

KATTY ELENA BARRIOS ROJAS

**BEHAVIOURAL DISCRIMINATION OF SOLITARY AND GREGARIOUS  
PHASES OF *Anticarsia gemmatalis* HÜBNER (LEPIDOPTERA:  
NOCTUIDAE)**

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

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Tathiana Guerra Sobrinho



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(Orientador)

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## ABSTRACT

ROJAS, Katty Elena Barrios, M.Sc., Universidade Federal de Viçosa, February, 2015. **Behavioural discrimination of solitary and gregarious phases of *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae)**. Adviser: Simon Luke Elliot. Co-adviser: Farley William Souza Silva.

*Anticarsia gemmatalis* is a species of Lepidoptera characterized by the capacity to express differentially its genotype as part of adaptation strategies to environmental changes. This phenomenon is called phenotypic plasticity and is considered one of the main reasons for the evolutionary success. Phenotypes or "phases" described for larvae of *Anticarsia gemmatalis* are: solitary, gregarious and intermediary. These phases differ not only in morphological parameters but also in physiological, ecological and behavioural parameters. As regards the behaviour, many studies have confirmed that this is a basic component of phenotypic plasticity and the first to be expressed when there is a phase change. For Lepidoptera, although some clues are known, the factors that induce and maintain the phase change process have not been fully elucidated. Thus, the objectives of our study were to describe the behavioural repertoires of solitary and gregarious phases of *Anticarsia gemmatalis* and test if specific mechanical and/or chemical stimuli induce the phase change in this species. For the description of the behavioural repertoire were used 50 solitary and 50 gregarious phase larvae. In an experimental arena, each larva was observed for 780s in order to register the different components of its behaviour. For the induction of phase change, solitary phase larvae were selected and divided into groups in which stimuli were applied. To apply the mechanical stimulus 120 solitary larvae were selected and divided into 4 groups of 30 which were stimulated with a nylon thread on the head, on the third abdominal segment or on the last abdominal segment, according to the group. The control group remained without stimulation. To apply chemical stimulus 160 larvae were selected and divided into four groups of 40. For the three stimulus groups cuticular hydrocarbon extracts of *Anticarsia gemmatalis* solitary phase larvae, *Anticarsia gemmatalis* gregarious phase larvae and *Spodoptera frugiperda* larvae were used, according to the group. For the control group we applied the organic solvent used in the extraction of cuticular hydrocarbons. The selected larvae were exposed to cuticular hydrocarbons by allowing them to walk on

a filter paper containing the extracts for 12 hours. For the first objective, the results indicated that there is a difference between the behaviours of *Anticarsia gemmatalis* solitary and gregarious phases. The solitary phase larvae are characterized by having a more sedentary behaviour and perform a larger number of head movements; meanwhile, gregarious phase larvae are characterized by being more active in the arena and by perform a greater number of walking events. Regarding the second objective, the results indicated that none of the treatments differed significantly ( $p < 0.05$ ), so none of the applied stimuli induced a phase change in this species. We believe that differences in behavioural patterns could be reflecting adaptive processes inherent to lifestyles of the phases and that these differences can have an impact on the biology, ecology and distribution strategies of *Anticarsia gemmatalis*.

## RESUMO

ROJAS, Katty Elena Barrios, M.Sc., Universidade Federal de Viçosa, fevereiro de 2015. **Discriminação comportamental das fases solitária e gregária de *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae)**. Orientador: Simon Luke Elliot. Coorientador: Farley William Souza Silva.

*Anticarsia gemmatalis* é uma espécie da ordem Lepidoptera caracterizada pela capacidade de expressão diferencial de seu genótipo como parte de estratégias de adaptação às mudanças ambientais. O fenômeno é denominado plasticidade fenotípica e é considerado uma das principais razões do seu sucesso evolutivo. Os fenótipos ou “fases” descritas para as larvas de *Anticarsia gemmatalis* são: solitária, gregária e intermediária. Estas fases se diferenciam não só em parâmetros morfológicos, mas também em fisiológicos, ecológicos e comportamentais. No que respeita ao comportamento, diversos estudos confirmam que este é um componente básico da plasticidade fenotípica e uns dos primeiros em expressar-se quando ocorre uma mudança de fase. Para lepidópteros, embora se conheçam algumas pistas, os fatores que induzem e mantem o processo de mudança de fase não foram completamente elucidados. Diante disso os objetivos do nosso trabalho foram: descrever os repertórios comportamentais das fases solitária e gregária de *Anticarsia gemmatalis* e, testar se determinados estímulos mecânicos ou/e químicos induzem a mudança de fase nesta espécie. Para a descrição do repertório comportamental foram utilizadas 50 larvas em fase solitária e 50 em fase gregária. Em uma arena experimental cada uma das larvas foi observada durante 780s, registrando assim diferentes componentes do comportamento. Para a indução da mudança de fase foram selecionadas larvas em fase solitária e divididas em grupos nos quais foram aplicados estímulos. Para aplicar o estímulo mecânico foram selecionadas 120 larvas solitárias e divididas em quatro grupos de 30. Foram feitas uma serie de toques com nylon na cabeça, no terceiro segmento abdominal ou no último segmento abdominal, segundo o grupo. O grupo controle permaneceu sem estímulo. Para aplicar o estímulo químico foram selecionadas 160 larvas e divididas em quatro grupos de 40. Foram usados extratos de hidrocarbonetos cuticulares de: larvas em fase solitária de *Anticarsia gemmatalis*, larvas em fase gregária de *Anticarsia gemmatalis* e larvas de *Spodoptera frugiperda*, segundo o grupo. Para o grupo controle foi utilizado o

solvente orgânico usado na extração dos hidrocarbonetos. As larvas selecionadas foram expostas aos hidrocarbonetos cuticulares colocando-as para caminhar sobre um papel filtro que continha os extratos durante 12 horas. Para o primeiro objetivo, os resultados indicaram que existe sim uma diferença entre o comportamento das larvas em fase solitária e fase gregária. As larvas em fase solitária se caracterizaram por apresentar comportamento mais sedentário e por realizar um maior número de movimentos com a cabeça; pelo contrário as larvas em fase gregária se caracterizaram por serem mais ativas na arena e por realizar um maior número de eventos de caminhada. Com respeito ao segundo objetivo, os resultados indicaram que nenhum dos tratamentos foi diferente significativamente ( $p < 0.05$ ), o seja nenhum dos estímulos aplicados induziu a mudança de fenótipo nesta espécie. Acreditamos que as diferenças nos padrões comportamentais poderiam estar refletindo processos adaptativos inerentes ao estilo de vida de cada fase e também, que estas diferenças podem ter um impacto sobre a biologia, ecologia e estratégias de distribuição de *Anticarsia gemmatalis*.

