2 hours. However, there was no difference in the amount of pheromone produced regarding the female age. The calling behavior was synchronized with the pheromone production, as expected. But this so late calling pattern and pheromone production is an unusual behavior, being reported for the first time in *H. armigera*. Few other moths present this late calling rhythm reported in the literature. Other *H. armigera* populations around the world call more frequently in the second half of scotophase, but it is common that the activity begins in the first half of the scotophase. The invasive nature of this insect in Brazil opens a new range for research of the reproductive behavior of *H. armigera*. Its close relationship with the native species *Helicoverpa zea* is something that should receive attention in the coming years.

**Keywords:** diel periodicity, sexual communication, invasive species, cotton bollworm

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# 1 Introduction

*Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae: Heliothinae) is one of the most harmful insect pest in the world. Highly polyphagous, it was reported in more than 100 host plants of a broad spectrum of families (Fitt, 1989; Czepak *et al.*, 2013; Tay *et al.*, 2013). The worldwide annual estimated losses and control costs is about US$5 billion (Lammers & MacLeod, 2007). Until 2013, its presence was limited to the Old World (Europe, Asia and Africa) and Oceania (EPPO, 2014). In the 2012/2013 crop, an outbreak of *H. armigera* in soybean and cotton crops in Brazil evidenced the invasion of the American continent (Czepak *et al.*, 2013). An invasion period prior to the 2012/13 Brazilian *H. armigera* outbreak is consistent with some studies (Tay *et al.*, 2013; Leite *et al.*, 2014) and indicated by Sosa-Gómez *et al.* (2016) that at least since 2008 it was present in Brazil.

*Helicoverpa armigera* caused losses of more than US$500 million in corn, cotton and soybean crops around the country in 2013 (Czepak *et al.*, 2013; Specht *et al.*, 2013). Now, it has been registered in all regions of Brazil (Leite *et al.*, 2014; Sosa-Gómez *et al.*, 2016), also in citrus and tomato crops (Bueno *et al.*, 2014; Pratissoli *et al.*, 2015) and has even crossed the Amazon basin (Mastrangelo *et al.*, 2014), reaching the far north of Brazil. Furthermore, it has already been found in other countries of the Americas, like Argentina (Murúa *et al.*, 2014), Paraguay (SENAVE, 2014) and Puerto Rico (NAPPO, 2014). The arrival to the United States is practically just a matter of time (Kriticos *et al.*, 2015), since *H. armigera* is a facultative migratory species (Fitt, 1989; Wu & Guo, 2005), capable of long flights of more than 1000 km in 2 or 3 nights (Gregg *et al.*, 1993). Also, the plasticity in some behaviors (for instance, in reproduction) increases the potential of *Helicoverpa armigera* to adapt to regions from different climate zones on the globe (Wu & Guo, 2005).

In moths, the sex pheromone production, the calling behavior, the male response and mating are governed by a circadian rhythm (Groot, 2014). There is a natural variation in timing of sexual activity within and between species, which is ruled by an endogenous circadian clock but affected mainly due changes in exogenous factors such as temperature and photoperiod, but also humidity, age and mating status (see Delisle & McNeil, 1987b; Groot, 2014). Also, a differential adaptation in a new habitat can happen so that the interference in the chemical signaling of closely related species is minimized. For example, three interfertile sibling species of *Euxoa* (Lepidoptera: Noctuidae) have the same sex pheromone but does not overlap their calling periods (Teal *et al.*, 1978), this being a reproductive isolation mechanism.

The calling behavior of *H. armigera* occurs only during the scotophase and also varies according to the population and its geographical distribution (Kou & Chow, 1987; Rafaeli & Soroker, 1989; Casimero *et al.*, 1999; Zhao *et al.*, 2007). For instance, the calling behaviour can range from an early calling in the first scotophase in some regions of China to late callings in Malawi, where females can start calling in its eighth scotophase (Kou & Chow, 1987; Colvin & Gatehouse, 1993; Casimero *et al.*, 1999; Zhao *et al.*, 2007). Although, the peak in calling activity have been observed occurring only after the second half of the scotophase (Kou & Chow, 1987; Rafaeli & Soroker, 1989; Casimero *et al.*, 1999; Zhao *et al.*, 2007). Unlike the number of calling behavior studies, to our knowledge, only Rafaeli & Soroker (1989) investigated the diel rhythm of pheromone production, in an Israeli population, where they found that it was synchronized with the calling behavior.

*Helicoverpa armigera* has a closely related species in Brazil, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae: Heliothinae), with its distribution restricted to the American continent. They share the same sex pheromone components (Klun *et al.*, 1979, 1980; Pope *et al.*, 1984; Vetter & Baker, 1984) and are capable of forming fertile hybrids in laboratory conditions (Laster & Hardee, 1995; Laster & Sheng, 1995). However, it is not yet known the dynamics of the calling behavior of both species in Brazil, which is especially important now that, for the first time, they can be found in the same habitat (Leite *et al.*, 2014). There are reports of traps containing *H. armigera* sex pheromone catching *H. zea* males in United States (Behere *et al.*, 2007a) and Argentina (Murúa *et al.*, 2014), an issue that needs to be taken into account in the monitoring of *H. armigera* in Brazil.

The reproductive behavior is an essential factor that influences the gene flow level of geographical populations (Zhao *et al.*, 2007). So, our main aim was to determine the diel periodicity of the calling behavior and sex pheromone production in a Brazilian population of *H. armigera*, in order to understand its possible relationships with other populations and other heliothines occurring in Brazil.

# 2 Methods and Materials

## 2.1 Insects

A colony of *H. armigera* was established by larvae collected in cotton fields in Luís Eduardo Magalhães, Bahia State, Brazil. The confirmation of the species identity was initially assessed through the male genitalia analysis (Pogue, 2004). Later, the molecular identification using DNA barcoding was made and the identity of the species was confirmed. The rearing was maintained in climate-controlled chambers at 25 ± 2°C and 70 ± 10% relative humidity. The moths were maintained under a reverse photoperiod (14L:10D; onset of scotophase at noon). The adults were kept in PVC cages (40 x 20 cm) with white paper napkin on the inner walls and the upper opening as an oviposition substrate. They were fed with a 10% sugar and 1% ascorbic acid solution soaked in cotton. It was offered *ad libitum* and changed every two days. Eggs were removed from the cage every two days and stored in plastic bags until hatching. Groups of neonates were transferred to an artificial diet with bean as the main ingredient (modified from Greene *et al.* (1976)) in plastic containers of 7.5 L until the 2nd instar. Then, they were individually placed in 16-cell PVC trays (Advento do Brasil Ind. e Comércio de Plásticos Ltda) with the same diet until pupation. The pupae were collected, separated by sex and placed separately in plastic petri dishes inside an acrylic cage (30 x 30 x 30 cm) until adult emergence. The pupae were acclimatized at the experiment sites for at least 7 days before it starts.

## 2.2 Calling Behavior

During the calling behavior, the females evaluated assumed a stationary position with the abdomen elevated, continually exposing the ovipositor together with the sex pheromone gland. In climate-controlled chambers (25.3 ± 0.08°C, 72.5 ± 0.2% relative humidity and 14L:10D reverse photoperiod), the calling behavior of *H. armigera* was assessed to determine when females emit the sex pheromone. Newly emerged females were individualized in 70 ml translucent plastic pots with the tops covered with cheesecloth fabric to allow air circulation. They were fed with the same sugar and ascorbic acid solution described previously, replaced every day. The females only emerged after the onset of the scotophase and the observations began on the next scotophase.

Sixty-seven females were observed throughout the scotophase every 10 minutes, from the first to the sixth calling day. Of these, 46 females called at least once. If a female was calling, it was assumed that she called for a duration of 10 minutes. We evaluated the average age at which they begin to call, the mean onset time of calling, the mean number of calling bouts (number of times that the female singly exposes the pheromone gland) and the mean time spent calling. Since some Noctuidae change their calling behavior after they start to lay infertile eggs (Turgeon & McNeil, 1982) and due to the fact that *H. armigera* also lay infertile eggs, it was assessed whether the oviposition affects the age at which the females begin to call, the total number of days that they call and their lifetime.

We compared females based on their calling age (age at which females start calling) (Turgeon & McNeil, 1982) instead of comparing females of the same chronological age (age after emergence), i.e., females calling for the first time were compared among themselves, regardless of their chronological age, and so on. It was necessary since the females start calling at different chronological ages. The observations were made using a Mini Maglite® flashlight covered with four layers of filter paper and a red color filter.

