FÁBIO DE ASSIS PINTO

SYMBIONTS AND IMMUNE DEFENCE IN LEPIDOPTERAN CROP PESTS

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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RESUMO

PINTO, Fábio de Assis. D.Sc., Universidade Federal de Viçosa, Julho de 2016. **De identificação ao controle: análises moleculares e bioquímicas como ferramentas em estudos de noctuídeos pragas.** Orientador: Simon Luke Elliot. Coorientadores: Kenneth Wilson e Robert Graham

Anualmente, lepidópteros noctuídeos são responsáveis por bilhões de dólares em perdas na agricultura mundial. Várias espécies estão em constante dispersão ao redor do mundo e geram grande preocupação devido à resistência a pesticidas químicos e culturas Bt. Esses fatores reforçam a importância no desenvolvimento de tecnologias que permitam desde o rápido diagnóstico dessas pragas no campo a métodos de controle específicos e seguros ao meio ambiente. Aqui, demonstramos que ferramentas de biologia molecular e bioquímica podem ser de grande auxílio em diferentes vertentes no estudo dessas pragas. Inicialmente, utilizando PCR, PCR-RFLP e sequenciamento, confirmamos a presença da lagarta do velho mundo *Helicoverpa armigera* no estado de Minas Gerais e avaliamos a prevalência e diversidade genética de simbiontes relevantes no controle biológico de noctuídeos como densovírus, baculovírus e *Wolbachia*. Em segundo, verificamos que expressão gênica de profenoloxidase, uma enzima crucial na defesa imune de noctuídeos, não varia de acordo com a idade e densidade populacional em condições naturais. Adicionalmente, verificamos que metodologias com base em RNA de interferência (RNAi) não silenciaram esse gene, enquanto que uma das técnicas contrariamente aumentou sua expressão. Por fim verificamos que em *Spodoptera littoralis*, noctuídeo que apresenta profilaxia dependente da densidade e plasticidade fenotípica, quando criados de forma gregária, apresentam maiores níveis de defesa imune somente na presença de um invasor na hemocele. Verificamos também que a atividade bioquímica das principais defesas humorais varia de acordo com a espécie e condição da bactéria invasora, demonstrando sinais de especificidade em seu sistema imune.

ABSTRACT

PINTO, Fábio de Assis. D.Sc., Universidade Federal de Viçosa, July, 2016. **From identification to control: molecular and biochemical tools in noctuid pests studies.** Adviser: Simon Luke Elliot. Co-advisers: Kenneth Wilson and Robert Graham

Noctuid lepidopterans are responsible for billions of dollars in economic losses across worldwide agriculture. These pest species are constantly being dispersed and generate great concern due their resistance against chemical pesticides and Bt crops. These factors emphasize the importance to the development of technologies for rapid field diagnosis and specific environmentally-safe pest control. Here we show that different molecular and biochemical tools can be widely useful in studies of these pests. First, using PCR, PCR-RFLP and gene sequencing, we confirmed the presence of the Old World bollworm *Helicoverpa armigera* in Minas Gerais state and identified the prevalence and genetic diversity of symbionts with potential use for noctuid biocontrol such as densovirus, baculovirus and *Wolbachia*. Secondly, we identified that in non-infected individuals prophenoloxidase gene expression (a crucial enzyme of noctuid immune defence) did not vary with age or population density. Additionally, we verified that gene knockdown methodologies based on interference RNA (RNAi) did not silence this gene; in fact one of them contrary increased its expression. Lastly, we found that *Spodoptera littoralis*, another noctuid that exhibits density-dependent prophylaxis and phase polyphenism, presents higher immune levels in the presence of a haemocoel invader when reared gregariously. We also found that biochemical activity of main humoral immune defences of this species vary according to species and condition of invader bacteria showing signals of immune specificity.

SUMÁRIO

Introdução

Insetos noctuídeos (Lepidoptera: Noctuidae) são considerados uma das principais pragas na agricultura mundial (Culliney 2014). Estima-se que espécies como a lagarta do velho mundo *Helicoverpa armigera* (Hübner, 1805), recentemente identificada no Brasil, cause mais de cinco bilhões de dólares de prejuízos anuais com seu controle (Lammers & Macleod 2007). De modo geral, vários noctuídeos pragas como *H. armigera*, *Spodoptera frugiperda* (Smith, 1797) e *Chrysodeixis includes* (Walker, 1857) possuem diversas características que dificultam seu controle. Várias dessas espécies são polífagas atacando diversas culturas de interesse econômico, podendo também utilizar como refúgio diferentes espécies de plantas não cultiváveis (Ramnath, Chitra & Uthamasamy 1992; Specht, Paula-Moraes & Sosa-Gómez 2015). Além disso, muitas dessas possuem habilidade de se deslocar centenas de quilômetros em uma única noite através de correntes de vento (Widmer & Schofield 1983; Feng *et al.* 2004).

Durante décadas, pesticidas químicos foram utilizados como principal forma de controle de pragas ao redor do mundo. Entretanto, problemas relacionados à resistência de insetos a vários princípios ativos levaram a busca de novas estratégias de controle (Casida & Quistad 1998). No início dos anos 90, através da utilização da tecnologia de transgenia, iniciou-se o desenvolvimento de linhagens de plantas que expressam toxinas originalmente produzidas pela bactéria *Bacillus thuringiensis* (Berliner, 1911) (Roessing & Lazzarotto 2015). Inicialmente a utilização de culturas de milho e soja Bt mostrou-se eficaz no controle de noctuídeos, porém sinais de resistência a esse método geram grandes preocupações no futuro do manejo dessas pragas (Fox 1996). Em diferentes partes do mundo há relatos de populações de espécies como *H. armigera* e *S. frugiperda* resistentes a diferentes toxinas Bt (Tabashnik *et al.* 2003; Tabashnik *et al.* 2008; Tabashnik, Brévault & Carrière 2013). Recentemente, populações de *S. frugiperda* resistentes à toxina Bt Cry1Fa foram também identificadas no Brasil (Farias *et al.* 2014), demonstrando que o fenômeno se torna cada vez mais recorrente.

Como alternativa aos métodos químicos e transgênicos, o controle biológico por sua vez possui diversas vantagens como: (i) especificidade (ii) menores riscos de aquisição de resistência e (iii) possibilidade de utilização com outros métodos de controle, porém ainda possui certas limitações (Huffaker 2012). Por sua vez, ferramentas de biologia molecular mostram-se aliadas promissoras para o aumento da acessibilidade e eficácia do controle biológico. Através da técnica de reação de cadeira da polimerase (PCR), estudos são capazes identificar espécies de pragas (Leite *et al.* 2014), linhagens resistentes a pesticidas (Rajagopal *et al.* 2009) e até mesmo entomopatógenos específicos (Graham & Wilson 2012), que podem ser usados no controle biológico. Com a utilização de técnicas de PCR e sequenciamento, agentes de controle biológico que são utilizados com grande sucesso no controle de noctuídeos, como baculovírus *Spodoptera littoralis nucleopoliedrovirus* (Breitenbach *et al*. 2013) foram identificados e isolados. Tais ferramentas possuem também grande utilidade em aspectos aplicados. Estudos focados no material genético de baculovírus identificaram genes relacionados às toxinas de diferentes linhagens desse vírus, proporcionando a recombinação gênica de diferentes linhagens e aumentando a eficácia desses agentes no campo (Blissard & Rohrmann 1990).

Em uma outra abordagem, estudos dos mecanismos de defesa em noctuídeos são também de grande importância para o sucesso do controle biológico. Atuantes no sistema imune humoral, enzimas como fenoloxidase e lisozima possuem papel crucial na defesa contra agentes de controle biológico (Grizanova *et al.* 2014). Estudos demonstram que a eficácia e tempo de ação de biopesticidas podem ser comprometidos pela ação desses mecanismos de defesa (Ambethgar, 2009), afetando assim competitividade do controle biológico com demais métodos de controle. Nesse contexto, o conhecimento de fatores específicos de imunidade em noctuídeos poderia por exemplo avaliar em qual idade esses insetos possuem níveis mais baixos de defesas humorais, consequentemente proporcionando aumento de eficácia no controle biológico (Bulmer *et al.* 2009).

Recentemente, com o desenvolvimento de técnicas baseadas em RNA de interferência (RNAi), metodologias para o silenciamento de genes relacionados ao sistema imune de várias pragas estão sendo desenvolvidas (Terenius *et al.* 2011), o que proporciona uma grande oportunidade para o aprofundamento no conhecimento das defesas humorais e como elas interagem com agentes de controle biológico. Através da síntese de uma fita simples de RNA complementar ao mRNA do gene alvo, torna-se possível bloquear a expressão de enzimas como a profenoloxidase e identificar seu efeito na susceptibilidade a agentes de controle biológico como bactérias entomopatogênicas. Embora exista um longo caminho para o emprego dessa tecnologia no controle de pragas no campo, como perspectiva essas moléculas silenciadoras atuariam aumentando a eficácia dos agentes de controle biológico, permitindo a diminuição do volume de aplicações, algo que pode representar um novo patamar no uso de microrganismos como forma controle de pragas (Di Lelio *et al.* 2014; Thakur, Mundey & Upadhyay 2016).

A utilização de tecnologias que possibilitem desde a identificação de novos agentes de controle biológico ao conhecimento dos mecanismos de defesa das pragas deve ser considerada como ponto principal em estratégias futuras para o controle de pragas. A popularização e aumento de acessibilidade de técnicas de biologia molecular por sua vez proporciona grandes perspectivas para alcançar esse objetivo. Assim, com base no controle biológico, poderá ser cada vez mais possível alinhar estratégias de controle de pragas que são eficazes, financeiramente viáveis e seguras ao meio ambiente.

Objetivos Gerais

Nesse contexto, os trabalhos aqui apresentados exploraram a utilização de ferramentas bioquímicas e moleculares em diferentes vertentes de estudos com noctuídeos pragas. Os objetivos foram:

Capítulo 1: confirmar a presença da lagarta do velho mundo, *H. armigera* no estado de Minas Gerais através de marcadores moleculares e avaliar a possibilidade de coexistência no campo com a lagarta da espiga *Helicoverpa zea*.

Capítulo 2: avaliar a presença, prevalência e diversidade genética, através de diferentes ferramentas moleculares e filogenéticas, de simbiontes relevantes ao controle biológico como densovírus, nucleopoliedrovírus (baculovírus) e *Wolbachia*.

Capítulo 3: padronizar uma técnica de silenciamento do gene relacionado à expressão de profenoloxidase. Adicionalmente, através de PCR quantitativo, avaliar se expressão de profenoloxidase varia de acordo com a idade e densidade populacional em *Spodoptera littoralis*.

Capítulo 4: primeiramente, testar a hipótese que larvas de noctuídeos expressando profilaxia dependente de densidade necessitam da presença de agentes invasores para apresentarem diferenças em suas defesas humorais. Em segundo, verificar se níveis de resposta imune humorais variam de acordo com diferentes espécies de bactérias (comensais e patogênicas) e sua condição (vivas ou mortas).

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CHAPTER 1

The Old World Bollworm (*Helicoverpa armigera*) is present and coexisting with corn earworm (*Helicoverpa zea*) in Minas Gerais, Brazil

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Abstract: *Helicoverpa armigera* (Hübner), one of the world’s most polyphagous and destructive crop pests, was first documented in Brazil in 2013. Within a few months it had spread over the Northeast and Central-west of Brazil, causing great losses. Our aims here were (i) to confirm its presence in the State of Minas Gerais, one of the most important agricultural regions in Brazil, and (ii) to assess the co-occurrence of this pest with the congeneric corn earworm *Helicoverpa zea* (Boddie). Using molecular screenings, we confirmed the presence of *H. armigera* in Bt-crops of soybean and cotton, and non-Bt-crops of soybean, cotton and maize. Mixed infestations of *H. armigera* with *H. zea* were found in non-Bt maize (Viçosa, southeastern Minas Gerais). These results highlight the need for adequate control strategies for *H. armigera* in Brazil, to deal with its polyphagous feeding habits, high capacity for dispersion and possible risks of hybridization with congeneric species.

Keywords: *Helicoverpa armigera*, Noctuidae, pest management, PCR-RFLP, mitochondrial DNA.

Introduction

The Old World Bollworm *Helicoverpa armigera* (Hübner) is considered one of the most important agricultural pests in the world. This polyphagous species is widely distributed throughout Asia, Africa, Europe and Australasia, and has been reported attacking several economically important crops, such as soybean, maize, rice and tomato (Reed & Pawar 1982). The larvae feed on both vegetative and reproductive structures of the plant, damaging leaves, stems, buds, inflorescences, fruits and pods. It is estimated that worldwide annual costs of this pest (combining yield losses with the costs of control efforts) are more than US$ 5 billion. In India and China, 50% of the total use of pesticides in agriculture is directed at *H. armigera* (Mengech, Saxena & Gopalan 1995; Lammers & Macleod 2007).

Until recently, *Helicoverpa armigera* was considered absent in the Americas. In 2013, though, it was officially recorded in Brazil (Czepak *et al.* 2013; Specht *et al.* 2013). It is worth noting that its morphological similarity to *Helicoverpa zea* (Boddie) is likely to have delayed the detection of its arrival in the Americas (Kriticos *et al.* 2015). Within a few months, it was shown that *H. armigera* was spread across the Northeast and Central-West of Brazil, causing billions of dollars of losses in the 2012/13 soybean and cotton harvest (de Freitas Bueno & Sosa-Gómez 2014). One year after the first report, *H. armigera* was also found in citrus and tomato in the Southeast of Brazil, specifically the states of São Paulo and Espírito Santo (Bueno *et al.* 2014; Pratissoli *et al.* 2015). More recently, it has been reported noctuids causing problems in Minas Gerais (one of the principal agricultural regions in Brazil), which calls attention to the possibility of these insects are actually *H. armigera* (Brasil 2013). According to this report, intense infestations of *Helicoverpa* sp. larvae in different crops, such as soybean, maize, cotton and carrot, have resulted in large economic losses.

The presence of *Helicoverpa* species in this region along with the proximity to other regions with already confirmed infestations, suggests that *H. armigera* may also be present in Minas Gerais. However, specific identification is complex and there is the potential for misidentifications due to morphological similarities in the *Helicoverpa* species complex, as is found between *H. armigera* and the corn earworm *Helicoverpa zea* (Behere *et al.* 2008). For these reasons, molecular analysis (for example PCR-RFLP) is necessary in most cases to distinguish these two species (Behere *et al.* 2008). Thus, our aim here was (i) to confirm the presence of *H. armigera* in economically important crops in Minas Gerais and (ii) to assess whether *H. armigera* and *H. zea* are to be found co-occurring in this region.

Materials and Methods

Helicoverpa samples were collected in three locations in Minas Gerais. All sampling was authorized by the farmers and the agencies responsible for such collections of biological materials (IBAMA licence no. 15BR017065/DF). Approximately 15 to 30 caterpillars were collected in each sampling event, and these were stored in individual micro-tubes with absolute ethanol. A total of 212 caterpillars were collected in 10 different sites, mainly in soybean, cotton and maize crops. Previously identified *H. armigera* laboratory samples, originally from Espírito Santo, São Paulo (southeastern Brazil) and Bahia (northeastern Brazil) were used such as positive control for genetic identifications.

DNA extractions were performed using Wizard® Genomic DNA Purification (Promega®) following the manufacturer's protocol for extraction of genomic DNA from plant tissue, but adding an RNAse step. *Helicoverpa* samples were identified (as *H. armigera* or *H. zea*) with polymerase chain reaction (PCR) by amplifying a 511 base pair (bp) fragment of COI mitochondrial gene using the following pair of primers: COI-F02 (5’ CTC AAA TTA ATT ACT CCC CAT C 3’) and COI-R02 (5’ GGA GGT AAG TTT TGG TAT CAT T 3’) (Behere *et al.*, 2008). The PCR conditions were as follows: (1) 94 °C for 5 min, 1 cycle; (2) 94 °C for 30 s, 50 °C for 60 s, 72 °C for 60 s, 35 cycles; and (3) 72 °C for 10 min, 1 cycle. PCR amplification of individual DNA samples was carried out in a total of 25 µl reaction volume, containing *ca.* 100 ng of genomic DNA, 0.2 mM of each primer and 1x Taq PCR Master Mix (Qiagen). The amplifications were confirmed by running 5 µl of the amplified samples on 2% agarose with 1:10,000 GelRed® (Uniscience).

In order to distinguish between *H. armigera* and *H. zea*, a 5 µl-aliquot of each amplified PCR product was digested with ten units of restriction enzyme (BstZ17I) in a 20 µl reaction volume according to the manufacturer's instructions (New England Biolabs). Upon incubation for 6 h at 37 ºC, the digested products were separated by electrophoresis and photo-documented under UV transillumination. Within partial COI sequences, a single base pair mutation is present at the BstZ17I recognition site (GAATAC) in *H. armigera*, but not in *H. zea* (GTATAC). Therefore, the restriction endonuclease reaction digests the 511 bp COI PCR product for *H. armigera*, giving two bands of 318 bp and 193 bp, while the fragment for *H. zea* is fully conserved (see Fig. 1A; Behere *et al.,* 2008).

