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SISTEMA DE DEFENSA DE LAS LARVAS DE GALLINA CIEGA CONTRA LOS HONGOS ENTOMOPATÓGENOS

Jhony Navat Enríquez Vara, Dr.

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Las larvas de gallina ciega son una plaga importante en cultivos de gramíneas en México. Dentro de las estrategias de manejo de esta plaga se ha considerado el uso del control microbiano con hongos entomopatógenos. Sin embargo, se han observado variaciones en la susceptibilidad de las especies de gallina ciega contra los hongos entomopatógenos. Se considera que uno de los factores importantes responsables de esta variación es el sistema de defensa (barreras inmunitarias y no inmunitarias) específicos de cada hospedero. Tomando como modelo de estudio a larvas gallina ciega de las especies *Phyllophaga polyphylla* y *Anomala cincta*, se estudió: a) si las variaciones de supervivencia entre ambas especies de gallina ciega contra *Metarhizium pingshaense* y *Beauveria bassiana* se deben a barreras inmunitarias; b) si las barreras inmunitarias de *P. polyphylla* varían fenológicamente (en 2011 y 2012) y c) si las barreras no inmunitarias protegen a *P. polyphylla* de la infección con *M. pingshaense*. Con larvas de ambas especies colectadas de campo, e inoculadas con ambas especies de hongos entomopatógenos, se registró la supervivencia, la actividad del fenol oxidasa (FO), producción de óxido nítrico (ON) y actividad lítica, tres componentes de las barreras inmunitarias. En el primer objetivo se encontró que *P. polyphylla* tuvo una mayor actividad de FO y una mayor supervivencia que *A. cincta*, pero esta última tuvo más ON que *P. polyphylla*, pero en ambas especies de gallina ciega no se encontraron diferencias significativas entre los grupos testigo e inoculados con los hongos. Con respecto a las diferencias fenológicas en el hospedero (*P. polyphylla*), las larvas colectadas en 2011 mostraron más actividad de la FO y su enzima no activada (proFenoloxidasa, pFO) que en 2012; sin embargo, nuevamente no se encontraron diferencias entre los grupos control e inoculados. Por último, se investigó el papel del integumento como barrera no inmunitaria, para lo cual se inyectaron directamente blastosporas de hongo en el hemocele de los insectos, a pesar de que no se observaron diferencias en los niveles de ON, actividad FO y actividad antimicrobiana entre larvas tratadas con el hongo y testigo, se observó una infección del 100%, comparada con larvas inoculadas topicalmente con condios del hongo. Se discute, el papel de las barreras inmunitarias, y la importancia potencial de las barreras no inmunitarias en la interacción del insecto y los hongos entomopatógenos. Se propone que estudios como estos, en el área de control biológico, permitirán analizar la importancia del sistema de defensa de los insectos plaga en el impacto de los hongos entomopatógenos en poblaciones de larvas de gallina ciega.

Palabras clave: ecoimmunología, Melolonthinae, Rutelinae, *Metarhizium pinshaense*, *Beauveria bassiana*, respuesta inmunitaria, barreras no inmunitarias.

DEFENSE SYSTEMS IN WHITE GRUBS AGAINST ENTOMOPATHOGENIC FUNGI

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Colegio de Posgraduados, 2013

White grub larvae are important pests of graminaceous crops in Mexico. Entomopathogenic fungi are among the most important control strategies. However, variation and inconsistent results have been reported when the susceptibility to entomopathogenic fungi of diverse populations of white grub larvae were compared. The defense system (immune and non-immune barriers) is considered one of the most important factors responsible for this variation. A series of experiments were done to investigate this using white grub larvae of the species *Phyllophaga polyphylla* and *Anomala cincta*. Firstly the relationship between immune barriers and variation in survival of the two white grub species when challenged with the entomopathogenic fungi *Metarhizium pingshaense* and *Beauveria bassiana* was quantified. Secondly, phenological variation in the immune barriers of *P. polyphylla* were investigated. Thirdly, the role of non-immune barriers (cuticle) in protection of *P. polyphylla* against *M. pingshaense* infection was evaluated. Following inoculation of both white grub species with each entomopathogenic fungi their survival and three components of the immune barrier (phenol oxidase activity (FO), nitric oxide (NO) and lytic activity) were recorded. *Phyllophaga polyphylla* larvae had greater PO activity and a better survival rate than *A. cincta* larvae, which showed greater NO levels compared to the former species. When larvae were collected in two different years (2011 and 2012), greater PO and pPO (enzyme not active of PO) were recorded in 2011, although no differences were detected between fungal and control treatments, the greatest survival rate was recorded in larvae collected in 2011. Finally, when *M. pingshaense* blastospores were injected directly into the haemocoel of *P. polyphylla* larvae, 100 % infection was observed and there were no differences in NO levels, PO and antimicrobial activity between fungal and control treatments; however, the high infection level obtained after injection compared to when larvae were surface inoculated, confirmed the important role of the cuticle in defence against fungal infection. The role of immune barriers as well as the importance of non-immune barriers in defence against fungal infection in white grub larvae are discussed in the context of the biological control of this pest.

Key words: ecoimmunology, Melolonthinae, Rutelinae, Metarhizium pinshaense, Beauveria bassiana, immune response, non-immune barrier.

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Capítulo 1. Introducción general

Los insectos continuamente están expuestos a parásitos y patógenos que impactan drásticamente su supervivencia y éxito reproductivo (Thomas *et al.* 2009), por lo que han desarrollado defensas contra ellos, éstas incluyen armas químicas, fisiológicas, morfológicas, de comportamiento o la combinación de estas (Schmid-Hempel 2011). Todos estos mecanismos de defensa se pueden clasificar en barreras no inmunitarias e inmunitarias (Parker *et al.* 2011; Schmid-Hempel 2011).

Dentro de las barreas no inmunitarias, la primera línea de defensa es el comportamiento que permite a los hospederos evitar el contacto directo con los patógenos (Siva-Jothy *et al.* 2005). Después del contacto, las barreras morfológicas como la cutícula proporcionan protección, ya sea inhibiendo el desarrollo de sus enemigos o impidiendo físicamente su paso al hemocele (Bogus *et al.* 2007; Ment *et al.* 2012). Además, cuando los patógenos han penetrado en el insecto se puede ejercer anorexia inducida por el desarrollo de la enfermedad (Adamo *et al.* 2010) o automedicación (Singer *et al.* 2009); en la primera, el hospedero reduce el consumo de alimento para que el transporte de lípidos no interfiera con la defensa, y en la segunda pueden consumir plantas con una alta concentración de metabolitos secundarios que reducen el desarrollo o eliminan a los patógenos.

Las barreras inmunitarias se activan cuando un patógeno penetra en el cuerpo del insecto. En esta fase, las moléculas asociadas al patógeno (MAP) son reconocidas por patrones moleculares de reconocimiento del hospedero (PMR) y esto desencadena una respuesta humoral y celular. En la respuesta humoral se activan las cascadas de la ruta Toll y/o IMD, y su activación estimula a las células del cuerpo graso para producir péptidos antimicrobianos contra

hongos, bacterias Gram positivas y Gram negativas (Tzou *et al*. 2002; Lemaitre y Hoffman 2007). Otro componente de la respuesta humoral es la cascada de la fenol oxidasa (FO), en esta ruta los patógenos activan a las serin proteasas, y éstas a su vez inducen la activación de fenol oxidasa (FO) a partir de la enzima no activada llamada profenoloxidasa (pFO) (Cerenius *et al.* 2008; Kanost y Gorman 2008). La FO genera melanina mediante una reacción no enzimática, pero también produce radicales libres que son tóxicos para los patógenos (Zhao *et al*. 2011). Por otra parte, en la respuesta celular los hemocitos se adhieren a las paredes de los patógenos para fagocitarlos, encapsularlos o melanizarlos. Durante la fagocitosis los hemocitos engullen a las bacterias, protozoarios o células infectadas con virus, y dentro de su fagosoma los atacan con una gran cantidad y variedad de compuestos tóxicos (Lemaitre y Hoffman 2007). La formación de cápsulas de melanina, considerada como la responsable de matar a hongos, nematodos y huevos de parasitoides (Carton *et al.* 2008; Castillo *et al.* 2011), se debe a la adherencia de los hemocitos sobre la superficie de los patógenos y la activación de FO (Strand 2008). Las cápsulas de melanina asfixian a los patógenos o parásitos por medio de la barrera física que imponen los hemocitos entre el enemigo y el cuerpo del insecto, o también los matan dentro de la cápsula con la producción de radicales libres generados por la activación de la FO (Nappi y Christensen 2005).

Para entender el sistema de defensa en la interacción insecto-patógeno se debe estudiar la inmunidad desde un enfoque holístico, es decir que se deben tomar en cuenta sus dos componentes: las barreras inmunitarias y no inmunitarias (Parker *et al.* 2011). Recientemente se ha encontrado que ambas defensas interactúan en el combate de las infecciones por patógenos y parásitos pero dependen de las condiciones ambientales (Smilanich *et al.* 2009a; Parker *et al.* 2011). Se ha reportado que los insectos pueden favorecer el aumento de su temperatura corporal

para incrementar la rapidez de su respuesta inmunitaria (Stahlschmidt y Adamo 2013), por ejemplo, cuando *Gryllus texensis* es inoculado con bacterias Gram negativas y se expone a altas temperaturas (33 °C) tienen más actividad de FO y lisozimas, y viven más que los grillos expuestos a temperaturas de 26 °C (Adamo y Lovett 2011).

Otro componente de complejidad en el sistema de defensa de insectos es su enorme variación dentro y entre las especies (Rolff y Siva-Jothy 2003; Schmid-Hempel 2005; Parker *et al.* 2011). Estas diferencias se deben a que las defensas contra patógenos y parásitos son costosas de producir y mantener, de manera que si se usan los recursos para montar un sistema de defensa, entonces se puede reducir los recursos destinados a incrementar y/o mantener la eficiencia en la expresión de otros componentes de la historia de vida del insecto como la reproducción (Siva-Jothy *et al.* 2005; Schmid-Hempel 2011). El razonamiento anterior es la base de la ecoinmunología, una rama de la biología que estudia la variación de la respuesta inmunitaria con respecto a los factores bióticos y abióticos (Rolff y Siva-Jothy 2003; Schmid-Hempel 2003, 2005; Schulenburg *et al.* 2009). Bajo este escenario, las variaciones en la respuesta inmunitaria son parte de un ajuste estratégico debido a que los individuos tienen la disyuntiva de invertir sus recursos en múltiples opciones, y la teoría de las historias de vida predice que la forma y magnitud de la respuesta inmunitaria reflejan el contexto ambiental (Ardia *et al.* 2011). Algunos estudios han demostrado que la respuesta inmunitaria de los invertebrados puede variar por factores bióticos y abióticos (Lazzaro *et al.* 2008; De Block y Stoks 2008; Triggs y Knell 2012). Por ejemplo, la temperatura (Catalán *et al.* 2012), la disponibilidad de alimento (Ponton *et al.* 2013), la calidad de las plantas de las cuales se alimentan los herbívoros (Klemola *et al.* 2007; Smilanich *et al.* 2009b; Vogelweith *et al.* 2011), el sexo (Zuk y Stoehr 2002) y las condiciones fisiológicas (González-Santoyo y Córdoba-Aguilar

2012) afectan la respuesta inmunitaria en varios grupos de insectos. Además, la respuesta inmunitaria puede variar durante la vida de un individuo en diferentes etapas de desarrollo (Eleftherianos *et al*. 2008; Shi y Sun 2010; Laughton *et al.* 2011; Srygley 2012) y la época del año (Córdoba-Aguilar *et al.* 2008; Contreras-Garduño *et al.* 2008).

Desde la perspectiva de la ecoinmunología se pueden estudiar las variaciones en la respuesta de los insectos plaga a los agentes de control biológico (entomopatógenos y parasitoides) para tener mejores predicciones en su uso. Los entomopatógenos y parasitoides pueden ser utilizados como reguladores de las poblaciones de insectos plaga (Hawkins *et al.* 1997; Lacey *et al.* 2001). Tal es el caso de bacterias, hongos, baculovirus y nematodos (Lacey y Kaya 2007). Así que el éxito o fracaso de los entomopatogenos en el control biológico podría deberse en gran medida al sistema de defensa del hospedero. Por ejemplo, Tunaz y Stanley (2009) encontraron que los insectos de áreas agrícolas comúnmente son desafiados por patógenos y logran recuperarse de las infecciones, esto fue más común en insectos colectados del suelo que los del follaje. Por tal motivo, el sistema de defensa inmunitario puede limitar el alcance y la eficacia de los agentes de control biológico en las áreas agrícolas. De acuerdo con lo anterior, es posible que en las áreas agrícolas se observen variaciones en la eficacia de los agentes de control biológico, pero, si se investigan las causas de las variaciones en esta defensa se pueden hacer mejores predicciones sobre el control biológico de plagas de importancia agrícola (Smilanich *et al.* 2009a).

La presión de selección que ejercen los patógenos sobre las poblaciones de insectos puede favorecer el sistema de defensa inmunitario y no inmunitario, y un modelo para analizar esto son las larvas de escarabajos comúnmente llamadas gallinas ciegas y sus hongos entomopatógenos. Las larvas de gallina ciega son un complejo de especies que se encuentran en

diversos hábitats como los cultivos agrícolas y zonas forestales (Morón 1997; Jackson y Klein 2006). Viven bajo el suelo durante varios meses, por lo que están en contacto con microorganismos entomopatógenos (Ritcher 1958; Jackson y Glare 1992; Klingen y Haukeland 2006). Por ejemplo, *Beauveria bassiana* y *Metarhizium anisopliae* s.l. dañan severamente a estas especies (Ansari *et al*. 2004; Rodríguez del Bosque *et al*. 2005; Béron y Díaz 2005), y se ha propuesto su uso en el combate contra esta plaga (Shah y Pell 2003). No obstante, uno de los problemas en el control microbiano de gallina ciega es que cohabitan varias especies en una misma localidad (Móron 1997; Ahrens *et al.* 2009), y existe variación interespecífica en las infecciones por hongos (Keller *et al.* 1999; Rodríguez del Bosque *et al.* 2005; Morales-Rodríguez *et al.* 2010; Nong *et al.* 2011; Guzmán-Franco *et al.* 2012). También existe variación fenológica anual en la resistencia (Morales-Rodríguez y Peck 2009). Esto sugiere una compleja interacción entre insectos y entomopatógenos, y los resultados pueden ser modificados por diferentes factores que hay que entender antes de hacer aplicaciones inundativas en campo con hongos entomopatógenos para el control de gallina ciega (Jackson 1999; Jaronski 2007).