## 2.3 Pheromone extraction

Newly emerged adult females were individualized as in the previous experiment. The quantification and periodicity of pheromone production were obtained through sex pheromone gland extractions from virgin females of 1, 3 and 5 days old. The extractions were made every 2 hours, starting 2 hours before the onset of the scotophase and finishing 2 hours after the end, with a total of 8 treatments (n = 5 glands per treatment). The sex pheromone glands, situated on the intersegmental membrane between the 8° and 9° abdominal segment (Jefferson *et al.*, 1968), were excised with micro scissors after the extrusion of the gland by a gently pressure on the females abdomen. The individual gland was then transferred to a conical vial with 20 $\mu$l of HPLC grade hexane and 40 ng of n-heptyl acetate as internal standard for 20 minutes. The extracts were then concentrated by evaporation to about 5 $\mu$l and stored at -20 °C until analysis.

### 2.3.1 Gas Chromatography and Quantification

Extracts were analyzed by gas chromatography with flame ionization detector (GC-FID) using a Shimadzu GC-17A equipped with a Rtx-5 Crossbond 5% diphenyl-95% dimethyl polysiloxane capillary column (30 m, 0.25 mm i.d. and 0.25 $\mu$m film thickness; Thames Restek UK Ltd). One $\mu$l of each sample was injected in splitless mode with the injector at 250°C. The column oven was maintained at 50°C for 1 min and then the temperature was increased to 280°C at 10°C/min, where it was held for 10 min. Helium was used as the carrier gas at a constant flow rate of 8 ml/min. Also, the Kovats retention indices (KI) (Kovats, 1958) were calculated using the retention times of n-alkanes (C7-C30) .

A Shimadzu GC 2010 gas chromatograph equipped with a Rtx-5MS Crossbond 5% diphenyl-95% dimethyl polysiloxane capillary column (30 m, 0.25 mm i.d. and 0.25 $\mu$m film thickness; Thames Restek UK Ltd) coupled to a Shimadzu GCMS-QP2010 SE mass spectrometer was also used in order to correctly identify the sex pheromone gland components by comparing the mass spectrum of the likely peak to those in the NIST database and by comparing its retention time with that of pure standard. The injector mode and temperature program were the same as those described above.

The concentration of the major pheromone compound, (Z)-11-hexadecenal (Z11-16Ald), was determined based on an analytical curve at 0.5, 1, 5, 10, 20, 40, 60, 80, 100 and 120 ng/$\mu$l with an authentic standard of Z11-16Ald.

## 2.4 Statistical analysis

The variables observed during the calling behavior were subject to a linear mixed-effects modelling, since it contained both fixed (female identity) and random effects (calling age). We performed a Chi-square test followed by a contrast analysis to determine which means differed (Crawley, 2007).

To determine if the age at which the females begin to lay eggs affects the age at which they begin to call, the total number of days that they call and their lifetime, we built a generalized linear model (GLM) with a quasipoisson distribution of errors. Next, a Chi-square test was performed.

The mean amount of Z11-16Ald in each treatment of the pheromone extractions was analyzed with a GLM with a negative binomial distribution of errors. We performed a Chi-square test followed by a contrast analysis to determine which means differed (Crawley, 2007).

All analyses were performed with the R statistical program (R Development Core Team, 2011).

# 3 Results

## 3.1 Calling Behavior

There was not a single chronological age that stood out over the others regarding the onset of the calling behavior (Figure 1), with most of the females beginning to call between 2 and 3 days old, which supports the use of calling age for the analysis. There was a significant effect of the calling age on the mean onset time of calling ($\chi^2 = 22.793$, df = 5, $p < 0.001$) and on the mean time spent calling ($\chi^2 = 22.033$, df = 5, $p < 0.001$). Moths on their first day of calling had a later onset time of calling and spend less time calling compared with older females (from 2 to 6 days old) (Figures 2 and 3). The mean number of calling bouts was not affected by the calling age (mean = 1.47 bouts; $\chi^2 = 7.1854$, df = 5, p = 0.20). The calling behavior only ocurred in the last quarter of the scotophase, reaching the highest frequency in the last hour of the dark period, ending immediately after the onset of the photophase (Figures 4 and 5).

The age at which the females began to lay infertile eggs (mean = 3.62 days) didn't affect the age at which they began to call (mean = 3.13 days; n = 46, $\chi^2 = 20.737$, df = 41, p = 0.33), the total number of days that they called (mean = 5.06 days; n = 46, $\chi^2 = 81.241$, df = 41, p = 0.26) and their lifetime (mean = 10.97 days; = 46, $\chi^2 = 62.341$, df = 41, p = 0.67).

## 3.2 Pheromone production

The components of the sex pheromone gland were confirmed as being the (Z)-11-hexadecenal (Z11-16Ald, KI = 1819), (Z)-9-hexadecenal (Z9-16Ald, KI = 1813), (Z)-7-hexadecenal (Z7-16Ald, KI = 1811) and hexadecenal (16Ald, KI = 1830). The females of 1 and 3 days old had the highest amount of Z11-16Ald at the last 4 hours of the scotophase ($\chi^2 = 30.88$, df = 34, $p < 0.001$ and $\chi^2 = 48.03$, df = 32, $p < 0.001$, respectively) while the 5 days old females had it at the last 2 hours ($\chi^2 = 41.23$, df = 32, $p < 0.001$) (Figure 6), all of them declining its production after the onset of photophase. There was practically no difference in the mean amount of Z11-16Ald produced by females of different ages (Table 1). The average ratio of Z11-16Ald and Z9-16Ald, the two essential sex pheromone components, was 97.6:2.4.

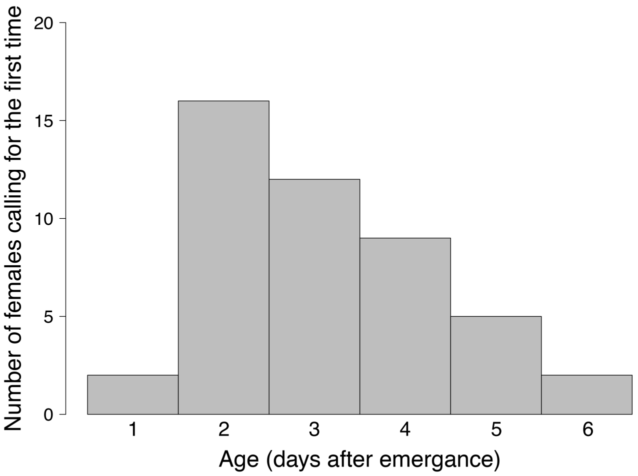

Figure 1. Frequency of *Helicoverpa armigera* virgin females calling for the first time in function of their age (n = 46).

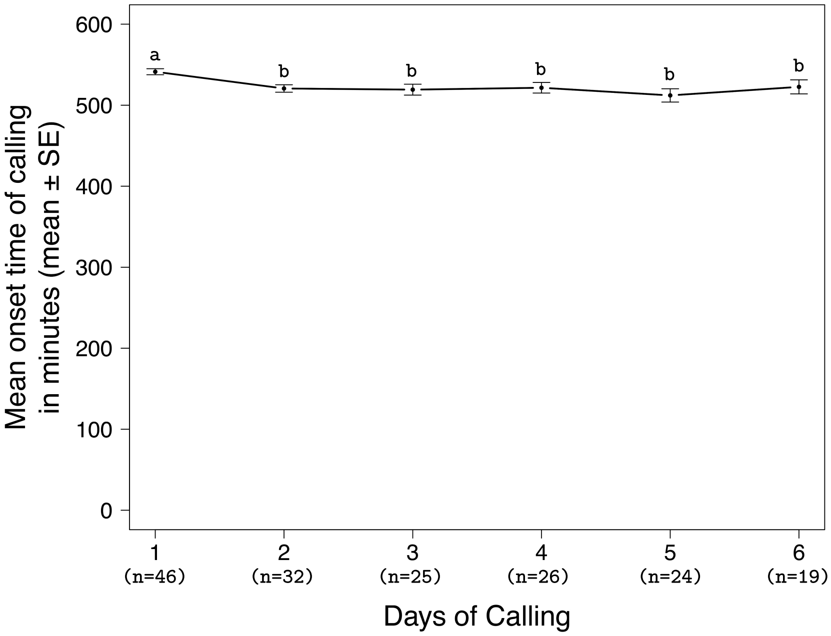

Figure 2. Mean onset time of calling of *Helicoverpa armigera* virgin females at six consecutive calling days. “n” indicates the number of females calling at each calling age. Means followed by different letters are significantly different (Linear mixed-effects modelling, Chi-square test followed by a contrast analysis, $p < 0.05$). SE = Standard error.