Results

Samples previously identified (by visual inspection in field; Fig. 1B, C and D) as *Helicoverpa* were indeed confirmed genetically as species of this genus. The restriction enzyme analysis confirmed the presence of *H. armigera* at all 10 sampling sites (Fig. 2). Individuals identified as *H. armigera* were found in soybean, cotton and maize crops attacking leaves and pods in soybean; leaves, flowers and bulbs in cotton; and exclusively ears in maize. Meanwhile, *H. zea* was found only in two sites, both in Viçosa, southeastern Minas Gerais (Zona da Mata), and exclusively on maize ears (Fig. 2). In both of these cases, it co-occurred with *H. armigera* in the same crops (Fig. 2). All samples from the three laboratory colonies, previously identified as *H. armigera*, were confirmed as such by molecular analysis.

Discussion

Our results extend our knowledge of the presence and plant hosts of *H. armigera* in Brazil, with particular reference to Minas Gerais. This pest can now be considered to have invaded all states of southeastern Brazil, being present in economically important dicotyledon hosts, such as tomato, soybean, and cotton, as well as in monocotyledons, such as maize and sorghum (Czepak *et al.* 2013; Specht *et al.* 2013; Bueno *et al.* 2014; Pratissoli *et al.* 2015). Biological and socioeconomic factors can explain the fast spread of this pest insect in other regions of Brazil. Adults of *H. armigera* can migrate as far as 2,000 km (Widmer & Schofield 1983), and larvae are able to feed on at least 60 and 67 crop and non-crop plant species respectively (Fitt 1989). In addition, the expansion of maize, soybean, and cotton crops in the previously infested regions could have facilitated the dispersal of this pest in southeastern states.

One of the sampling sites (Paracatu in northwestern Minas Gerais), where individuals of *H. armigera* were found, is located 80 kilometres from the border of Goiás (midwestern Brazil), where this pest was first reported in Brazil (Czepak *et al.* 2013). Besides both regions having extensive cultivation of soybean and cotton, massive shipments, especially of agricultural produce, are taken from midwestern Brazil across Minas Gerais towards the coastal ports (to the states of Rio de Janeiro and Espírito Santo) for export. Beside this, native plant hosts surrounding crops might support herbivorous populations during periods between harvests, which could facilitate persistence and spread of invasive organisms. *Helicoverpa armigera* has recently being recorded in other South American countries, a further demonstration of its invasive capacity. Murúa *et al.* (2014) found *H. armigera* adults in two different sampling sites of chickpea crops in the province of Tucumán, Argentina. Farmers from Chile, Uruguay and Paraguay have reported lepidopteran attacks in several crops, and it is likely that those species are also *H. armigera*, although no confirmation was possible yet due to the difficulties inherent in purely visual identification (Formentini *et al.* 2015).

In all soybean and cotton sampling sites, *H. armigera* was the only *Helicoverpa* species found. In contrast, *H. zea* was exclusively found on maize crops, from Viçosa region, where mixed infestations with *H. armigera* were observed. Note that maize was not sampled in the other regions, so we cannot preclude the possibility that mixed infestations occurred there. The fact of mixed infestations between these two species is very concerning due to the possibility of hybridization between these species in field conditions. PCR-RFLP is commonly used to identify these species yet cannot detect hybrids such as might arise between these two *Helicoverpa* species. For this reason, we cannot exclude the possibility that some of the insects we sampled are actually such hybrids. The close relationship and highly genetic similarity between *H. armigera* and *H. zea* is reflected in shared morphological and behavioural features (Behere *et al.* 2007). For example, *Helicoverpa* species share the same chemical compounds in sex pheromones, hence allowing male *H. armigera* moths to be attracted by sex pheromones produced by female *H. zea* moths (Berg, Zhao & Wang 2014). Under laboratory conditions, *H. armigera* and *H. zea* have been able to mate and produce fertile offspring (Laster & Sheng 1995). However, the possibility of hybridization in controlled conditions does not mean that this phenomenon happens in the field due to a series of unpredictable factors in natural conditions. Nevertheless, these two species could lead to heterosis, promoting hybrid lineages with characteristics that may present serious risks for crops, with the need of new strategies of control (Tay *et al.* 2013).

A further concern is that many of the *H. armigera* larvae found in this study were found in Bt-soybean and Bt-cotton crops. The rapid dispersion and the possibility of the development of resistance to insecticides and Bt crops of *H. armigera* mean that this pest may represent a major threat to Brazilian agriculture. In South-American countries, efforts have recently begun to monitor pest species and resistance to insecticides and Bt (dos Santos 2015; Formentini *et al.* 2015), however there is still a long path to obtain a complete and adequate screenings to monitor and contain insecticide resistance.

In conclusion, our field study indicates that *H. armigera* is present and established in different regions and host plants from Minas Gerais, and that it can co-occur with its close relative, *H. zea.* Since many populations of *H. armigera* around the world have been selected for resistance to chemical pesticides and Bt crops (Alvi *et al.* 2012), it is possible that the populations that originally invaded Brazil were already resistant. Due to these facts, the development of monitoring strategies for this pest and alternative control methodologies based on biological control may be key tools for future management of *H. armigera* in Brazilian crops.

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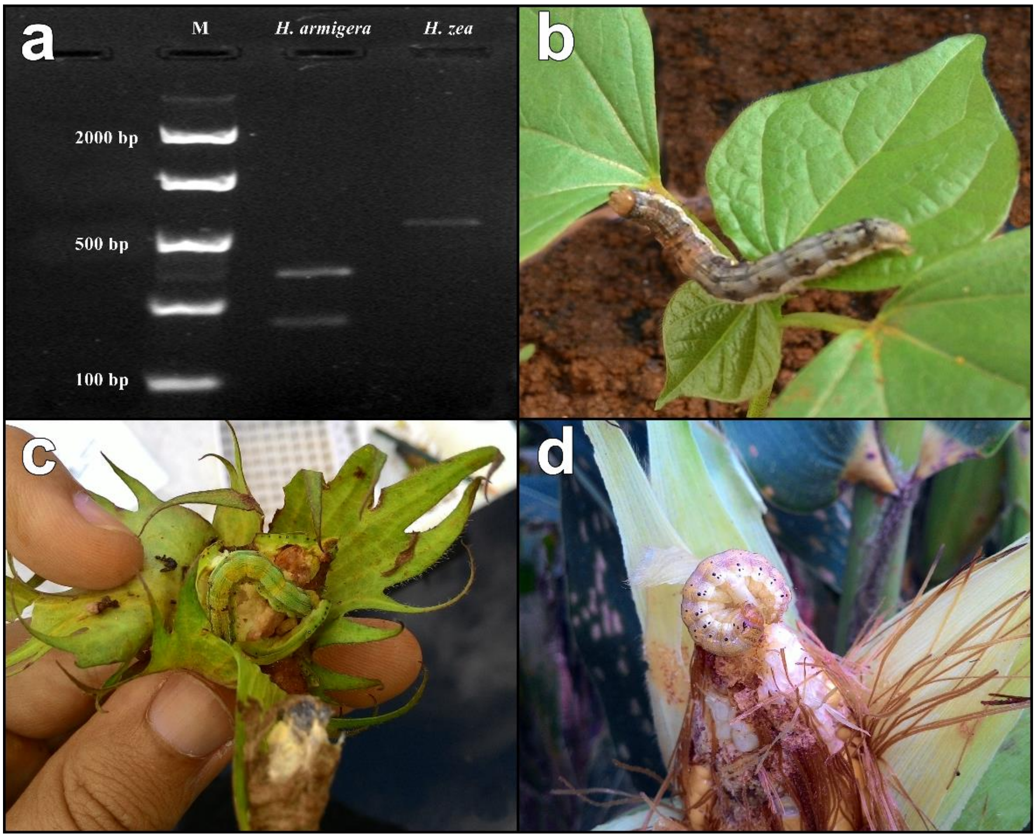

Figure 1: (a) Partial COI (511 bp) RFLP pattern (digested with BstZ17I) of *Helicoverpa armigera* and *Helicoverpa zea*, with bands separated on 2% agarose Gel. M= DNA ladder; (b-d) *Helicoverpa* larvae were found attacking leaves and pods in (b) soybean; (c) leaves, flowers and bulbs in cotton; and (d) maize ears. In soybean and cotton crops, only *H. armigera* was found, but in maize both *Helicoverpa* species were found indicating possibilities of hybridization. For cotton and soybean, *H. armigera* was also found in Bt crops.

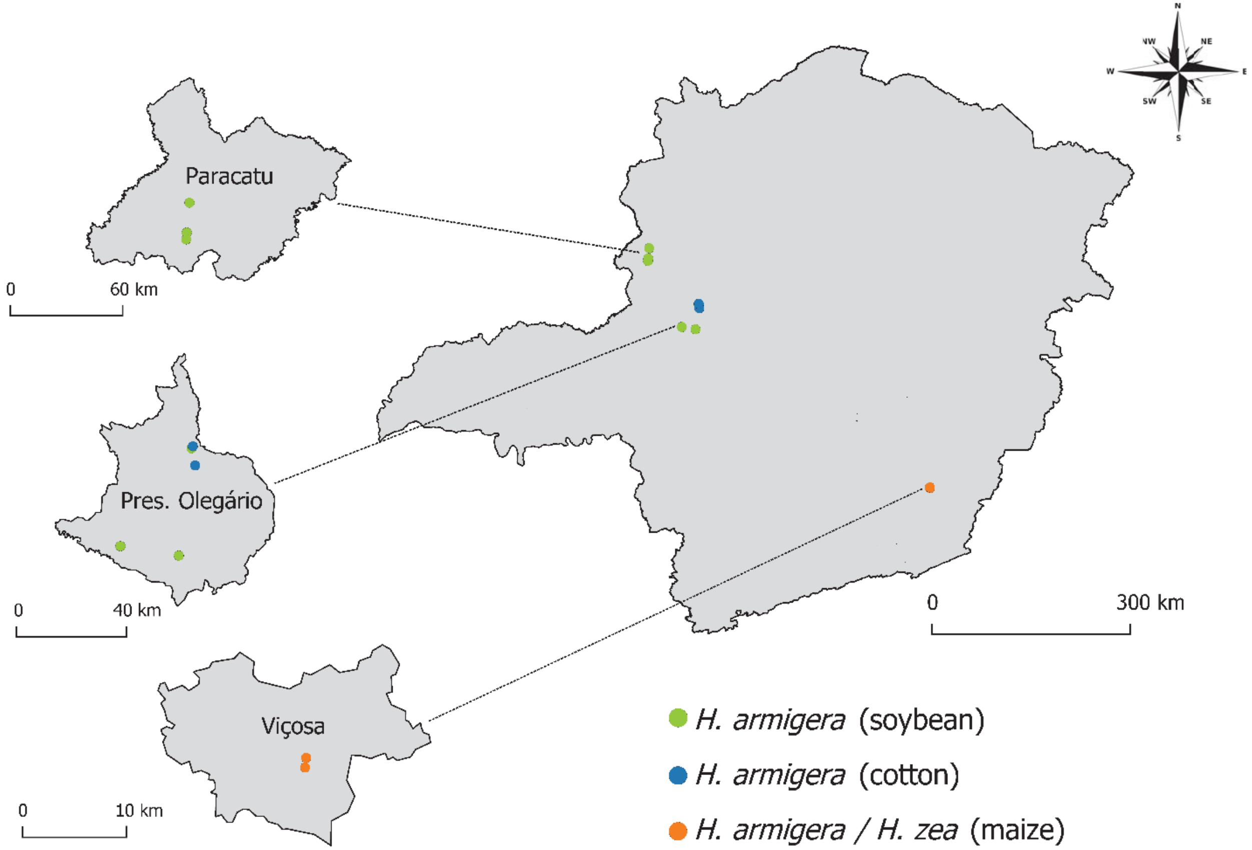

Figure 2: Sampling sites in the State of Minas Gerais where *Helicoverpa armigera* and *Helicoverpa zea* were collected in the study. The ten samplings sites were focused in three municipalities (Highlighted maps: Paracatu, Presidente Olegário and Viçosa). Individuals of *H. armigera* were found in 6 soybean (green circles) and 2 cotton (blue circles) sampling sites, including Bt and Non-Bt crops. Mixed infestations between *H. armigera* and *H. zea* were found in 2 sampling sites with non-Bt maize crop (orange circles).

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CHAPTER 2

Presence, prevalence and genetic diversity of relevant symbionts in noctuid pests (Lepidoptera: Noctuidae) from Minas Gerais, Brazil

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Abstract: Microbial symbionts play important roles on different aspects for insects including nutritional benefits, sex determination and defence. Conversely, some groups promote several negative effects on their hosts, which can be explored to control pest species that cause massive agricultural losses. Developing our understanding of diversity and prevalence of relevant symbionts for biological control as well their interactions on field community is an important step for new insights in biological control. Here, focusing in the region of Minas Gerais in Brazil, we address the following objectives: (1) to identify the natural presence on population and prevalence of infections of three relevant symbionts (*Wolbachia,* densovirus (DNV) and baculovirus virus (NPV)) in noctuid pests; (2) to evaluate effects of insect host species and location on these symbionts and (3) to evaluate the genetic diversity of *Wolbachia* strains inferring the nature of symbiotic interaction (parasite/mutualist) with their host. We demonstrate that the symbionts are widely present in different noctuid species and locations including soybean, maize and laboratory colonies. In turn, symbiont prevalence also varied greatly indicating that both host species and location are related with prevalence levels. The genetic studies for *Wolbachia* isolates identified three different strains, which one belongs to the supergroup F, observed for the first time in Noctuidae; and the other two to supergroup B, which presents several strains with negative effects for hosts as male killing and increasing of susceptibility against pathogens. These results provide a general screening of symbionts with perspectives for novel approaches in pest management, such possible bacterial symbionts to enhance the impact of microbial biopesticides, providing a new target for intelligent pest management strategies.

Keywords: bollworm, arthropod, baculovirus, *Wolbachia*, densovirus, nucleopolyhedrovirus, parasite, Noctuidae.

Introduction

Insects are considered the biggest and most diverse group between animal clade in number of species, ecological habits, and biomass (Stork 1988; Basset *et al.* 2012). Recently, studies emphasize that diversification and evolutionary success of this group have depended partially of their diverse range of relationships with symbionts, which are known to act directly in insect nutrition and defence (Klepzig *et al.* 2009; Engel & Moran 2013). Due to the relevance of symbionts in several functions for their hosts, the understanding of interactions in aspects such as specificity, pathogenicity, sub-lethal effects and behavioural manipulation, are highly relevant in several perspectives, linking to medicine, ecology and crop protection (Foster & Harris 1997; Kikuchi *et al.* 2012; Engel & Moran 2013). In several studies with insect pests, it has been observed that symbiotic microorganisms can promote beneficial effects such as increasing host fecundity (Weeks & Stouthamer 2004), protecting the host against pathogens (Hedges *et al.* 2008; Kikuchi *et al.* 2012; Xu *et al.* 2014) and acting directly in insect resistance against pesticides (Kikuchi *et al.* 2012). On the other hand, negative effects such as host mortality (Graham *et al.* 2012), alteration in sex ratio (Hurst & Jiggins 2000; Charlat, Hurst & Merçot 2003) and modification of feeding behaviour (Aly 1983; Chen *et al.* 2008) can also be attributed to symbionts, which may promote excellent perspectives for pest control.

In Brazil, insects of the Noctuidae family are responsible for massive agricultural losses upon many economically important food crops. Intensive cultivation of soybean, maize and cotton in different regions of the country are specially affected by *Helicoverpa* sp., *Chrysodeixis includens*, *Spodoptera frugiperda*, *Spodoptera eridania* and *Heliothis virescens* (Silvie *et al.* 2007; Moreira & Aragão 2009). Additionally, in 2011/2012 and 2012/2013 growing seasons, it was detected high infestations of *Helicoverpa armigera*, one of the most destructive noctuid pests in the world. Of course, it resulted in significant economic losses and great concern due to the historical difficulties controlling this pest in other regions (Czepak *et al.* 2013).

Several noctuid species, as it is the case of *S. frugiperda* and *H.* armigera, already present resistant populations against many chemical pesticides around the world (Heckel *et al.* 1997; Sivasupramaniam *et al.* 2007; Wu 2007). Recently in Brazil, it was observed populations of *S. frugiperda* resistant to Bt corn on field conditions, leading to concerns for the future of Cry toxins for pest control (Monnerat *et al.* 2015). Even so, strategies mainly based upon chemical pesticides and Bt crops have been utilized to avoid the spread and reduce the damage of *H. armigera* and other noctuid species in the country.

Due a series of problems related to chemical pesticides as high toxicity (Kamrin 1997; Hernández *et al.* 2013), lack of selectivity (Carlile 2006; Dively *et al.* 2015) and insect resistance (Heckel *et al.* 1997; Roush & Tabashnik 2012), alternative methods such as the use of symbionts to direct control and to enhance efficacy of other methods can achieve more effective results in integrated pest control (Graham *et al.* 2012). There is an increasing interest in other forms of pest control, including the use of host-specific viral pesticides, derived from densoviruses, small RNA viruses and baculoviruses (Allaway & Payne 1984; Christian *et al.* 2005; Gu *et al.* 2011; Scholefield 2015). In addition, it was recently observed that some *Wolbachia* strains can enhance the susceptibility of *Spodoptera exempta*, a major pest in Africa, against entomopathogenic viruses used in biological control strategies (Graham *et al.* 2012). Thus, the knowledge about the natural presence and the web of interactions between these symbionts can be an important step on development of efficient strategies to control noctuid pests in Brazil.