Considerando la variación que existe en la susceptibilidad de las gallinas ciegas contra los hongos entomopatógenos, se analizó su sistema de defensa a nivel interespecífico (en *Phyllophaga polyphylla* y *Anomala cincta*) e intraespecífico (tomando en cuenta a *P. polyphylla).* Así que la presente tesis está organizada en tres capítulos:

En el capítulo dos se investigó si existían diferencias en la respuesta inmunitaria de dos especies de gallinas ciegas (*Phyllophaga polyphylla* y *Anomala cincta*) que cohabitan el mismo nicho, contra dos hongos entomopatógenos (*Metarhizium pinshaense y Beauveria bassiana*) que fueron aislados de la misma región de donde se recolectaron las larvas. Después de la inoculación con los hongos, se tomaron muestras de hemolinfa para registrar la actividad de

fenoloxidasa (FO) y la producción de óxido nítrico (ON). Se encontraron diferencias entre especies: el porcentaje de supervivencia en larvas de *P. polyphylla* fue mayor en comparación con *A. cinct*a, mientras que *P. polyphylla* generó más FO pero menos ON que larvas de *A. cincta*. No obstante, estas diferencias solamente se encontraron de manera basal, ya que la inoculación con hongos no tuvo ningún efecto. Esto sugiere que los hongos entomopatógenos tardan varios días en penetrar el exoesqueleto y no activan la respuesta inmunitaria o que los hongos nunca lograron penetrar el exoesqueleto. Estos puntos se discuten en este segundo capítulo.

Otro aspecto que se estudió en el tercer capítulo fue la variación que puede existir en la respuesta inmunitaria en larvas colectadas en dos diferentes años. Para esto, larvas de *P. polyphylla* se inocularon con blastosporas de *M. pinshaense* y se determinó la actividad de pFO y FO. Se encontró que las larvas colectadas en el año 2011 tuvieron una mayor cantidad de pFO y FO, comparadas con larvas colectadas en 2012. Nuevamente no existieron diferencias significativas entre los insectos inoculados y su control. Como causas probables de estos resultados se consideró a la calidad del alimento disponible o la mayor incidencia de entomopatógenos, como posible consecuencia de la mayor precipitación durante 2011 comparado con 2012.

Debido a que los resultados del segundo y tercer capítulo sugirieron que los hongos entomopatógenos no penetraron la cutícula, en el capítulo cuatro se investigó la posible contribución de la cutícula de las larvas de gallina ciega, barrera no imunitaria, para evitar la infección por hongos entomopatógenos. Para esto, primero se inyectaron blastosporas de *M. pinshaense* a las larvas de *P. polyphylla*, con diferentes dosis, para determinar la DL_{50}. Posteriormente, se inyectó la DL_{50} a otro grupo de larvas y 24 horas después se registró la actividad antimicrobiana, FO y ON. De los tres parámetros registrados, solamente se activó la

FO, lo que sugiere que es costoso producir y mantener al mismo tiempo la actividad antimicrobiana, el ON y la FO. Después de comparar los resultados del porcentaje de mortalidad entre los métodos de inoculación, inyección e inmersion, y la activación de la respuesta inmunitaria, en el capítulo tres se discute que posiblemente las gallinas ciegas inviertan más en una respuesta no inmunitaria como el grosor de la cutícula que en una respuesta inmunitaria.

En resumen, a lo largo de esta investigación se describen evidencias experimentales relacionadas con factores que producen variación en la respuesta inmunitaria de larvas de gallina ciega contra hongos entomopatógenos, además, se discute el impacto que tedrían estas variaciones en la interacción hongo-insecto en el campo, y las implicaiones de las mismas para diseñar un esquema de manejo microbiano de esta plaga con hongos entomopatógenos.

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Capítulo 2. Is survival after pathogen exposure explained by host's immune strength? A test with two species of white grubs (Coleoptera: Scarabaeidae) exposed to fungal infection[1]

Abstract

It is usually assumed that a host's survival after pathogen exposure should correlate with the host's immune strength. In the laboratory and using two species of white grubs, *Phyllophaga polyphylla* (Bates) exhibited a higher survival than *Anomala cincta* (Say), when inoculated with *Metarhizium anisopliae* and *Beauveria bassiana* (two fungus pathogens of the above white grub species). We tested whether such survival difference correlates with differential expression of immune ability. Thus, immune response (phenoloxidase -PO- and nitric oxide production - NOP) and survival after experimental fungal infection were compared among and within (challenged vs control groups) white grub species. As expected, results showed that *P. polyphylla* had higher PO and survival values compared to *A. cincta*. However, only *A. cincta* produced NOP. Thus, our study provides support for the idea that survival correlates with host's basal immune strength that nevertheless only applies to PO, but not to NOP. The interspecific difference in PO and NOP may be due to the distinct pathogenic pressures that each grub species faces according to their different feeding regimes. Paradoxically, no differences in survival and immune response were found within each white grub species. This suggests that the difference observed between species is better explained by their basal immune response but not to challenge. We discuss possible scenarios to explain why white grubs are attacked by fungus in the field but not in our laboratory conditions.

[1]Enríquez-Vara, J. N., Córdoba-Aguilar, A., Guzmán-Franco, A.W., Alatorre-Rosas, R., Contreras-Garduño, J. 2012. Is survival after pathogen exposure explained by host´s immune strength? A test with two species of white grubs (Coleoptera: Scarabeidae) exposed to fungal infection. **Environmental Entomology** 41: 959-965.

2.1 Introduction

In the last two decades, evolutionary biologists have tried to explain variation in host's immune ability using both ecological and physiological approaches (reviewed by Schulenburg et al. 2009). One key assumption is that immune ability is a costly trait closely linked to host's fitness and given that pathogens are a prevailing evolutionary force, hosts are expected to invest substantially in immune ability (Schmid-Hempel 2003, 2005; Schulenburg *et al.* 2009). Therefore, one would expect that host's survival following infection should correlate with immune performance. Despite this, a number of studies in invertebrates have found that immune strength is not necessarily a good indicator of survival and resistance after pathogen exposure (Adamo 2004, Kanost and Gorman 2008, González-Santoyo and Córdoba-Aguilar 2012). Some explanations for this are that: 1) there have been methodological problems during quantification of immune response; and, 2) some studies have used dead pathogens, artificial challenges, or both that are likely to provide a different immune response than when real and natural pathogens are used (Adamo 2004, Boughton *et al.* 2011, Demas and Nelson 2012, González-Santoyo and Córdoba-Aguilar 2012). The former explanation is important because different methods of quantification could affect the result and interpretation of the same immune marker (Boughton *et al.* 2011). According to the latter explanation, natural pathogens will trigger a more robust immune response compared to an artificial challenge (Adamo 2004). Thus, whether or not immune performance predicts survival is still an unsettled question (González-Santoyo and Córdoba-Aguilar 2012).

White grub larvae are major pests of agriculture and horticulture in many countries (Jackson and Klein 2006). Soil dwelling pests such as white grubs continuously interact with a large variety of pathogens (Jackson and Glare 1992). Among these pathogens, entomopathogenic

fungi have been considered a potential microbial control agent of white grubs, as these microorganisms are common pathogens of insects (Roy *et al.* 2006). A fungal infection usually starts when conidia of entomopathogenic fungi attach to the insect cuticle, germinate and form an infective hypha known as the "appressorium" (Clarkson and Charnley 1996). The appressorium penetrates the cuticle by mechanical pressure and enzymatic degradation. Once inside the insect, the appressorium produces yeast-like blastospores that eventually turn into hyphae that colonize the haemocoel (Gillespie *et al.* 2000). More than one white grub species may co-exist in the same habitat (Morón 1997), and given that each species has a particular life history, interspecific variations in their response to pathogens are expected. For example, despite the fact that *Phyllophaga polyphylla* (Bates) and *Anomala cincta* (Say), two common white grub species in Mexico, cohabit in the same agroecosystem (Marín 2001), they show mortality differences when inoculated with *B. bassiana* and *M. anisopliae* conidia, two natural soil borne pathogens (Meyling and Eilenberg 2007). More specifically, mortality proportion differed between insect hosts and fungi species: more than 90% of *A. cincta* larvae became infected by *M. anisopliae* compared whit = 30% by *B. bassiana*; conversely, *P. polyphylla* larvae infection was never above 30% for both pathogens (Guzmán-Franco *et al.* 2012). Related to this, differential susceptibility of white grubs species to fungal infection has been reported elsewhere (Rodríguez del Bosque *et al.* 2005, Morales *et al.* 2010), but there is no information of whether such differential susceptibility is related to white grubs' immune response. According to theory, biotic factors that explain survival between or within species can only be explained either by predators or pathogens (Demas and Nelson, 2012). Thus, if predation is controlled, potentially different survival ability expressed by both white grub species should be linked to immune strength.

In invertebrates, the presence of parasites and pathogens in the hemocoel activates the immune response, including the enzymatic cascade of phenoloxidase (PO) (Cerenius and Söderhäll 2004) and nitric oxide production (NOP) (Faraldo *et al.* 2005). PO leads to melanization and killing of pathogens by encapsulation or generation of toxic molecules (Nappi and Christensen 2005), while NOP affects DNA synthesis and repairing mechanisms, and therefore protein synthesis of pathogens (Rivero 2006). Considering the large variation between *P. polyphylla* and *A. cincta* in their susceptibility to infection by the entomopathogenic fungi *B. bassiana* and *M. anisopliae* as indicated by Guzmán-Franco *et al.* (2012) we have tested whether such susceptibility difference correlates with PO activity and NOP between and within host species in laboratory conditions by using natural pathogens. After fungal infection, we compared PO and NOP between both white grub species, and expected that both immune parameters should show higher values in *P. polyphylla* than in *A. cincta*. We also compared PO and NOP within each white grub species, and expected that infected insects should show higher values. Finally, we compared survival both between and within species to relate it with to their immune response.

2.2 Material and Methods

Third-instar *P. polyphylla* and *A. cincta* were collected from corn fields in Guanajuato, Mexico (20º02’30.1” N, 100º28’36.4’’W) in October 2010. They were taken to the laboratory and maintained individually in plastic cups (100 ml) at 20 º C with moistened peat moss as a substrate for four weeks, before they were used in the experiment.

Two isolates were used, GC01 (*M. anisopliae*) and GC15 (*B. bassiana*). Isolate GC01 was obtained from an unidentified infected white grub larva and GC15 from a *Phyllophaga* sp

infected larva (J.N.E.-V., unpublished data). Isolates were preserved at -80°C by using 10% glycerol as crio-protectant. For experiments, isolates were grown in Sabouraud dextrose agar (SDA) plates (Bioxon, Becton Dickinson de Mexico, Cuatitlan Izcalli, Mexico) at 25 °C during 20 d. Conidial suspension were produced as described below, and the same method was used for both isolates. 10 ml of sterile 0.03% Tween 80 were added to a Sabouraud dextrose agar plate containing a fungal colony. Using a glass rod, conidia and mycelia were removed from the plate and deposited into a 50 ml centrifuge tube and vortexed for 15 min at maximum speed. Conidia were separated from hyphae by filtrating the suspension through a sterile cloth into a new centrifuge tube. Conidia concentration was estimated using a haemocytometer. A $1x10^8$ conidia/ml suspension was produced for each isolate. The percentage of viable conidia was determined prior to the experiment following the method proposed by Goettel and Inglis (1997). According to this method, normal viability must be greater than 90 %, which was the case of our study.

While still being in their third instar, one group of *P. polyphylla* larvae was washed in distilled water, dried on filter paper, and then dipped in 100 ml of $1x10^8$ conidia/ml suspension of *M. anisopliae* for 20 s (from now on, the PM group). A second group, *P. polyphylla* larvae were dipped in 100 ml of $1x10^8$ conidia/ml suspension of *B. bassiana* (from now on, the PB group). A third group of *P. polyphylla* was dipped in 0.03% Tween 80 (Sham) (from now on, the PS group) while a fourth group was not manipulated (Control) (from now on, the PC group). The same groups were established, but with *A. cinta* larvae and were termed AM, AB, AS and AC respectively according to what we describe above. All inoculated larvae, were transferred individually to each of 12 wells of a cell culture plate (COSTAR^L, Corning Inc. NY, USA). This contained a 2 cm diameter filter paper that had been previously moistened with 150 µl of sterile

distilled water. Two culture plates were used for each treatment. A small slice of carrot was placed as food source and replaced as required. The 12-well plates were incubated at 23 ± 1ºC and 90 % RH for 35 d. The methodology and treatments were the same for each white grub species.

The experiment was carried out using a completely randomized design where all treatments were carried out the same day. Each experiment was repeated three and two different occasions for *P. polyphylla* and *A. cincta,* respectively. Because we discarded the larvae from which we did not obtain enough haemolymph (=20 µl), the final number of larvae per treatment and white grub species was unbalanced. Therefore, resulting sample sizes were PM = 42, PB = 40, PS= 35, PC = 35, AM = 23, AB = 21, AS = 24 and AC = 20.

Haemolymph samples were obtained from each larva 24 h after fungal inoculation. Each individual larva was surface-sterilized with 70% ethanol and then rinsed twice using sterile distilled water. Haemolymph samples were obtained by cutting the third thoracic leg and three drops (approximate 20 µl) of haemolymph were collected using sterile, prechilled Eppendorf tubes (1.5 ml) containing 100 µl of ice-cold phosphate buffered saline (PBS, Sigma, pH 7.4), which were vortexed for 10 s. The mixture was centrifuged for 10 min at 10,000 rpm at 4ºC, and the supernatant was divided in two 50 µl aliquots. The first aliquot was mixed with 50 µl of PBS and protease inhibitors (PBS-IP), and the second aliquot was mixed with 50 µl of PBS. The first subsample was used to measure protein haemolymph content and PO activity, and the second subsample was used to record NOP. All measurements were carried out immediately after haemolymph collection.