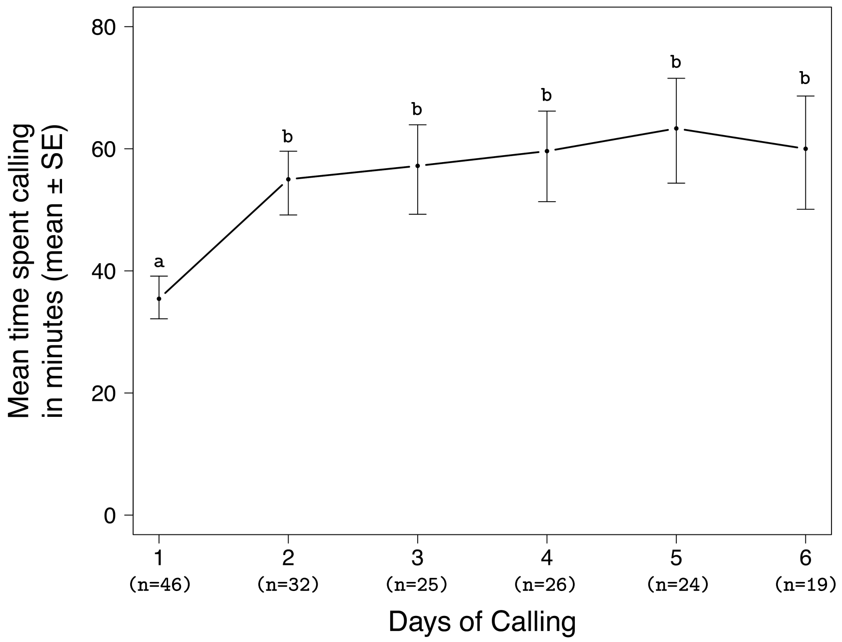

Figure 3. Mean time that virgin females of *Helicoverpa armigera* spent calling during six consecutive days. “n” indicates the number of females calling at each calling age. Means followed by different letters are significantly different (Linear mixed-effects modelling, Chi-square test followed by a contrast analysis, $p < 0.05$). SE = Standard error.

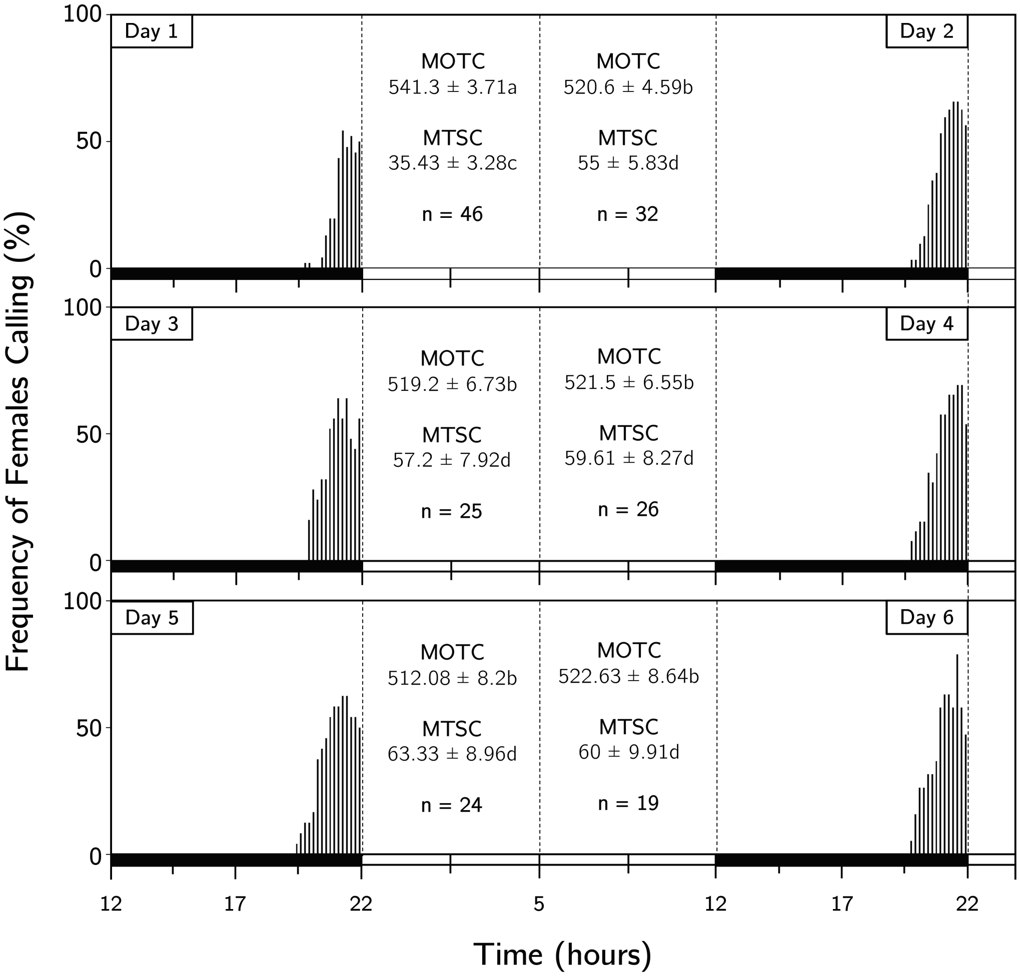

Figure 4. The calling pattern of *Helicoverpa armigera* virgin females at six consecutive calling days. The vertical bars indicate the calling frequency percentage for each observation. The dark bar below the graphs indicates the 10h scotophase while the blank bar indicates the 14h photophase between two scotophases (reverse photoperiod; onset of scotophase at noon). The mean onset time of calling (MOTC) (minutes ± Standard error (SE)), mean time spent calling (MTSC) (minutes ± SE) and number of females calling at each calling age (n) are respectively indicated on each graph. Means followed by different letters are significantly different (Linear mixed-effects modelling, Chi-square test followed by a contrast analysis, $p < 0.05$).

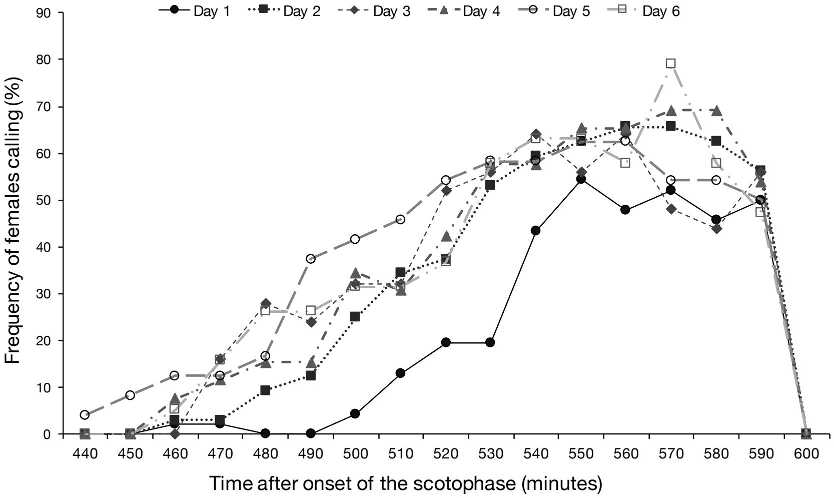

Figure 5. The frequency of females calling at the last quarter of the 10h scotophase of *Helicoverpa armigera* virgin females at six consecutive calling days. Each line represents a calling age (in days), as indicated at the top of the graphic. Every 10 minutes the frequency of females calling (in %) was assessed, which is represented by the markers in each line.