# 1. GENERAL INTRODUCTION

## 1.1 Phenotypic plasticity

Environmental changes generate strong selective pressures in populations (Komers, 1997; Fusco & Minelli, 2010). Features that allow individuals to generate an adaptive response to these changes and to contribute to the fitness of future generations are favoured by natural selection (Pigliucci, 2005; Kowalski *et al.*, 2014). One of these characteristics is phenotypic plasticity and has its genetic basis in the capacity of one genotype to produce more than one phenotype when exposed to different environments (Miner *et al.*, 2005; Fusco & Minelli, 2010; Pfennig *et al.*, 2010; Dingemanse & Wolf, 2013; Simões *et al.*, 2013).

There are two different types of phenotypic plasticity: reaction norm and polyphenism (De Jong & Bijma, 2002; Nijhout, 2003). Reaction norm refers to a gradual phenotypic change in response to an environmental variable (Stearns, 1989; Fusco & Minelli, 2010). Meanwhile, the term polyphenism defines a continuous range of phenotypic variation that exists between two extremely different phases (Pener, 1991; Fescemyer & Erlandson, 1993; Sword, 2002; Fusco & Minelli, 2010). Phase polyphenism is very common in insects and can be considered one of the main reasons for their evolutionary success especially in species considered pests (Hochkirch *et al.*, 2008; Simpson *et al.*, 2011; Simões *et al.*, 2013). This process has been described in species of large groups such as Hemiptera, Phasmatodea, Orthoptera, Coleoptera and Lepidoptera (Applebaum & Heifetz, 1999; Silva *et al.*, 2013). In the literature, the most prominent examples of polyphenism are: the

different stages of the life cycle in holometabolous insects; the presence or absence of winged females aphids; the castes of social insects and, the different phases in the development of some species of Lepidoptera and Orthoptera (Canfield *et al.*, 2008; Suzuki & Nijhout, 2008; Fusco & Minelli, 2010; Beldade *et al.*, 2011; Simpson *et al.*, 2011). This last type of polyphenism is known “phase polyphenism” and was the focus of our research.

## 1.2 Phase polyphenism-inducing factors

The mechanism used by the individual to perceived and translated environmental stimuli, as well as the nature of these stimuli have been the focus of research and discussion for a long time (Kaiser *et al.*, 2014). Within the phenomenon of phase polyphenism, some induction mechanisms are known (Badyaev, 2005; Whitman & Agrawal, 2009). Generally, hormonal processes are pointed out in the literature as induction mechanisms in several species (Nijhout, 1999; Boerjan *et al.*, 2011). Hormones such ecdysteroids and juvenile hormone (JH) contribute to the phase change in *Schistocerca gregaria* (Hägele *et al.*, 2004) and *Anticarsia gemmatalis* (Fescemyer & Hammond, 1988) respectively.

Other factors such as the availability of resources and population density have been suggested as factors (Gunn, 1998; Bailey *et al.*, 2005). Population density has been considered as a key factor for the induction of several changes in the phenotype, genotype and behaviour of animals (Roessingh *et al.*, 1998; Leo Lester *et al.*, 2005; Cotter *et al.*, 2008; Ma *et al.*, 2011).

According to Applebaum and Heifetz (1999), Boris Uvarov was the first to register density-dependent phase polyphenism in *Schistocerca gregaria* (locust) and

ever since this has been one of the most studied examples of phase polyphenism (Sword & Simpson, 2000; Simpson *et al.*, 2011). *Schistocerca gregaria* belongs to a group of grasshoppers of the family Acrididae (Roessingh *et al.*, 1993). One of the most distinctive characteristics of this group is the capacity to express two different phases through its development in response to changes in population density (Simpson *et al.*, 1999). Uvarov defined these phases that vary in different features including morphology, physiology, ecology and behaviour (Simpson *et al.*, 1999; Silva *et al.*, 2013). Uvarov ascribed the solitary phase to that with green nymphs with relatively sedentary behaviour and a tendency to avoid conspecifics (Simpson *et al.*, 1999). He ascribed the gregarious phase to that conformed by black nymphs with yellow or orange patches which have more active behaviour and a tendency to join with conspecifics (Bailey *et al.*, 2005; Boerjan *et al.*, 2011; Simpson *et al.*, 2011). Uvarov also noted that it is possible to find a large number of intermediate phenotypes between (Pener & Yerushalmi, 1998).

Some work with *Schistocerca gregaria* has demonstrated that density is in fact the main stimulus that induces the change (Simpson *et al.*, 1999; Rogers *et al.*, 2014). However, this process is not only about the number of individuals in a territory. An increase in density generates intraspecific competition for food, predation pressures and allows stimulus such as volatile and contact pheromones, visual and mechanical contact to contribute to this process (Sword *et al.*, 2010). Of these stimuli, mechanical contact has emerged as one of the most important (Hägele & Simpson, 2000).

Many other organisms are models for density dependent phase polyphenism research, for example, some species of aphids (Hemiptera) and Lepidoptera

(Noctuidae). In aphids, a higher density of individuals on a plant usually facilitates or increases inter- and intraspecific interactions and generates responses such as the expression of a winged phenotype in females (Prokopy & Roitberg, 2001; Castañeda *et al.*, 2010; Simpson *et al.*, 2011).

In addition, species of Noctuidae (Lepidoptera) such as *Anticarsia gemmatalis*, have density-dependent phase polyphenism that is characterized by two well-differentiated phases: solitary and gregarious (Silva *et al.*, 2013). When the larvae are reared in isolation, their tendency is to be light green and when are reared together their tendency is to be black (Fescemyer & Erlandson, 1993). There is also a wide spectrum of intermediary phenotypes in the population and some of the phase features have been considered analogous to locusts (Silva *et al.*, 2013). For *Anticarsia gemmatalis*, different factors such as plant age, humidity, temperature, have been mentioned as inducers of morphological and physiological changes (Fescemyer & Hammond, 1986; Fescemyer & Erlandson, 1993). Recently, Silva *et al.* (2013) suggested that another factor that could be critical in the phase change induction is mechanical contact; however, this has not been studied in detail.