Here, focusing in Minas Gerais state, we aimed to: (1) identify the natural presence and prevalence of three relevant symbionts (*Wolbachia,* densovirus (DNV) and specific baculovirus (NPV)) in the most relevant noctuid pests; (2) evaluate the effects of insect species, host plant and location on the diversity of these three symbionts and (3) analyse the genetic diversity of *Wolbachia* present in noctuid host species, identifying possible negative effects of the strains found.

Materials and Methods

Samples were taken in nine different locations focused in three municipalities of Minas Gerais State (Fig. 1). Permission for all samples was allowed by the growers and the Brazilian agency responsible for research with biological materials (IBAMA license n. 15BR017065/DF). General information related with the samples, including GPS coordinates of each location are listed in Table 1. A total of 287 caterpillars of different species and origins were processed. From these, 212 were *H. armigera, H. zea, C. includens* and *Spodoptera* sp. obtained in soybean and maize crops. In addition, we obtained 75 noctuid larvae of three different laboratory colonies originally from Espírito Santo, Bahia and São Paulo.

For each sampling event described above, 15 to 30 caterpillars were collected in individual micro-tubes and stored in absolute ethanol. DNA extractions were performed using Wizard® Genomic DNA Purification (Promega®) following the manufacturer's protocol for extraction of genomic DNA from plant tissue, but with increased centrifugation time and adding an RNAse step. All DNA samples were used to evaluate the presence of *Wolbachia*, DNV and NPV. The screening for *Wolbachia* was performed by amplifying the wsp region with the primers set 81F and 691R (Table 2), which were shown to be able to amplify the wsp gene fragment from more than 28 different lineages (Zhou, Rousset & O'Neill 1998). These primers amplify a DNA fragment ranging from 590 to 632 bp depending on the individual *Wolbachia* strain. PCR was carried out using ~500 ng of DNA, with the following reaction conditions: (1) initial denaturation at 94 °C for 5 min; (2) 40 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s; (3) final extension at 72 °C for 5 min.

To detect DNV, specific primers amplifying a 496 bp fragment, DVVPF/DVVPR (Table 2) were used. The PCR program was as follows: (1) initial denaturation at 94 °C for 5 min; (2) 40 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; (3) final extension at 72 °C for 5 min. In turn, for detection of NPV, a pair of specific primers amplifying a fragment of 445 bp, NPVF/NPVR (Table 2) were used with the following reaction conditions: 1) initial denaturation at 94 °C for 5 min; (2) 40 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s; (3) final extension at 72 °C for 5 min. All positive samples for the three symbionts were purified and sequenced by Sanger method (Eurofins Genomics, UK). Subsequently, the sequences were analysed and edited using BioEdit to remove universal primer regions. Preliminary identifications against previously published sequences were provided by Nucleotide BLAST. Sequence alignment was performed with MEGA 6 using default parameters.

Data Analysis

To evaluate whether insect species and location (sampling location and kind of crop/laboratory) affected the prevalence of each symbiont, we used generalised linear models (GLM) with binomial errors. Chi-square tests were used to examine the relation of the explanatory variables on the prevalence of each symbiont (*Wolbachia*, DNV and NPV). Reported statistics are for minimal models following model simplification, such that all terms in the model caused a significant reduction in model residual deviance and no additional terms significantly improved the fit of the model. Throughout the text, means ± standard errors (SE) are presented. All statistical analysis cited above were conducted using R software v3.2.0 (R Core Team 2015).

For *Wolbachia*, due to the diversity of lineages amplified by the same pair of primers, a Maximum-Likelihood phylogenetic analysis was performed having selected the most appropriate model based on lowest AIC score.

Results

Wolbachia: Presence, prevalence and strains found

Wolbachia was detected in all sampling sites, except in one laboratory colony (Table 1). Mean of *Wolbachia* prevalence was 30.46% (n= 46) in soybean, 3.33% (n= 2) in maize and 17.11% (n= 13) in laboratory samples. *Wolbachia* was also detected in all noctuid species evaluated (*H. armigera*: 21%, n= 20; *H. zea*: 5.9%, n= 1; *C. includens*: 34.8%, n= 32 and *Spodoptera* sp.: 5.2%, n= 3) presenting a total prevalence of 21% (n= 61) (Fig. 2A, 2B). In turn, prevalence varied significantly between the noctuid species, crop species and sampling locations (GLM: Noctuid species: χ^2= 16.26, df= 3, P< 0.001; Crop species: χ^2= 13.81, df= 2, P< 0.001; GPS location: χ^2= 31.26, df= 11, P< 0.001).

Three strains of *Wolbachia* were isolated, which we refer to as strains wNoc1, wNoc2 and wNoc3. Strains wNoc1, wNoc2 and wNoc3 were observed in two (16.6%), four (33.3%) and one (8.3%) of the 12 sampling sites respectively, with one sampling site containing all three strains, two with two and four with just one. Based on wsp gene sequencing, strain wNoc1 was most closely matched to AY331114.1, a phenotype belonging to the supergroup F. In turn, wNoc2 was most closely matched to HQ404773.1 and wNoc3 to AB094202.1, both belonging to supergroup B (Fig. 4).

Evaluation of presence and prevalence for Densovirus (DNV) and Baculovirus (NPV)

DNV was also observed in four sampling sites, which were all soybean crops and in two laboratory colonies of *H. armigera* evaluated (Table 1). Mean DNV prevalence was 12.58% ± 2.71 in soybean and 11.84% ± 3.76 in laboratory samples. The prevalence in noctuid species was 10.83% (n= 13) in *H. armigera,* 15.21% (n= 14) in *C. includens* and 1.75 (n= 1) in *Spodoptera* sp. No positive samples were found in *H. zea* (Fig. 2C, 2D). The prevalence of densovirus varied with insect species, crop species and sampling location (GLM: Noctuid species: χ^2= 11.34, df= 3, P< 0.01; Crop species: χ^2= 13.81, df= 2, P< 0.001; GPS location: χ^2= 31.26, df= 11, P< 0.001).

NPV was also observed in four sampling sites of soybean crops and in two of the three laboratory colonies of *H. armigera* evaluated (Table 1). Mean of NPV prevalence was 7.95% ± 2.21 in soybean and 1.32% ± 1.32 in laboratory samples. Prevalence in noctuid species was 5.83% (n= 7), 2.17% (n= 2) and 7.01% (n= 4) in *H. armigera*, *C. includens* and *Spodoptera* sp., respectively (Fig. 2E, 2F). For NPV, the sampling location and crop species also affected the prevalence, however it was not affected by the noctuid hosts evaluated (GLM: Noctuid species: χ^2= 4.64, df= 3, P= 0.24; Crop species: χ^2= 9.21, df= 2, P< 0.01; GPS location: χ^2= 24.72, df= 2, P< 0.01).

For both viruses, no interactions between the explanatory variables were observed, indicating that noctuid species, sampling location and crop species affected the viral prevalence independently. There were no observed multiple infections of DNV and NPV in insects samples, while multiple infections with *Wolbachia* presented a total prevalence of 2.86% (n= 10) and 2.79% (n= 8) for DNV and NPV respectively.

Discussion

Presence, prevalence and diversity of *Wolbachia* strains

Here, we explored ecological and phylogenetic aspects of *Wolbachia* in noctuid pests from Minas Gerais state. The presence of this symbiont was confirmed in more than 80% studied sampling sites, being found in all noctuid species sampled in maize, soybean and laboratory colonies. Although widely present, *Wolbachia* prevalence was highly variable between sampling sites and insect species, which emphasizes that both taxonomic groups and ecological niches may affect its prevalence (Zug & Hammerstein 2012). As observed here, the prevalence of this bacteria is strongly affected by its host species being reported as 56% in *Spodoptera exempta* (Graham *et al.* 2012), 47% in *Polytremis fukia* (Jiang *et al.* 2016), 11% in *Neonympha mitchellii mitchellii* (Hamm *et al.* 2014) and 3.4% in *Pieris rapae* (Tagami & Miura 2004).

Differences in prevalence and diversity of strains in *Wolbachia* infections between locations and taxa can also occur due some species being more exposed to infections than others (West *et al.* 1998). New *Wolbachia* strains can invade and be spread to new host species through cross breeding, which is phenomenon regularly observed in noctuids such *Helicoverpa* species also sampled in this study (Laster & Sheng 1995; Wang & Dong 2001). Additionally, another important aspect is that prevalence surveys inevitably may underestimate/overestimate the number of species infected by *Wolbachia* for some reasons: 1) the presence of *Wolbachia* in a given species can vary according to their population, (2) infected and uninfected individuals will coexist in a same population, which can affect a screening for presence / prevalence (Cook & Butcher 1999) and (3) samplings with no sex identification can give false negatives due natural absence in males for some *Wolbachia* strains.

We found three different *Wolbachia* strains belonging to two different supergroups (B and F). Supergroup B is normally found in insects Orders including Lepidoptera, which is known for causing several phenotypic alterations on their hosts, including parthenogenesis induction, feminization, cytoplasmic incompatibility, and male killing (Lindsey *et al.* 2016). In turn, "F" is a relatively recent supergroup most common in nematodes, termites and lice species (Lo *et al.* 2002; Covacin & Barker 2007). According with phylogeny based in wsp gene, the strain wNoc2 was most related with a *Wolbachia* strain of *Cotesia glomerata*, which is a generalist parasitoid wasp (Geervliet *et al.* 2000) and wNoc3 to strains isolated from leafhoppers.

Recent studies have demonstrated that horizontal transmission mediated by plant host is also possible for some vertically transmitted symbionts (Caspi-Fluger *et al.* 2012), which could explain the relation of *Wolbachia* lineages found in our samples with the other host species. Another possibility is that an infection-transmission event may also have occurred by interference of intermediary organisms as generalist parasitoids (Cook & Butcher 1999; Gehrer & Vorburger 2012). By the other side, possibilities of contaminations cannot be excluded, which generally is not discussed in many studies involving *Wolbachia.* Sampled larvae hosting parasitoids infected with *Wolbachia* could also explain the similarities between the strains found with those from parasitoids. Another important point is that *Spodoptera* genus exhibits cannibal behaviour and are opportunist predators (Rodríguez-del-Bosque *at al.* 2012). Thus, we also cannot exclude the possibility that not related *Wolbachia* lineages found were obtained through ingestion of an insect prey and are not really infecting these noctuids.

Here, we did not aim to test the host effects related with the isolated strains, however male-killing *Wolbachia* phenotype is generally found in Lepidoptera (Hurst *et al.* 1999; Fujii *et al.* 2001; Sasaki, Kubo & Ishikawa 2002; Graham & Wilson 2012). Apart from this characteristic, there is a growing literature on the capacity of some *Wolbachia* strains to protect its host from viral infections (Charlat, Hurst & Merçot 2003; Hedges *et al.* 2008; Teixeira, Ferreira & Ashburner 2008; Zélé *et al.* 2012). On the other hand, for other strains, this scenario can be totally different. Graham *et al.* (2012) showed via laboratory and field studies that *Wolbachia* may increase the susceptibility of African armyworms to its endemic baculovirus by 6 to 14 times. Besides the negative results related with sex ratio and sterilization caused by *Wolbachia*, recent results with different host species including noctuid pests emphasize that some strains can increase host susceptibility to baculovirus and other pathogens (Graham *et al.* 2012; Dodson *et al.* 2014; Hughes, Rivero & Rasgon 2014). This aspect presents interesting perspectives to be explored in integrated control programs, inclusive due the presence of co-infection between *Wolbachia* and both viruses screened in this study (DNV and NPV).

DNV and NPV presence and prevalence:

In our snapshot for DNV, it was found in *H. armigera*, *Chrysodeixis includes* and *Spodoptera* sp. from soybean and laboratory colonies being absent in *Helicoverpa zea* and maize crops. This group of virus is widely found in noctuid species being present in different regions and crops (El-Mergawy *et al.* 2003; Huynh *et al.* 2012; Xu *et al.* 2014), however only few studies emphasize the aspects of its symbiotic interactions. Some DNVs are reported as pathogenic to their hosts, being considered as potential biological control agents of insect (Gu *et al.* 2011), in turn contrary to the general idea about viruses, which have long been considered an obligatory parasite harmful to their host, apparently some DNV lineages can present non-virulent relationships with their hosts (Roossinck 2011; Roossinck 2015). Recently, it was observed a mutualistic relationship between Chinese populations of *H. armigera* and a specific DNV lineage, which is both vertically and horizontally transmitted and presents similar features with mutualist *Wolbachia* lineages, such as positive effects on development, fecundity and protection against pathogens (Xu *et al.* 2014). It is still unknown if DNV found in our samples also plays a role in insects defence against baculoviruses or other entomopathogen, however this can be an important subject for future research due the relevance in biocontrol.

The baculovirus NPV was present in both laboratory and field-collected samples, however with less expressive values than other symbionts studied. This was expected due differences in virulence and transmission dynamics strategies of NPV compared to other studied symbionts. During our samplings, the population of noctuids screened was considerably low and we did not presence any outbreaks, which can explain the low levels found. In most of the cases baculovirus prevalence on field is strongly related with fluctuations of host population, showing extremely high levels in outbreaks and being almost undetectable at low populations (Dwyer *et al.* 2002; Cory & Myers 2003).

NPVs have been used in several success for programs of biological control against noctuid pests around the world (Buerger, Hauxwell & Murray 2007; Grzywacz *et al.* 2008; Kumari & Singh 2009; Szewczyk *et al.* 2009). This can be associated to its important features such high virulence, specificity and persistence on field, which promote these viruses as an excellent option for microbial biopesticide (Jones 1993). In Brazil it is already possible to find biocontrol products based on NPV (Perini *et al.* 2016), however due viral specificity surveys to identify specific lineages for each pest species along with large-scale field trials to identify efficacy and safety can be the key for an efficient alternative to chemical insecticides.

Conclusion

Our field study indicates that Wolbachia, although found in most studied sites, presents highly variable prevalence between species and locations from Minas Gerais state. The strains found could be classified in three phylogenetic lineages belonging to supergroup B, which is normally found in insects and presents several parasite lineages, and supergroup F, observed for the first time in noctuids. Both DNV and NPV were also widely present, but with lower and less variable prevalence. Overall, our results provide a general screening of symbionts with perspectives for novel approaches in pest management, such as the possibility of bacterial symbionts to enhance the impact of microbial biopesticides, as well as the mutualist viruses that can be a new target for intelligent pest management strategies.

Table 1: General table. Field samples were realized in Soybean and Maize crops from three cities of Minas Gerais State (Paracatu, Presidente Olegário and Viçosa). In addition, samples from three different noctuid laboratory colonies were used (Alegre, Patos de Minas and Viçosa). The number of samples (n) varied according to availability of insects on field. The DNA of each sampled insect was extracted and used in three different PCR reactions to evaluate the presence of *Wolbachia*, Densovirus (DNV) and Nucleopolyhedrovirus (NPV). The prevalence of each symbiont (%) was evaluated for each location according to sample size.

Location	Origin of samples	GPS coordinates	Nº samples (n)	*Wolbachia* (%)	DNV (%)	NPV (%)
Alegre	Laboratory colony	-20.761537, -41.535274	15	6.66	0	0
Paracatu	Soybean	-17.225960, -47.020210	30	40	33.33	10
Paracatu	Soybean	-17.376572, -47.033677	5	40	20	0
Paracatu	Soybean	-17.411394, -47.037058	19	63.16	26.32	10.53
Patos de Minas	Laboratory colony	-18.618622, -46.516000	30	0	3.33	3.33
Pres. Olegário	Soybean	-18.372100, -46.525400	5	40	0	20
Pres. Olegário	Soybean	-18.052950, -46.290810	30	20	0	0
Pres. Olegário	Soybean	-18.389290, -46.535700	30	30	20	0
Pres. Olegário	Soybean	-18.422009, -46.334577	32	25	12.50	15.63
Viçosa	Laboratory colony	-20.758620, -42.868570	30	43.33	6.67	3.33
Viçosa	Maize	-20.767565, -42.869529	6	16.67	0	0
Viçosa	Maize	-20.767642, -42.869633	54	1.85	0	0

Table 2: Primers used in this study. Columns represent names of primers given by authors, related primer sequences and target species respectively.