Given that high variation in protein content among samples may bias PO measurements (Contreras-Garduño *et al.* 2007), protein determination was first recorded to homogenize protein content so that the PO content in each sample could be measured. The BCA assay kit (Pierce Biotechnology, Rockford, IL) was used to determine protein concentration in each sample by using the instructions provide by manufacturer. In 96 well microplates, 10 µl of each sample, 40 µl of PBS and 150 µl of a mixture 50:1 of the reagents A and B (provided with the kit) were added and incubated for 30 min at 37ºC. Absorbance was recorded at 562 nm in a micro-plate reader (Varioskan Flash Thermo Scientific). A known concentration of Bovine Serum Albumin (BSA) was used as a standard curve (provided with the kit) that was compared with our sample to estimate the amount of protein in each experimental sample. Absorbance was recorded at 562 nm in a micro-plate reader (Varioskan Flash Thermo Scientific). To estimate PO activity in each sample, an aliquot that contained 40 µg of protein was taken and deposited in 96 well microplates (Corning Inc., Corning, NY), and these were dose-titered to a volume of 50 µl of sample and PBS. To this mixture, 50 µl of L-DOPA (4 mg/ml) was added to obtain a final volume of 100 µl. PO activity was determined indirectly by oxidation of L-dihidroxifenilalanina (L-DOPA, Sigma). As blanks, only 50 µl of PBS and 50 µl of L-DOPA (4 mg/ml) were used. The slope of the curve was calculated by using the optical density at 490 nm. Optical density readings were taken every minute for 1 hour at 30° C.

A colorimetric nitrate/nitrite assay kit (SIGMA) was used to prepare the standard curve and to estimate NOP in each sample by using the instructions provided by manufacturer. The basis of this technique is that the nitric oxide is a highly unstable radical that rapidly reacts with other oxygen-reactive species to form stable products, such as nitrites, nitrates and toxic radicals, such as peroxynitrite. Hence, the total nitrate, or nitrite content can be used to estimate indirectly

the amount of nitric oxide in each sample. These quantifications were compared with a standard curve to know the amount of nitrate or nitrite content in µM. Readings were performed at 540 nm in a Varioskan Flash microplate reader.

After haemolymph extraction that allowed us determining PO and NOP, survival was assessed: n = 150 of the three replicates for *P. polyphylla* and n = 87 of the two replicates for *A. cincta*. For this, larvae were checked every other day for 5 wk to record the number of dead larva per day. To observe sporulation and thus confirm or not death by fungal infection, the surface of each dead larvae was sterilized, and placed in a petri dishes (4 cm diameter), lined with damp filter paper, and then incubated at 25 °C and 100 % RH for 10 d (see also Lacey and Brooks 1997).

Statistical analyses. For each immune parameters measured, we performed a univariate general linear model with species of white grub and treatment as fixed factors, and replication experiment as random factor. Haemolymph protein concentration, PO activity and NOP were set as dependent variables. Notice that haemolymph protein concentration was included in this analysis as this variable is usually correlated with immune strength and animal condition (e.g. Contreras-Garduño et al. 2007). PO activity was ln-transformed to meet assumption of normal distribution. Different transformation methods were applied to NOP data set, but normality was never reached. Therefore, data were analyzed using Kruskall-Wallis tests. It has to be clarified, however, that only *A. cincta* (but not *P. polyphylla*) larvae produced NOP, thus, only treatments from this species were compared. A comparison between replicates was also carried out before to combine all data per replicate (data not shown). Normal distribution and equality of variances were tested using Kolmogorov-Smirnov and Levene tests respectively.

Kaplan-Meier survival curves were constructed using daily mortality, and log-rank test was used to measure statistical differences between species and treatments. A Cox regression analysis was used to determine whether data for species, treatments, or experimental replicates influenced survival significantly (Selman *et al.* 2011). Analyses were made using the following procedures: PROC GLM, PROC NPAR1WAY, PROC LIFETEST and PROC PHREG (SAS 9.0 statistical software; SAS Institute, Cary, NC).

2.3 Results

There were differences in haemolymph protein concentration between *P. polyphylla* and *A. cincta* (F = 34.93; df = 1,229; P<0.05; Fig. 2.1.), which had higher values in the former species. However, there were no differences according to treatment (infected and control animals) within species (F = 0.35; df = 3,229; P = 0.79; Fig. 2.1.). However, the interaction between treatments and species of white grubs were not significant (F = 0.16; df = 3,229; P = 0.92).

P. polyphylla showed higher values of PO than *A. cincta* (F = 64.47; df =1,229; P<0.05; Fig. 2.2.), but no differences were found according to treatment (infected and control animals) within species (F = 0.60; df = 3,229; P = 0.59; Fig. 2.2.). The interaction between treatments and species of white grubs was not significant (F = 0.74; df = 3,229; P = 0.52). Given that only *A. cincta* produced NOP, data from treatments (infected and control animals) within this species were compared according to treatment. However, no significant differences were found among treatments (X^2= 6.55; P = 0.08).

There were survival differences between species with *P. polyphylla* showing higher values than *A. cincta* (X^2 = 62.05; P < 0.05; Table 2.1.; Fig. 2.3.). The Cox regression analysis

indicated that treatment (X^2 = 0.49; P = 0.48) and experimental replicates (X^2 = 0.49, P = 0.48) did not have a significant effect on survival. However, the effect of species on survival was highly significant (X^2 = 31.19; P < 0.05; Fig. 2.3.).

2.4 Discussion

Our results provided partial support for the hypothesis that immune strength is directly linked to survival ability. We found that PO and survival had higher values in *P. polyphylla* than in *A. cincta*. This is coherent with the protein concentration values we found for both species given that it is usually found that protein concentration and PO values are correlated (Contreras-Garduño *et al.* 2007). However, NOP was found only in *A. cincta.* We believe that previous feeding history and pathogen exposure of the larvae we used may explain such interspecific differences in immune values. Although both species coexist in the same agroecosystem, each species shows different food preferences (reliant on resource availability) and micro-habitats (Marín 2001). For example, *Phyllophaga* species are normally found in soils containing roots that are used for feeding. Many *Anomala* species prefer soil containing large organic matter contents, and larvae may or may not feed on roots (Najera and Jackson 2010). The differences in feeding habits and habitats may affect their basal immune response according to two different processes: 1) food may directly affect the immune response, 2) differences in microhabitats could lead each species to differential parasite selective pressures, or both. A recent paper that states that in phytophagous insects, their immune response is determined by their feeding habits (Vogelweith *et al.* 2011) provides support for the first hypothesis. The second process could be supported because it has been proposed that *Phyllophaga* species are exposed to a large diversity of pathogens, such as bacteria, nematodes, parasites and fungi (Jackson and Glare 1992, Jackson and Klein 2006, Meyling and Eilenberg *et al.* 2007), while *Anomala* species could be exposed to

bacteria mainly (Alexander 1977, Villalobos *et al.* 1997). Further differences in mechanisms underlying immune response can be put forward to explain interspecific host differences and pathogen exposure. For example, PO is a key enzyme used extensively during melanization of the cuticle, wound repair, and encapsulation of pathogens and parasites such as bacteria, nematodes, fungi and parasitoids (Cerenius and Söderhäll 2004, Kanost and Gorman 2008, González-Santoyo and Córdoba-Aguilar 2012). Nitric oxide is a very reactive and unstable gas that damages pathogen's DNA, and is commonly induced by bacterial infection (Foley and O`Farrel 2003). Probably, the greater expression of PO in *P. polyphylla* compared to *A. cincta* is related to the larger diversity of pathogens (bacteria, nematodes, parasitoids and entomopathogenic fungi) that may infect *P. polyphylla*. The greater values of NOP found in *A. cincta* compared to *P. polyphylla* could be a consequence of the former species interacting more with bacteria. Both hypotheses await for further investigation.

Interestingly, the immune response based on PO activity and NOP between inoculated and non-inoculated larvae within each species were similar. The fungi's ability to penetrate and their effects on insect immune response may explain this. After fungal infection but within 24 to 48 h, crucial processes occur that include adhesion of conidia to the epicuticle, germination, formation of appressorium and penetration (Perkul and Grula 1979, Gillespie *et al*. 2000, Wang and St. Leger 2005). It is already known that cuticle damage elicits immune reactions (Brey *et. al.* 1993), and it could be expected that invading fungal structures could also elicit humoral response. For example, when the germinative tube of a fungus penetrates the cuticle, melanized points are observed at locations of penetration (Golkar *et al.* 1993) and PO is a key molecule to generate melanine. Upon arriving to the hemocele, the fungus induces humoral and cellular responses (Vilcinskas and Götz 1999). According to this scenario, an increase in PO and NOP

levels should be observed in the fungus-inoculated insects compared with control groups, but we did not detect this. We propose two explanations: either PO and NOP are not good indicators of a fungus-induced immune response, fungus did not penetrate the insect cuticle, or both. Related to the first explanation and as far as we know, this is the first time that NOP is recorded against entomopathogenic fungi, so we do not know how much effective NOP is as an immune marker. However, PO has been previously measured against entomopathogenic fungi and results are contradictory. For example, some studies have found a positive relationship between PO, survival against fungal infections, or boths (Wilson *et al.* 2001), wheras others have not (e.g. Schwarzenbach and Ward 2007). These opposing results could be explained by pathogen's virulence, host's resistance, or both, which can lead to variation in fungal resistance among insect species (see a similar rationale in Rantala *et al.* 2011). Therefore, further experiments must test whether other immune markers apart from PO and NOP, correlate with fungal resistance and survival in white grubs against *M. anisopliae* and *B. bassiana*. However, given that survival was not different between challenged and control groups within species of white grubs, we propose that fungus did not penetrate the insect (the second explanation). In the field, it has been reported that *M. anisopliae* and *B. bassiana* are infecting white grubs (Glare 1992) and this is also the case for all species that were used in this study in the same agroecosystem. Perhaps the complex interaction between the host-parasite dynamics and their natural environment could favor fungal penetration in the field. Given that the insect cuticle represents an efficient barrier against entomopathogenic fungi (Boucias and Pendal 1998, Villani *et al.* 1999), we suggest two hypotheses whose basis relies on the cuticle damage that favors fungal infections in the field. The first hypothesis is that cuticle could be damaged during larval movements in response to food and soil moisture in the field (Marrone and Stinner 1983): cuticle abrasion increases the

opportunity for surrounding entomopathogenic fungi to penetrate the larvae. However, this way of infestation does not seem likely (Villani *et al.* 1999). The second hypothesis is that fungi could infect the insect during molting (Vey and Fargues 1977). For example, Vey and Fargues (1977) found that *B. bassiana* infected *Leptinotarsa decemlineata* (Say) larvae during molting. How this latter hypothesis operates needs further testing preferably under field conditions so that soil characteristics that may affect white grub molting can be used.

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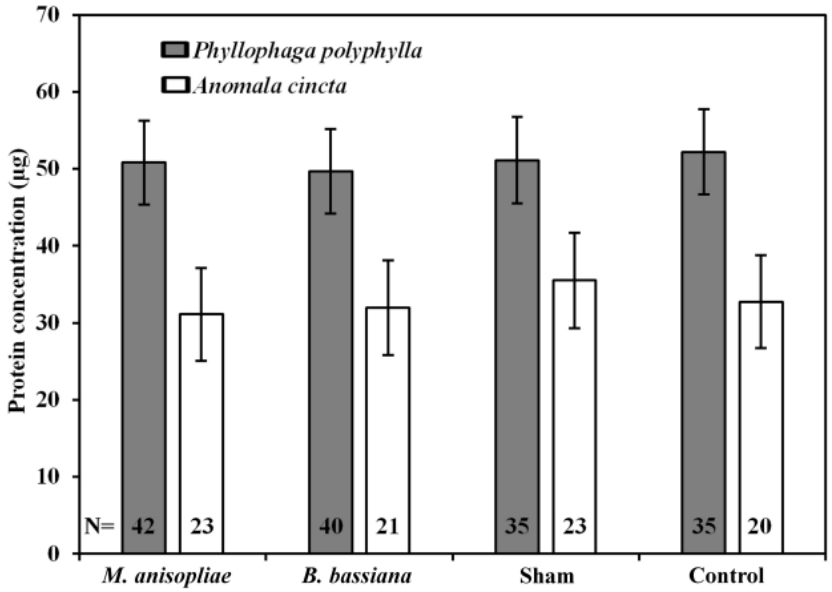

Figure 2.1-Hemolymph protein concentration according to treatment. Sample size is shown in each bar. Each bar indicates mean ± SE.

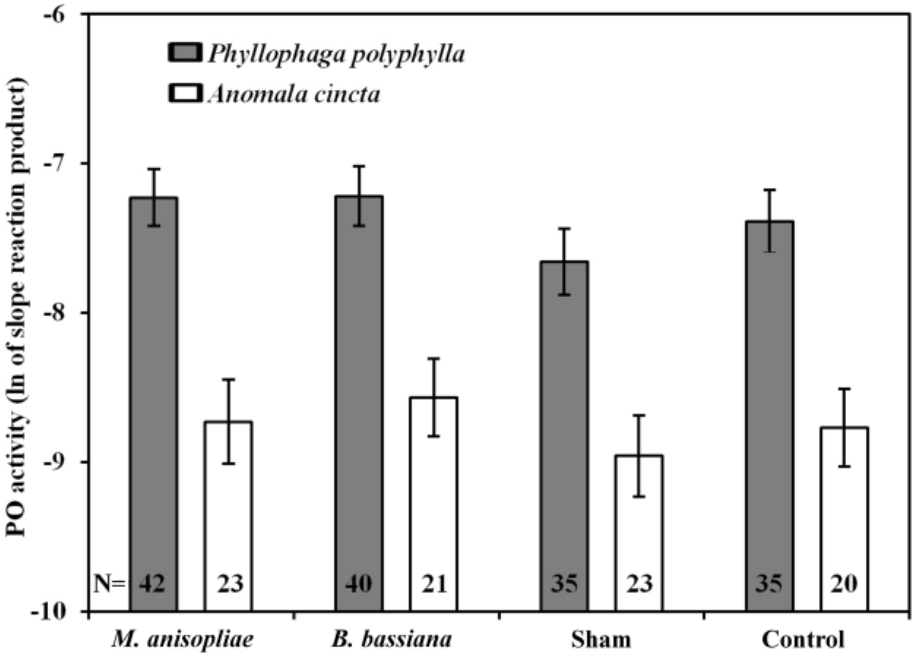

Figure 2.2-Slope PO activity expression according to treatment. Sample size is shown in each bar. Each bar indicates mean ± SE.