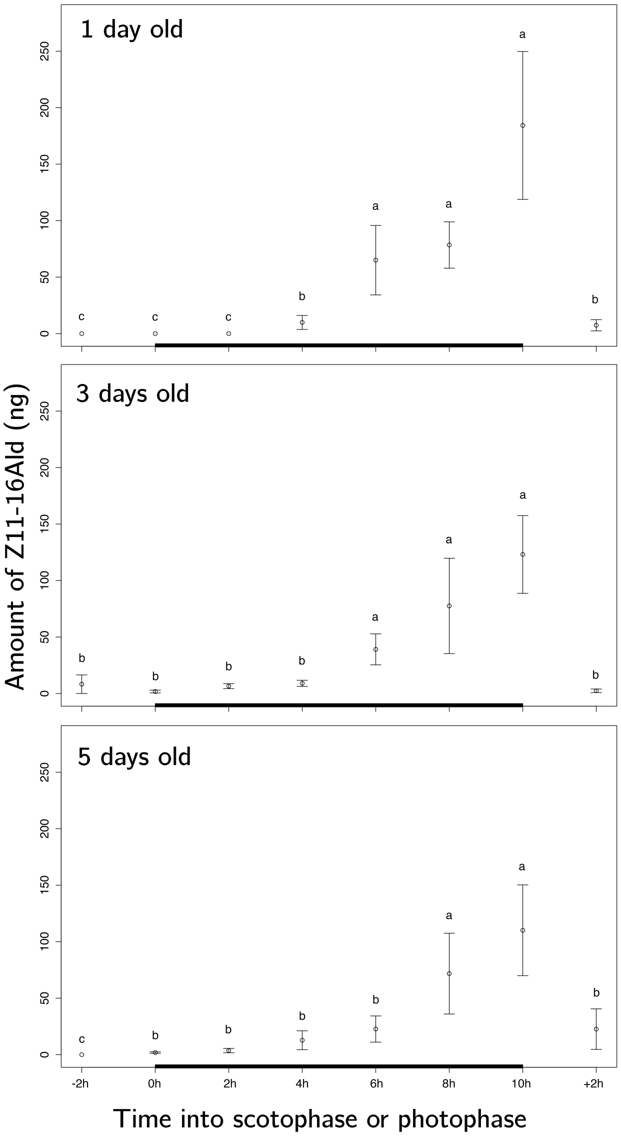

Figure 6. Mean amounts (ng/female) ± SE of the major sex pheromone compound, Z11-16Ald, produced during the scotophase by females of 1, 3 and 5 days old, respectively. The extractions were made every 2 hours, starting 2 hours before the onset of the scotophase and finishing 2 hours after the end (n = 5 glands per time point). Means followed by different letters are significantly different (GLM with a negative binomial distribution of errors, followed by a Chi-square test and a contrast analysis, $p < 0.05$). The scotophase is represented by the dark line on the time axis. SE = Standard error.

Table 1. Mean amount of Z11-16Ald in the sex pheromone glands of *H. armigera* before, during and after the scotophase. Data within a line were compared (GLM with a negative binomial distribution of errors, followed by a Chi-square test and a contrast analysis). Means followed by different letters are significantly different at $p < 0.05$. SE = Standard error.

| | Amount of Z11-16Ald (ng/gland) (mean ± SE, n = 5) | | | | | |
|---|---|---|---|---|---|---|
| Time | 1 day | 3 days | 5 days | df | $\chi^2$ | p |
| 2h before onset of scotophase | 0a | 8.23 ± 8.22a | 0a | 13 | 222.58 | 1 |
| **Into scotophase** | | | | | | |
| 0h | 0a | 1.75 ± 1.25a | 1.82 ± 0.62b | 13 | 14.253 | 0.034 |
| 2h | 0a | 6.48 ± 2.16a | 3.58 ± 1.92a | 13 | 14.369 | 0.09 |
| 4h | 9.89 ± 6.19a | 9.02 ± 2.69a | 12.75 ± 8.35a | 13 | 18.434 | 0.73 |
| 6h | 65.01 ± 30.75a | 39.1 ± 13.73a | 22.67 ± 11.59a | 13 | 18.223 | 0.15 |
| 8h | 78.43 ± 20.49a | 77.51 ± 42.21a | 71.71 ± 35.75a | 13 | 18.357 | 0.9 |
| 10h | 184.27 ± 65.45a | 123.09 ± 34.41a | 110.08 ± 40.17a | 13 | 16.049 | 0.21 |
| 2h after onset of photophase | 7.39 ± 4.97a | 2.41 ± 1.59a | 22.60 ± 17.93a | 13 | 16.357 | 0.28 |

# 4 Discussion

Our results are consistent with many others that show that moths call earlier and for a longer period when they are older (Turgeon & McNeil, 1982; Kou & Chow, 1987; Kamimura & Tatsuki, 1993; Mazor & Dunkelblum, 2005), which is supposed to be a way these females increase their chances of mating (Delisle, 1995). The mean number of calling bouts was lower than expected and remained the same at all calling ages. Other populations of *H. armigera* show a greater number of calling bouts than observed by us (Kou & Chow, 1987; Hou & Sheng, 2000; Zhao *et al.*, 2007). In our observations, most of the females exposed their pheromone glands a single time during the calling behavior and for a long time. Usually, it is expected that the calling behavior of moths starts with short bouts followed by longer ones, at least in other species (Turgeon & McNeil, 1982; Kou & Chow, 1987; Kamimura & Tatsuki, 1993). According to Conner *et al.* (1985), frequent calling bouts suggest a rhythmic pulsed emission of the sex pheromone, which may be a specific recognition factor. Turgeon & McNeil (1982) proposed that a high concentration of pheromone is released during the short bouts, which would increase the female's active space. The low frequency of calling bouts with the gland almost constantly exposed in this *H. armigera* population does not fit the suggested hypotheses. The reason for this unusual pattern remains to be elucitaded.

The vast majority of moth species studied so far show its calling behavior during the scotophase (see Groot, 2014), which was not different in *H. armigera*. However, this Brazilian population presents a remarkable calling rhythm, calling only during the last quarter of scotophase, with a peak frequency in the last hour. Other *H. armigera* populations around the world call more frequently in the second half of scotophase, but it is common that the activity begin in the first half of scotophase (Table 2). To our knowledge, such rhythm of late calling was observed only in three other moth species, all of them from the Noctuidae family: *Mythimna unipuncta* (Delisle & McNeil, 1987a), *Mamestra configurata* (Gerber & Howlader, 1987) and *Euxoa rockburnei* (Teal *et al.*, 1978). *Mythimna unipuncta* and *M. configurata* behave differently depending on the photoperiod and temperature (Delisle & McNeil, 1987a; Gerber & Howlader, 1987). Most likely, the late calling behavior in *H. armigera*, together with a nearly intermittent gland exposure, is displayed according to these two conditions – photoperiod and ambient temperature are the main exogenous factors that affect the sexual activity in moths (see Groot, 2014). Also, the chemical environment around can alter these patterns. For example, a pre-exposure of sex pheromone to females modify the calling rhythm in noctuids and tortricids (Sadek *et al.*, 2012; Stelinski *et al.*, 2014). We individualized the *H. armigera* females during the observations, with no pre-exposure to the pheromone. So, their contact with other females may lead to a change in their calling pattern, as it has been hypothesized for some heliothinae (Groot *et al.*, 2005).

The compounds found in the sex pheromone gland of this Brazilian population were the same as those found in other *H. armigera* populations around the world (Piccardi *et al.*, 1977; Nesbitt *et al.*, 1979, 1980; Dunkelblum *et al.*, 1980; Kehat & Dunkelblum, 1990). Z11-16Ald and Z9-16Ald are considered the only active components, and have been found in very similar proportion to populations from Israel and China (Kehat & Dunkelblum, 1990; Wu *et al.*, 1997), although the mean amount of Z11-16Ald obtained in the Brazilian population was higher. Further studies, as electroantennography response to the pheromone blend emitted by females and the wind tunnel response of males are needed for a better understanding and confirmation of the active compounds occurring in Brazilian populations.

It is expected a synchronization between the production of sex pheromone and the calling behaviour of virgin female moths (Pope *et al.*, 1984; Delisle & McNeil, 1987a; Babilis & Mazomenos, 1992). In *H. armigera*, the pheromone production rate was consistent with the calling behavior, starting slowly before the end of the first half of scotophase, way before the onset of calling, with peak production after the second half of scotophase and declining after the onset of photofase, when the calling ends. In an Israeli population, Rafaeli & Soroker (1989) found a peak of pheromone production during the early-mid and mid-late scotophase, declining before the end of the calling (Table 2), which is a more frequent behavior in moths (see Groot, 2014). Synchronization between production and calling appears to be related to species with *de novo* pheromone synthesis every night (Groot, 2014), which appears to be the case of heliothine moths (Pope *et al.*, 1982, 1984; Rafaeli & Soroker, 1989; Heath *et al.*, 1991; Park *et al.*, 1996).

Can our data be relevant in terms of reproductive isolation of *H. armigera*? As said before, *H. armigera* is an invasive insect that started its spread through the New World in Brazil (Czepak *et al.*, 2013). Another heliothine, *H. zea*, is a moth distributed throughout the American continent, being one of the most hazardous pests in United States (Capinera, 2008), but a secondary pest of corn, cotton and tomato in Brazil (Cruz *et al.*, 2012). It is suggested that *H. zea* populations were stablished in the continent via a founder event from *H. armigera* about 1.5 million years ago (Mallet *et al.*, 1993; Behere *et al.*, 2007b). This so close relationship between the two species is evidenced by the morphological similarity between them. They can't be reliably identified without assessment of the male genitalia or via molecular techniques (Pogue, 2004; Gilligan *et al.*, 2015). Moreover, they share the same sex pheromone (Klun *et al.*, 1979, 1980; Pope *et al.*, 1984; Vetter & Baker, 1984). Few studies have evaluated the pheromone production and calling behavior in *H. zea* (Pope *et al.*, 1984; Raina *et al.*, 1986), but they show that an overlap between the time of these activities in *H. armigera* and *H. zea* is very likely to happen. This allow us to evaluate a scenario where the exotic species *H. armigera* and the native species *H. zea* are in sympatry, with regard to the chemical communication of both species. For this, we suggest be necessary to answer the following questions:

Are they occurring in sympatry and synchrony in Brazil?