Finally, is also important to consider that besides these stimuli, the process can be also influenced by past experiences of parental generation, genotype variations and features of development (body size, reproductive patterns) (Dingemanse & Wolf, 2013).

### 1.3 Phase change, behavioural characteristics

In phase polyphenism, the change of phase involves a number of characteristics, these mainly morphological, physiological and behavioural (Simpson

*et al.*, 1999). When behavioural characteristics are expressed, usually the species show two or more types of behaviour. The capacity that allows individuals within a population to adjust their behavioural repertoire in response to changes in environmental conditions has been defined as “behavioural plasticity” (Geva *et al.*, 2010; Kaiser *et al.*, 2014).

There are two types of behavioural plasticity: developmental and activational. The first one refers to the adaptive change when a genotype expresses different behavioural phenotypes in different environments because of different developmental pathways induced by those environments. This process includes changes in morphology and physiology. The second refers to learning process that involves changes in the nervous system because of experience. This process includes structural and functional changes in neural networks (Snell-Rood, 2013).

For our work, we focus the attention on the behavioural plasticity related to development. Simpson *et al* (1999) pointed out that the study of behaviour represents improved tool to study phenotypic plasticity because despite other more conspicuous phase change characteristics such as colour, behavioural changes are the first to be expressed during the process and they are not dependent upon moulting, so can be studied over the life of the insect.

#### 1.4 Adaptive significance of the phase change

Based on the examples cited above, phase change appears to be a positively selected in response to different selection pressures (Hochkirch *et al.*, 2008). However, situations of high density can also be stressful for these species. Increases in population density could also be reflected in food depletion or increased risk of

diseases transmitted by contact. These factors could be considered adverse for the population fitness eventually (Cotter *et al.*, 2004).

Depending on the situation, there are some hypotheses to explain the adaptive value of the phenotype change in high-density conditions (Cotter *et al.*, 2004). For example, for morphological changes such colour, Simpson *et al* (1999) suggest that in locusts the colour change can serve as a visual cue to intraspecific processes such as aggregation of nymphs. Another alternative explanation for colour change is density-dependent prophylaxis (DDP) (Stearns, 1989; Barnes & Siva-Jothy, 2000; Sword, 2002; Cotter *et al.*, 2004). In this hypothesis, the authors associated high levels of melanisation with high levels of the enzyme phenoloxidase enzyme and disease resistance (Elliot *et al.*, 2003; Silva *et al.*, 2013). They proposed that individuals belonging to the gregarious phase invest more in immune defence than those of the solitary phase and so combat the increased risk of infection at high densities (Elliot & Hart, 2010; Silva *et al.*, 2013).

For other situations such as behavioural changes, other adaptive advantages such as thermoregulation, counter-predation behaviour (aposematism or crypsis) or protection against parasitism are highlighted in the literature, but they have not been fully elucidated (Prokopy & Roitberg, 2001). Komers (1997) pointed out that what refers to behaviour; the main cause for those adaptive advantages will depend on each situation and each organism.

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**BEHAVIOURAL DISCRIMINATION OF SOLITARY AND  
GREGARIOUS PHASES OF *Anticarsia gemmatalis* HÜBNER  
(LEPIDOPTERA: NOCTUIDAE)**

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### 3. INTRODUCTION

For many organisms the capacity to deal with environment changes has an influence in their survival and reproductive success (Via *et al.*, 1995; Komers, 1997; Foster, 2013). Many species have the capacity to express their genotype in a differential way and to show different phenotypes according to their interaction with the environment (Nijhout, 1999; Simpson *et al.*, 2011). These species stand out for their great capacity to adapt to different selective pressures (Nijhout, 2003). The capacity is called "phenotypic plasticity" and is closely related to the evolutionary success of these species. It allows organisms to deal with a variety of environmental pressures, to occupy different ecological niches, to extend their geographical distribution, and to decrease the probability of extinction (Pfennig *et al.*, 2010; Snell-Rood *et al.*, 2010; Simpson *et al.*, 2011).

One type of phenotypic plasticity is called "Phase polyphenism" and is a widespread phenomenon in insects occurring in species of Lepidoptera, Orthoptera, Coleoptera and Hemiptera (Simpson *et al.*, 2011; Silva *et al.*, 2013). The phenomenon is induced by several stimuli (Komers, 1997). The increase in population density is one of the most mentioned in the literature as phase change-induction stimuli. The induction in this case may correlate with the increased likelihood of inter- or intraspecific interactions (Anazonwu & Johnson, 1986; Silva *et al.*, 2013; Vilcinskas, 2013). Examples of insects that have different density dependent phases are *Spodoptera littoralis*, *Spodoptera exempta*, *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) and *Schistocerca gregaria* (Orthoptera: Acrididae) (Silva *et al.*, 2013).

The larvae of *Anticarsia gemmatalis* are characterized by expressing three principal phases in response to changes in the environmental conditions: solitary, gregarious and intermediary (Fescemyer & Hammond, 1988; Silva *et al.* 2013). The solitary phase is composed of individuals with cuticle and cephalic capsule light green and without any black patterns. Individuals that have black cuticle and yellowish cephalic capsule compose the gregarious phase. The intermediary phase has a spectrum of colour combinations (Fescemyer & Erlandson, 1993; Silva *et al.*, 2013).

The phase change involves a complex set of characters including morphology, physiology and behaviour (Hägele & Simpson, 2000; Silva *et al.*, 2013; Simpson *et al.*, 1999). Within these characteristic one of the principal to be studied is behaviour because it is the first feature to be expressed in the process of phase change and is continuous throughout the life of an insect (Komers, 1997; Simpson *et al.*, 1999; Sword, 2003). In species of locusts for example, one of the earliest distinguishing features of phase change from solitary to gregarious is the interaction with conspecifics (Simpson *et al.*, 1999; Simpson *et al.*, 2001). When the locusts nymphs are in solitary phase they exhibit a clear repulsion by conspecifics but after phase change is induced they are attracted to conspecifics (Sword & Simpson, 2000).