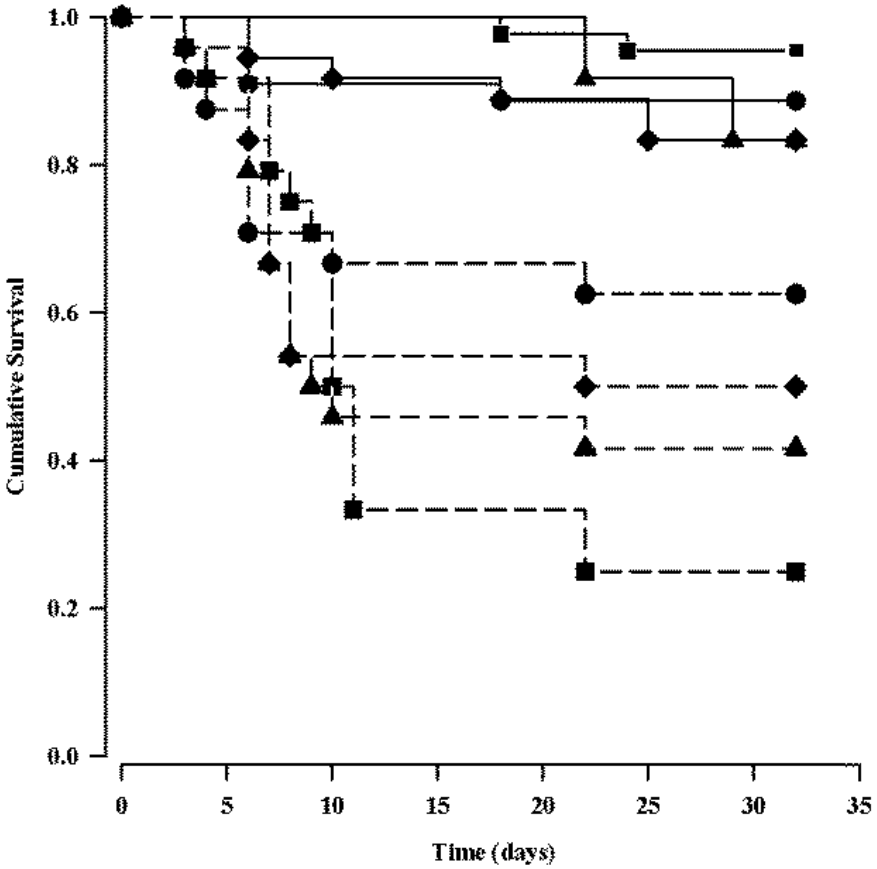

Figure 2.3-Survival of third-instar larvae of *Phyllophaga polyphylla* (solid lines) and *Anomala cincta* (broken lines) according to treatment. ■=larvae infected with *Metarrhizium anisopliae*, ▲=larvae infected with *Beauveria bassiana*, ●=larvae dipped in 0.03% Tween 80 (sham) and ♦= larvae not manipulated (control).

Table 2.1 Cox regression model results comparing survival of white grub larvae according to species, replicate, treatment and immune variable (PO and NOP).

Factors	B[a]	s.e.	Wald	d.f.	p	odds[b]
Species	-1.73567	0.31076	31.1940	1	0.0001	0.176
Experiment	0.14585	0.20787	0.4923	1	0.4829	1.157
Treatment	-0.08084	0.11466	0.4971	1	0.4808	0.922
PO	53.74922	30.55290	3.0948	1	0.0785	2.2E23
NO	0.02502	0.01654	2.2900	1	0.1302	1.025

[a]Regression coefficient of overall survival functions for variable.
[b]Odds ratio of variable relative to reference category (=exp(b)).

Capítulo 3. Temporal differences in the immune response of *Phyllophaga polyphylla* larvae to entomopathogenic fungi[2]

Abstract

Larval stages of root-feeding insects are major pests of agricultural crops. Entomopathogenic fungi have been used as biological control agent of these pests, however, inconsistent results suggest that a more basic understanding of the factors affecting insect/fungal interactions are needed. One of the main barriers to infection is the immune system, but little is known about the factors affecting immune efficacy. We found variation in survival of *Phyllophaga polyphylla* (Bates) (Coleoptera: Melolonthidae) larvae collected in 2011 and 2012, and that this was correlated with differences in phenoloxidase (PO) activity, prophenoloxidase (ProPO) activity and protein concentration in the haemolymph. Larvae were injected with 1. viable blastospores of *Metarhizium pingshaense* Q.T. Chen & H.L. Guo (Hypocreales: Clavicipitaceae), 2. non-viable blastospores of *M. pingshaense*, 3. PBS+Tween, or 4. received no injection. Overall, PO, proPO and protein concentration in larvae after 12 h were similar amongst treatments within each year of collection. However, larvae collected in 2011 showed greater PO and ProPO activity but lower protein concentrations compared with larvae collected in 2012. This suggests a stronger immune response in larvae collected in 2011 compared with 2012. This was confirmed in a survival study when, following a non-infective injection (non-viable blastospores), larvae from 2011 survived for 35 while larvae from 2012 survived only two days. Temporal variation in immune response could result in different survival rates and influence the efficacy of fungal pathogens when applied as control agents. Potential causes of this variation in immune response are discussed.

[2] Enríquez-Vara, J.N., Contreras-Garduño, J., Guzmán-Franco, A.W., Córdoba-Aguilar, A., Alatorre-Rosas, R. and González-Hernández, H. 2013. Temporal differences in the immune response of *Phyllophaga polyphylla* larvae to entomopathogenic fungi. Journal of Invertebrate Pathology (**submitted**).

3.1 Introduction

Root-feeding white grubs (Coleoptera: Melolonthidae) are found in diverse habitats including agricultural and forest areas (Jackson and Klein, 2006). The larval stage of these insects lives below ground for several months, where contact with soil-borne pathogens is very likely to occur (Jackson and Glare, 1992; Ritcher, 1958). The entomopathogenic fungi *Beauveria bassiana* s. l. (Bals-Criv.) Vuill. and *Metarhizium anisopliae* s. l. (Metsch.) Sorok. are common soil inhabitants (Meyling and Eilenberg, 2007), and can cause high rates of mortality in a number of white grub species (Berón and Díaz, 2005; Rodríguez-del-Bosque *et al.,* 2005), suggesting that biological control of white grubs using entomopathogenic fungi has great potential. *Metarhizium pingshaense* Q.T. Chen & H.L. Guo, a species with a close phylogenetic relationship with *M. anisopliae* s. s. (Bischoff *et al.,* 2009) has recently been found infecting white grub larvae in Mexico (Carrillo-Benitez *et al.,* 2013) and its pathogenicity against two economically important white grub species, including *Phyllophaga polyphylla* (Bates), has been evaluated (Enríquez-Vara *et al.,* 2012; Guzmán-Franco *et al*., 2012).

Interactions between insects and pathogens are complex, and the factors that influence the outcomes of these interactions need to be understood before successful field-scale applications as microbial control agents are possible. Most studies have addressed variation amongst fungal species against one insect species for isolate selection purposes (e.g. Gindin *et al*., 2006; Ondiaka *et al*., 2008). However, little has been done regarding variation in susceptibility to pathogens within insect populations of the same species over time (Noma and Stricker, 1999). Preliminary field experiments in Guanajuato, Mexico showed variation in the levels of infection achieved by *M. pingshaense* in white grub populations on maize in different years (unpublished data).

Although the cause of these results was unclear, the important role of variation in the immune capability of the white grubs merited further consideration.

Insects can activate different cellular and humoral defense mechanisms against infection, one of the most important being the prophenoloxidase system (ProPO) (González-Santoyo and Córdoba-Aguilar, 2012). Surface molecules of invading microorganisms activate conversion of ProPO into active phenoloxidase (PO), a key enzyme in the cascade leading to melanisation of the intruder (Söderhäll and Cerenius, 1998). Active PO has a tendency to adhere to cell surfaces, including haemocytes, to form aggregates (González-Santoyo and Córdoba-Aguilar, 2012) that are assumed to be related to pathogen resistance (Cotter *et al.*, 2004; Mucklow and Ebert 2003; Rantala and Roff, 2007).

The effectiveness of the immune response in invertebrates can be greatly affected by biotic and abiotic factors (De Block and Stoks, 2008; Lazzaro *et al*., 2008; Triggs and Knell, 2012), and such variation may result in resource allocation trade-offs, as the maintenance and deployment of an efficient immune response may take resources from other functions such as reproduction or longevity (Moret and Schmid-Hempel, 2000; Schmid-Hempel, 2011; Siva-Jothy et al., 2005). The species and virulence of pathogens (Schmid-Hempel, 2011), food availability and weather conditions (De Block and Stoks, 2008; McKean et al., 2008; Ponton *et al*., 2013; Seppala and Jokela, 2011; Yourth *et al*., 2002) have been reported as the main factors modifying the effectiveness of the immune response.

Success in the implementation of a microbial control programme will depend on the availability of information that aids an understanding of the factors that affect or modify the interaction between entomopathogenic fungi and the insect immune system. To contribute to this, in 2011 and 2012 we measured PO, proPO and protein concentration in the haeomolymph

of *P. polyphylla* larvae inoculated with viable and non-viable blastospores of *M. pingshaense*. We also assessed the fitness cost in terms of survival of larvae collected in both years following injection with a non-infective challenge (non-viable blastospores).

3.2 Material and Methods

3.2.1 *Phyllophaga polyphylla* larvae

For experiments third-instar *P. polyphylla* larvae were collected manually from the same maize fields in San Lorenzo, Guanajuato, Mexico in October 2011 and 2012. Collected larvae were deposited in a plastic container filled with damp peat moss (Growing Mix®, Canada) and returned to the laboratory. Each larva was placed individually into plastic cups (100 mL) and maintained at 20 °C with damp peat moss for four weeks before being used in experiments. All larvae were identified as described by Guzmán-Franco *et al*., (2012) and weighed before experimentation.

3.2.2 Production of *M. pingshaense* blastospores

On each occasion that an experiment was done, *M. pingshaense* isolate MGC01 (Carrillo-Benitez *et al*., 2013) was retrieved from storage at -80 °C and grown on Sabouraud Dextrose Agar (SDA) in 9 cm triple-vented Petri dishes for 20 days at 25 ºC and in complete darkness. For each experiment and replicate, the isolate was always retrieved from storage and never sub-cultured. Using a sterile scalpel, conidia (and associated mycelium) were scraped into a sterile 50 mL volume centrifuge tube containing 30 mL of 0.03% Tween 80 and vortexed for 5 min. Conidia were separated from the mycelium by filtration through a sterile cloth, resuspended in sterile 0.03% Tween 80 in a new sterile 50 mL centrifuge tube and the concentration estimated using a haemocytometer. A conidial suspension of known concentration was inoculated into a sterile 250 mL Erlemeyer flask containing 50 mL of sterile liquid medium containing yeast

extract, sucrose and Tween 80 (2:2:0.4 p/v) (YST) to achieve a final concentration of $1x10^6$ conidia mL^1. This was incubated on a shaker at 120 r.p.m. and 28 °C for three days. Blastospores were harvested by filtration through sterile cloth to remove the mycelium. The resulting suspension was centrifuged at 10 000 g for 10 min and the supernatant discarded. The pellet containing blastospores was washed three times with a solution of phosphate buffered saline (PBS) (pH 7.4) and 0.03 % Tween 80 (PBS+Tween), centrifuged at 10 000 g for 10 min, and finally resuspended in the PBS+Tween solution. The concentration of blastospores was determined using a haemocytometer. Prior to use in an experiment, the percentage of viable blastospores was estimated using the plate count technique on SDA (Goettel and Inglis, 1997). On all occasions that the experiment was done, more than 95 % of blastospores were viable.

Non-viable blastospores were obtained by incubating viable blastospores in 1.5 M NaOH solution for 30 min at 70 °C (Gottar *et al*., 2006). The resulting mixture was vortexed for 1 min and then incubated again under the same conditions. Following this the mixture was centrifuged for 10 min at 10 000 g and 4°C, and finally resuspended in PBS+Tween. Resulting suspensions of non-viable blastospores were washed and viability assessed as described before. On all occasions there was no germination of blastospores recorded.

3.2.3 Immune response of *P. polyphylla* larvae after inoculation with the fungus *M. pingshaense* in 2011 and 2012

3.2.3.1 Experimental design

Four treatments were evaluated against individual larvae (n=5 per treatment in 2011 and n=20 in 2012): treatment 1, each larva received a 10 µL injection of PBS+Tween; treatment 2, each larva received a 10 µL injection of PBS+Tween containing 1 x 10^3 viable blastospores; treatment 3, each larva received a 10 µL injection of PBS+Tween containing 1 x 10^3 non-viable

blastospores; treatment 4, each larva received no injection (control). All larvae were first anesthetised and immobilised on ice for 15 minutes. All injections were made into the larval haemocoel through the dorsal surface at the junction between the second and third abdominal segments using a 30-gauge needle fitted to a 1 mL syringe mounted on a calibrated micro-applicator. All treated larvae were transferred individually to 12-well cell culture plates (COSTAR®, Corning Inc. NY, USA) (1 larva per well), each well contained a 2 cm diameter filter paper moistened with 80 µl of sterile distilled water. The 12-well culture plates were incubated at 25 °C in complete darkness. PO activity, ProPO activity and total protein concentration were estimated in the haemolymph of larvae 12 h after treatment application (see 3.2.3.2, 3.2.3.3, 3.2.3.4 below). This post-treatment period of time was selected from preliminary studies on PO and ProPO activity done 0, 6, 12, 24 and 48 h post treatment which showed that the greatest values were obtained after 12 h (data not shown). Each year the complete experiment was repeated on three occasions, each separated by one week and during the same period of the year.