Yes. According to Leite *et al.* (2014), *H. armigera* is mainly found on soybean, bean and cotton crops, while *H. zea* is mainly found in corn crops. But in winter crops of millet and cotton they found both species occurring simultaneously. They were also found occurring simultaneously in corn crops (Araújo *et al.*, Chapter 2). Since both species are highly polyphagous (Fitt, 1989) and have long distance migratory capacity (Fitt, 1989; Westbrook *et al.*, 1997; Wu & Guo, 2005), it is reasonable to assume that they will be found together in other hosts besides the already mentioned.

Are they capable of forming hybrids in the field?

Although it may look unnatural, it is speculated that at least 10% of the animals hybridize (Mallet, 2005). For the moth *Ostrinia nubilalis* (Lepidoptera: Crambidae), mating occurs naturally in the field between the Z– and E–pheromone strains when in sympatry, with a proportion of up to 15% of hybrids (Dopman *et al.*, 2010). According to Laster & Hardee (1995) and Laster & Sheng (1995), North American *H. zea* is capable of forming fertile hybrids with Chinese and Russian *H. armigera*, but this was only observed in laboratory conditions. In the field, mate finding is mediated through long distance sex pheromones emitted mainly by the female, which leads us to the next complementary question.

Can males of a moth species be attracted by the sex pheromone of another species female?

The answer to this question depends on factors such as sex pheromone composition, sympatric occurrence and reproductive isolation mechanisms. In the genus *Heliothis* and *Helicoverpa* (Heliothinae subfamily), Z11-16Ald comprehend the major pheromone component in most of the studied species (see Berg *et al.*, 2014), except for *Helicoverpa assulta* (Z9-16Ald) (Cork *et al.*, 1992) and *Helicoverpa gelotopoeon* (16Ald), where Z11-16Ald is not even produced by the female (Cork & Lobos, 2003). A secondary minor compound is also a part of the active pheromone blend, being critical to location of the female (Nesbitt *et al.*, 1980; Vetter & Baker, 1983, 1984). The blend can vary qualitatively and quantitatively, which helps to ensure the specificity of pheromone. In addition, inhibitory compounds can also be emitted by females. Nevertheless, cross-attraction can occur in some cases. For example, *H. armigera* males fly upwind to the sex pheromone blend of *H. assulta* in wind tunnel (Zhao *et al.*, 2006), although they have opposite ratios of the binary sex pheromone blend. In *O. nubilalis*, once the female is locked on the pheromone plume, it reduces its specificity, i.e., a blend with a different ratio can become attractive (Kárpáti *et al.*, 2013). Since *H. armigera* and *H. zea* have similar ratios of their sex pheromones, we can speculate that a cross-attraction has a good chance of happening. There are reports of traps containing *H. armigera* sex pheromone catching *H. zea* males in United States (Behere *et al.*, 2007a) and Argentina (Murúa *et al.*, 2014).

In conclusion, our data reveal a reproductive behavioral pattern rarely found in the moths so far studied. Some geographic variation of the diel periodicity of this behavior may occur due to adaptation to new environments. Considering that we have at least two maternal lineages of *H. armigera* in Brazil that are rapidly expanding (Tay *et al.*, 2013; Leite *et al.*, 2014), it is possible to have different behavioral patterns occurring in Brazilian populations. Also, its close relationship with *H. zea* is something that should receive attention in the next years. So, it is a new challenge for the national and continental agriculture, and the anticipation of what may happen puts us one step forward to seek more efficient ways to the pest management.

Table 2. Timing of sexual activities of female *Helicoverpa armigera* from different populations around the world. Adapted from Groot (2014).

| Ocurrence | | Host[a] | T (°C)[b] | L:D[c] | Activity[d] | Mode of observation[e] | Time of day[f] | | | | | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Country | Population Origin | | | | | | midday | mid-late A | start S | early-mid S | mid-late S | end S | early-mid M | |
| Israel* | | Bean, chikpea, corn, cotton, tomato | 25 | 14:10 | Pher | | | | | ■ | ■ | ▒ | | Rafaeli and Soroker, 1989 |
| | | | | | Calling | Group | | | | ▒ | ■ | ■ | | |
| Taiwan | Tainan | Corn, tomato | 25 | 16:8 | Calling | Individual | | | | ▒ | ■ | ■ | | Kou and Chow, 1987 |
| China | Beijing | | 26 ± 1 | 16:8 | Calling | Individual | | | ▒ | ▒ | ▒ | ■ | | Zhao et al., 2007 |
| | Kashi | | | | | | | | ▒ | ▒ | ■ | ■ | | |
| | Haikou | | | | | | | | | ▒ | ■ | ■ | | |
| Japan | Ushimado | Cabbage | 25 ± 1 | 16:8 | Calling | Individual | | | | | ▒ | ■ | | Casimero et al, 1999 |
| | Souja | | | | | | | | | | ▒ | ■ | | |
| | Okinawa | Okra | | | | | | | | ▒ | ■ | ■ | | |
| Brazil | Luís Eduardo Magalhães | Cotton | 25,3 ± 0,08 | 14:10 | Pher | | | | | ▒ | ■ | ■ | ▒ | This work |
| | | | | | Calling | Individual | | | | | | ■ | | |

[a] Hosts where the insects were first collected.

[b] Temperature during the experiments.

[c] Light:Dark hours in the experiment.

[d] Daily activity patterns; Pher, Pheromone titers in the female sex pheromone gland; Calling, female calling.

[e] Observations during the calling behavior. Group, 10 grouped females observed; Individual, individualized females observed.

[f] Activity at specific times of the day. Midday, Mid-late Afternoon, Start Scotophase, Early-mid Scotophase, Mid-late Scotophase, End Scotophase, Early-mid Morning. White, no activity; Gray, some activity; Black, peak activity.

* Presumable

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# Chapter 2

# A promising attract-and-kill method for managing *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in corn crops

# A promising attract-and-kill method for managing *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in corn crops

Hernane Dias Araújo[1], Antônio Cláudio Ferreira da Costa[2] and Eraldo Lima[3]

## Abstract

The study of the chemical ecology of insects led to the development of efficient and sustainable pest management techniques using semiochemicals. One of these techniques is the attract-and-kill, that consists of attracting the insect to a lure in combining with an insecticide that kills the insect. Here, we assessed the efficacy of the plant-based attractant NOCTOVI® as an attract-and-kill method against noctuid corn pests, mainly *Spodoptera frugiperda* and *Helicoverpa* spp., when used together with 2% of the insecticide methomyl. The product was hand sprayed in a linear range of 92 m in every treated portion of a commercial corn crop. The total mean amount of *S. frugiperda* males collected in pheromone traps was higher in areas without application than in areas with NOCTOVI® and with the NOCTOVI® + methomyl mixture. The density of *Helicoverpa* spp. males was not different between the treatments. Dead moths from the families Noctuidae and Erebidae were found only in the areas treated with the insecticide, most of them being *S. frugiperda*. It was observed no mortality of non-target organisms. The mixture of NOCTOVI® and methomyl proved to be suitable for use as attract-and-kill, evidenced by fewer males of *S. frugiperda* being caught in this treatment than in the control one. Thus, NOCTOVI® is shown to be a promising product for *S. frugiperda* and other noctuid corn pests control.

**Keywords:** fall armyworm, *Helicoverpa* spp., feeding attractant, NOCTOVI®

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# 1 Introduction

In the last years, the motivation for sustainable management of agricultural insect pests has shifted from concern only with agricultural products and the environment to the urgency of increasing food security (Witzgall *et al.*, 2010). More selective methods of pest control have been one of the major objectives of the study of behavior-modifying chemicals (semiochemicals), so as to decrease the use of insecticides (El-Sayed *et al.*, 2009). For several decades, the knowledge gained from studying the chemical ecology of insects has led to efficient and sustainable control methods through different techniques (see Witzgall *et al.*, 2010).

One of these techniques is the attract-and-kill. It consists of attracting the insect to a lure in combining with a trap or an insecticide that promotes its death. Since the 1950s this technique has been used against tephritid fruit flies with hydrolyzed protein as attractant. However, this material has poor selectivity, attracting most insects, including natural enemies (Jones, 1998). In the following years, synthetic pheromones (selective intraspecific attractants) also began to be used as lures. The method has been successfully used for the control of some moth pests, like *Cydia pomonella* (Lepidoptera: Tortricidae) (Charmillot *et al.*, 2000) and *Pectinophora gossypiella* (Lepidoptera: Gelechiidae) (Beasley & Henneberry, 1984) (see also El-Sayed *et al.*, 2009; Witzgall *et al.*, 2010). In most of the cases a synthetic sex pheromone was used as attractant, which has benefits but also limitations. On the one hand, it has high selectivity and minimal impact on non-target organisms. On the other hand, it mostly attracts males and its efficacy decreases in high densities of the pest due to the competition of the lure with more females, making the cost benefit of the technique more relevant for populations in low density (El-Sayed *et al.*, 2009).