As well as locusts, *Anticarsia gemmatalis* is of great importance. From a biological point of view this is because the different phenotypes expressed confer a greater capacity for to generate an adaptive response to environmental changes. From an economic point of view this capacity has allowed them to exploit diverse crop plants such as lettuce, peanuts, rice, peas, beans, wheat and soybean, causing major problems and yield losses (Sosa-Gómez, 2004; De Bortoli *et al.*, 2005; Fugii *et*

*al.*,2005; Praça *et al.*, 2006). Despite this neither the induction mechanisms nor the behavioural repertoires of each phase have been described.

For these reasons, the objectives of our research were: (1) to determine the behavioural repertoires of the two extreme phases of *Anticarsia gemmatalis*: solitary and gregarious, and (2) to evaluate how mechanical and chemical stimuli applied to solitary phase larvae might induce changes in behavioural or morphological characteristics (cuticle and cephalic capsule larval colour).

## 4. METHODOLOGY

### 4.1 *Anticarsia gemmatalis* rearing conditions

In the laboratory, the *Anticarsia gemmatalis* population was established using first instar larvae. First, we put each larva in a 50 ml disposable plastic cup with artificial diet *ad libitum*. The diet was made according to Hoffman-Campo *et al.* (1985). Plastic cups were used to reduce the risk of contamination and to reduce labour in the laboratory (Hoffman-Campo *et al.*, 1985).

The cups containing the larvae were kept in an incubator under controlled conditions ( $25 \pm 5$  °C and 12:12 light-dark photoperiod) until the final instar. The pupae were placed in glass cages and kept in a rearing room ( $25 \pm 5$  °C and  $70 \pm 5\%$  RH with a 12:12 light-dark photoperiod), until adult emergence. Once emerged, moths remained in the glass cage and were fed daily with water, beer, honey and sucrose solution (Hoffman-Campo *et al.*, 1985). White paper sheets were placed inside the cage to serve as substrate for the oviposition of the moths. Paper sheets were exchanged approximately every two days with a new one. The paper sheets with eggs were separated, placed in a 500 ml plastic container and, maintained with artificial diet under controlled conditions until hatching. The whole process was repeated in order to maintain the laboratory population.

For the experiments, we needed a continuous supply of larvae of the two phases: solitary and gregarious. These phases were obtained by rearing the larvae in densities of one (solitary) and four (gregarious) per cup. The two groups were maintained in a physical, visual and olfactory isolation but with the same temperature, humidity, photoperiod and artificial diet. For the bioassays we used larvae of third and fourth instar i.e. approximately eight days old.

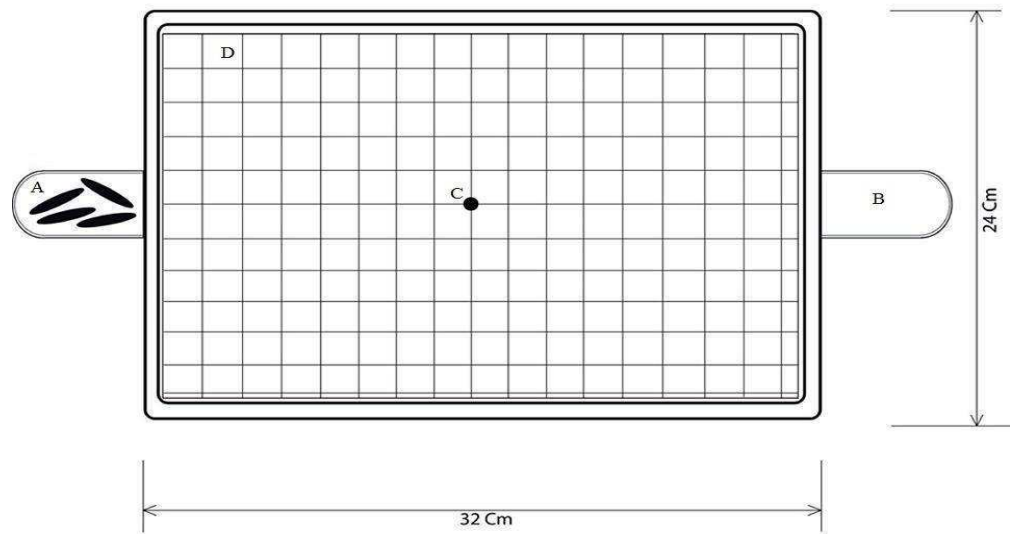
## 4.2 Behavioural assays

### 4.2.1 Bioassay 1: Behavioural characterization of solitary and gregarious phases of *Anticarsia gemmatalis*.

To accomplish the first objective we set up an experimental arena using a white plastic tray with the following dimensions: 32 cm x 24 cm x 7 cm. In the right and left sides of the tray, we made a hole in which were placed the stimulus and no-stimulus test tubes. The stimulus tube contained 25 gregarious phase larvae and the no-stimulus tube was empty (adapted from Simpson *et al.*, 1999). The position of these tubes was changed between test individuals. The tray walls were covered with tape and with red cellophane paper, we made a cover to prevent light stimulus and at the same time facilitate observation.

For the test, we selected a single larva and then we placed it in the centre of the experimental arena. We kept it under observation for 780 seconds and recorded by hand all the behaviours shown by the individual. In this fashion, 100 larvae were evaluated (50 of each phase). Figure 1 shows the experimental arena.

During a pilot test we record the following behaviours characteristics: 1) position of the stimulus in the arena, 2) walking distance, 3) time for the larvae to start walking in the arena, 4) number of walking events, 5) number of head movements, and 6) final position of the insect in the arena with respect to the stimulus. Therefore, these were the behavioural characteristics used during the bioassay 4.2.1 to describe the behaviour of the each phase.



**Figure 1.** Basic schema of the experimental arena (top view). In the scheme the letters A, B, C and D. represent the principal points of the experimental arena. A) Tube with the stimulus group B) Tube without the stimulus group C) Point of larvae positioning in the beginning of each bioassay, and D) Graph paper located at the bottom of the arena to help with the record of the direction and total distance walked by the larvae. Were evaluated 100 larvae of *Anticarsia gemmatalis*, 50 reared alone and 50 reared in groups. The stimulus group was made up of 25 gregarious larvae.

Laboratory conditions during observations were similar in lighting, humidity and temperature. The number of walking events and number of head movements were measured using a tally counter. The time was measured using a stopwatch. The distance and final position were measured with the aid of a numbered millimetre paper placed on the floor of the experimental arena. Each animal was tested only once in the behavioural assay. It is important to note that with some individuals behavioural bioassays were ended in less than 780 seconds. This was applied under the following conditions: 1) if the larva came into contact with the stimuli tube or 2) after 180 continues seconds of larval inactivity. These last data was discarded. In Table 1 a detailed description of each behavioural variable is given.