3.2.3.2 Haemolymph collection from larvae

Each larva was surface sterilised with 70% ethanol and rinsed twice in sterile distilled water. Haemolymph samples were obtained by cutting the third thoracic leg of each larva from which 10 µL of haemolymph could be collected into sterile, precooled Eppendorf tubes (1.5 mL) containing 90 µL of PBS solution (pH 7.4), and vortexed for 10 s. The mixture was centrifuged for 10 min at 10 000 rpm and 4 ºC to remove haemocytes and cell debris. The supernatant was used to measure protein concentration, prophenoloxidase (ProPO) and phenoloxidase (PO) activity as described in 3.2.3.3 and 3.2.3.4.

3.2.3.3 Estimation of haemolymph protein concentration in larvae

Protein was quantified using the Bicinchoninic Acid Assay kit (BCA) (Pierce Biotechnology, Rockford, IL) following the manufacturer's instructions. Briefly, 10 µL of each supernatant sample (see 3.2.3.2) was added to 40 µL of PBS and 150 µL of the kit reagents. The mixture was incubated for 30 min at 37ºC and then the absorbance measured on a MultiSkan Ascent reader (Thermo-Fisher Scientific, Suwanee, GA, USA) at 562 nm. A known concentration of Bovine Serum Albumin (BSA) was used as a standard curve (provided with the kit) that was compared with each sample to estimate the amount of protein present. The complete process was done in duplicate for each sample. The protein concentration in samples was expressed in µg/µL of haemolymph.

3.2.3.4 Measurement of phenoloxidase (PO) and prophenoloxidase (ProPO) activity in larvae

PO activity was measured using a modified version of the method described by Enriquez-Vara et al., (2012). Briefly, an aliquot of haemolymph containing 10 µg of protein was placed in a 96 well microplate (Corning Inc, Corning NY) and then diluted with PBS to achieve a volume of 50 µL. To this mixture, a further 50 µL of PBS and 50 µL of L-dihydroxyphenylalanine (L-DOPA; [4 mg/mL]) (Sigma-Aldrich Corp. St. Louis, MO, USA) were added to obtain a final volume of 150 µL. For blank wells, only 100 µL of PBS and 50 µL of L-DOPA (4 mg/mL) were used. The reaction was allowed to proceed for 60 min at 25 °C. Readings of absorbance were taken every 5 min on a MultiSkan Ascent microplate reader at 492 nm. Enzyme activity was measured as the slope achieved during the linear phase of the reaction when the enzyme catalysed the transition from L-DOPA to dopachrome. The slope was used as the unit of activity of the enzyme as it was related to enzyme concentration. For example, higher values for the slope result from a faster reaction speed for the catalysis of L-DOPA to dopachrome, which are a

result of a higher PO concentration and activity. Estimation of PO activity was done in duplicate for each larva.

ProPO activity was measured using a method modified from Adamo (2004). This method used the enzyme α-chymotrypsin to activate all the ProPO present in the haemolymph and that which had been converted into PO. The reaction mixture contained 10 µg of protein (adjusted to 50 µL with PBS as above) and 50 µL of 1.3 mg mL^{-1} α-chymotrypsin (Sigma-Aldrich Corp. St. Louis, MO, USA) diluted in PBS. The mixture was incubated in a 96-well plate for 20 min at room temperature. Then, 50 µL of L-DOPA (4 mg/mL) were added to obtain a final volume of 150 µL and the activity measured as described above.

3.2.3.5 Statistical analyses

Differences in the haemolymph protein concentration were compared among treatments and year (2011 and 2012), and the interaction between these two factors determined using a general linear model (GLM); no data transformation was required. PO and ProPO data were transformed using a Box-Cox (PO activity: $\lambda = 0.2$; ProPO: $\lambda = 0.3$) procedure to meet assumptions of normality and homogeneity of variance. The effects of treatment and year (2011 and 2012) were analysed separately for PO and ProPO activity using GLM. All analyses were performed using the statistical package R (R Development Core Team, 2011).

3.2.6 Survival of larvae following injection of a non-infective challenge

When viable blastospores of *M. pingshaense* were injected into *P. polyphylla* larvae, all insects died within 72 h of injection (data not shown), making it very difficult to detect any effect of treatment and year on larval fitness, as measured by survival. Therefore, a non-infective challenge (non-viable blastospores) was used to determine how stimulation of the immune system alone affected survival. There were five experimental treatments: treatment 1, each larva

(n=15) received a 10 µL injection of PBS+Tween; treatment 2, each larva (n=15) received a 10 µL injection of PBS+Tween containing 1 x10^3 non-viable blastospores; treatment 3, each larva (n=15) received a 10 µL injection of PBS+Tween which was then repeated after 7 and 15 days; treatment 4, each larva (n=15) received a 10 µL injection of PBS+Tween containing 1 x10^3 non-viable blastospores which was then repeated after 7 and 15 days; treatment 5, each larva (n=15) received no injection (control). All larvae were manipulated and incubated as described in 2.3.1. Mortality was assessed every 24 h for 35 days. Dead larvae were incubated at 25°C and 100 % RH for 7-10 days to determine the cause of mortality; larvae dying due to fungal infection would sporulate and produce conidia. The complete experiment was repeated on two occasions each year.

3.2.6.1 Statistical analysis

Only data from larvae collected in 2011 could be analysed as injected larvae collected in 2012 all died within 48 h of injection, except larvae from the control treatment. Assessment of variation between replicates showed no significant differences and allowed the data from the two separate replicates to be pooled (data not shown). Data were subjected to Kaplan-Meier analysis with survival curves constructed using mortality data recorded every 24 h for each treatment, with statistical comparisons made using the log-rank test. Firstly the PBS+Tween treatments (single and multiple injections combined) were compared with non-viable blastospores treatments (single and multiple injections combined), and secondly comparisons were made between single and multiple injections separately for each treatment. The fifth treatment (control) was not formally included in the analysis as no mortality was recorded during the course of the experiment (in fact, only 10 % of larvae had died after 40 days of incubation in this

treatment [data not shown]). All analyses were performed using the statistical package R (R Development Core Team, 2011)

3.3. Results

3.3.1 Estimation of PO, ProPO levels and protein concentration in larvae from *P. polyphylla* larval populations collected in 2011 and 2012 and following inoculation with *M. pingshaense*

There were no significant differences in PO activity amongst all treatments ($F_{3, 127}$=2.46, P=0.0660, Fig. 3.1.a); however, overall PO activity were significantly different when comparing between 2011 and 2012 ($F_{1, 127}$=13.85, P=0.0003); PO values were larger for larvae collected in 2011 than for larvae collected in 2012, which was consistent across all treatments ($F_{3, 127}$=0.91, P=0.4385; Fig. 3.1.a).

There were no significant differences in ProPO activity amongst all the treatments ($F_{3, 127}$=0.43, P=0.7287), but, as for PO activity, there were significant differences found between years ($F_{1, 127}$=32.29, P=0.0001). Again, greater activity was found for larvae collected in 2011 compared with larvae collected in 2012 (Fig. 3.1.b), and this difference was consistent across treatments ($F_{3, 127}$=1.16, P=0.3291) (Fig. 3.1.b).

There were no significant differences in protein concentration amongst all treatments ($F_{3, 127}$=1.9890, P=0.1189); however overall protein concentrations were significantly different between years ($F_{1, 127}$=5.4680, P=0.0209) and, in contrast to PO and ProPO activity, were significantly greater in 2012 than 2011. This difference was consistent across treatments ($F_{3, 127}$=0.5137, P=0.67355) (Fig. 3.2.).

3.3.2 Survival of larvae following injection with a non-infective challenge

Overall, there was no significant difference in survival of larvae injected either with PBS+Tween or with non-viable blastospores (χ_1^2=3.303, P=0.069). Also, there was no

significant difference in larval survival in relation to whether they were injected once or three times with either PBS+Tween or with non-viable blastospores (χ_1^2=0.246, P=0.62 and χ_1^2=2.542, P=0.111 respectively) (Fig. 3.3.). Although no analysis could be done on larvae collected in 2012 as all injected larvae died within 48 h, the control mortality was only circa 10 % after 35 days confirming that dead larvae in the treatments was due to the inoculations of either PBS+Tween or non-viable blastospores.

3.4. Discussion

Our results showed that injection of *M. pingshaense* blastospores or PBS resulted in similar PO and proPO activity in *P. polyphylla* larvae but that this varied between years in the same population. Although the estimations of PO and proPo activity cannot be related specifically to pathogen challenge, they do provide a good estimation of the overall immune response of larvae, and how this changes in the same insect population with time.

The lack of differences in the PO and ProPo levels in larvae from the different treatments may be as a consequence of the larvae self-regulating this process. For example *M. anisopliae* secretes proteases and chitinases that aid fungal penetration of the insect cuticle (Manalil et al., 2010) and these proteases are considered to contribute to activation of proPO (González-Santoyo and Córdoba-Aguilar, 2012). However, it has also been reported that cellular responses may limit the activation of proPO as, during conversion of proPO into PO, cytotoxins are produced that may negatively affect the insect (Franssens et al., 2008; Zhao et al., 2011). Additionally, the activation of the PO/ProPO system is considered costly for the insect in terms of energy investment (e.g. Ardia et al., 2012). In relation to this, when non-viable blastospores or PBS+Tween, were injected into *P. polyphylla* larvae, large mortalities occurred, even though both were considered as non-infective challenges. This, suggests that a lot of energy had been

invested by the insect in a capability to suppress any foreign body detected, and that this resulted in low survival. There are several reports suggesting that investing in an immune response can be costly in terms of fitness (Schmid-Hempel, 2011), leading sometimes to death of the insect (Manalil et al., 2009) or to autoreactivity caused by cytotoxins (Sadd and Siva-Jothy, 2006).

Interestingly, we found differences in PO and ProPO activity between larvae collected in different years; the greatest values were found in larvae collected in 2011 compared to larvae collected in 2012, suggesting that larvae collected in 2011 were more likely to survive immune challenge (not only pathogen-related), compared with larva collected during 2012. This was confirmed when a non-infective challenge was injected into larvae collected in 2012, and they all died within 48 h.

The biotic and abiotic factors modulating the quantity of resources invested to establish an effective immune response in different years are diverse, and our data do not allow us to identify these factors specifically. However, we observed that precipitation levels were different in the two years evaluated with 495 and 300 mm of rain in 2011 and 2012 respectively. Although we could not confirm it experimentally, we suggest that in 2011, the greater precipitation levels could have resulted in higher quality plants providing better nutrients for larvae (e.g. Brown and Gange, 1990). Despite this, larval body weight, an indicator of animal body condition, did not statistically differ between both years (0.76±0.12 g) (analysis not shown). When protein concentration was measured, this showed an opposite pattern to PO and proPO and one explanation for the dissimilarity between the immune components and protein concentration may be as a consequence of water stress on plants. It is known that maize roots exposed to water stress (which we propose happened with maize plants in 2012), produce numerous secondary metabolites (Erb and Lu, 2013; Ribau and Pilet, 1991), causing the insect to

produce more detoxification enzymes (Després et al., 2007). This may have increased the overall concentration of protein in the haemolymph in 2012 (Smilanich et al., 2009), although this remains to be confirmed.

In summary, our results have important implications for the use of entomopathogenic fungi for white grub control. Our results suggest that virulence is not the only factor influencing the infection capacity of a pathogen, but that the life history of an insect population may also affect its susceptibility and should be considered in the decelopment of microbial control programmes for soil-dwelling pests. Understanding the variables influencing larval life histories could be used to predict the conditions necessary to ensure that pest survival in response to pathogen challenge can always be impaired, thus enhancing pest control.

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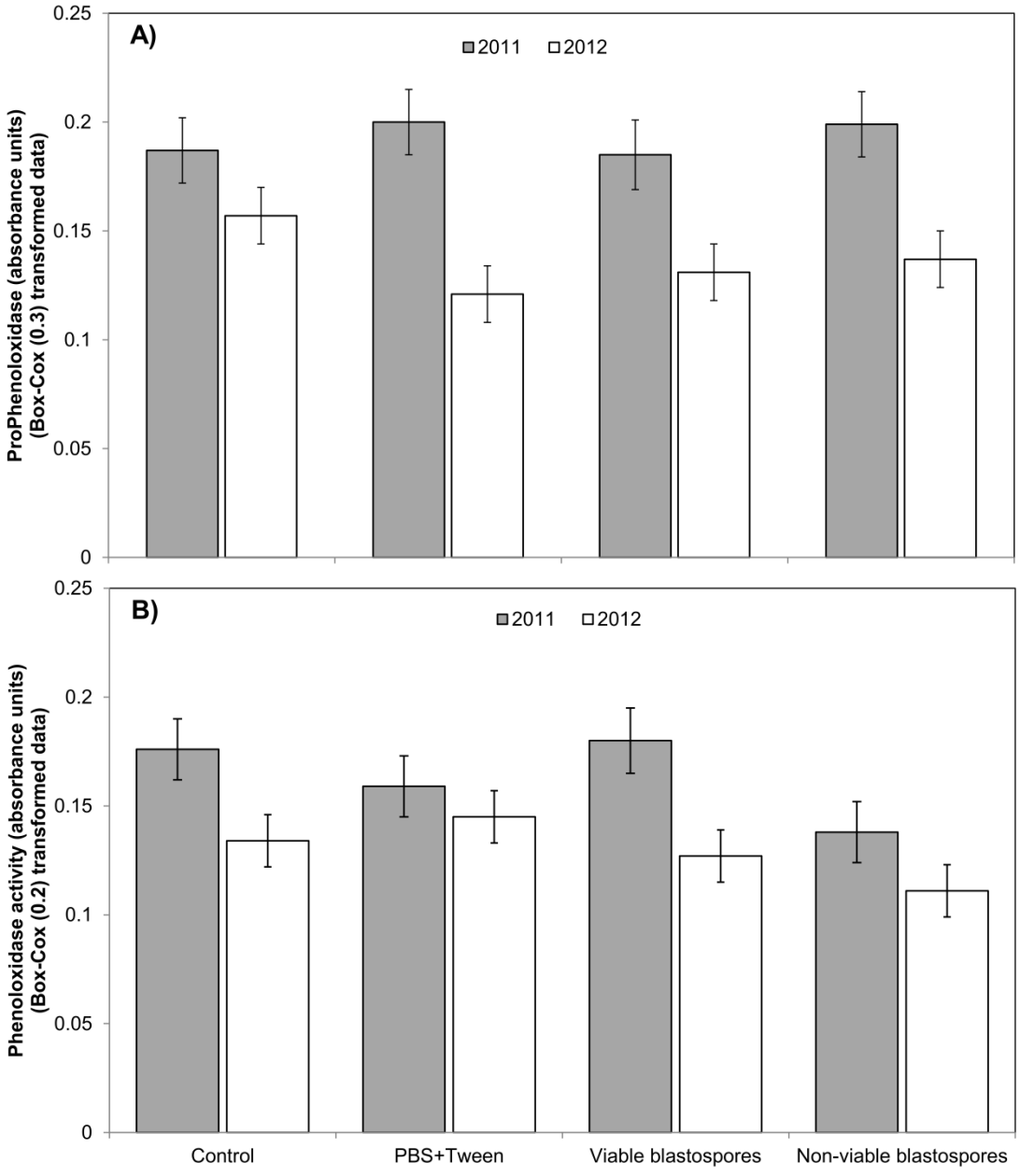

Figure 3.1-Prophenoloxidase (A) and Phenoloxidase (B) activity in relation to treatments and year. Error bar represents mean±S.E.