Although the use of pheromones works well in pest management for some cases, for insects capable of multiple mating may not work very well. It would be necessary to remove many males from the population (Gregg *et al.*, 2016b). So, female-specific or bisexual attractants should overcome the weaknesses of the use of pheromone. In Australia, the attractant Magnet®, based on plant volatiles, was successfully developed for the control of *Helicoverpa armigera* and *Helicoverpa punctigera* (Lepidoptera: Noctuidae) in cotton. It is a bisexual attractant, although it has been more attractive to females. The differential of this attractant is that it doesn't mimic any particular plant, being a blend of empirically tested compounds (Britton *et al.*, 2002; Del Socorro *et al.*, 2010a,b; Gregg *et al.*, 2010, 2016a,b).

Another attractant, NOCTOVI® (ISCA Technologies, Inc.), has a similar approach. It consists of oleoresins that attract the insect and sugars that act as phagostimulants. The manufacturer markets the product for the control of *H. armigera*, having tested its efficiency in cotton and soybean crops. They also claim that the product is effective against other noctuids of the genera *Helicoverpa* and *Heliothis*, besides being attractive to *Spodoptera* spp..

In Brazilian corn crops, the main pest is the noctuid fall armyworm, *Spodoptera frugiperda* (Cruz *et al.*, 2012b). Control of moths of this genus has been done through chemical insecticides, but mismanagement has led to resistance problems (Guerrero *et al.*, 2014). The use of genetically modified (GM) plants, which express the insecticidal protein of *Bacillus thurigiensis* (Bt), reduced the use of chemical control (Lu *et al.*, 2012). However, the lack of adequate management also led to the development of resistant insects (Santos-Amaya *et al.*, 2015).

The use of semiochemicals can be an efficient alternative control method and a tool to cope with the development of resistance. The synthetic sex pheromone of the fall armyworm is commonly used in the population monitoring to decide whether to apply insecticide or not (Cruz *et al.*, 2012a). To our knowledge, no attract-and-kill method is used for the control of *S. frugiperda*, especially using plant-based volatiles. Thus, the objective of this study was to investigate the potential of NOCTOVI® as a bisexual attractant for use in attract-and-kill to the control of noctuid corn pests, mainly *S. frugiperda*.

# 2 Methods and Materials

## 2.1 Study site

The experiment was conducted in the city of Janaúba, Minas Gerais State, Brazil (15°43’60”S; 43°25’28”W). The experimental area contained approximately 15 ha, irrigated by sprinkler and planted with two varieties of GM Bt corn: Herculex® I (expressing Cry1F protein) and VT PRO 2™ (which expresses Cry1A.105 and Cry2Ab2 proteins). The area did not have other adjacent plantations, only native flora. There was another Bt corn crop about 500 m from the area, but in a more advanced phenological stage. Visible damage (scraped and holed leaves plants) caused by *S. frugiperda* larvae was observed before the experiments (Cruz *et al.*, 2012b). The corn was planted with a spacing of 50 cm between rows and 40 cm between plants. A herbicide application was made in the area a week before the beginning of the experiments. Most of the corn were 6 weeks old at the beginning of the experiment.

## 2.2 Experimental plot

The experimental area was divided into 12 plots of 0.68 ha each. Three treatments were performed, divided into 4 plots by treatment: only NOCTOVI® application, application of NOCTOVI® mixed with the carbamate insecticide methomyl (Brilhante BR, Ouro Fino Química Ltda.) at 2% and a control area, without any product applied. Areas with different treatments were 50 m distant from one another and areas with the same treatment were 25 m distant from each other. NOCTOVI® and the mixture of NOCTOVI® plus insectide were sprayed over the plants using a 5 L hand pump sprayer coupled to a cone nozzle without filter (SS, Brudden®, Brudden Equipamentos Ltda.) in the dosage of 1L/ha. The product was applied by making zig-zag movements, so as to cover the application line of 92 m in each treated plot with a range of 1 m width of the product or mixture (Figure 1). The treatments were applied one day before the beginning of the sampling and repeated on the fifth day after the beginning of the sampling, always in the afternoon and without the activity of the sprinklers. Due to logistic difficulties in the application of the product and therefore evaluation, the areas tested were not randomized. However, the plots were distant enough from one another to avoid any interference among the treatments. In addition, the population density of *S. frugiperda* was homogeneous in the total area before the beginning of the experiment (see results ahead).

To test the efficiency of NOCTOVI®, the following parameters were evaluated: the density of *S. frugiperda* and *Helicoverpa* spp. males in each plot through catches in sex pheromone traps, before and after the onset of applications; and the presence, counting and identification of dead moths found on the ground in 4 sampling areas of 9 $m^2$ per plot equally distributed along the application line (Figure 1). The synthetic sex pheromone of *S. frugiperda* consisted of a blend of (Z)-7-dodecenyl acetate, (Z)-9-tetradecenyl acetate and (Z)-11-hexadecenyl acetate (ChemTica Internacional, S.A.) and the one of *Helicoverpa* spp. (marketed as specific to *Helicoverpa armigera*) consisted of a blend of (Z)-11-hexadecenal and (Z)-9-hexadecenal (ISCA Technologies, Inc.). A sex pheromone delta trap of each type was placed on each plot above the crop. The sticky liner was replaced with a new one after each evaluation.

Also, to check for differences in the attractiveness of NOCTOVI® between males and females, *S. frugiperda* individuals found on the ground were separated by gender. The *Helicoverpa* spp. individuals caught in pheromone traps were identified to species through the male genitalia analysis (Pogue, 2004). Data collection took place between 19 and 27 September 2016.

## 2.3 Statistical analysis

The mean number of moths caught per trap in total and per sampling day in each treatment was analyzed using generalized linear models (GLM) with quasi-poisson distribution of errors. We conducted a Chi-square test followed by a contrast analysis to determine which means differed (Crawley, 2007). All analyzes were performed in the statistical program R (R Development Core Team, 2011).