**Table 1.** Description of the behavioural variables evaluated for *Anticarsia gemmatalis* larvae in the experimental arena.

<b>Behavioural variables</b>	<b>Description</b>
Stimulus position	Position of the stimulus in the sides of the experimental arena (right or left). Exchanged between each test.
Walking distance	Number of centimetres walked by larvae during the total time in the arena.
Number of walking events	Number of times when the larva walked. A new event was considered when the larva did not walk for at least 5 seconds.
Initial time at rest	Time in seconds for which the larva remained motionless in the centre of the arena before its first walk event.
Number of head movements	Number of times that the larva moved its head to one side or another.
Final Larva position	Position of the larva at the end of the bioassay. This variable was registered with values 0 or 1. The zero value indicated that the larva was in the opposite direction of the stimulus and the one value indicated that direction of the larva was in favour of the stimulus.

#### 4.2.2 Bioassay 2: Colour characterization of the head capsule and cuticle of different phases of *Anticarsia gemmatalis*.

In order to have a control that help with the recognition and discrimination of the *Anticarsia gemmatalis* larvae used for the experiments, ninety larvae were selected, thirty from each phase: green, black and intermediary. These larvae were photographed one by one, with a Power Shot A640 camera attached to the Zeiss Axioskop 40 light microscope.

Then, using the ImageJ program 1.42q the photos were converted to grey scale, which allowed us to measure the mean value of lightness on a scale. The lightness scale ranges in values for 0 to 255, where values closer to zero are considered darker or near to black. The degree of lightness was measured for two body regions of the each larva: cuticle and head capsule.

#### 4.2.3 Bioassay 3: Behavioural responses of *Anticarsia gemmatalis* to chemical stimulation.

To evaluate the response of the *Anticarsia gemmatalis* larvae to chemical stimulation, 160 uniformly light green larvae were selected. Those larvae were divided into four groups of 40. Three groups were used for treatment and one for control. The three treatments were performed using cuticular hydrocarbons as stimulus. The cuticular hydrocarbons were extracted by submerging approximately 30 larvae in 50 ml of dichloromethane for 10 minutes (adapted from Hägele & Simpson, 2000). Using a graduated pipette the extract was placed on a filter paper that was at the bottom of a Petri dish and the larvae were placed to walk for 12 hours.

For treatment 1, the cuticular hydrocarbons were extracted from *Anticarsia gemmatalis* black phase larvae. For treatment 2 these were extracted from larvae of *Spodoptera frugiperda* (reared under the same conditions as *Anticarsia gemmatalis*). For treatment 3, these were extracted from *Anticarsia gemmatalis* green phase larvae. The control was done by just applying dichloromethane on filter paper. After 12 hours having installed the experiment, the larvae were evaluated in order to find indicators of a possible phase change. For this purpose we evaluated the behaviour and the final colour of the larvae after the completed bioassay.

To evaluate behaviour we used the same method as in the bioassay 4.2.1 and evaluating the same variables. Once the behavioural analysis was completed we evaluated the colour. To do this, photographic images of each larva were recorded using the same method as in the bioassay 4.2.2.

#### 4.2.4 Bioassay 4: Behavioural responses of *Anticarsia gemmatalis* to the mechanical stimulation

To evaluate the induction of phase change by a mechanical stimulus we selected 120 uniformly light green larvae. The larvae were divided into four groups, three with a stimulus and a fourth without stimulus (control). The groups with stimulus were denominated A, B and C. In group A, the stimulus was applied to the head of the larva. In group B this was applied in the third abdominal segment (at the height of the first pair of legs), and in group C this was applied in final segment of the abdomen (adapted from Simpson *et al.* 2001).

The stimulus consisted in 30 touches made with a nylon wire (simulating contact). The stimulus was done every half hour for 4 continuous hours (adapted from Simpson *et al.*, 2001). After finishing the test, we evaluated the larvae in order to find indicators of a possible phase change. For this purpose we evaluated the behaviour and the final colour of the larvae. The behavioural repertoire of each larva was described using the same method and measuring the same variables as in the bioassay 4.2.1. After 24 hours, larvae were also photographed to measure the lightness of the cuticle and head capsule using the same method as in the bioassay 4.2.2. It was evaluated 24 hours later to try to register possible late effects on colour changes (Simpson *et al.*, 2001).

#### 4.3 Statistical analysis

#### 4.3.1 Selection of behavioural discriminators of the solitary and gregarious phases of *Anticarsia gemmatalis*.

In order to know if the solitary and gregarious phases of *Anticarsia gemmatalis* are different in their behaviours, the data collected in the experimental arena was analysed. For this analysis we performed a logistic regression using the statistical program SPSS. The program regressed the response variable against a series of explanatory variables (behavioural parameters). The explanatory variables used were: stimuli position, walking distance, number of walking events, initial time at rest, number of head movements and final larva position. The response variable was the phase. This regression type is the most appropriate in this case because the response variable "phase" is binary or dichotomous. The logistic regression analyses are widely recommended for such behavioural studies (Roessingh *et al.*, 1993; Rogers *et al.*, 2014; Simpson *et al.*, 1999). These analyses make no assumptions about the distribution of the data (Roessingh *et al.*, 1993).

#### 4.3.2 Selection and model fit

The first step in the logistic regression analysis was to create an initial model including all explanatory variables as a function of the response variable (Simpson *et al.*, 1999). After obtain the *p*-values for each variable, the initial model was simplified to its most parsimonious using the 'stepwise elimination' procedure, which is commonly recommended by statistical texts and manuals (Hardy & Field, 1998). That final version was called final model.

Once the final model was established and the behavioural predictors selected, the adjustment of the model was estimated. For this we performed the Hosmer and Lemeshow test. This test measures the correlation of observed and

predicted values of the response variable. In this case, the best model adjustment is indicated by a smaller difference between the observed and predicted values. A good model fit is indicated by a chi-square value not significant (Hosmer *et al.*, 1997).