Primer name	Primer sequence (5'-3')	Target species	Reference
wsp81F	TGG TCC AAT AAG TGA TGA AGA AAC	*Wolbachia*	Behere *et al.* 2008
wsp691R	AAA AAT TAA ACG CTA CTC CA		
DVVPF	GGA TTG GCC TGG GAA ATG AC	Densovirus (DNV)	Xu *et al.* 2014
DVVPR	CGT TGT TTT TAT ATC CGA GG		
NPVF	AAA AGA AAA GCC AGC AAC G	Baculovirus (NPV)	Xu *et al.* 2014
NPVR	ACG ATT GAA TGT CAC CAC G		

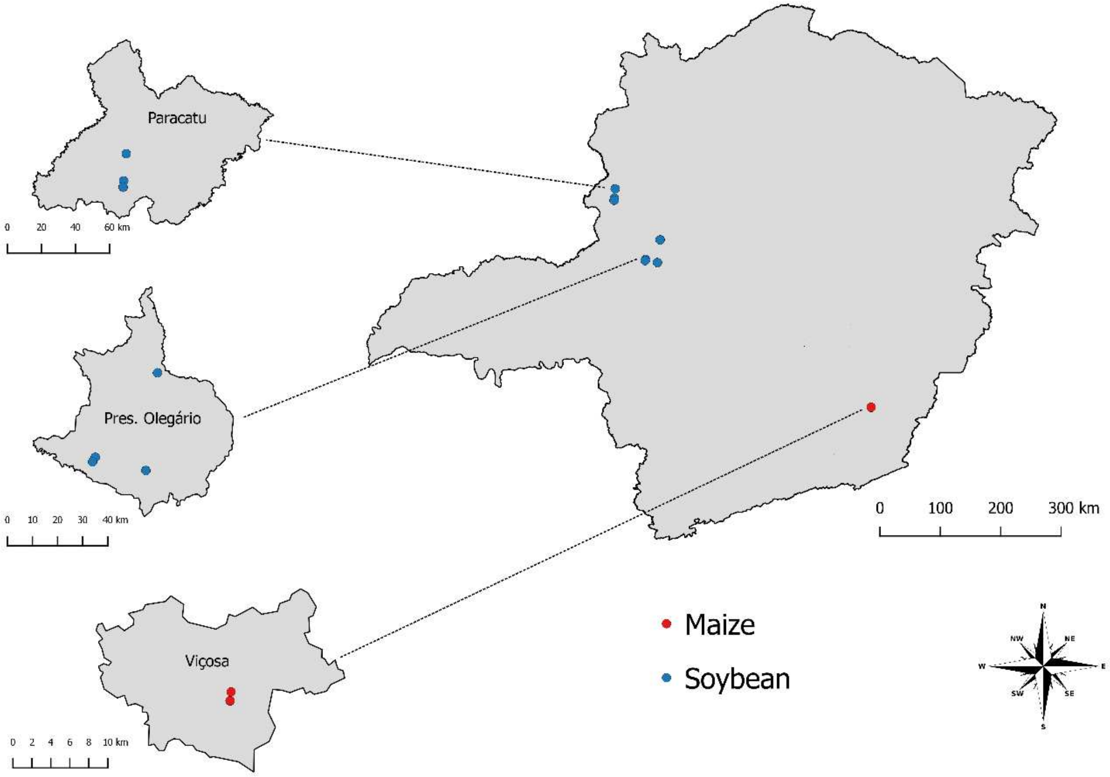

Figure 1: Minas Gerais map emphasizing the nine sampling sites from maize (red) and soybean (blue) crops which the noctuid pests were obtained for this study. The sampling s were focused in three municipalities (Paracatu, Presidente Olegário and Viçosa). For soybean crops, three populations were obtained in Paracatu and four obtained in Presidente Olegário. For maize, two populations were obtained in Viçosa. Each sample was used to detect the presence of *Wolbachia*, DNV and NPV.

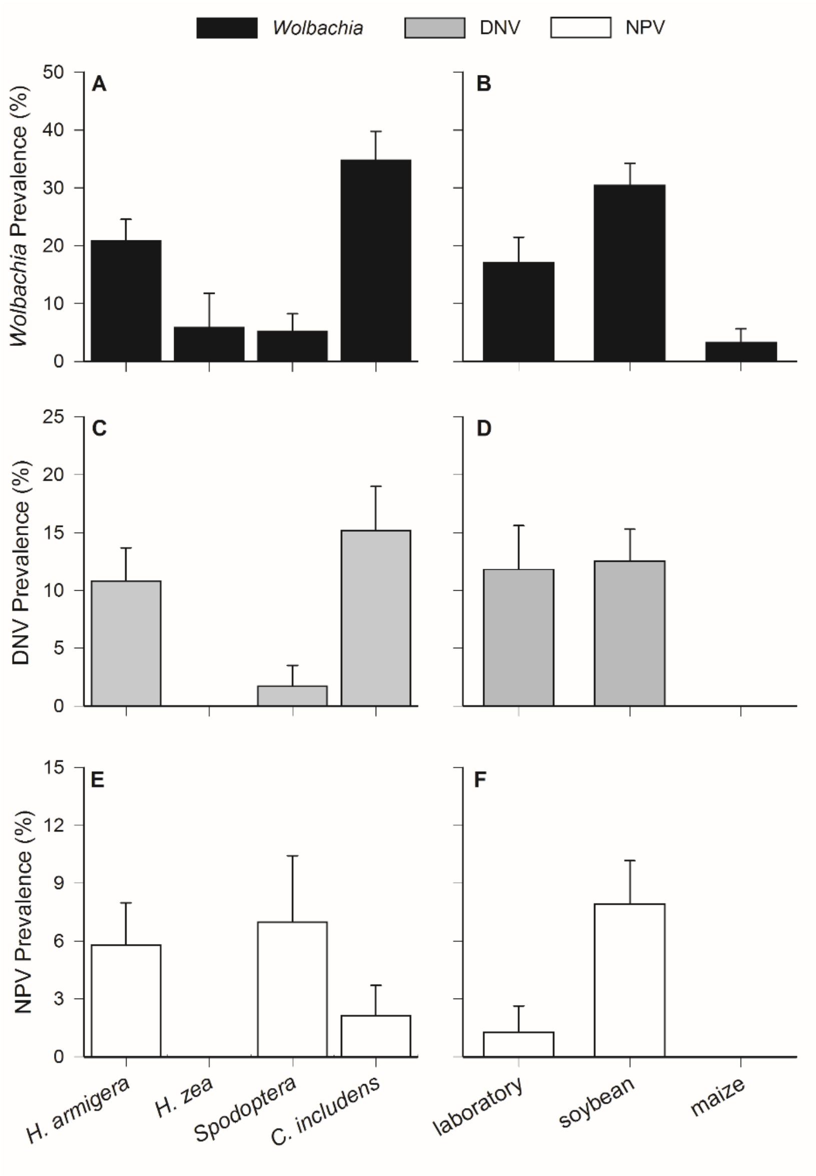

Figure 2: Prevalence of *Wolbachia*, DNV and NPV according to host species and origin of samples (soybean, maize and laboratory colony). *Wolbachia* prevalence both varied significantly between noctuid host species studied (A) χ^2= 16.26, df= 3, P< 0.001 and origin of samples (B) χ^2= 31.26, df= 11, P< 0.001; DNV prevalence was also affected by host species (C) χ^2= 11.34, df= 3, P< 0.01 and habitat (D) χ^2= 13.81, df= 2, P< 0.001. In turn, NPV prevalence was not affect by host species (E) χ^2= 4.64, df= 3, P= 0.24, however it also varied according to habitat (F) χ^2= 9.21, df= 2, P<0.01.

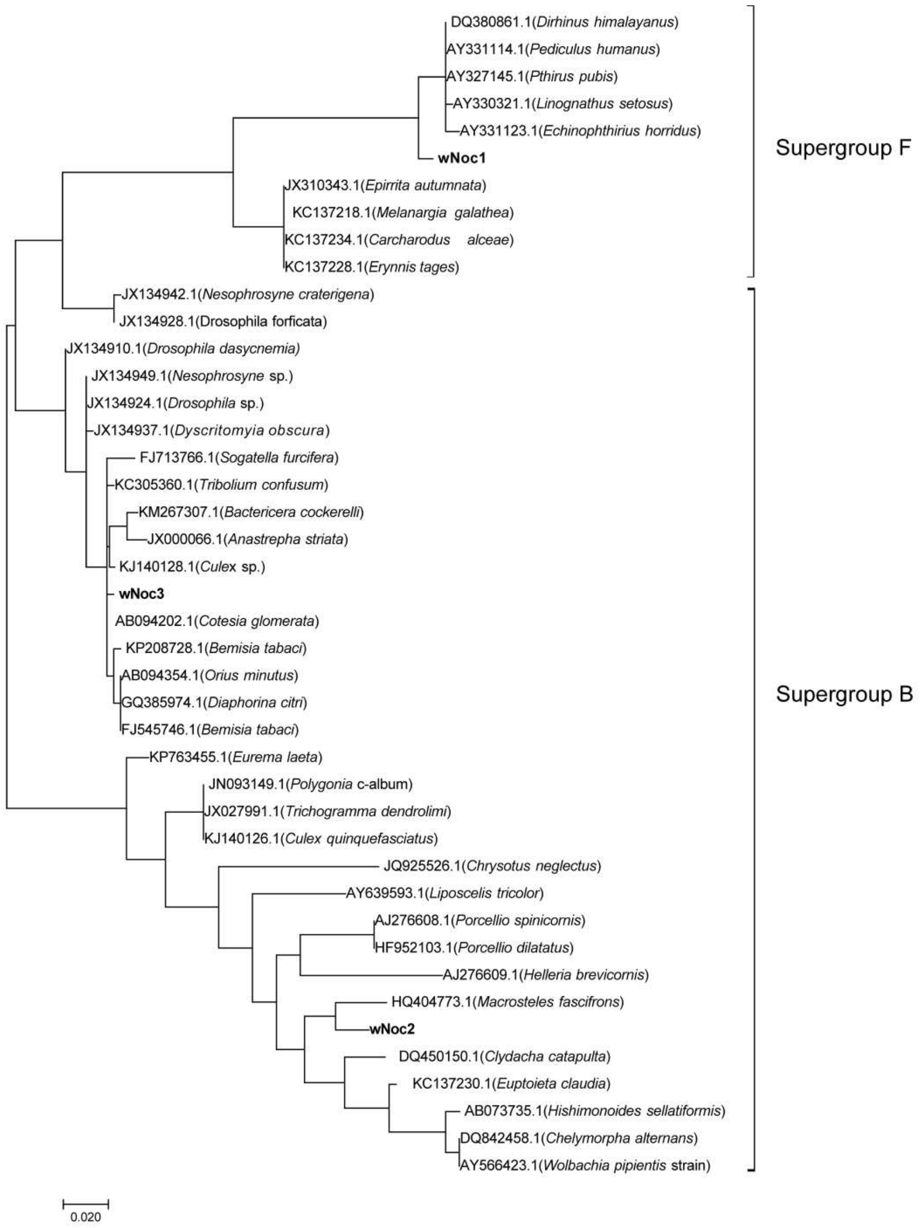

Figure 3: Maximum-Likelihood (GTR+G+I model) phylogenetic tree for the *Wolbachia surface protein* (WSP) gen of *Wolbachia* isolates. The three strains isolated from noctuids (wNoc1, wNoc2, wNoc3) in this study are emphasized in bold. NCBI codes followed by host species are given for each sequence used. The scale bar represents a 2% estimated difference in nucleotide sequence. Numbers given at each node correspond to the percentage bootstrap values (for 1000 repetitions).

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CHAPTER 3

Absolute quantification of Prophenoloxidase (PPO) gene expression and upregulation by dsRNA in the Cotton Leafworm *Spodoptera littoralis*

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Abstract: Phenoloxidase (PO), considered a primary defence mechanism, PO is produced in an inactive form called Prophenoloxidase (PPO). It is activated by disturbances to host organism homeostasis such as infections and injuries. In lepidopterans, the activation and effects of this enzyme can vary according to population densities and larval age, however only few studies focus in molecular aspects, such as gene expression of PO. Here, we first standardize a qRT-PCR technique for absolute quantification of PPO gene expression in *S. littoralis*. We found that PPO gene expression did not differ in larvae raised alone or in groups of three individuals (solitary or gregarious) and also between larval age (48 to 144h post-eclosion). Later, we tested three different administration methodologies for double-stranded PPO RNA (dsRNA) to evaluate prospects for gene silencing. Pellet feeding and soaking methodologies did not determine PPO expression, although the droplet feeding methodology surprisingly resulted in increasing of gene expression. Assuming that PPO is a precursor of primary defence mechanism, we believe that variations can occur in PO expression during infectious process. However, PPO apparently remains stable in natural conditions regardless of larval age and population density. Failures in knockdown are relatively common in lepidopterans; on the other hand, even rare gene overexpression can be explained by previous studies showing that dsRNAs can affect multiple signalling and transcription pathways. The potential risks for abnormal effects in this technique including gene activation and non-target genes knockdown must be carefully considered in the design of RNAi for experiments with practical prospects applications such as pest control.

Keywords: Lepidoptera, RNAi, Real-Time PCR, Noctuidae, siRNA, Phenoloxidase

Introduction

Phenoloxidase (PO) is considered the key enzyme on reaction chain leading to melanin synthesis in insects. This process has a multifunctional role on defence actuating against pathogens, wound healing and cuticle pigmentation (Ashida 1997; Zufelato *et al.* 2004). PO is usually synthesized as an inactive zymogen called Prophenoloxidase (PPO), which is cleaved to generate active PO as a result of proteolytic cascade activation. PO-mediated melanin synthesis leads to a local increase of free radicals and quinones, the mediators of protein cross-linking and the precursors of the melanin polymer, playing a fundamental role in encapsulation of particles and microorganisms non-phagocytized (Cerenius, Lee & Söderhäll 2008; Di Lelio *et al.* 2014).

In some species, it is observed that PPO expression vary drastically during larval instar and is expressed only after post-embryonic stage (Fan *et al.* 2011). In honey bees, the expression and activity of PPO on haemolymph is increased during pupal age and apparently plays an important role in cuticle melanisation (Zufelato *et al.* 2004). On the other hand, the aspects rated with expression and activity of this enzyme may be totally different in different groups, which emphasizes the importance on studies with different groups of insects and other invertebrates.

With techniques, such as gene silencing and synthetic inhibitors, it has been observed that PO also has a crucial role in cuticle development and immune resistance in some species (González-Santoyo & Córdoba-Aguilar 2012). Using synthetic inhibitors of PO, Bai *et al.* (2014) showed negative effects on larval development and adult emergence in the oriental fruit fly *Bactrocera dorsalis*. In another example, Rodriguez-Andres *et al.* (2012) observed that blocking PO activity leads to increased arbovirus replication and mortality in *Aedes aegypti.* On the other hand, little is known about the expression of this gene and its precursor in Lepidoptera, including possible effects of silencing this gene in development and defence against microorganisms.

Recently, gene silencing by RNAi has been shown to be a promising tool for investigations of gene functions in many lepidopteran species (Terenius *et al.* 2011; Kotwica-Rolinska *et al.* 2013; Di Lelio *et al.* 2014). In this process, an mRNA molecule is targeted for degradation by a dsRNA with a strand complementary to a fragment of the mRNA, promoting the inhibition of gene expression (Belles 2010). Silencing of PPO genes can generate relevant information about this enzyme such as its specific role in immune system and development in this group of insects. However, it is often difficult to identify effective methods for knocking down the expression of target genes in Lepidoptera (Terenius *et al.* 2011; Yu *et al.* 2013). The failures in gene silencing and abnormal effects apparently are related with the target tissue, dsRNA molecule and methodology for administration of RNAi (Belles 2010; Terenius *et al.* 2011; Yu *et al.* 2013). On the other hand, several studies have demonstrated good results using this technique with methodological adjustments to suit the species and the target gene, which is encouraging for using this technique in applied settings such as pest control (Sivakumar *et al.* 2007; Terenius *et al.* 2007; Bautista *et al.* 2009; Masumoto, Yaginuma & Niimi 2009; Rodriguez-Cabrera *et al.* 2010; Di Lelio *et al.* 2014).

Here, we first developed a standardization of a real-time PCR methodology for absolute quantification of *Spodoptera littoralis* (Boisduval, 1833) PPO. This species is an important polyphagous pest in different regions of the world (Cabi, 2015). Such as other noctuids, this *S. littoralis* presents density-dependent polyphenism, where insects reared at high densities tend to present higher levels of PO activity than insects reared alone (Cotter, *et al*. 2004). However, there is no information about gene expression of PPO related with population density for this species. In this context, we evaluated the levels of gene expression reared at different densities (solitary and gregarious), also during different larval ages. With the same standardized technique, we also tested three different administration methodologies for a synthetized double-stranded PPO RNA (dsRNA) to evaluate prospects for gene silencing.

Materials and Methods

Insect Material

All larvae used on this study were obtained from colonies of the insect and parasite ecology laboratory, University of Lancaster, Lancaster UK. During larval stage, the insects were reared on artificial diet (72 g/L wheat germ, 33 g/L casein, 29.25 g/L sugar, 14.25 g/L yeast extract, 9.37 g/L Wesson's salt, 1.5 g/L ascorbic acid, 0.93 g/L cholesterol, 0.93 g/L P-hydroxybenzoic acid, 1.87 g/L linseed oil, 0.093 g/L vitamin mix, 0.93 g/L choline and 18.75 g/L agar), at 25 ± 1 ºC and 70 ± 5% RH, with 16:8 h light-dark period.

RNA extraction and cDNA synthesis

Total RNA was extracted from *S. littoralis* larvae using RNAesy® Mini Kit (Qiagen) as follows. The larvae were macerated in a 1.5 mL microtube with 350 µl lysis buffer (3.5 µl β-mercaptoetanol and 346.5 µl RLT buffer supplied with the RNAeasy kit) and vortexed for 10 minutes. Suspensions were further homogenized by passage through a QIAShredder (Qiagen) mini-spin column according to the manufacturer's instructions. The homogenates were then subjected to RNA extraction procedure of the Qiagen RNAeasy kit with a final elution in RNAse-free water. RNA quality and quantity were assessed with a NanoDrop 2000c spectrophotometer (Fisher Scientific). A total of 0.5 ng of total RNA per larvae was reverse transcribed using Omniscript RT Kit (Quiagen). The reaction was performed containing 1 µl transcriptase reverse enzyme, 4 µl of RT buffer 5x, 1 µl RT primer mix and 14 µl of RNA template.