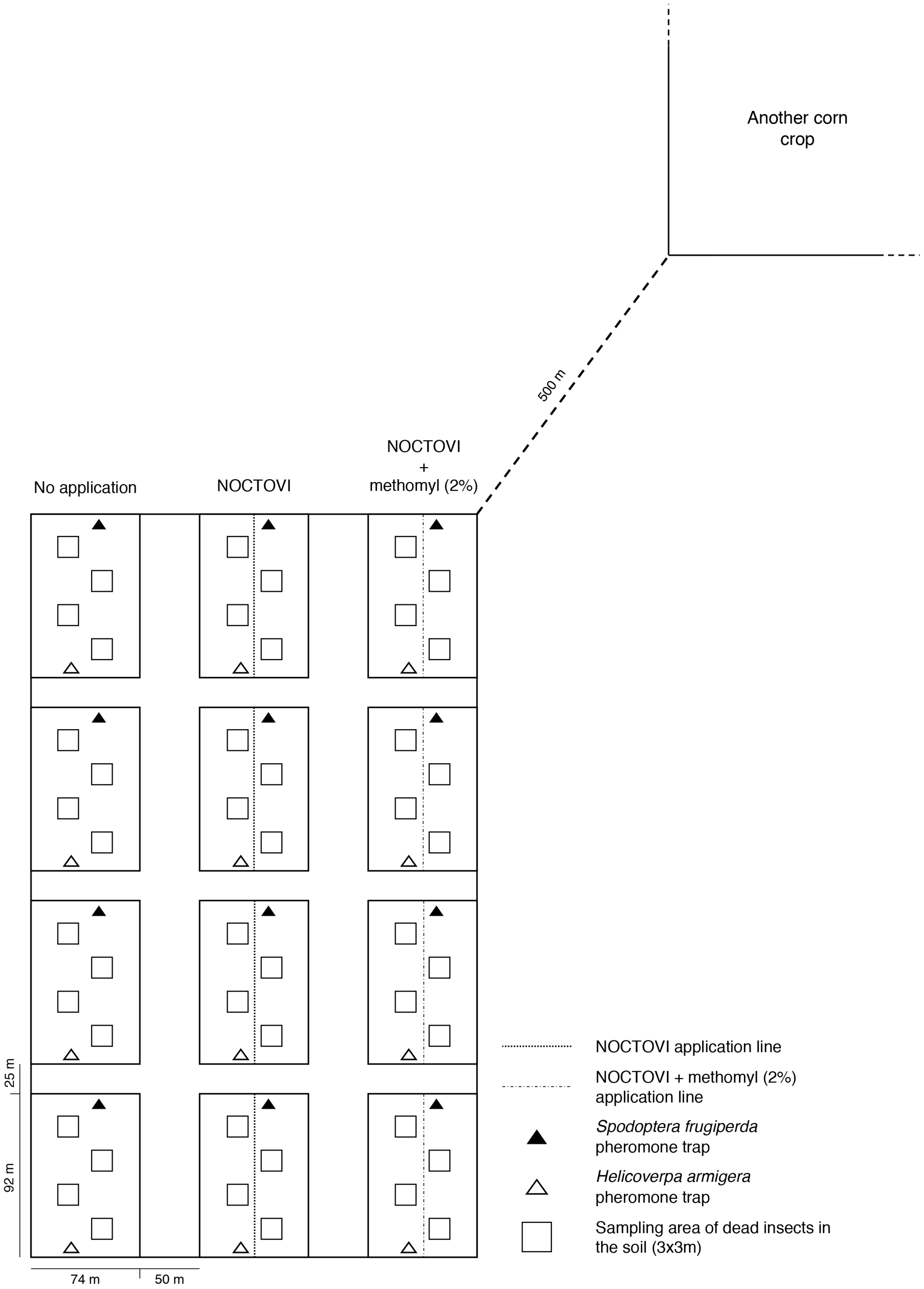

Figure 1. Sketch of the experimental area.

# 3 Results

## 3.1 Pheromone traps

Prior to product application, the density of moths was homogeneous throughout the area. There was no difference in the mean number of *S. frugiperda* ($df = 9$, $\chi^2 = 12.76$, p = 0.77) and *Helicoverpa* spp. ($df = 9$, $\chi^2 = 17.98$, p = 0.15) males collected in sex pheromone traps between different plots before the start of the experiment.

After the application of the treatments, the mean amount of *S. frugiperda* males collected in pheromone traps was higher in areas without application than in areas with NOCTOVI® and with the NOCTOVI® + methomyl mixture ($df = 9$, $\chi^2 = 67.15$, p = 0.03) (Figure 2). There was no difference in the density of *Helicoverpa* spp. ($df = 9$, $\chi^2 = 4.14$, p = 0.57). The analysis of the catch of *S. frugiperda* males per sample day (Figure 3) is summarized in Table 1.

Of the 43 males collected in sex pheromone traps for *Helicoverpa* spp., 39 (90.7%) were identified as *H. zea* and 4 (9.3%) as *H. armigera*.

## 3.2 Dead moths on the ground

Dead moths were observed on the ground only in the plots treated with the NOCTOVI® + methomyl mixture. The specimens were separated by morphospecies and identified to family, and whenever possible, to genus or species. They all belonged to the superfamily Noctuoidea, divided between the families Noctuidae and Erebidae (Table 2). The ratio of *S. frugiperda* males and females found on the ground are summarized in Table 3. It is noteworthy that the assessed region contained many ants, which carried the carcass of dead moths. Thus, the number of moths affected by the mixture is possibly higher than found.

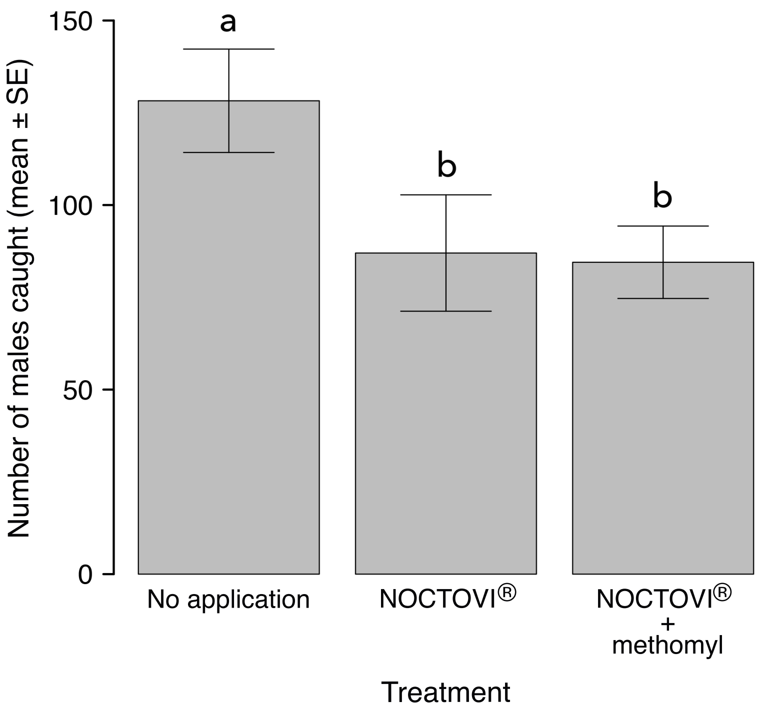

Figure 2. Mean number of *S. frugiperda* males caught in sex pheromone traps per treatment over sampling days after application of the product (n = 4 traps per treatment). Means followed by different letters are significantly different (GLM, Chi-square test followed by a contrast analysis, $p < 0.05$). SE = Standard error.

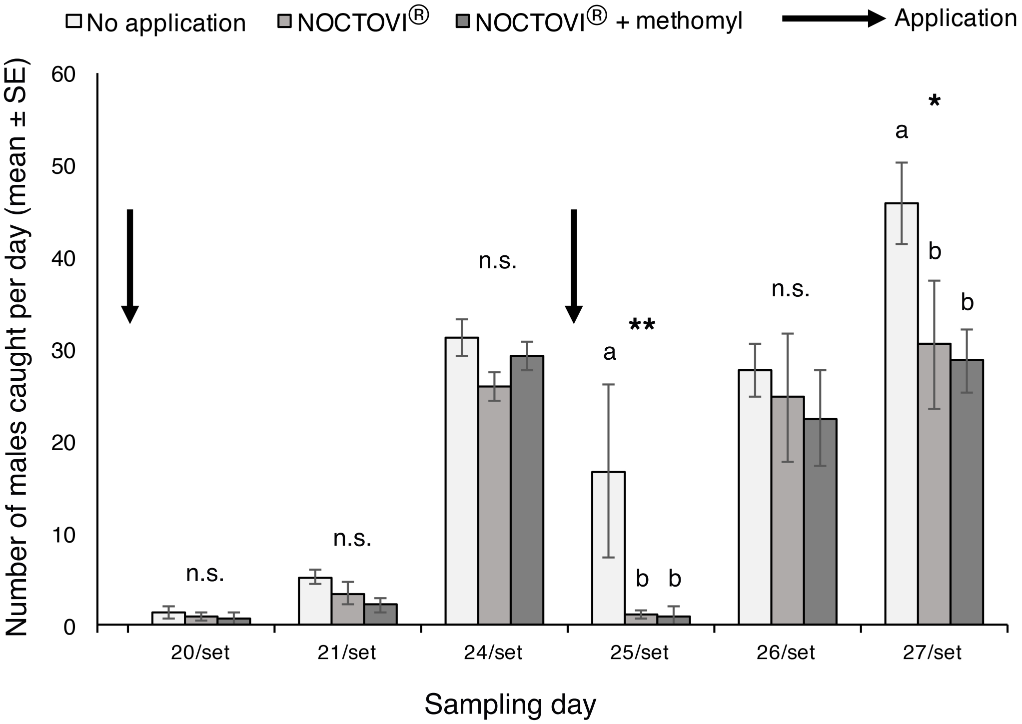

Figure 3. Mean number of *S. frugiperda* males caught in sex pheromone traps per sampling day before and after the start of treatments (n = 4 traps per treatment). Means followed by different letters are significantly different (GLM, Chi-square test followed by a contrast analysis). n.s. = not significant ($p > 0.05$); SE = Standard error. * $p < 0.05$; ** $p < 0.01$. The arrow indicates when the treatments were applied.