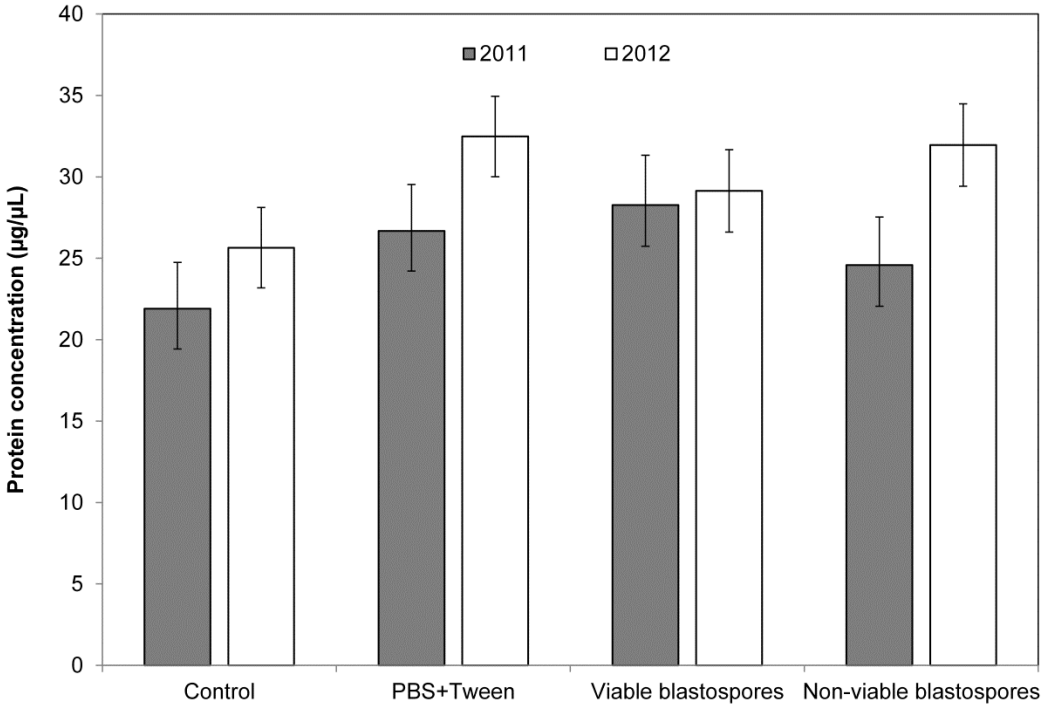

Figure 3.2-Mean protein levels according to treatments and year. Error bars represents mean ± S.E.

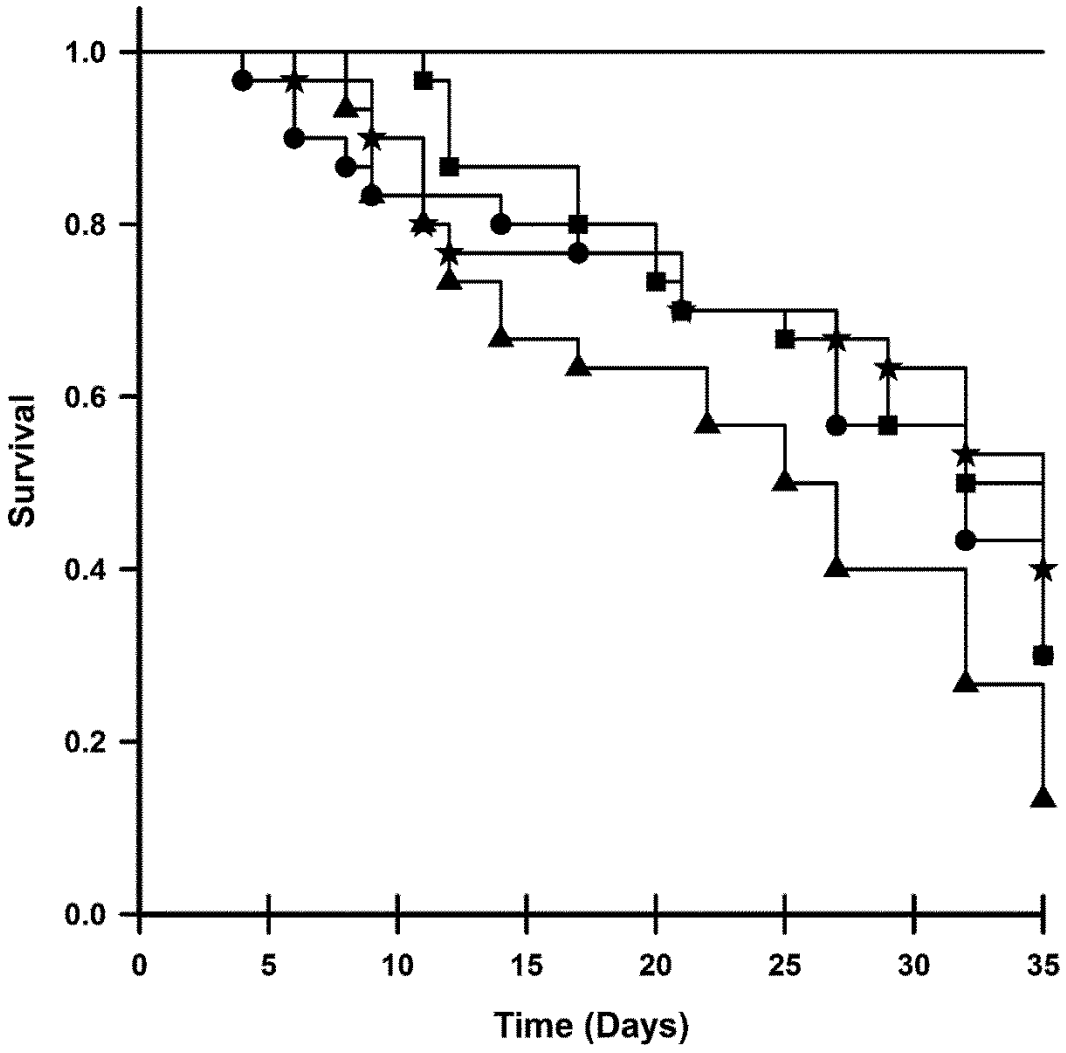

Figure 3.3-Survival of *P. polyphylla* larvae injected: three times with non-viable blastospores (▲), one time with non-viable blastospores (●), three times with PBS+Tween (■), one time with PBS+Tween (★) and one control treatment (—). Only data from larvae collected in 2011 are presented.

Capítulo 4. Is the immune response of *Phyllophaga polyphylla* larvae an effective barrier against *Metarhizium pingshaense*?[3]

Abstract

Insects protect themselves by either or both, immunological and non-immunological barriers, but their relative contribution to resistance has been rarely investigated. This is particularly important in insect pest species to know how they combat its bio-control species. To test this, we used *Phyllophaga polyphylla* and *Metarhizium pingshaense.* To overcome the cuticle as barrier against infection, we injected different concentrations of blastospores into the hemocoel of *P. polyphylla* larvae. Firstly, we estimated a median lethal dose (LD50) of blastospores to be injected into the hemocoel in order to exposed larvae to the fungus but at the same time ensure the larvae remained alive for long enough to allow further estimation of immunological parameters. Secondly, we injected the estimated LD50 of blastospores into the hemocoel of larvae to quantify PO, NO and antimicrobial activity as a response against fungal invasion. Contrary to a previous report that showed that *M. pingshaense* is unable to kill *P. polyphylla* after topical applications, (a) when blastospores were injected into the hemocoel of *P. polyphylla* larvae, 100 % mortality was obtained and (b) when injecting the LD50 into the hemocoel of the larvae, immune response did not differ with control. These results suggest that cuticle could be the main barrier against infection in *P. polyphylla* against *M. pingshaense*. These results highlight the importance of studying immunological and non-immunological barriers in insect pests against its potential bio-controls. The role of our findings to understand how *M. pingshaense* combat its host is discussed.

[3]Enríquez-Vara, J.N., Guzmán-Franco, A., Alatorre-Rosas, R., González-Hernández, H., Córdoba-Aguilar, A. and Contreras-Garduño, J. 2013. Is the immune response of *Phyllophaga polyphylla* larvae an effective barrier against *Metarhizium pingshaense*? Bulletin of Entomological Research (**submitted**).

4.1 Introduction

Understanding the basis of host resistance is an intriguing biological phenomenon given that parasites and pathogens are ubiquitous and impose a strong selective pressure on their host (Schmid-Hempel 2011). In both vertebrates and invertebrates, resistance consists of both non-immunological and immunological barriers (Hart 2011; Parker et al. 2011). The former could be a behavioral, mechanical and/or hostile environment against invaders (Smilanich et al. 2009). On the other hand, the immunological defence prevents foreign agents to cause infection by humoral and cellular components. For example, once an intruder has been detected, the fruit fly *Drosophila melanogaster* produces a wide arsenal of immune responses; these fruit flies defend themselves against Gram-negative bacteria by the activation of the IMD or against fungi and Gram-positive bacteria by the Toll pathways. Both pathways originate the production of antimicrobial peptides that kill the invaders. Alternatively, encapsulation or melanization can be directed against nematodes, fungus and parasitoids (Lemaitre and Hoffmann 2007; Castillo et al. 2011).

Although it is well known that immunological and non-immunological barriers confer host resistance to parasites and pathogens its interaction has scarcely been studied (Parker et al. 2011) and insects are good model systems to understand this relationship. For example, entomopathogenic fungi, widely used as biological control agents (Clarkson and Charnley 1996), use mechanical pressure and enzymatic degradation to damage the insect cuticle and penetrate the host (Hajek and St. Leger 1994). Hence, the cuticle represents the first barrier against entomopathogens (Wilson et al. 2001). Insect cuticle is composed of epicuticle, exocuticle and endocuticle (Chapman 1998), but the mechanical (thickness and strength) and biochemical (i.e. production of humoral response as PO) traits of both exocuticle and endocuticle are responsible

to protect insects against fungi (Siva-Jothy et al. 2005). For example, the lipids of the epicuticle protect ticks from *Metarhizium anisopliae* infection (Ment et al. 2010). After injury (i.e. by the fungus penetration), production of antimicrobial peptides and cellular response are activated to protect the hemolymph from invasion (Lemaitre and Hoffmann 2007). After the pathogen penetrates the cuticle, the immune response (humoral and/or cellular) in the hemolymph attacks the fungi by phagocytosis, lytic activity and the activation in phenoloxidase (PO), the latter producing nodule formation, encapsulation and melanization (Lavine and Strand 2005; Bogus et al. 2007).

Given that both immunological (Schulenburg et al. 2009) and non-immunological barriers (Parker et al. 2011) are costly, it should be expected that an increase in the effectiveness of one would decrease the expression of the other (Parker et al. 2011). White grubs are a good model for testing this hypothesis. These soil dwelling herbivore insects continuously interact with a large variety of pathogens (Jackson and Klein 2006), including entomopathogenic fungi. The wide use of these microorganisms to regulate white grub species (Shah and Pell 2003) has demonstrated a differential susceptibility of these insects to fungal infection (Rodríguez del Bosque et al. 2005; Morales et al. 2010), evidenced by the fact that some species have proved less susceptible (Nong et al. 2011; Guzmán-Franco et al. 2012). This difference in susceptibility could be due to the differences in cuticle structure and/or the effectiveness of the immune response in the different insect species, respectively. One example is that of *Phyllophaga polyphylla* which had more PO activity and longer survival times when facing a challenge by *Metarhizium pingshaense* (Enríquez-Vara et al. 2012) compared to *Anomala cincta*. A second study confirmed the result with *P. polyphylla* as only 20% of larvae were infected by *M. pingshaense* (Guzmán-Franco et al. 2012). However, is not clear if the infection that *M.*

pingshaense may have produced was avoided inside the host by the immune response or by the cuticle. One approach for testing the relative contribution of each factor is by artificially by-passing the mechanical barrier imposed by the cuticle by injecting blastospores (the fungal form that multiply inside the insect) into the host haemocoel. Once inside the host, the immune response and survival can be measured. To test the hypothesis that the effectiveness of immunological barriers is traded-off with effectiveness of non-immunological barriers we predicted that if the cuticle is an effective barrier to avoid infection, then the immune response would not perform an effective function. Therefore, we analysed the survival rate and immune response (phenoloxidase, lytic activity and nitric oxide) of *P. polyphylla* larvae after injection with different doses of the entomopathogenic fungus *M. pingshaense*.

4.2 Materials and methods

Insects

Third-instar *Phyllophaga polyphylla* larvae were collected from corn fields in Guanajuato, Mexico (20° 02´30.12" N, 100 ° 28´36.4"). Once collected the larvae maintained individually in plastic cups (100 mL) at 20 °C with damp peat moss (Growing Mix®, Canada) for 4 weeks before they were used in the experiment.