Primer design and amplification tests

The pair of primers (PPOF, PPOR, Table 1) was designed to amplify the general mRNA of *Spodoptera* PPO gene through reverse transcription polymerase chain reaction (RT-PCR). The primer design was based on different sequences previously published in the Gen-Bank database (accession numbers AY703825, DQ289582 and EF684939). To test the efficiency of the primers, a PCR amplification of target gene was performed in a 10µl reaction mixture containing 2 µl of cDNA derived from the reverse transcription reaction, 5 µl of Taqman Fast Universal PCR master mix (2X), 0.5 µl (0.3 µM) of Probe, 0.5 µl (0.25 µM) of each specific primer, and 1.5 µl of sterile H2O. The EF1 primers (Table 1) were used as controls to evaluate potential problems related with the cDNA template. The qRT-PCR reactions were performed on a 7500 Fast Real-Time PCR System (Applied Biosystems) using the following conditions: 95 °C (20 s), 40 cycles of 95 °C (03 s), 60 °C (30 s).

Standard Curve and quantification

A standard curve was constructed with the cDNA amplicon obtained from conventional RT-PCR and confirmed by gel electrophoresis (Fig. 1). To quantify the number of genomic copies, first it was evaluated the concentration of a purified amplicon sample with a NanoDrop 2000c spectrophotometer (Fisher Scientific). With this information, the number of genomic copies was estimated by calculating from the molecular weight and cDNA amount based on the assumption that the average weight of a base pair (bp) is 650 Daltons. This means that one mole of a bp weighs 650 g and that the molecular weight of any double stranded DNA template can be estimated by taking the product of its length (in bp) and 650. The inverse of the molecular weight is the number of moles of template present in one gram of material. Using Avogadro's number, 6.022 x 10^{23} molecules/mole, the number of molecules of the template per gram can be calculated: mol/g x molecules/mol = molecules/ g. Finally, the number of molecules or number of copies of template in the sample can be estimated by multiplying by 1 x 10^{9} to convert to ng and then multiplying by the amount of template (in ng) (Staroscik 2004; Moeller 2012). Using the known concentration amplicon sample, serial 10-fold dilutions (10 to 10^{6} copy number) were used as positive samples for the development of a standard curve. The efficiency of the method was tested over 120 PPO positive samples previously confirmed.

PPO gene expression in larvae of different ages and population density

Using the standardized real time PCR protocol, the absolute quantification of PPO gene expression was assessed on larvae from different ages (hours post eclosion) and population conditions. For this assay, around 120 24h old larvae of *S. littoralis* were placed in 25 mL pots, either singly ('solitary' treatment) or in groups of three individuals ("gregarious" treatment). Larvae were provided with artificial diet *ad libitum* and reared at 25 ± 1 ºC and 70 ± 5% RH, with 16:8 h light-dark period. Twelve larvae from each treatment were chosen randomly every 24h for total RNA extraction and qRT-PCR analysis. PPO expression was evaluated in larvae from 48 – 144 hours post-eclosion.

dsRNA synthesis

Total RNA purified from *S. littoralis* larvae was retro-transcribed with the protocol cited above and a 238 bp cDNA fragment was obtained by PCR using the primers PPOF/PPOR (Table 1). This cDNA fragment was used as a template for a nested-PCR reaction, performed with primers containing at their ends the T7 polymerase promoter sequence T7PPOF and T7PPOR (Table 1). The resulting PCR product served in turn as template to synthesize dsRNA using the RiboMAX™ Large Scale RNA Production Systems (Promega), according to the manufacturer's instructions. Two complementary RNA strands were synthesized from the nested PCR product. The resulting RNA strands were annealed after the transcription reaction to form dsRNA. Remaining single-stranded RNA and DNA template were removed with a nuclease digestion step. The dsRNA was then purified by isopropanol precipitation and quantified by measuring their absorbance at 260 nm with a NanoDrop 2000c spectrophotometer (Fisher Scientific). Products were run on 2% agarose gels to confirm their integrity (Fig. 2).

dsRNA administration tests

For each methodology assay, a total of 60 (30 control / 30 treatment) *S. littoralis* larvae were used. After the administrations, the larvae were maintained in diet cups at 28 ± 1 °C. Below are the specifications of each methodology:

Pellet Feeding and Soaking: For these two methods, L3 larvae reared individually in diet cups were used. For pellet feeding, 2 µl of dsRNA solution (450 ng/µl) with food dye (4%) was administered daily over 5 days by providing the larvae with a 0.5cm-diameter diet-plug each day. For control group, it was provided a food pellet with ultrapure water with food dye (4%). In soaking methodology, one group of larvae (treatment) was soaked in 450 ng/µl dsRNA solution and another one (control) was soaked in ultrapure water, both for five minutes daily. On these two methodologies, every 24 h after application, six larvae from each group (dsRNA and control) were chosen randomly for RNA extraction and qRT-PCR analysis.

Droplet feeding: neonate larvae were starved for 24 hours and submitted to a modified droplet feeding method (Hughes, Van Beek & Wood 1986; Bouwer & Avyidi 2006). In this method larvae were transferred to the centre of 90mm Petri surrounded by drops of 1 µg of dsRNA containing 4% blue food dye. As a control, starved larvae were exposed to droplets of sterile distilled water containing 4% blue food dye. After 30 minutes, the larvae that ingested the solution were identified by body colour change (the same of food dye) and immediately transferred into diet cups. Total RNA was isolated from larvae at 24 h post-feeding for qRT-PCR analysis.

Data Analysis

Regression analysis were used to determine the relation between cycles of threshold (Cts) and genomic copy numbers. This information was then used to establish a standard curve for qPCR and evaluate the efficiency of this technique. In gene expression and dsRNA experiments, the genomic copy numbers obtained from qPCR analysis were used such as response variable to evaluate effects of larval age, rearing condition and dsRNA. The data were log-transformed and evaluated using lineal models (LMs) with normal errors. All statistical analyses were conducted using R v3.2.0 (R Core Team 2015).

Results

Absolute quantification by standard curve

The methodology of qRT-PCR using standard curve method successfully amplified and quantified the expression of PPO gene in *S. littoralis* larvae. Using a dilution series of 10 to 10^6 copies of PPO DNA-amplicon, even less than 10 copies of DNA could be reproducibly and readily detected. The Cts were linearly proportional to the input copy number showing an adequate pattern for quantifying (Fig. 1). The slope (−3.44) of the linear regression standard curve presented a strong dependence between Ct and starting quantity (R^2= 0.99). Additionally, the efficiency of PCR reaction was 95.11%, according to the formula: efficiency = $(10^{-1/\text{slope}}) - 1$.

All 120 samples used such as positive controls for PPO were successfully amplified, with a strong dependence between Ct and starting quantity (R^2= 0.98) (Fig. 2) following the same pattern shown in the standard curve. PPO gene expression could be detected in all larval ages analysed in the experiment (48 – 144 hours post-eclosion), however no differences were observed between the groups (LM: larval age: F= 0.992, df= 4, P= 0.41) (Fig. 3A). PPO expression was also not affected by population density (LM: population solitary / gregarious: F= 0.332, df= 1, P= 0.56) (Fig. 3B).

DsRNA methodologies

Gene expression of PPO in *S. littoralis* larvae was not affected by the first two dsRNA methodologies (LM: dsRNA / pellet feeding: F= 0.378, df= 1, P= 0.54; LM:

dsRNA / soaking: F= 0.09, df= 1, P= 0.76), which was evaluated on larvae from 24 to 144 hours after dsRNA exposure (Fig. 4A). Unlike the first two, the experiment using droplet feeding administration methodology showed a slight increase in PPO expression (Fig. 4B) for larvae exposed to dsRNA (F= 6.328, df= 1, P< 0.01).

The results were further verified by qRT-PCR using EF1α as reference gene to examine possible effects of dsRNA on non-target genes and problems related with equipment accuracy. The analysis revealed that EF1α remained unchanged between dsRNA-fed and control larvae for the three different dsRNA methodologies (Fig. S3).

Discussion

PPO expression in *S. littoralis*

The relevance of PPO activation in aspects of physiology and immunity has been intensively studied for many years, however very little is known about molecular and biochemical aspects of this important factor in invertebrate organisms (Shi *et al.* 2014). Here we characterized the gene expression of PPO in *S. littoralis* larvae, which according to our results is apparently expressed since the first instar, maintaining low levels of expression and with no apparent increasing related to population densities. Apparently, the expression of genes related with this molecule varies between species. In brine shrimps genes related with PPO are inactive in embryonic phase beginning only at emergence (Fan *et al.* 2011), whereas in *Anopheles* it is expressed throughout all life stages from egg to adult phase (Lee *et al.* 1998).

In our study levels of PPO expression were equally low in all larval ages and population densities. Perhaps the lack of immune challenges in our study may be related with results shown. For each species, specific factors can trigger PPO expression (Ashida 1997). PPO, as a precursor of the cascade reaction of humoral defences reacts in the presence of pathogen antigens, increasing its expression

(Iwanaga & Lee 2005). For example, in *Drosophila* flies it was observed that expression of PPO is strongly related with PO activity, which is against bacterial and fungal infections (Binggeli *et al.* 2014). PPO expression can also be affected in symbiont – vector relationships such in interactions between arboviruses and *Aedes albopictus* where viruses can activate PPO cascade generating anti-viral mechanisms (Rodriguez-Andres *et al.* 2012).

Several studies emphasize that for some insects, different phenotypes and population size can affect immune traits, including the PPO complex. It is also observed that some noctuid species tend to invest more in defence mechanisms when reared in high densities (see Wilson & Reeson 1998). However, the expression of PPO may not be totally linked with population size as we observed in this study. In the absence of triggering factors, differences on immune investment related with density depend of prophylaxis may not be shown. Additionally, for some species high population densities with no infections can also generate opposite effects, decreasing immune defences such PPO activity (Piesk *et al.* 2013).

dsRNA effects in *S. littoralis* PPO

Even using three different methodologies we did achieve a successful knockdown for PPO gene of *S. littoralis.* Differently from other insect groups such as beetles and bees, the use of RNAi methodologies in lepidopterans present several problems issues (Terenius *et al.* 2011). The causes of these problems are not totally clear and lack of publications related with these failures lead to expenditure of time and resources replicating studies with methodologies that will not silence genes (Yu *et al.* 2013). It is speculated that failures can be due to a combination of different factors, such as the amount of dsRNA administrated, insect age/instar, administration form and also the gene itself (Hong *et al.* 2005; Charoensapsri *et al.* 2009; Yu *et al.* 2013). We checked a history of failures of RNAi knockdown before choosing characteristics of methodologies analysed. For example, micro-injection of dsRNA is often used with success; however this kind of methodology generally is more suitable for embryos and does not present good results in larvae and later stages (Charoensapsri *et al.* 2009; Terenius *et al.* 2011). Another important factor is the quantity of dsRNA administrated. With the exception of the droplet feeding technique, we precisely controlled the quantity of dsRNA administered considering that both lower and higher concentrations can result in failures (Hong *et al.* 2005; Terenius *et al.* 2011).

Differently from soaking and pellet feeding methodologies, insects exposed to dsRNA via droplet feeding presented an increasing of PPO gene expression. This process can be characterized as a mediated gene upregulation, i.e. an increasing of gene expression through a trigger factor, including dsRNA molecules. Several studies have emphasized the possibility of upregulation by RNAi, however in most of them, the effect is observed in non-target genes that are affected in different ways due to silencing methodologies (Jackson *et al.* 2003). For example, in a study to identify RNAi effects in non-target genes of honey bees, Jarosch and Moritz (2011) identified several unspecific results in a several other genes. Additionally, RNAi methodologies based in dsRNAs can also induce transcriptional activation, a process where the target gene can be overexpressed due histone modification and / or DNA methylation with participation of Ago proteins (Li *et al.* 2006).

The knockdown methodology based in dsRNA acts such as Trojan horse transporting RNAi molecules to intracellular space, which can be related with an infection process shown in dsRNA viruses (Wagner 2011). Moreover, in a related study with the shrimp *Penaeus monodon*, dsRNA also promoted upregulation of PPO instead of silencing (Paria *et al.* 2013). In our study, especially for the droplet feeding methodology where first instar larvae were exposed to dsRNA *ad libitum,* we believe that this molecule has activated the immune system increasing expression of genes related with defence, including PPO.

Molecular studies related with immune genes such as PPO have an important role not only to better understanding defence processes in invertebrates, but also for an applied point of view. Recently, perspectives of using RNAi to pest control are being discussed due several advantages of this method such as specificity and lack of risks to non-target organism (Tian *et al.* 2009; Surakasi, Mohamed & Kim 2011; Estep III, Becnel & Sanscrainte 2016; Thakur, Mundey & Upadhyay 2016). In theory, RNAi molecules reducing immune competence or affecting vital physiological processes could enhance the impact of natural enemies, and may also improve the efficacy of biopesticides (Di Lelio *et al.* 2014). However, as we observed in our study, unexpected results from RNAi methodologies may also happen, which emphasize the necessity of extensive laboratory tests to evaluate critical points for application of RNAi.

Conclusion

In conclusion, we successfully standardized a qPCR methodology for absolute quantification of *S. littoralis* PPO gene. With this technique, we could evaluate that on absence of immune challenges, the expression of this gene is not affected by larval age and population density. With this same standardization, we could also identify failures on gene silencing of PPO, even using different RNAi methodologies. Standardization for absolute quantifications of genes can promote an important step on immune studies of pest insects. The set of information about how immune defences actuate on organism can help to increase biocontrol efficacy bringing great perspectives for the future of pest control.

Table 1: Primers used in this study

Primer name	Sequence (5' - 3')	Target
PPOF	CCG TGA TGA CAC CAA GAA TG	*Spodoptera* sp. Prophenoloxidase (PPO) mRNA
PPOR	ATG CCA ATG CCA GTG ATG TA	
EF1F	TCA AGA ACA TGA TCA CTG GAA CCT	General EF1α mRNA
EF1R	CCA GCG GCG ACA ATG AG	
T7PPOF	TAA TAC GAC TCA CTA TAG GCC GTG ATG ACA CCA AGA ATG	T7 primers for PPO dsRNA
T7PPOR	TAA TAC GAC TCA CTA TAG GAT GCC AAT GCC AGT GAT GTA	

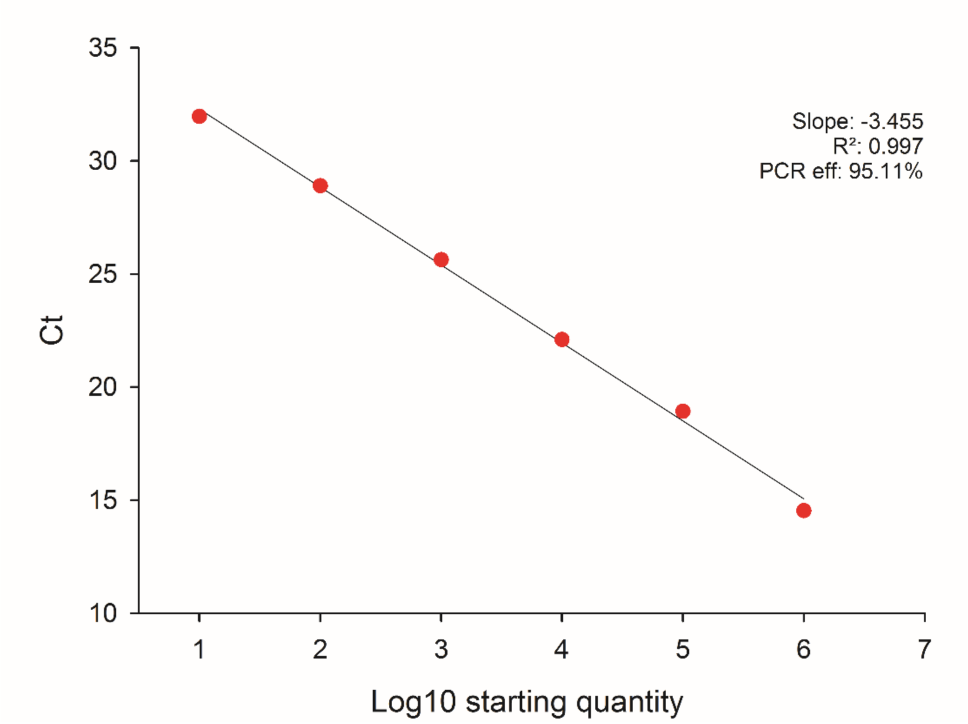

Figure 1: Standard curve obtained by plotting the Cycle of Threshold (Ct) value vs. the initial number of genomic copies. The red circles represent the log10 transformed initial number of genomic copies added in reaction. The regression line represents the negative relation between the initial number of genomic copies and cycle of threshold (i.e. lower initial number of genomic copies will generate higher Cts). Slope= regression coefficient; R^2= coefficient of determination; PCR Eff= Efficiency of amplification.