Table 1. Effect of the treatment (NOCTOVI®, NOCTOVI® + methomyl and no application) on the male *S. frugiperda* density per sampling day (GLM, Chi-square test followed by a contrast analysis). * $p < 0.05$; ** $p < 0.01$.

| Day | n | *df* | $\chi^2$-value | p-value | |
|---|---|---|---|---|---|
| Sept 20 | 12 | 9 | 15.58 | 0.71 | |
| Sept 21 | 12 | 9 | 9.65 | 0.08 | |
| Sept 24 | 12 | 9 | 3.83 | 0.09 | |
| Sept 25 | 12 | 9 | 68.25 | 0.003 | ** |
| Sept 26 | 12 | 9 | 45.56 | 0.78 | |
| Sept 27 | 12 | 9 | 31.74 | 0.04 | * |

Table 2. Total number of moths collected on the ground from the sampled areas in the different treatments.

| Moths collected on the ground | | | | |
|---|---|---|---|---|
| | | Amount | | |
| Family | Species | NOCTOVI® + methomyl | NOCTOVI® | No application |
| Noctuidae | *Spodoptera frugiperda* | 110 | 0 | 0 |
| | *Mythimna* sp. | 14 | 0 | 0 |
| | *Helicoverpa* sp. | 3 | 0 | 0 |
| | *Agrotis* sp. | 1 | 0 | 0 |
| | unidentified | 1 | 0 | 0 |
| Erebidae | unidentified | 4 | 0 | 0 |
| | *Melipotis* sp. | 1 | 0 | 0 |
| | *Utetheisa ornatrix* | 1 | 0 | 0 |
| | unidentified | 1 | 0 | 0 |
| | unidentified | 1 | 0 | 0 |
| | unidentified | 1 | 0 | 0 |
| | unidentified | 1 | 0 | 0 |
| | unidentified | 1 | 0 | 0 |

Table 3. Total number of dead *Spodoptera frugiperda* collected on the ground by gender.

| Gender | Amount | Percentage |
|---|---|---|
| Male | 88 | 80% |
| Female | 9 | 8.2% |
| Indistinguishable | 13 | 11.8% |

# 4 Discussion

The application of NOCTOVI® + methomyl quickly promoted the reduction of the population of *Spodoptera frugiperda*, which persisted until the end of the experiment. The dead moths collected on the ground evidences the efficiency of the method as a form of control of adult insects of the Noctuoidea superfamily. However, due to the fast predation of moths on the ground by ants, it was difficult to estimate satisfactorily the number of insects that was removed from the environment by this method. Carbamate insecticides cause high mortality rapidly at low concentrations and methomyl has been shown to be effective in controlling adult moths. Gregg *et al.* (2016b) tested the efficiency of different insecticides for use in attract-and-kill and concluded that the most efficient was methomyl. One of the desirable aspects of an insecticide for this purpose is the lack of repellent or deterrent effects, which was the case of methomyl in the experiment mentioned but not directly tested in our experiment. However, methomyl has high toxicity in mammals and should be handled with caution (Del Socorro *et al.*, 2010b).

NOCTOVI® is water soluble and irrigation of the area may explain the increase of *S. frugiperda* capture in the days following application. The product manufacturer has a more moisture-resistant formulation, which should be more suitable to similar areas. Nevertheless, the reduction in population density in the treated areas was visible in the short term.

We observed no mortality of non-target organisms, such as pollinators and natural enemies, which indicates a possible selectivity of the attractant. If this does occur, it corroborates with the idea that the attract-and-kill technique is compatible for use in integrated pest management programs (El-Sayed *et al.*, 2009).

Dead insects were found only in the treatment containing insecticide, which demonstrates that the attractant does not cause the death of moths. However, there was no difference in the density of *S. frugiperda* caught in sex pheromone traps between the treatment with and without insecticide. Thus, it is possible that occurred an interference of the NOCTOVI® volatiles in the perception of the synthetic pheromone by male moths. The sex pheromone is usually detected in a complex volatile environment. Party *et al.* (2013) showed that the odor background can interfere in the walking response of *Spodoptera littoralis* males to the female sex pheromone. They postulate that this can occur in two ways: by masking, when a strong odor interferes negatively in the detection of the pheromone, interfering in the olfactory system, reducing the intensity of the perceived pheromone; or merely by distraction, when the perception of a background odor can reallocate the finite attention of the animal to its olfactory environment, acting as a disturbing stimulus. A similar pattern may have occurred in our studied system. When the odors of NOCTOVI® and the synthetic sex pheromone arrive simultaneously on the antenna of the insect, by any of the factors mentioned above may be occurring interference in the perception of odors. So, an interference in this perception may lead to greater difficulty in locating calling females in field by males, which would lead to fewer mating.

NOCTOVI® + methomyl caused mortality of *S. frugiperda* adults of both sexes, although the great majority of the insects found dead were males. When testing another feeding attractant, Del Socorro *et al.* (2010b) found the opposite. They observed that mated females of *H. armigera* and *H. punctigera* were more attracted than males. The fact that there was no difference in the treatment with and without insecticide in captures in pheromone traps may indicate that males do indeed have a high preference for NOCTOVI®, to the point of competing with the synthetic pheromone. Electrophysiological and behavioral studies may help to elucidate whether males are more attracted than females to the attractant. The electroantenographic response may answer with which intensity each sex perceives the NOCTOVI®, and there may also be differences between virgin or mated insects. In a wind tunnel, where the insect is evaluated in free flight, it can be assessed an agonist or antagonist behavior of each sex in the presence of the attractant.

According to El-Sayed *et al.* (2009), one of the weaknesses of the attract-and-kill method, when the attractant is a pheromone, is that its efficacy decreases when there is a high density of the pest as there would be a greater competition between attractant and calling wild females. In addition, it can be laborious and not financially advantageous. So, the use of empirically developed attractants can be a counterpoint to these weaknesses. They are cheaper and can be easily applied on the crop without the need for a trap (Gregg *et al.*, 2016b). Because they attract both sexes, they are also more advantageous, since the removal of the female from the population has a more direct impact on its future density (Gregg *et al.*, 2016b). In our system, if the attractant does indeed interfere with the encounter of a calling female, it would become a further advantage of using these volatiles rather than synthetic pheromones.

The presence of visible damage in the used area, the lack of refuge area and reports of resistant insects to Cry1F in Brazil (Farias *et al.*, 2014) indicates that the population of *S. frugiperda* studied is resistant, even the plantation containing also pyramided Bt corn (Santos-Amaya *et al.*, 2015). The selection of insects resistant to Bt may cause, due to pleiotropic effects, different levels of efficiency of the method (Poullot *et al.*, 2001). Differences in the reproductive behavior of resistant and susceptible insects may occur. In a *H. armigera* population, Zhao *et al.* (2009) showed that the selection pressure due to cotton CryAc changed the female calling behavior. Thus, it is possible that resistant and susceptible individuals respond differently to the attractants, which should be considered in the evaluation of the efficiency of the method. For GM crops, a better adjusted attractant could act in the selective removal of resistant moths to reduce the frequency of resistant alleles (Del Socorro *et al.*, 2010b).

An important point to discuss is the relationship between *H. zea* and *H. armigera.* Although marketed as specific, the synthetic sex pheromone of *H. armigera* attracted both species to the traps, which was expected since they share the same sex pheromone (Klun *et al.*, 1979, 1980; Pope *et al.*, 1984; Vetter & Baker, 1984). This has also been reported in the United States and Argentina (Behere *et al.*, 2007; Murúa *et al.*, 2014). This information becomes relevant once a pest misidentification can lead to unnecessary and/or inefficient control measures. Unlike United States, *H. zea* is not a very harmful pest in Brazil (Capinera, 2008; Cruz *et al.*, 2012b). But the close related species *H. armigera* caused serious damage in different cultures across Brazil (Czepak *et al.*, 2013; Specht *et al.*, 2013). Thus, caution is required to correctly identify the species caught in those traps. Leite *et al.* (2014) found both species occurring simultaneously in crops of millet and cotton. For the first time, it is reported that both species also occur in sympatry in corn crops. This situation may raise questions about possible cross-attraction and hybrid formation between the two species.

As suggested by Del Socorro *et al.* (2010b), the attract-and-kill method may be a viable form as a substitute for control targeting larvae. Although it was not the objective of this work, long-term evaluations of oviposition, number of larvae in the next generation and yield of the crop can be also important to a better acceptance of the method. Besides, irreversible sublethal effects of insecticides, that removes the individual from the population as well as its death, should also be evaluated (Krupke *et al.*, 2002). The use of attractants instead of synthetic pheromones in the method turns it more affordable. Although not as specific as pheromones, the attractants have proven to be selective enough for safe use in integrated pest management programs (Gregg *et al.*, 2016a) and NOCTOVI® has proved to be efficient to attract *S. frugiperda* in corn crops.

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# General Conclusions

The moths of the Noctuidae family are among the world's most relevant agricultural pests. The results presented here contributed to shed light on semiochemicals and associated behaviors related to some major noctuid pests. We showed an example of an initial experiment in insect chemical ecology and an experiment of the practical use of the knowledge acquired through basic research. For the first time it was described a late pheromone production and calling behavior in *Helicoverpa armigera*. In the case of the American continent, it is now necessary to evaluate its relationship with the close related native species *Helicoverpa zea* and possible new emergent challenges. More sustainable methods of pest control should be sought, especially those with greater selectivity. NOCTOVI® showed to be efficient to be used in an attract-and-kill method for the control of *Spodoptera frugiperda* adults in corn crops, even in high population density. In addition, because of its selectivity, it is unlikely to be harmful to non-target organisms, making it a more sustainable solution for the management of noctuid pests.