Production of blastospores

The fungus *M. pingshaense* isolate GC01 was used. Enriquez-Vara et al. (2012) and Guzman-Franco et al. (2012) used this isolate against *Phyllophaga polyphylla*. In both works the isolate GC01 was referred as *Metarhizium anisopliae* (morphospecie) but Carrillo-Benítez et al. (2013) used molecular methods to demonstrate that the isolate GC01 is *Metarhizium pingshaense*. Hence we will refer to the isolate GC01 as *Metarhizium pinshaense*. First, conidia were produced in petri dishes containing Saburaud Dextrose Agar medium (SDA). After 20 days

of incubation at 25 ºC in complete darkness, conidia from the medium were harvested with a sterile scalpel. Conidia and mycelium were deposited into a sterile 50 mL volume centrifuge tube containing 30 mL of 0.03% Tween 80. The mixture of conidia and mycelium was stirred for 15 min. Conidia were separated from mycelium by filtration trough sterile cloth and deposited into a new sterile 50 mL volume centrifuge tube. Conidia concentration was estimated using a haemocytometer. Conidial suspension was then inoculated and grown in 50 mL of sterile liquid medium containing yeast extract, sucrose and Tween 80 (2:2:0.4 p/v) (YST). Liquid medium contained in a 250 mL Erlenmeyer flask and with a concentration of $1x10^6$ con mL^{-1} was incubated on a shaker at 120 r.p.m. at 28 °C for three days. Blastospores were harvested by filtration through sterile cloth and, to remove any remaining liquid medium, the suspension was centrifuged three times at 10,000 rpm for 10 min and suspended in phosphate buffered saline solution pH 7.4 (PBS) (Sigma). The concentration of blastospores was determined using a haemocytometer. The percentage of viable blastospores was estimated prior to experiments using the plate count technique on SDA (Goettel and Inglis 1997). In all cases more than 95 % were viable.

Survival of *P. polyphylla* larvae injected with *M. pingshaense* blastospores

Different groups of 30 third-instar *P. polyphylla* larvae were injected with different doses of blastospores of *M. pingshaense* (10^3,10^4,10^5 and 10^6 blastospores, in a total volume of 5 µL per larva) suspended in PBS. Before injection, white grubs were anesthetized on ice and immobilized. The blastospores suspension was injected into the larvae hemocoel through the dorsal surface at the junction between the second and third abdominal segments. Injections were carried out using a 30-gauge needle fitted to a 1 mL syringe mounted on a calibrated micro-applicator. As control group, larvae were only injected with PBS. The larvae were transferred

individually to 12-well cell culture plates (COSTAR ®, Corning Inc. NY, USA) (1 larvae per well), which contained a 2 cm diameter filter paper which had been moistened with 80 µl of sterile distilled water. The 12-well culture plates were incubated at 25 °C in complete darkness and mortality assessed every 24 h for 10 days. Dead larvae were incubated at 25º C and 100 % RH for 7-10 days, to encourage sporulation thereby allowing fungal infection to be confirmed.

Data were analysed using Kaplan-Meier survival curves, and the log-rank test was used to evaluate statistical differences between white grubs injected with PBS only or with different doses of blastospores of *M. pingshaense*. Kaplan-Meier survival curves were constructed for each treatment. The log-rank test was used to compare survival amongst curves constructed for each treatment.

Immune response of *P. polyphylla* against *M. pingshaense* infection

The immune response of *P. polyphylla* against *M. pingshaense* infection was estimated by quantifying the production of NO, PO and antimicrobial activity in the insect´s hemolymph as a response to infection (see below). To achieve this, a lethal dose (LD_{50}) concentration of blastospores was injected into the hemolymph. Injecting a LD_{50} increased the survival time before death thereby allowing immune parameters to be quantified. The LD_{50} was estimated by dose-response assays.

Estimation of LD_{50}

The estimation of LD_{50} was obtained using the same methodology described above with some modifications. Twelve third-instar *P. polyphylla* larvae were exposed to four doses. Based on the results of the previous experiment, a different set of doses was selected $5x10^3$, $1x10^4$, $5x10^4$ and $1x10^5$ blastospores of *M. pingshaense* in PBS. The complete experiment was repeated on two different occasions. Larval mortality was recorded every 24 hours for five days. Mortality

was corrected using Abbott´s formula (Abbott, 1925). Data from the bioassays were analysed using a generalized linear model (GLM) with binomial error and probit link in the statistical package GenStat v. 8.0 (Payne et al. 2005). The numbers of infected larvae were assumed to follow a binomial distribution with sample sizes equal to the number of larvae tested. Before combining two replicates, a parallel model analysis was done for each replicate. First, a single line was fitted to data from replicates. Second, intercepts were allowed to vary amongst the replicates and third, slopes were also allowed to vary amongst the replicates. If the single line model was the best for each replicate, then data from the two replicates could be combined.

Concentration causing 50% infection (LD_{50}) of larvae was estimated from best fit model and confidence interval (CI) for LD_{50} was calculated according to Fieller's theorem (Fieller 1944).

Quantification of immune parameters

Five groups of 12 larvae each were injected with the LD_{50} estimated previously ($5x10^3$ blastospores) to quantify NO, PO and microbial activity production. These parameters were estimated in the hemolymph of larvae at five different times after injection (0, 2, 6, 12 and 24 h). The immune response of each of the five different times after injection was estimated in a different group of 12 larvae. A different set of five groups of 12 larvae were injected with only PBS and treated as described before. A total of 120 larvae were used for both treatments and times of quantification. All treated larvae were maintained as described before until hemolymph was collected.

Hemolymph collection

For the hemolymph collection, the integument of each larva was surface sterilized with 70% ethanol and then rinsed twice using sterile distilled water. Hemolymph samples were

obtained by cutting the third thoracic leg of each larva and four drops (approximate 30 µL) of hemolymph were collected into sterile and precooled Eppendorf tubes (1.5 mL) containing 100 µL of phosphate buffered saline (PBS), and vortexed for 10 s. The mixture was centrifuged for 10 min at 10, 000 rpm and 4 ºC to remove hemocytes and cell debris. The supernatant was divided into three aliquots, two of 50 µL and one of 30 µL. The two 50 µL aliquots were mixed separately with 50 µL of PBS, while the third was placed in a 0.5 mL Eppendorf tube and kept at -80 °C until required. The first 50 µL subsample was used to measure protein hemolymph content and phenoloxidase activity (PO). The second subsample was used to estimate nitric oxide (NO) production, and the 30 µL sample was used to estimate antimicrobial activity. All measurements were carried out immediately after hemolymph collection.

Protein content

Proteins were measured using the BCA (Pierce Biotechnology, Rockford, IL) protein assay kit with BSA as the protein standard. Two replicates of 10 µl of hemolymph/PBS mixture were used to measure the protein in each sample (see Enriquez-Vara et al. 2012). The absorbance was measured on a Varioskan Flash microplate reader (Thermo Fhiser Scientific, Waltham, MA) at 562 nm.

Phenoloxidase activity (PO)

Hemolymph PO activity was measured using the method described by Enriquez-Vara et al. (2012). Briefly, an aliquot that contained 40 µg of protein was placed in a 96 well microplate (Corning Inc, Corning NY), and then dose-tittered to a volume of 50 µL of sample and PBS. To this mixture, 50 µL of L-DOPA (4 mg/mL) was added to obtain a final volume of 100 µL. PO activity was assayed spectrophotometrically with dopamine as a substrate. The slope of the curve

was calculated by using the optical density at 490 nm. Optical density readings were taken every minute for one hour at 30 °C (Enríquez-Vara et al. 2012).

Nitric oxide (NO) production

A colorimetric nitrate/nitrite assay kit (SIGMA) was used to prepare the standard curve and to estimate NO in each sample following the manufacturer's instructions. The basis of this technique is that nitric oxide is a highly unstable radical that rapidly reacts with other oxygen-reactive species to form stable products, such as nitrites, nitrates and toxic radicals (i.e., peroxynitrite). Hence, the total nitrate and nitrite content is used to indirectly estimate the amount of nitric oxide in each sample. The amount of NO (µM) in samples was estimated by extrapolation with a standard curve of known concentrations. Readings were performed at 540 nm.

Antimicrobial activity

Antimicrobial activity in the haemolymph samples was measured using the cup agar-diffusion assay technique (Mohring and Messner, 1968). The microbial activity was measured according to the methods described by Bogus et al. (2007), with some modifications. Briefly, the assays were performed in 90-mm Petri dishes containing 66 mM Sörensen buffer, pH 6.4 (10 mL), using *Micrococcus lysodeiticus* (7 mg) as substrate, agar (100 mg) and streptomycin sulfate (0.7 mg). Hemolymph samples (4 µL) free of insect cells were added to the Petri dishes, samples formed a circle of three mm in diameter. The diameters of the lytic zones around the three mm diameter samples were measured after incubation of the Petri dishes for 24 h at 37 °C. The antimicrobial activity of the insect hemolymph samples was expressed in equivalents of chicken egg white lysozyme. Increasing concentrations of lysozyme (10-1000 ug/mL) were used as a standard for comparisons.

The quantities NO and antimicrobial activity found were insignificant, therefore not included in the analysis. Data from PO activity were analysed using analysis of variance (ANOVA) in the statistical package SAS v. 9.0 (SAS, North Carolina,USA). We compared PO measurements between PBS (control) and injected with blastospores treatments, and their interaction with the time after inoculation. PO data were ln-transformed to meet assumption of normal distribution and equality of variances.

4.3 Results

Survival of *P. polyphylla* larvae injected with *M. pingshaense* blastospores

Significant differences were found in the survival rates of *P. polyphylla* larvae amongst all treatments compared (X^2 =145.86; P < 0.001; Fig. 1). When the larvae were injected with $1x10^5$ and $1x10^6$ blastospores of *M. pingshaense*, some died within the first 48 hours post-injection and 100% mortality was recorded at 120 hours. An intermediate effect in survival was obtained when the larvae were injected with $1x10^4$ blastospores (Fig.1). The survival rate in larvae injected with $1x10^3$ blastospores and larvae in the PBS control were similar and mortality was never greater than 10% (Fig. 1).

Immune response of *P. polyphylla* against *M. pingshaense* infection

Estimation of LD_{50}

No evidence of non-parallelism (χ_1^2=0.22, P=0.637) or differences in intercepts (χ_1^2=2.93, P=0.087) amongst replicates were found, justifying the pooling of data from separate replicates for further analyses. The LD_{50} value estimated for *M. pingshaense* was 5.2 x 10^3 ± 3.3-7.5 x 10^3 (CI) blastospores. Therefore, larvae were injected with the LD_{50}, estimated to assess immune response parameters.

Quantification of immune parameters

After injection of blastospores only PO production was activated. No antimicrobial or NO activity was recorded. PO production was similar in larvae injected with blastospores and PBS ($F_{1,105}$ = 0.16, P = 0.6896, Fig. 2), and this result was consistent throughout all measurements times (($F_{4,105}$ = 1.75, P = 0.1438, Fig. 2).

4.4 Discussion

The interaction between immunological and non-immunological barriers has scarcely been studied (Parker et al. 2011). Previous studies showed that *P. polyphylla* larvae is highly resistant to infection by *M. pingshaense* when immersed in a conidial suspension of this fungus, with mortality never exceeding 20% after 36 days of incubation (Enríquez-Vara et al. 2012; Guzmán-Franco et al. 2012). However, by injecting blastospores directly into the hemocoel, greater mortality was found in inoculated larvae as nearly 100% of larvae were infected after 120 hours of incubation at the greatest doses. This supports the hypothesis that the cuticle of *P. polyphylla* is a physical barrier to fungal infection by the natural co-occurring pathogen *M. pingshaense*. Bogus et al. (2007) found that mortality associated with the topical application of conidia is related to the cuticle thickness in three insect larvae. The cuticle may be important as a barrier in soil systems because it prevents the negative impact of abiotic factors (i.e., the damage to the cuticle due to friction with the soil), and therefore may favour resistance against a wide variety of pathogens and parasites (Villani et al. 1999). As the epicuticle is more variable in its components than the procuticle, this could be implicated in the differential insect resistance to invaders (see Golebiowski et al. 2008). The mechanical (thickness and strength) and biochemical (PO and lipids) traits of both exocuticle and endocuticle could protect the insect host against fungal infections (Siva-Jothy et al. 2005; Ment et al. 2010).

In order to test the above hypothesis, larvae were injected with *M. pingshaense*. After being injected with the LD_{50} of fungus, only the phenol oxidase (PO) activity of larvae was activated with no change in nitric oxide (NO) or antimicrobial activity. Indeed, immunological and non-immunological barriers against infection are costly to produce (Schmid-Hempel 2005; McKean and Lazzaro 2011; Parker et al. 2011). For example, Ardia et al. (2012) found that immune response entails specific energetic and corresponding physiological costs when insects produced encapsulation as a response. The encapsulation response led to increased levels of phenoloxidase and CO_2 production and decreased levels of lysozyme. On the other hand, in *Achaeta domesticus* the investment in cuticle thickness (a non-immunological barrier) decreased egg production (Bascuñan et al. 2010). In addition, in species in which the non-immunological barriers are effective at combatting parasites or pathogens, the immunological barriers are reduced (Parker et al. 2011). For example, wood ants (*Formica paralugubris*) use conifer resins (a non-immunological barrier) to protect themselves from *Pseudomonas fluorescens* and *Metarhizium anisopliae* (Chapuisat et al. 2007), and the presence of resin in nest material was associated with a decrease in the immune response (Castella et al., 2008). Finally, Dubovskiy et al. (2013) found that PO and lytic activity were lower in hemolymph that cuticle in *G. mellonella* against *M. anisopliae*. Our results suggest that the cuticle, a non-immunological barrier is playing a key role in the resistance of *P. polyphylla* against *M. pingshaense* infection, and is traded-off with NO and antimicrobial activity, but not with PO. This result suggests that some but not all immune markers are traded-off with non-immunological response. Although it is likely that the cuticle, specifically the thickness, may be variable amongst white grub species and modifying the immune response, this requires further experimental evidence.