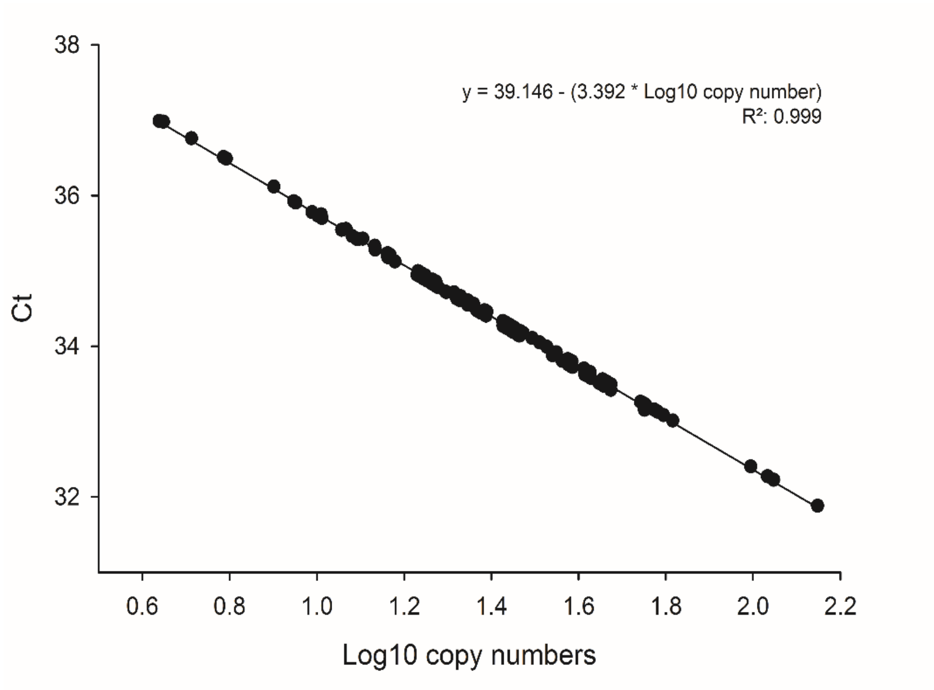

Figure 2: Validation of standardized qPCR methodology for PPO absolute quantification. Regression curve shows the linear relationship between the cycle of threshold (Ct) and initial number of genomic copies from 120 samples used such as controls. Negative exponential regression (R^2=0.98) gives y= 32.42 + 4.77e(-0.35x).

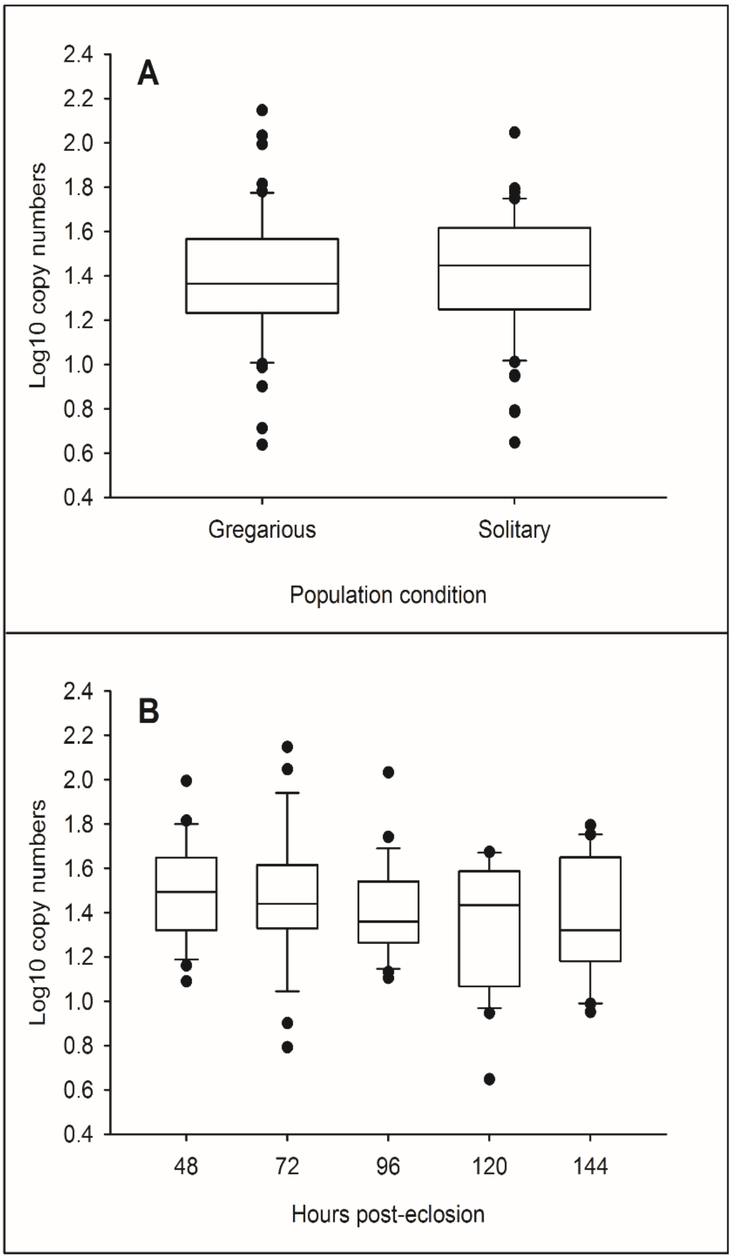

Figure 3: A - Box plot of the log-transformed genomic copy numbers of PPO gene expression in larvae with 48 to 144 hours post-eclosion (48h: n= 24; 72: n= 24; 96: n= 24; 120: n= 24; 144: n= 20). B - Box plot of the log-transformed genomic copy numbers for PPO gene expression related with larvae reared in solitary and gregarious conditions (solitary: n= 60; gregarious: n= 57). In both cases no differences were observed in PPO expression.

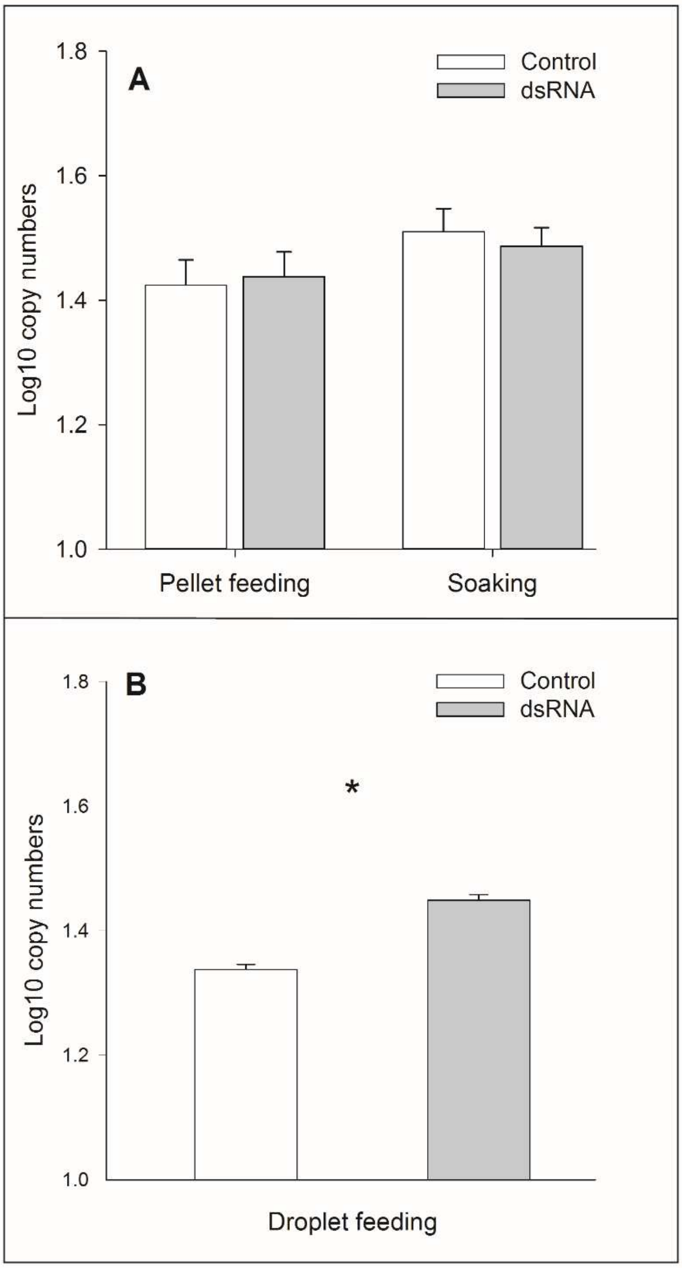

Figure 4: A - Log-transformed genomic copy numbers for PPO gene expression in larvae from pellet feeding and soaking dsRNA administration assays (both, control: n=30; dsRNA treatment: n=30). No differences were observed in PPO expression for both dsRNA administration methodologies. B - Log-transformed genomic copy numbers for PPO gene expression in larvae from dsRNA droplet feeding assay (control: n=30; dsRNA treatment: n=30). In this administration methodologies, the expression of PPO was higher on larvae exposed to dsRNA.

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CHAPTER 4

Density prophylactic response and immune specificity in the African cotton leafworm *Spodoptera littoralis*

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Abstract: The African cotton leafworm *Spodoptera littoralis* (Boisduval, 1833) demonstrates density-dependant phenotypic plasticity (DPP) with regards to pathogen resistance. Individuals of this species invest more in immune defence and are more resistant to infections when they are reared in crowded conditions compared to solitary conditions. However, it remains to be clarified whether insects can specifically regulate their immune investments in response to pathogen traits (such as virulence, viability and host specificity). Here, our initial experiment tested whether immune DPP differs during infection. Next, we focused on humoral immune responses, testing whether they showed pathogen specificity, by exposing larvae to three bacterial species, *Xenorhabdus nematophila* (host-specific pathogen), *Bacillus thuringiensis* (generalist pathogen) and *Bacillus subtilis* (non-pathogenic). We also tested whether this specificity depended on virulence (alive vs heat-killed). In the population density experiment, both prophenoloxidase (PPO) and phenoloxidase (PO) activity were directly affected by the presence of bacterial infection. Immune responses were higher in gregarious larvae infected with bacteria, but not different or even lower than in solitary larvae when not infected. In general, the three bacteria when injected alive presented higher levels of lysozyme, PPO and PO activity than controls, with slight differences between them. However, when inactivated by heat killing, only *X. nematophila*, a haemolymph pathogen, produced higher levels of lysozyme activity. Our results suggest that only with a triggering factor such as bacterial invasion, can trade-offs between immune investment and DDP be shown in *S. littoralis*. Independent of pathogenicity, presence of live bacteria in haemolymph can activate humoral immune defences. However, levels of immune responses can vary according to bacterial species and condition (live/dead) showing signs of specificity.

Keywords: Lepidoptera, insect immunity, melanisation, Noctuidae, gregarious, solitary, *Bacillus thuringiensis*, *Bacillus subtilis*.

Introduction

Insects have a set of mechanism which allow them to identify and defend themselves against foreign particles or live organisms (reviewed in Gillespie & Kanost 1997). These responses such as immune defences, act in a chain reaction starting with recognition of non-self through identification of specific chemical structures present in invaders. After identification, mediation of immune signals such as eicosanoids triggers cellular and humoral responses (Gillespie & Kanost 1997; Schmid-Hempel 2005). Cellular defences are typical unspecific immune responses performed by haemocytes, which generally phagocyte small particles and capsulate larger ones in a process by which the parasite is covered by a melanised capsule of haemocytes (Götz 1986; Au *et al.* 2004). In turn, insect's haemolymph also contains several peptides and enzymes with antimicrobial activity such as phenoloxidase (PO), lysozyme and antimicrobial peptides (Lambert *et al.* 1989; Cotter & Wilson 2002; Chapelle *et al.* 2009; Wang & Jiang 2010).

Some of these molecules such as PO also have other relevant functions in organism (such as in cuticle melanisation) and their expression levels can vary drastically according to insect species, age and population density (Wilson & Reeson 1998; Cotter & Wilson 2002; Rajagopal *et al.* 2005; Hegazi & Khafagi 2008; Fu *et al.* 2016). The cotton leaf worm *Spodoptera littoralis* (Boisduval, 1833), exhibits density-dependent phase polyphenism (Reeson *et al.* 1998; Cotter *et al.* 2004), which according with density-dependent prophylaxis (DDP) hypothesis increased levels of cuticular melanisation observed in crowd-reared insects are related with higher levels of immune defences (Wilson & Reeson 1998).

In DDP hypothesis, crowded individuals invest more in immune function than those reared solitarily in order to balance the risk of infections at high densities (Wilson & Reeson 1998). However, studies using different insects have been demonstrating that immune defences are costly and risky to be constantly maintained at higher levels (Sheldon & Verhulst 1996; Rigby, Hechinger & Stevens 2002; Schmid-Hempel 2005). Therefore, the investment and differences in defence performance between solitary and gregarious insects may be noted only with a triggering process such as a bacterial infection. In addition, trade-offs between immune defences may occur to minimize the total costs invested in immunity, such low levels of lysozyme contrasting with high PO activity (Cotter *et al.* 2008; Wang *et al.* 2010; Cotter *et al.* 2011).

Variations on immune investment can also be observed according to pathogen species, status and virulence (Lochmiller & Deerenberg 2000; Moret & Schmid-Hempel 2000). Zibaee and Malagoli (2014) found that levels of humoral responses in Asiatic rice borer *Chilo suppressalis*, varied according to species and virulence of different entomopathogenic fungi. Moreover, incubations of heat killed pathogenic bacteria *Xenorhabdus nematophila* and non-pathogenic *Bacillus subtilis* with lepidopteran haemocytes *in vitro* revealed differential cellular immune responses according with bacteria species (Giannoulis *et al.* 2007).

Here, we hypothesize that insects with phenotypic plasticity related with density, both reared solitary or in group, maintain indistinguishable levels of humoral defences on absence of a triggering factor, such as foreign particles and organisms. Additionally, independently of population density, we expect that humoral immune defences in *S. littoralis* present certain levels of specificity according to bacterial species, pathogenicity and bacterial status (dead or alive).

Materials and Methods

Insect Material

The insects used in all assays were obtained from colonies of the insect and parasite ecology laboratory, University of Lancaster, Lancaster UK. Larvae were maintained with artificial diet (72 g/L wheat germ, 33 g/L casein, 29.25 g/L sugar, 14.25 g/L yeast extract, 9.37 g/L Wesson's salt, 1.5 g/L ascorbic acid, 0.93 g/L cholesterol, 0.93 g/L P-hydroxybenzoic acid, 1.87 g/L linseed oil, 0.093 g/L vitamin mix, 0.93 g/L choline and 18.75 g/L agar), at 25 ± 1 C and 70 ± 5% RH, with 16:8 h light-dark period.

Experiment 1: Relevance of bacterial infection in variations of population density and investment in immune defences

Moths were sexed and couples individualized in plastic pots and fed *ad libitum* with 5% sucrose solution. The couples were allowed to oviposit on sheets of absorbent paper and eggs were collected daily and kept in small plastic pots (50 mL) containing artificial diet (described above). Upon egg hatching, caterpillars were promptly placed in 100ml plastic and separated in two groups: caterpillars were kept alone or three individuals per pot. After 10 days, larvae that reached 6th instar were injected with 5 µl of solution containing 10^3 per ml of *Xenorhabdus nematophila* (sublethal dose) in their abdominal pleural region using a 50 µl Hamilton syringe with a micro-injector. Four distinct groups were established in this study (Solitary larvae + bacterial infection, solitary + control solution, gregarious + bacterial infection, gregarious + control solution). After 24 hours, haemolymph from all larvae was collected and kept in 1.5ml microtubes containing anticoagulant buffer (van Sambeek & Wiesner 1999) stored −20 C until use. Around 50 insects were used per treatment. The bacteria were cultured in tryptic nutrient broth (Sigma-Aldrich, USA) at 25°C for 24 h. After growing, bacteria were quantified with a Neubauer improved cell counting chamber and treatment dose was prepared.

The immune defences assessed here were: lysosome-like inhibition activity, prophenoloxidase (PPO) and phenoloxidase (PO) relative activity. These parameters were chosen due their relevance as humoral defence mechanisms against several pathogens including bacteria. Lysozyme-like activity was evaluated by antibacterial activity on agar (Lambert *et al.* 1989). Agar plates containing 10 ml of 1% agar with 5 mg per ml freeze-dried *Melissococcus lysodeikticus* were prepared. For each plate, 20 holes with a diameter of 2 mm were punched in the agar and 1 µl of haemolymph was placed in each well, two replicates per sample. The plates were incubated at 33 C for 18 hours then photographed using a Canon PowerShot G6 digital camera (Canon, Tokyo, JAPAN). The diameter of the clear zones was calculated using Image Pro Plus software (Media Cybernetics). A serial dilution of hen egg white lysozyme (0.01 to 2%) was used as positive control of the methodology.

For PPO and PO activity, 8 µl of haemolymph from each larva were diluted in 360 µl of NaCac and split in two portions. The first one was used to evaluate PO activity, which were added 20µl of NaCac buffer + 90 µl of dopamine. Reactions were carried out in duplicate along with negative control samples. During the reaction, the enzyme catalyses the conversion of L-DOPA to dopachrome; dopachrome can then be measured spectrophotometrically. Readings were taken every 12 s, at 25 C, for 10 min at 490 nm. Second, PPO was then evaluated adding 20µl of 20 mg/ml chymotrypsin in NaCac buffer + 90 µl of dopamine at same reading conditions. Chymotrypsin activates the zymogen PPO, and along with dopamine also produces dopachrome providing indirect evaluation of PPO activity.