#### 4.3.3 Probability of being classified as solitary

After stabilizing the final model and identify the behavioural variables that help to discriminate between behaviours of the *Anticarsia gemmatalis* phases we used a logistic algorithm derived from the final model to calculate the probability of larvae resulting for the bioassays 3 and 4 to be classified as solitary after chemical and mechanical stimulus (Hardy & Field, 1998). We compared the effect of the stimulus evaluating changes in larvae behaviour and colour. For this we performed an analysis of variance (ANOVA) using statistical software R. Throughout this study we used the term “Psolitary” to indicate the probability of being classified as solitary larvae.

#### 4.3.4 Colour characterization of the solitary and gregarious phases of *Anticarsia gemmatalis*.

With the colour results for bioassays 3 and 4 we was performed an analysis of variance (ANOVA) using statistical software R to compare the treatments of each bioassay.

## 5. RESULTS

### 5.1 Behavioural characterization of solitary and gregarious phases of *Anticarsia gemmatalis*.

The test in the arena allowed discriminated the behaviour characteristics of the solitary and gregarious phases of *Anticarsia gemmatalis*. In the table number 2, we present the results obtained in the initial model.

**Table 2.** Behavioural variables retained in the logistic regression initial model from solitary and gregarious larvae of *Anticarsia gemmatalis*.

Behavioural variables	$\beta$	S.E.	Wald	df	p-value	Exp( $\beta$ )
Stimulus position	0,013	0,432	0,001	1	0,975	1,013
Walking distance	0,006	0,007	0,731	1	0,393	1,006
<b>Number of walking events</b>	<b>-0,075</b>	<b>0,028</b>	<b>7,008</b>	<b>1</b>	<b>0,008</b>	<b>0,928</b>
Initial time at rest	-0,013	0,008	2,817	1	0,093	0,987
Number of head movements	0,007	0,004	3,297	1	0,069	1,007
Final larval position	-0,638	0,433	2,176	1	0,140	0,528
Constant	1,130	0,667	2,871	1	0,090	3,096

All these values are calculated for solitary phase *Anticarsia gemmatalis* larvae. The  $\beta$  value indicates the relation between the explanatory and response variable, the sign (positive or negative) indicates the direction of the relationship. S.E values are the standard errors. Wald is a statistical that allows comparing the maximum likelihood estimate of the behavioural parameters and its estimate standard error, higher values than four indicate that the explanatory variable explains the variable response. df values are the degrees of freedom of the model. p-value is the measure of significance of variable in the model. Exp( $\beta$ ) values indicates the strength of the relationship between variables, values more distant of 1 indicate a stronger numerical relationship. For an explanation of the behavioural variables, see table 1.

The results of Initial model indicated that the only variable that significantly explained the response variable was the number of walking events ( $p = 0.008$ ). Regarding to the negative sign of the coefficient  $\beta$  (-0,075) it indicates that solitary phase larvae are negatively related to this variable. This means that solitary phase larvae performed a significantly smaller number of numbers of walking events than

gregarious phase larvae. The Wald statistic value above 4 indicates that the explanatory variable is appropriate to describe the behaviour. Once after getting that result, they were removed one by one the variables in order to optimize the process. The results of the final model are in table 3.

**Table 3.** Behavioural variables retained in the logistic regression final model from solitary and gregarious larvae of *Anticarsia gemmatalis*.

<b>Behavioural variables</b>	<b><math>\beta</math></b>	<b>S.E</b>	<b>Wald</b>	<b>df</b>	<b>p-value</b>	<b>Exp(<math>\beta</math>)</b>
<b>Number of walking events</b>	<b>-0,061</b>	<b>0,023</b>	<b>6,968</b>	<b>1</b>	<b>0,008*</b>	<b>0,941</b>
<b>Number of head movements</b>	<b>0,008</b>	<b>0,004</b>	<b>4,590</b>	<b>1</b>	<b>0,032*</b>	<b>1,008</b>
Constant	0,316	0,480	0,433	1	0,511	1,371

All these values are calculated for solitary phase *Anticarsia gemmatalis* larvae. The  $\beta$  value indicates the relation between the explanatory and response variable, the sign (positive or negative) indicates the direction of the relationship. S.E values are the standard errors. Wald is a statistical that allows comparing the maximum likelihood estimate of the behavioural parameters and its estimate standard error, higher values than four indicate that the explanatory variable explains the variable response. df values are the degrees of freedom of the model. p-value is the measure of significance of variable in the model. Exp( $\beta$ ) values indicates the strength of the relationship between variables, values more distant of 1 indicate a stronger numerical relationship. For an explanation of the behavioural variables see table 1.

Logistic regression final model enable to select two of the six variables included in the initial model. These two variables: number of walking events (p=0,008) and number of head movements (p=0,032) are considerate as predictive variables for the behaviour of the larvae (Table 3).The results showed that although the two phases share a similar behavioural repertoire, there have also differences. Solitary phase larvae have a lower number of walking events than the gregarious phase larvae. On the contrary, the solitary phase larvae differ from gregarious phase for the large number of head movements.

The adjustment of the logistic regression model was calculated comparing the predicted values to the observed values using the Hosmer and Lemeshow test. The result ( $X^2=6,768$ ; p=0.562) indicated that the observed and estimate values are not significantly different, so the model is correctly fitted. The final model was able to classify 65% of the total assessed larvae correctly. It classified 29 as gregarious

phase larvae (58%) and 36 as solitary phase larvae (72%) of the initial 100 larvae (table 4).

**Table 4.** Observed and expected phase predicted values to *Anticarsia gemmatalis* phases and its percentage of correct classification based on the logistic regression final model.