Experiment 2: Immune specificity related with bacteria species

To assess the specificity on immune system of *S. littoralis*, a second assay was realized exposing 6th instar larvae to three different bacteria species: *X. nematophila*, a virulent gram-negative bacteria that infects several insect's haemolymph; B*. thuringiensis* a gram positive bacteria with cry toxin highly lethal to noctuid larvae; and *B. subtilis*, a non-pathogenic gram positive bacteria normally found in soil. The insect's condition for this experiment were the same for the first one, however only insects kept alone were used. The sub-lethal doses used for each species of bacteria were based on previous studies with these species, which were capable of activate the immune system of larvae without killing them in a period of 48h. To determine if only bacterial constituents can activate immune system and show signs of specificity, other group of larvae were injected with the same sub-lethal doses of heat-killed bacteria (72°C for one hour). Control larvae, both from alive and heat killed bacteria, were injected with 5 µl of sterile nutrient broth solution.

Bacterial injections were also done such as in the first experiment (for all bacteria: 5µl of 10^3 bacteria per ml solution), and after 24 hours, haemolymph from each insect was extracted and readily stored at −20 C until use. Fifty larvae were used for each treatment (Live bacteria: *X. nematophila*, *B. thuringiensis*, *B. subtilis* and negative control; Heat-killed bacteria: *X. nematophila*, *B. thuringiensis*, *B. subtilis* and negative control). Lysozyme-like inhibition activity, prophenoloxidase and phenoloxidase relative activity was also used to the specificity on *S. littoralis* innate immunity. Due a problem with dopamine, the heat-killed treatment for *B. thuringiensis* could not be evaluated for PO and PPO activity.

Data Analysis

For Experiment 1, we evaluated the effects of bacterial infection and population density on immune defence of *S. littoralis*. We first fitted full models using LM function due normality of data distribution. To evaluate the individual effects for each bacteria and population size treatments, contrast models were made. Final models were accepted when not significantly different from the previous models. Residuals of the final model were checked for suitability of the distribution.

In the second experiment, we tested the specificity level of immune defences exposing larvae to different species of bacteria, both alive and heat killed. Initially we fitted full models using LM function. After evaluating that main terms were significantly different, post-hoc pairwise comparisons were made via Tukey's test (glht function in multcomp, correcting P values for multiple comparisons by the single-step method). All Statistical analyses were conducted using R v3.2.0 (R Core Team 2015).

Results

Relevance of bacterial infection in variations of population density and investment in immune defences

On absence of bacterial infection, gregarious insects did not present higher levels of immune defences. In turn, when infected by *X. nematophila*, some immune defence evaluated here presented different aspects according to population density.

In general, lysozyme-like inhibition activity was not affected by population size (LM: df= 1, F= 0.025, P= 0.8745; Fig. 1). Evaluating each treatment separately, lysozyme activity was also not different between solitary and gregarious larvae both from control and infected with *X. nematophila* (LM: control: df= 1, F= 1.5306, P= 0.218 / *X. nematophila*: df= 1, F= 0.1049, P= 0.7467; Fig. 2). The general relative PPO activity was higher in gregarious larvae (LM: df= 1, F= 0.025, P= 0.8745; Fig. 1). However, evaluating each treatment separately was identified that PPO levels were higher in gregarious larvae only in the presence of bacterial infection (LM: control: df= 1, F= 3.5295, P= 0.062 / *X. nematophila*: df= 1, F= 8.9227, P= 0.003; Fig. 2). Following the same pattern, general PO relative activity was also higher in gregarious larvae (LM: df= 1, F= 7.872, P= 0.005; Fig. 1). However, PO presented an opposite pattern where the activity was higher in solitary larvae from control group, but also higher in gregarious larvae when they were infected with bacteria (LM: control: df= 1, F= 7.1218, P= 0.008 / *X. nematophila*: df= 1, F= 23.119, P< 0.001; Fig. 2).

Immune specificity related with bacteria species

Immune defences in *Spodoptera littoralis* varied activity according to bacteria species. Live bacteria have affected immune defences evaluated, but when heat killed in most of the cases no effects were observed. However, bacterial constituents of heat killed *X. nematophila*, the only specific haemolymph pathogen used in the assays, could also activate one of the defence parameters evaluated. Although we have not evaluated one of heat killed bacteria treatments for PPO and PO (see above), we could identify a clear difference in lysozyme activity.

In general, lysozyme inhibition activity, PPO and PO activity were affected by live bacterial infections (LM: lysozyme: df= 3, F= 50.481, P< 0.001; Fig. 3A / PPO: df= 3, F= 10.685, P< 0.001; Fig. 3C / PO: df= 3, F= 59.666, P< 0.001; Fig. 3E). Larvae infected by *B. thuringiensis* presented highest levels for lysozyme, followed by *B. thuringiensis* and *X. nematophila* with no difference between them, and control treatment with lowest levels (see lysozyme in Table 1). The levels of PPO were equally higher in *B. thuringiensis* and *B. subtilis* than *X. nematophila* and control (see PPO in Table 1). In turn, PO activity was also higher in *B.* thuringiensis, followed by *B. subtilis* and *X. nematophila* with no difference between them (see PO in Table 1).

In treatments which all bacteria were heat killed, only lysozyme inhibition activity differed between the treatments (LM: lysozyme: df= 3, F= 8.5855, P< 0.001; Fig. 3B / PPO: df= 3, F= 0.0438, P= 0.957; Fig. 3D / PO: df= 3, F= 1.5072, P= 0.223; Fig. 3F). In turn, only heat killed *X. nematophila* presented higher activity compared with controls (see lysozyme in Table 2).

Discussion

Relevance of bacterial infection in variations of population density and investment in immune defences

Humoral immune defences have a crucial role in defence against infections (Schmid-Hempel 2005; Grizanova *et al.* 2014). In DDP hypothesis it is proposed that insects reared at high densities tend to invest more in immune defences (Wilson & Reeson 1998), such as lysozyme, PPO and PO. However, our results indicate that differences on immune levels related with DDP could be shown only in the presence of a triggering invasion on insect haemocoel. In addition, the relationship of immune investment with DDP may depend of several factors including risks of infection, life history of insect species and nutritional condition (Wilson & Reeson 1998; Cotter *at al.* 2011). In our study PO levels were also higher in solitary than gregarious larvae when not infected with bacteria. Although this result is not in agreement with the general idea of immune investment and DDP, it matches previous results for this species. In a study with non-infected insects, Cotter *et al.* (2008) found higher levels of haemolymph PO in white solitary than black gregarious larvae of *S. littoralis*, however cuticle PO presented opposite results. It is speculated a possible trade-off between these two traits due large amounts of granular PO in dark-selected lines, which could reduce the copper availability for haemolymph PO (Wilson *et al.* 2001; Cotter *et al.* 2008). However, as observed in our study in the presence of bacterial infection, the activity of haemolymph PO was also higher in gregarious larvae.

Regardless of bacterial infection, lysozyme activity did not present differences between population densities. For some species, even presenting DDP, levels of some defence traits may not be affected by population density (Wilson *et al.* 2002; Wilson *et al.* 2003; Piesk *et al.* 2013). In the butterfly *Pieris napi*, which larvae are typically gregarious, Piesk *et al.* (2013) contrary found higher number of haemocytes and encapsulation in larvae reared alone than those reared in group (in absence of immune challenging). Silva and Elliot (2016) also observed that both encapsulation response and haemocyte activity were not affected by population size in the velvetbean caterpillar *Anticarsia gemmatalis*. Moreover, the relevance of both humoral and cellular defences on immune defences may vary between species, which can also influence their investments at high densities (Schmid-Hempel 2003). In *S. littoralis* both PPO and PO play an important role not only in insect immunity at high densities, but also in cuticle pigmentation and secondary processes (Ishaaya 1972). This may suggest that even in the presence of infection, gregarious larvae are unable to invest in all types of immune response simultaneously and tend to invest more in polyvalent molecules such PO (Cotter *et al.* 2004), which also actuate in melanisation and cuticle pigmentation.

Immune specificity related with bacteria species

Live bacteria when injected in *S. littoralis* haemocoel trigger responses such as lysozyme, PPO and PO activity. Moreover, each immune defence presented different levels between bacteria species. Differences in response of immune defences are generally attributed to bacterial species and levels of virulence (Howard, Miller & Stanley 1998; Giannoulis *et al.* 2007), however apparently the recognition and activation of humoral defences in insects are much more complex. Diverging from other bacteria and immune defences, *X. nematophila* did not affect levels of PPO. Previous studies have shown that both *X. nematophila* and *Bacillus* are recognized by insect immune system, however *X. nematophila* is capable of modify its surface properties to avoid some defence mechanisms such AMPs (Nielsen-LeRoux *et al.* 2012). In addition, haemolymph pathogens such as *X. nematophila* have a set of adaption to avoid or surpass humoral defences (Herbert & Goodrich-Blair 2007). These bacteria destroy haemocytes when phagocytized (Au *et al.* 2004) possibly affecting production of PPO, which is partially stored in these cells.

Bacterial cell wall is another important characteristic on humoral immune recognition and specificity (Lambert *et al.* 1989). Here we used the Gram- *X. nematophila* and Gram+ *B. thuringiensis* and *B. subtilis*. In insects, both Gram groups can trigger reactions on immune defences (Bulet *et al.* 1991). However, humoral immune responses can vary according to insect and also bacteria species

(Brookman, Ratcliffe & Rowley 1989). It is being shown that both *X. nematophila* and *Bacillus* can activate humoral defences in lepidopterans (Giannoulis *et al.* 2008; Tamez-Guerra *et al.* 2008; Fu *et al.* 2016). Peptidoglycans, which are widely found in cell wall of Gram+ bacteria, are considered the main responsible to immune recognition (Kang *et al.* 1998). However, the quantity of this molecule may be not totally related with intensity of response. In *Spodoptera exigua*, specific peptidoglycans along with the outer membrane of *X. nematophila* activates antimicrobial peptides, however the immune activity is even higher when dead bacteria of this species were injected (Ji & Kim 2004).

When heat killed, bacteria species presented contrasting results among the evaluated parameters and only *X. nematophila* affected one of immune defences evaluated. Bacterial parts related with immune recognition are generally thermostable, however studies show that immune recognition of heat killed bacteria show different responses of live bacteria (Brookman, Ratcliffe & Rowley 1989). Once inside the haemocoel, bacterial constituents of dead bacteria initially face the primal cellular defences (Lavine & Strand 2002); however some specific molecules can also trigger humoral defences. As observed here, other studies show that heat killed *X. nematophila* can increase lysozyme activity (Ji & Kim 2004; Giannoulis *et al.* 2007), whereas *B. thuringiensis* not (Wang *et al.* 2009). At first view, this fact could be attributed due differences in cell wall type (Gram+ or -), whereas studies show that other heat killed Gram+ bacteria such *Staphylococcus aureus* can increase lysozyme activity in lepidopterans (Gagen & Ratcliffe 1976; Wang *et al.* 2009). These aspects can indicate that specificity of humoral defence in *S. littoralis* may be related with the risk provided by invader. Once phagocytized, bacterial constituents of *Photorhabdus* and *Xenorhabdus* species destroy haemocytes and compromise cellular immune defences (Au *et al.* 2004). By the other side, cellular constituents of other species that we used here may be harmless to cellular defences of *S. littoralis*.

Conclusion

In conclusion, we have demonstrated that only with a triggering factor such as bacterial infection, variations between immune investment and DDP could be shown in *S. littoralis.* Independent of pathogenicity, the presence of live bacteria in haemolymph activated humoral immune defences. In turn, even without a clear sign of immune specificity related with virulence, levels of immune responses can vary according to bacteria species. Meanwhile, inactive bacterial constituents could identify a relevant signals in immune specificity of *S. littoralis*, being that only traces from a specific haemolymph pathogen has increased one of humoral defences evaluated. Recent studies have emphasized possible role of antimicrobial peptides (AMPs) on specific immune responses (Nielsen-LeRoux *et al.* 2012; Rahnamaeian *et al.* 2015), inclusive showing levels of immune priming (Wu *et al.* 2014). Accordingly, next studies should consider evaluations of AMPs on lepidopteran immune specificity. Understanding immune characteristics and mechanisms of insects can help in development of strategies to overcome defensive barriers increasing success of biological control.

Table 1: Pairwise comparisons between treatments of live bacteria assays for lysozyme, phenoloxidase and prophenoloxidase activity

Immune parameter	Linear Hypotheses	Estimate	Std. Error	t value	Pr(>|t|)
Lysozyme	*B. subtilis vs* control	0.153450	0.016898	9.081	**0.001*****
	B. thuringiensis vs control	0.193230	0.016898	11.435	**0.001*****
	X. nematophila vs control	0.148466	0.016984	8.741	**0.001*****
	B. thuringiensis vs B. subtilis	0.039781	0.016898	2.354	0.088
	X. nematophila vs B. subtilis	-0.004984	0.016984	-0.293	0.991
	X. nematophila vs B. thuringiensis	-0.044765	0.016984	-2.636	**0.043***
Phenoloxidase (PO)	*B. subtilis vs* control	0.016010	0.002221	7.207	**0.001*****
	B. thuringiensis vs control	0.028779	0.002216	12.988	**0.001*****
	X. nematophila vs control	0.020575	0.002227	9.239	**0.001*****
	B. thuringiensis vs B. subtilis	0.012769	0.002221	5.748	**0.001*****
	X. nematophila vs B. subtilis	0.004565	0.002233	2.045	0.173
	X. nematophila vs B. thuringiensis	-0.008204	0.002227	-3.684	**0.0014****
Prophenoloxidase (PPO)	*B. subtilis vs* control	0.003028	0.000918	3.297	**0.005****
	B. thuringiensis vs control	0.004419	0.000916	4.823	**0.001*****
	X. nematophila vs control	0.000399	0.000916	0.436	0.972
	B. thuringiensis vs B. subtilis	0.001391	0.000918	1.515	0.429
	X. nematophila vs B. subtilis	-0.002629	0.000918	-2.862	**0.022***
	X. nematophila vs B. thuringiensis	-0.004020	0.000916	-4.388	**0.001*****

Table 2: Pairwise comparisons between treatments of heat killed bacteria assays for lysozyme, phenoloxidase and prophenoloxidase activity

Immune parameter	Linear Hypotheses	Estimate	Std. Error	t value	Pr(>|t|)
Lysozyme	*B. subtilis vs* control	0.026745	0.017790	1.503	0.436
	B. thuringiensis vs control	0.002256	0.017790	0.127	0.999
	X. nematophila vs control	0.079291	0.017790	4.457	**0.001*****
	B. thuringiensis vs B. subtilis	-0.024488	0.017790	-1.377	0.514
	X. nematophila vs B. subtilis	0.052547	0.017790	2.954	**0.017***
	X. nematophila vs B. thuringiensis	0.077035	0.017790	4.330	**0.001*****
Phenoloxidase (PO)	*B. subtilis vs* control	0.003539	0.002376	1.489	0.298
	X. nematophila vs control	0.003606	0.002376	1.518	0.284
	X. nematophila vs B. subtilis	0.000067	0.002376	0.028	1
Prophenoloxidase (PPO)	*B. subtilis vs* control	0.000243	0.000994	0.244	0.968
	X. nematophila vs control	0.000265	0.000994	0.267	0.962
	X. nematophila vs B. subtilis	0.000022	0.000994	0.023	1

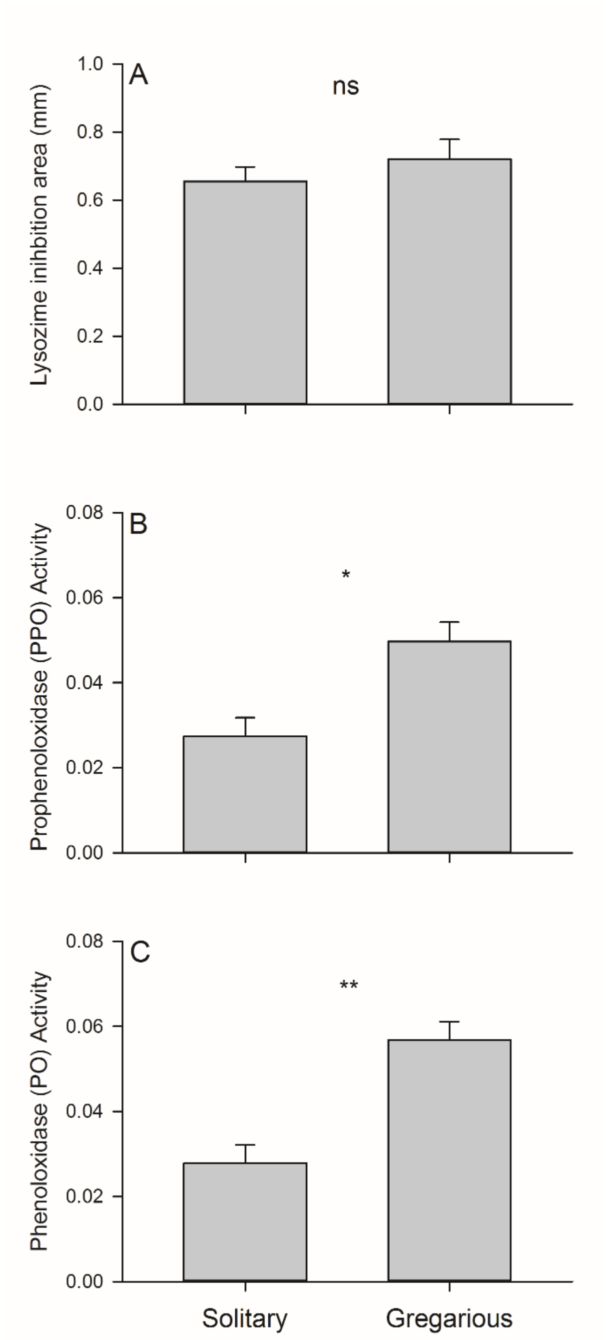

Figure 1: Effects of population size on immune defences: A= lysozyme inhibition activity; B= Prophenoloxidase; C= phenoloxidase enzymatic activity. Larvae were kept alone or in groups of three individuals from 1st to 6th instar. The graphs represent the general results (control + bacterial infection treatment). Error bars are standard errors. Asterisks indicate significant F-tests: *P < 0.05, **P < 0.01; and "ns" non-significant terms.