Observed phase	Expected phase		Correct percentage
	Gregarious	Solitary	
Gregarious	29	21	58%
Solitary	14	36	72%
Overall percentage			<b>65%</b>

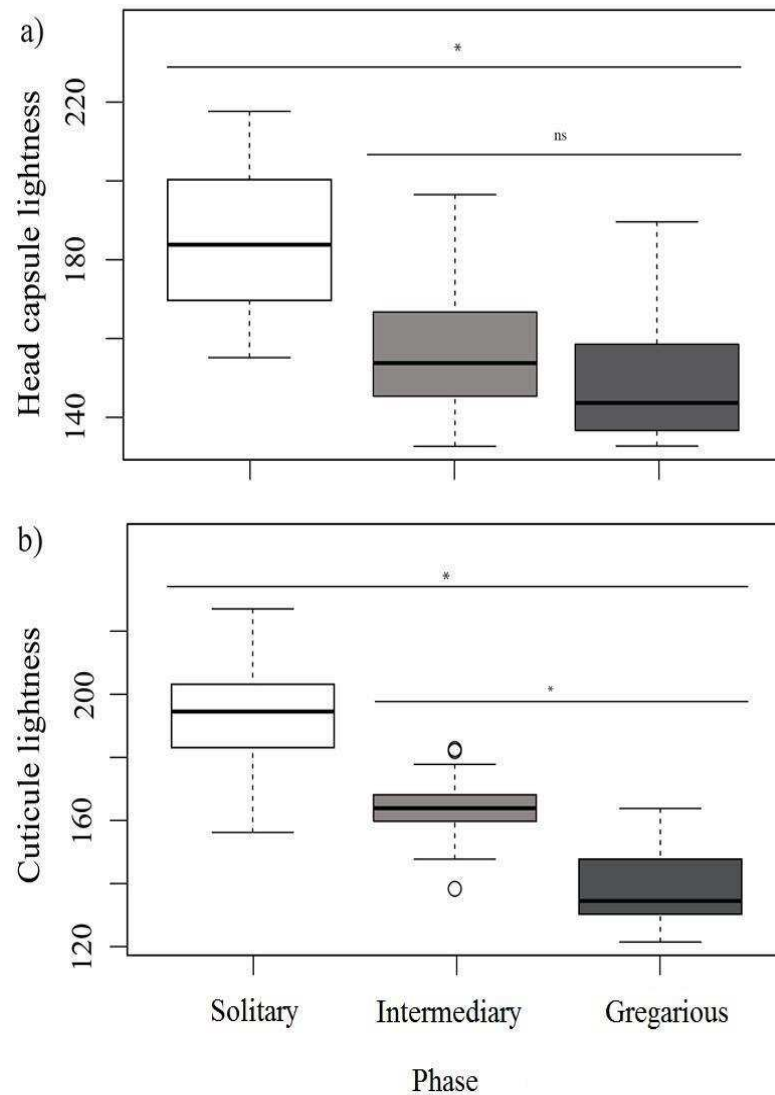
N=100 *Anticarsia gemmatalis* larvae (50 of each phase)

## 5.2 Colours characterization of solitary and gregarious phases of *Anticarsia gemmatalis*.

The results for a colour characterization of the *Anticarsia gemmatalis* phases are represent in the Figure 2. In this figure is possible to observe that indeed there is a significant difference between the lightness of the cuticle and the cephalic capsule from solitary, gregarious and intermediary phase larvae. These data reflects natural tendency of the species, so the solitary phase larvae are different both in cuticular and cephalic capsule lightness of the intermediary and gregarious phase larvae. The solitary phase larvae show lightness values closer to zero (or white) according with the established scale. In contrast, the gregarious phase larvae show lightness values closer to 255 (or black). The results were used as a control to have one objective morphological measure of each phase to compare them.

## 5.3 Phase change induction by mechanical and chemical stimulus

The results obtained for induced change are summarized in table 5. We calculated the mean values and standard error for each treatment and the treatments were compared by ANOVA analyses.



**Figure 2.** Distribution of lightness cuticle and the head capsule values calculated for three larval phases of *Anticarsia gemmatalis*: solitary, gregarious and intermediary. N = 30 for each phase. (\*) significantly different (ns) no significantly different.

In general, for mechanical stimulation there was no statistically significant response to the phase change, in any treatment. Neither in behaviour nor in colour change (lightness). No significant results were obtained for treatments relating to chemical stimulation either. In conclusion, these specific types of stimuli were not effective to induce the phase change in *Anticarsia gemmatalis*.

**Table 5.** Effect of mechanical and chemical stimulation on the induction of *Anticarsia gemmatalis* phase change.

<b>Variable</b>	<b>Stimulus</b>	<b>Mean</b>	<b>SD</b>
<b>Mechanical</b>			
P(Solitary) N=120	Control	0.5733	0.0174
	Head	0.5893	0.0138
	Third abdominal segment	0.5910	0.0141
	Last abdominal segment	0.5903	0.0154
		<b>p-value</b>	<b>Deviance</b>
ANOVA		0.8188	0.0065
<b>Chemical</b>			
P(Solitary) N=160	Control	0.5625	0.0116
	Treatment 3	0.5740	0.0079
	Treatment 1	0.5830	0.0137
	Treatment 2	0.5713	0.0103
		<b>p-value</b>	<b>Deviance</b>
ANOVA		0.6272	0.0086
<b>Mechanical</b>			
Head capsule lightness N=120	Control	179.3	5.4428
	Head	178.3	5.1612
	Third abdominal segment	167.8	4.6831
	Last abdominal segment	172.6	6.0409
		<b>p-value</b>	<b>Deviance</b>
ANOVA		0.5091	1600.1
Cuticle lightness N=120	Control	159.7	4.9076
	Head	162.3	4.2062
	Third abdominal segment	152.3	4.7521
	Last abdominal segment	156.0	5.2114
		<b>p-value</b>	<b>Deviance</b>
ANOVA		0.3993	2559.2
<b>Chemical</b>			
Head capsule lightness N=160	Control	166.35	4.4031
	Treatment 3	170.30	3.8503
	Treatment 1	172.0	3.9616
	Treatment 2	170.6	3.9964
		<b>p-value</b>	<b>Deviance</b>
ANOVA		0.7146	0.4543
Cuticle lightness N=160	Control	161.0	5.2448
	Treatment 3	163.4	4.8044
	Treatment 1	167.7	4.4262
	Treatment 2	160.3	5.3892
		<b>p-value</b>	<b>Deviance</b>
ANOVA		0.7836	0.3578

Results of behaviour and colour are presented like P(solitary) and lightness values respectively and are expressed as means and standard error and compared by ANOVA.

## 6. DISCUSSION

During this investigation we evaluated both the behavioural characteristics of the larvae of solitary and gregarious phases of *Anticarsia gemmatalis* as well as the role some chemical and mechanical stimuli in phase change induction in this species. Our findings provided evidence that the solitary and gregarious phases of *Anticarsia gemmatalis* that our laboratory population can be separated based on their behaviour in the bioassay arena.