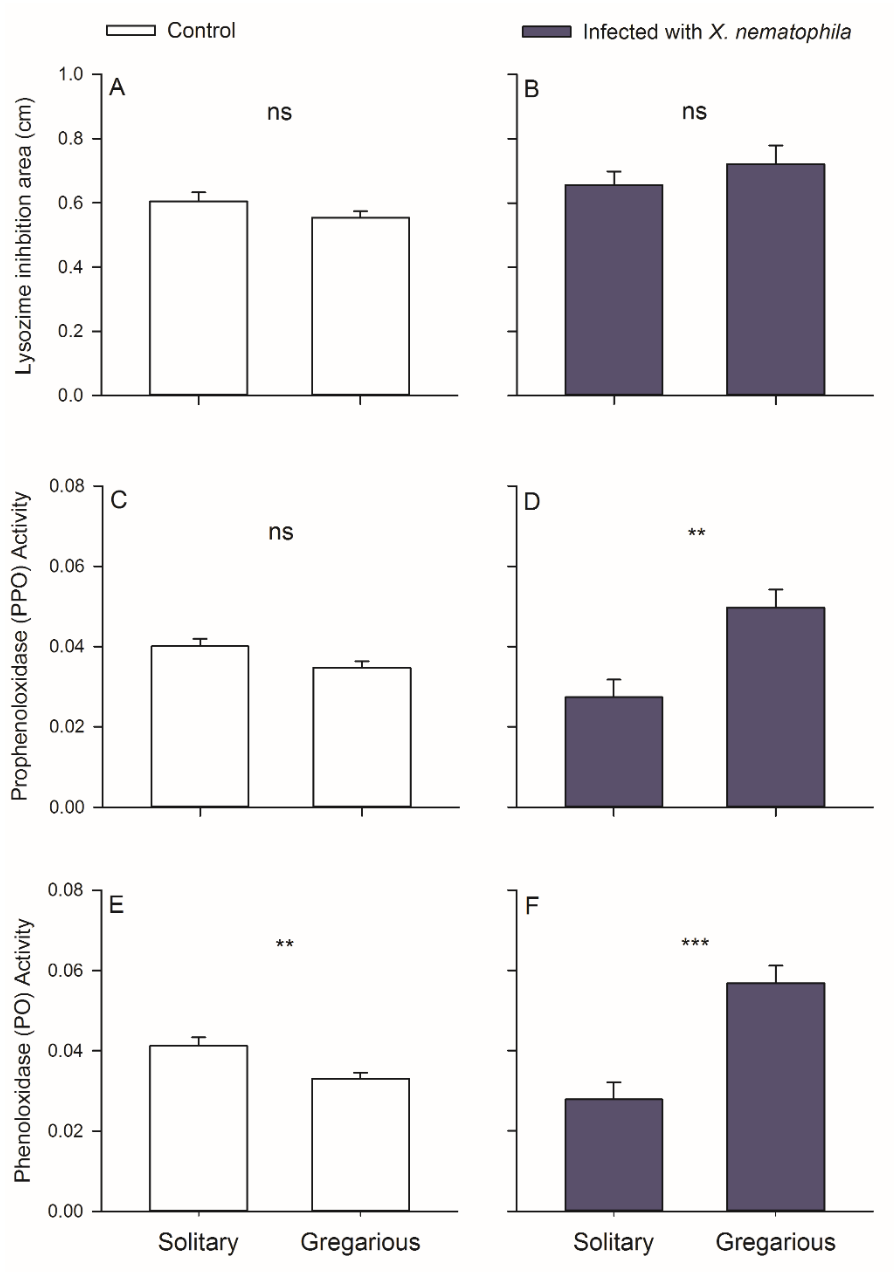

Figure 2: Relevance of bacterial infection on immune defences from larvae maintained in different population densities. Immune defence response on solitary and gregarious larvae not infected by bacteria (on the left panels), and infected by *Xenorhabdus nematophila* (on the right panels). Parameters evaluated: lysozyme inhibition activity (A, B), prophenoloxidase (C, D) and phenoloxidase enzymatic activity (E, F). Error bars are standard errors. Asterisks indicate significant F-tests: **P < 0.01, ***P < 0.001; and "ns" non-significant terms.

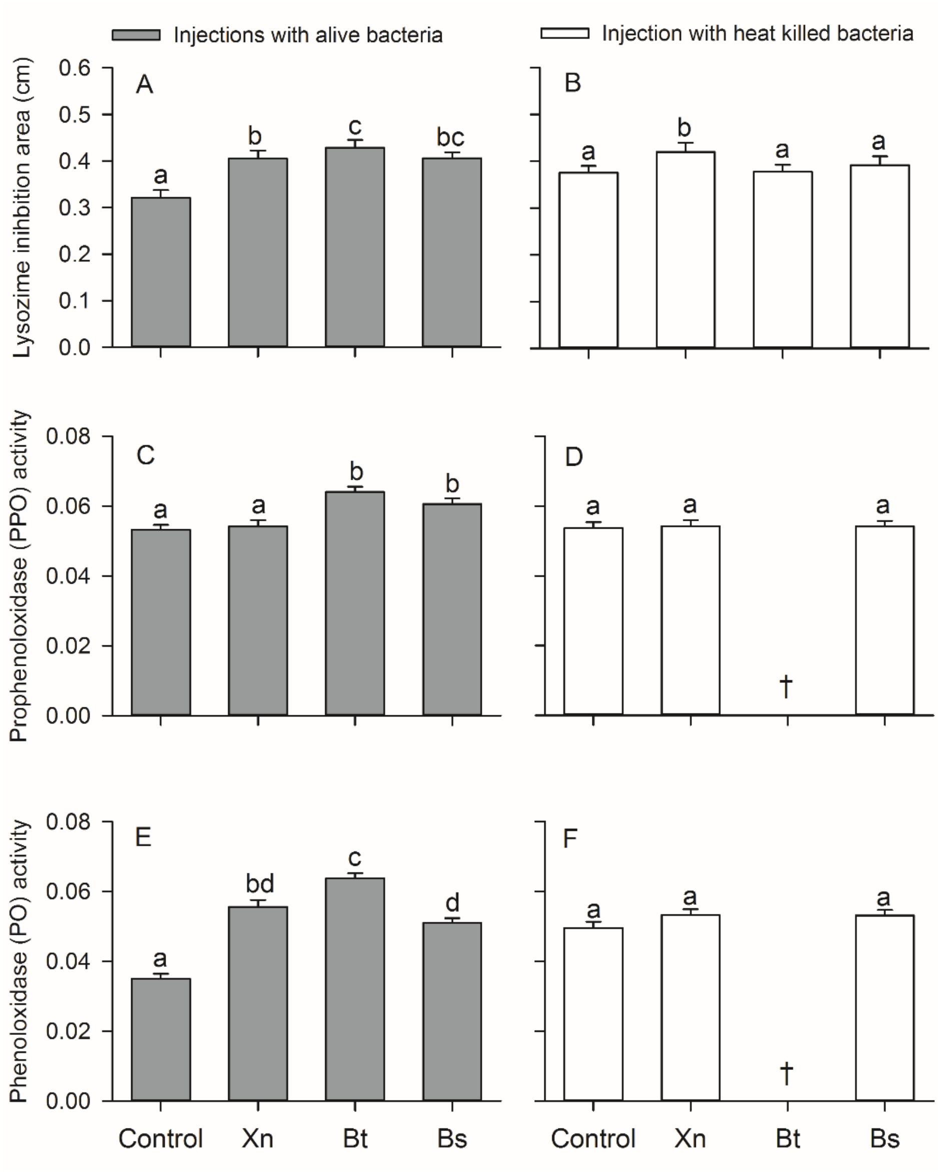

Figure 3: Immune specificity levels in *Spodoptera littoralis* larvae. Immune defence responses of larvae exposed to live bacteria (on the left panels), and exposed to cellular constituents of heat killed bacteria from the same species (on the right panels). Same parameters from the first experiment were evaluated: lysozyme inhibition activity (A, B), prophenoloxidase (C, D) and phenoloxidase enzymatic activity (E, F). Both alive and heat killed bacteria were injected on insect's haemolymph, which Control= only nutrient broth, Xn= *Xenorhabdus nematophila*, Bt= *Bacillus thuringiensis* and Bs= *Bacillus subtilis*. †= Heat killed *B. thuringiensis* treatment could not be evaluated for prophenoloxidase and phenoloxidase. Error bars are standard errors. Bars with different letters are significantly different according to post hoc Tukey tests in multiple comparisons analysis.

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CONCLUDING REMARKS

From genetic identification to applied studies on insect immunity, molecular and biochemical tools show great prospects for noctuid pest control. With the popularization and increasing of accessibility of molecular biology tools such as PCR, routine laboratories may easily identify and monitor both pest species and entomopathogens on field. On the other hand, theoretical studies focusing the immune defences of these pests can promote important information which can be used to increase the efficacy of entomopathogens. The combined use of these techniques can be useful on development of intelligent integrated pest management strategies based on biological control. Here we could show how some of these tools can be relevant on different aspects.

Main conclusions:

1. Through genetic markers, we found that the Old World bollworm *H. armigera* is present and spread in Minas Gerais state. This important pest can be found in different kinds of crops and in coexistence with the corn earworm H*. zea*, which generate concerns due hybridization possibilities.

2. The symbionts screened (DNV, NPV and *Wolbachia*) are widely present in different noctuid species and locations including soybean, maize and laboratory colonies. In turn, their prevalence was highly variable according to noctuid species and location. Additionally, three different lineages of *Wolbachia* could be found, including unseen genotypes for noctuid pests.

3. In *S. littoralis*, larval age and population size did not affect gene expression of prophenoloxidase (PPO) in absence of immune challenge. The three dsRNA methodologies testes did not silence our target gene, however droplet feeding

methodology generated the opposite result increasing PPO expression. This phenomenon should be discussed in future studies due perspectives of using RNAi in pest control.

4. We found that in *S. littoralis*, which present DDP and phenotypic plasticity, gregarious insects present higher humoral defences only in the presence of a haemocoel invader. Levels of humoral defences have variated according to bacteria species. When heat killed, only the specific haemolymph pathogen *Xenorhabdus nematophila* increased one of the humoral defences tested.

THESIS APENDIX

RELATED TO CHAPTER 2:

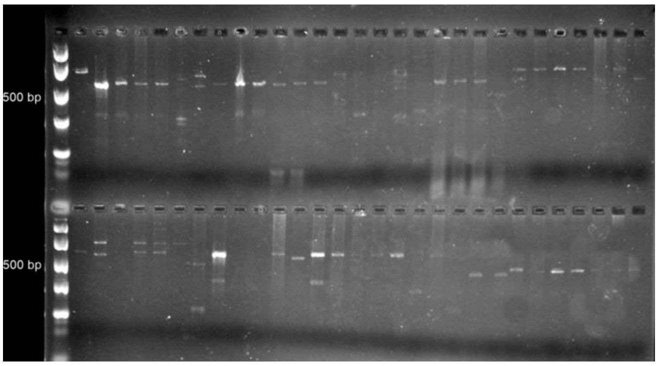

Fig. S1: *Wolbachia* positive samples amplified by wsp primers (81F/691) in 2% agarose gel. These primers amplify a DNA fragment with variable size depending on the individual strain.

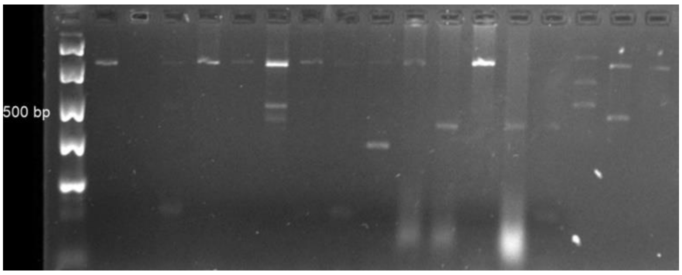

Fig. S2: DNV positive samples in 2% agarose gel. Variable product sizes were produced using DVVP primers due different viral lineages found and confirmed by sequencing.

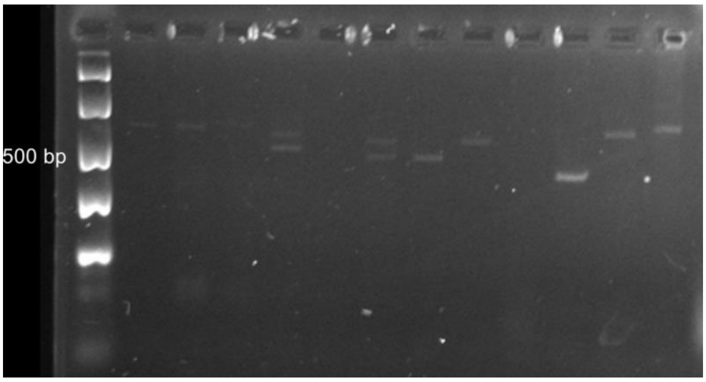

Fig. S3: NPV positive samples in 2% agarose gel. Variable product sizes were produced due to different baculovirus strains found and confirmed by sequencing.

RELATED TO CHAPTER 3:

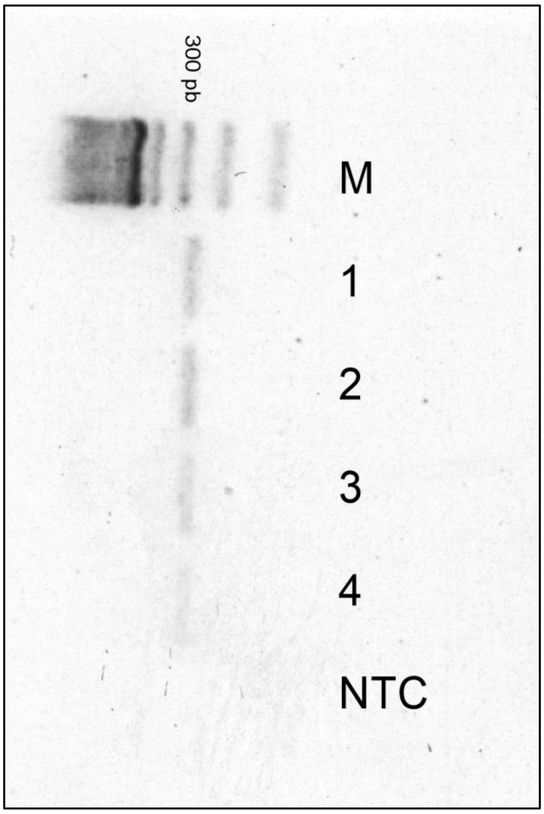

Fig. S1: Agarose gel of PPO dsRNA amplicons. M= DNA marker; 1-4 positive samples; NTC= non template control.

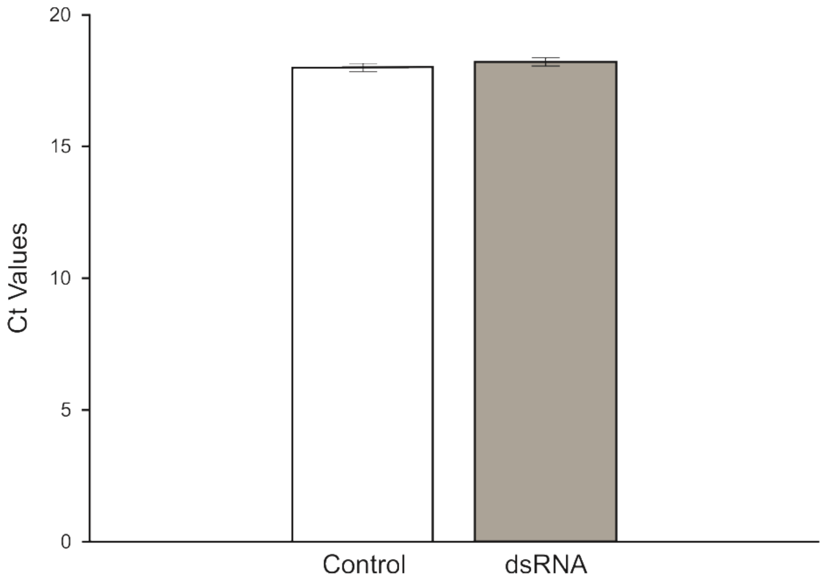

Fig. S3: Ct values for EF1α gene of larvae. This result indicated that dsRNA methodology to knockdown PPO gene which demonstrated that the dsRNA methodologies used did not detectably affect this non target gene (control: n=30; dsRNA treatment: n=30).