The results indicated that the behavioural repertoires of both phases are composed of at least six conspicuous behavioural variables. Two of those variables can be used to discriminate the phases. One is the number of walking events and the other the number of head movements. Although these are common behaviours, the frequency of each was different in each phase. The solitary larvae have a greater number of head movements and the gregarious larvae a greater number of walking events. According to Ewing & Manning (1967), this may be due to differences in genotype expression.

In the first place, we expect that the differences in the number of walking events and number of head movements could be associated with the lifestyle of each phase and could confer to individuals adaptive advantages related to the situations that are commonly experienced by them. For example, a smaller number of walking events could allow to green phase larvae to pass unnoticed in the presence of predators and this behaviour could be supported by its green colour that blends with the green of the leaves (Chambers *et al.*, 1996; Sword & Simpson, 2000).

On the other hand, gregarious phase individuals become more conspicuous to predators. The adaptive role of dark colour and active movement has been widely studied in locusts (Sword & Simpson, 2000). In this case, the colour could be associated with aposematism (Despland & Simpson, 2005b). Gregarious nymphs commonly feed on plants with toxins in contrast with solitary nymphs and are associated by predators with little or no palatability (Sword *et al.*, 2000; Despland & Simpson, 2005a).

In the case of *Anticarsia gemmatalis* there is so far no evidence of an aposematic function, so the colour can be explained in other ways (Sword & Simpson, 2000). As pointed out by some authors the dark colour patterns may be functioning as visual cues for the localization of conspecifics (Sword & Simpson, 2000; Despland & Simpson, 2005b). As *Spodoptera exempta*, *Anticarsia gemmatalis* larvae have a tendency to express black coloration at low population densities (Silva *et al.*, 2013) which might suggest that this is an important adaptive advantage for both species (Gunn, 1998).

With respect to the direction of walking into the experimental arena, neither of the two *Anticarsia gemmatalis* phases walks with any apparent pattern. In natural conditions some species follow temporal patterns of random movement while foraging whereas others move in directed spatial patterns (Shapiro-Ilan *et al.*, 2014). We believe that an increase in walking activity could be a strategy of gregarious phase larvae to compensate the apparent lack of olfactory location for conspecifics. A greater number of walking events without a pattern could result in a greater probability to find conspecifics randomly.

With respect to head movements, our results show that *Anticarsia gemmatalis* solitary phase larvae performed a higher number than gregarious phase larvae. Dethier (1942) pointed out that lepidopteran larvae are capable of detecting light using their sensory organs called stemmata. Both phases perform movements with their heads from one side to other. This behaviour is called searching in the literature and is done by the larvae in order to scanning their surrounding environment and orient themselves (Bell, 1990; Wicklein, 2003). Searching behaviour represents the confluence of three kinds of factors: biological characteristics and abilities to perceive sensory information of insects, environmental factors (availability of resources, risk of predation), internal factors, determining what an individual needs (Bell, 1990). Our results show differential performance of head movements between the phases which could suggest that there is a difference in the perception of stimuli between them (but have no evidence for this at the moment).

In general, these *Anticarsia gemmatalis* behavioural results are similar to the results found for *Spodoptera* larvae and *Schistocerca gregaria*. It has been reported that those larvae and nymphs are more active when they are reared in groups than when reared in isolation (Hodjat, 1970; Despland & Simpson, 2005b).

Regarding the method used to analyse the behaviour, the logistic regression final model predicted correctly the phase of 65% of the total larvae. This percentage indicated that the model is efficient to identify differences between the two phases, but could be improved. There are other factors that we considered important and could contribute the improvement of the model to a better discrimination between *Anticarsia gemmatalis* phases as for example the walking speed.

The second objective of our work was to assess whether certain mechanical and chemical stimuli contributed to the phase shift in this *Anticarsia gemmatalis*. Our results showed that neither chemical nor mechanical stimuli applied to solitary larvae had a primary role in the induction of phase change process and this may be due to several factors. For mechanical induction it is known that tactile stimuli is an important factor for phase changes in different species but their efficacy will depend on the stimulation site (Rogers *et al.*, 2003). We applied the stimuli in the head, the third abdominal segment and in the last abdominal segment. These body regions were selected for methodological practicality, but the stimuli do not have an induction response. These results could be reflect that body areas as: head, legs, thorax and abdomen should not cause pronounced behavioural phase change, because these structures are regularly stimulated by the animal itself during normal feeding, and walking activity (Simpson *et al.*, 2001). In addition, the intensity of the stimulus could be related, while some authors applied touch stimulus during five seconds every 60 seconds, we applied the touches every half hour.

For chemical induction, the cuticular hydrocarbons were used as stimulus due to their high specificity (Howard & Blomquist, 2005). However, our results showed no role for this type of chemical stimulation in induction of the phase change for *Anticarsia gemmatalis*. Because this method had positive results in other species (Heifetz *et al.*, 1996), we assumed that these results could be affected by the procedures for extracting the cuticular hydrocarbons. There were methodological differences between our experimental protocol and that of Heifetz *et al.*, (1996) as regarding to the cuticular hydrocarbons concentration applied.

About colour-change results, there is not a positive correlation between stimulus and the induction treatments. It has been reported the *Anticarsia gemmatalis* real phase did not appear until after it had moulted to the last instar (Fescemyer & Hammond, 1986). Because we measured the colour variations four hours later (mechanical stimulation) and 12 hours later (chemical stimulus) it could be that the time required for the result to be apparent and measurable was not enough in either case.

In general, other factors could be affecting our results. First, the rearing histories prior to capture of the *Anticarsia gemmatalis* larvae were unknown. There is a possibility that the variation in behaviour observed was due to uncontrolled differences in parental effects between populations (Sword, 2003). Secondly, it has been reported that larvae phase change occur in species that are actively gregarious, and although it has a tendency, *Anticarsia gemmatalis* is not considered a gregarious species (Anazonwu & Johnson, 1986). Third, the chance process could occur over many timescales some characters change rapidly while others change slowly through accumulation across generations (Simões *et al.*, 2013), however we evaluated only one instar of one generation. Fourthly, many insects have a critical period in development when the individuals are sensitive to induction stimuli, so there is the possibility that the stimulation period chosen (3rd-4th instar) was not adequate for this species.

In addition, phase change induction besides being the result of the correlation between the signal, the environment and the body (Simpson *et al.*, 1999; Sword, 2003) could be reflecting tendencies related to polymorphism. This means

distinct morphological differences occur simultaneously among conspecific individuals that are under the strict control of the genotype (Pener, 1991).

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