It is still unclear why the PO quantities did not differ between the PBS and blastospore treated larvae; however we propose that blastospores of *M. pingshaense* were not detected by the cellular or humoral innate immune response of *P. polyphylla.* The entomopathogenic fungi must be discreet to avoid being recognized as a foreign agent by the host immune response (Wang and St. Leger 2005; Vilcinskas 2010). Poprawski and Yule (1991) injected $3x10^6$ spores of *M. anisopliae* in *Phyllophaga anxia* larvae, obtaining 42% mortality. Interestingly, we injected only $1x10^6$ blastospores, which produced 100% mortality. It is likely that spores are better detected by the insect immune response than blastospores (Wang and St. Leger 2006), as well as by the fact that blastospores are the stage of replication of the fungus leading to a faster invasion of the host´s hemocoel, which is not the case for spores (Gillespie et al. 2000).

Finally, our results with *M. pingshaense* did not support the hypothesis that *Metarhizium* normally reduces PO activity, as demonstrated in *G. mellonella* larvae infected with *M. anisopliae* (Slepneva et al. 2003), but are in accordance with a recent work in *G. mellonella* against *M. anisopliae* (Dubovskiy et al., 2013). The immune response caused by a fungal infection may vary according to the distinct host and fungal pathogen species. When the fungus *Conidiobolus coronatus* were inoculated in different insect species, the immune response based on the PO levels determined varied. PO levels decreased when *G. mellonella* was inoculated whereas no modification was found in *Diprion pini* and an increase was observed in *Calliphora vicina* (Bogus et al. 2007). Further experiments are required to clarify why *M. pingshaense* blastospores apparently are not detected by *P. polyphylla* immune system, and whether these mechanisms vary according to the host species. The cuticle therefore appears to have been selected as the main barrier against infection in *P. polyphylla* rather that humoral defence. It is likely that modifying the cuticle structure accordingly is less costly than investing in more

sophisticated humoral defence mechanisms, although this requires further experimental evidence.

In conclusion, our results suggest that the non-immunological defence against *M. pingshaense* is more important than the immunological barrier in *P. polyphylla* larvae. Further studies will be required to determine the importance of cuticle thickness for white grub resistance to infection by entomopathogenic fungi. Previous studies demonstrated that cuticle thickness is costly (Bascuñan et al. 2010), therefore, it is important to determine how costly it is in *P. polyphylla* in comparison with immunological barriers. Finally, it is still required to determine how *M. pingshaense* avoids recognition by their host, this represent the first report of this fungal species infecting white grub, specifically *P. polyphylla* larvae. A better understanding of the defence mechanisms of insect pests against fungal infection should ensure a more effective use of these microorganisms in microbial control programs. We encourage research to improve the *M. pingshaense* molecular mechanisms penetration to overcome the *P. polyphylla* cuticle.

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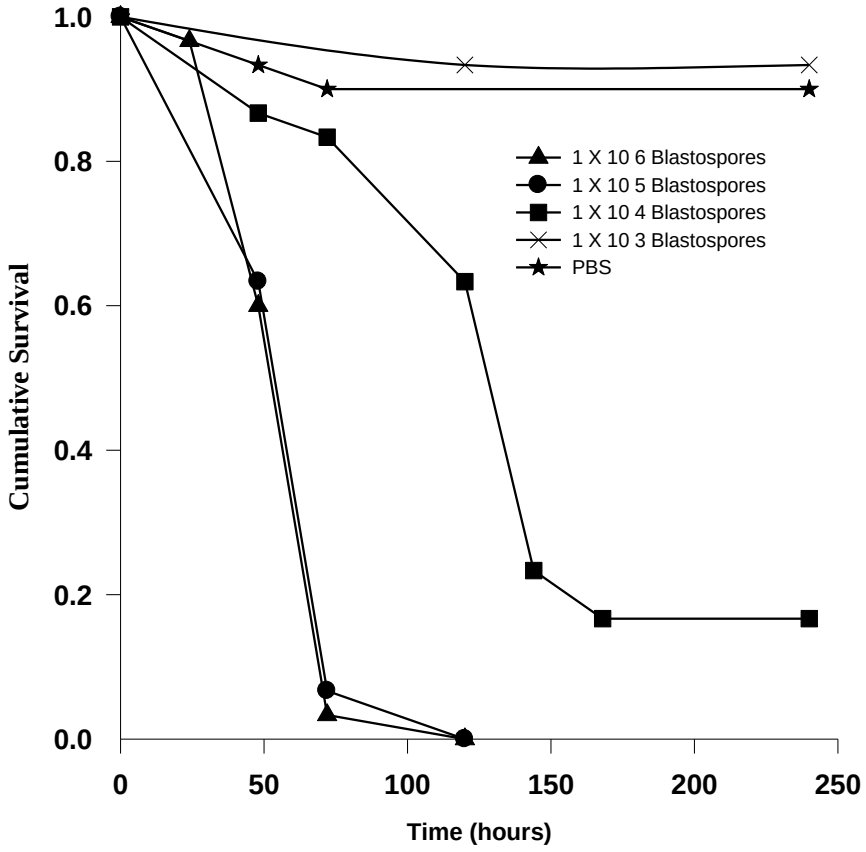

Figure 4.1-Survival of third-instar larvae of *Phyllophaga polyphylla* injected with different concentrations of blastospores of *Metarhizium pingshaense*.

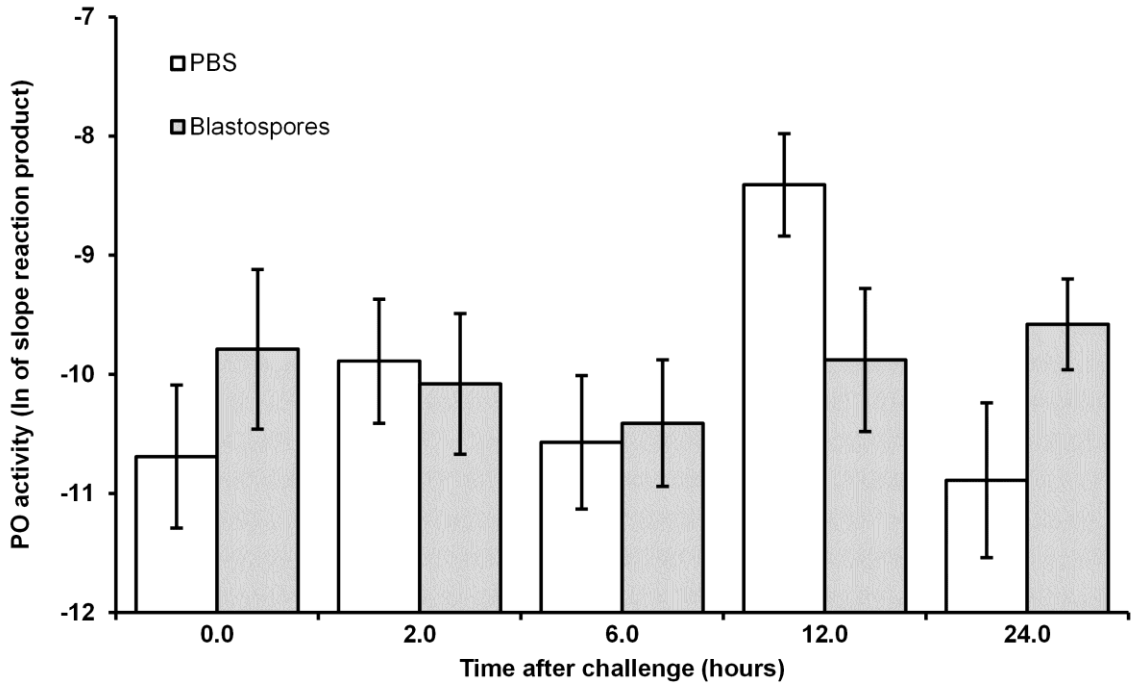

Figure 4.2-Slope PO activity expression according to PBS or blastospores and time. Sample size was 12 larvae per time point and treatment. Each bar indicates mean ± SE.

Capítulo 5. Discusión general

En el presente trabajo se investigó el efecto de la defensa inmunitaria y no-inmunitaria en la supervivencia de larvas de gallina ciega inoculadas con hongos entomopatógenos. Debido a la variación en la susceptibilidad entre las especies de *Phyllophaga polyphylla* y *Anomala cincta* contra los hongos entomopatógenos reportada previamente (Guzmán-Franco *et al.* 2012), se analizó la hipótesis de que la variación en la supervivencia entre las especies de gallina ciega se debe a diferencias en su sistema inmunitario.

La respuesta inmunitaria medida como la producción de óxido nítrico (ON) y la actividad de la fenoloxidasa (FO) parcialmente explica la hipótesis de que las variaciones en la susceptibilidad de las gallinas ciegas contra los hongos entomopatógenos se deba a la respuesta inmunitaria. Las larvas de *P. polyphylla* y *A. cincta* presentaron diferencias en su respuesta inmunitaria basal, y estas diferencias se relacionaron con la supervivencia de cada especie. Las diferencias en la FO y ON entre *P. polyphylla* y *A. cincta* podrían deberse a la presión de selección que los patógenos que comúnmente se encuentran en sus hábitats, ejercen sobre estos insectos asi como a sus preferencias alimenticias. Por ejemplo, las especies del género *Phyllophaga* normalmente se alimentan de raíces y se encuentran en suelos agrícolas, en estos suelos existen reservorios de patógenos como bacterias, nematodos, hongos y parasitoides (Klingen y Haukeland 2006; Meyling y Eilenberg 2007), y debido a esta exposición, una respuesta inmunitaria clave contra estos patógenos es la FO (Söderhäll y Cerenius 1998; Lemaitre y Hoffmann 2007; Kanost y Gorman 2008). En cambio, las especies del género *Anomala* se pueden alimentar de materia orgánica y/o raíces por lo que prefieren suelos con alto contenido de materia orgánica y en estos suelos suelen encontrarse una mayor cantidad de

bacterias (Alexander 1977), y una de las respuestas inmunitarias contra estos microorganismos es la producción de ON (Foley y O`Farrel 2003).

Por otra parte, en el capítulo dos se plantea la idea de que las diferencias en la suceptiblidad contra los hongos entomopatógenos entre *P. polyphylla* y *A. cincta* podrían deberse a la cutícula. Con respecto a esto, se ha reportado que el grosor de la cutícula puede retardar la penetración de los hongos entompatógenos (Bogus *et al*. 2007). Por lo que es posible que *P. polyphylla* tenga una cutícula más gruesa que *A. cincta*. Los reportes de Guzmán *et al*. (2012), Nong *et al*. (2011) y Rodriguez del Bosque *et al*. (2005) son consistentes en que las especies del género Phyllophaga son más resistentes que las especies del género Anomala a las infecciones por hongos entomopatógenos. Por tal motivo, en los capítulos tres y cuatro se utilizaron las larvas de *P. polyphylla* para investigar las variaciones anuales en las barreras inmunitarias y la interacción entre las barreras inmunitarias y no inmunitarias.

Las condiciones ambientales son importantes en la disponibilidad de recursos que afectan la defensa inmunitaria de los insectos, esto implicaría que la inversión en las defensas inmunitarias variará con el ambiente (Schmid-Hempel 2005). Cuando se midieron los niveles de pFO y FO en larvas de *P. polyphylla* tras haber sido inoculadas con blastsporas de *M. pingshaense*, se encontró una mayor cantidad de ambos compuestos en las larvas colectadas en 2011 en comparacion con 2012, sugiriendo una diferente inversión de energía en la defensa inmunitaria (profenoloxidasa (pFO) y FO) en el tiempo. No tenemos evidencias para asegurar cuál o cuáles factores ocasionaron estas diferencias; sin embargo, suponemos que la precipitación fue un factor clave. Es probable que en el año con mayor precipitación (2011), las plantas sufrieron de un menor estrés hídrico lo que que podría traducirse en alimento de mejor calidad para las larvas rizófagas (Brown y Gange 1990; Ponton *et al.* 2013) como es el caso de

P. polyphylla. Asimismo, es posible que durante 2012 las plantas produjeran metabolitos secundarios como respuesta a un mayor estrés hídrico, por lo que las larvas tuvieron que invertir más en recursos en mecanismos de desintoxicación hacia los metabolitos producidos por la planta, que en respuesta inmunitaria (Bukovinsky *et al.* 2009; Smilanich *et al.* 2009; Erb y Liu 2013); sin embargo, es necesario validar estas hipótesis mediante evidencias experimentales.

El hecho de que la respuesta inmunitaria no haya sido diferente entre larvas inoculadas por medio de inmersion y no inoculadas con hongos entomopatógenos, sugiere que probablemente los hongos no penetraron el integumento, lo que supone a la epicutícula como una barrera importante contra los hongos, esto puede ser soportado por los datos de baja mortalidad que se encontraron durante esta investigación en larvas de *P. polyphylla,* lo cual ya ha sido previamente reportado (Guzmán-Franco *et al*. 2012). Cuando las blastosporas de este mismo hongo se inyectaron directamente en el hemocele de las larvas, se encontró un 100% de mortalidad con la dosis más alta después de 120 horas de incubación, lo cual confirma la importancia de la cutícula del insecto para evitar infecciones. Anteriormente ya se había reportado el papel de la cutícula para evitar infecciones, ya que larvas con cutícula gruesa impiden la penetración de los hongos e invierten poco en su respuesta inmunitaria p.e. producción de péptidos antimicrobianos, encapsulación, FO, lisoenzima y fagocitosis, en comparación con las larvas de insectos con cutícula delgada, en las cuales se observó una entrada de los hongos entomopatógenos (Bogus *et al.* 2007). Sin embargo, no podemos descartar el hecho de una cercana coevolución de hongos e insectos, lo cual se podría traducir en mecanismos que el hongo emplea para no ser detactado por el sistema inmune de la larva, como la producción de una proteína de colágeno sobre la pared celular de las blastosporas de los hongos entomopatógenos para enmascarar los carbohidratos, y de esta manera evadir el sistema

inmunitario de los insectos (Wang y St. Leger 2006). Este hecho también podría explicar la falla en la activación de la respuesta inmunitaria de las larvas al ser injectadas con blastosporas de *M. pinshaense*, ya que el sistema inmunitario sería incapaz de reconocer al hongo. Por este motivo, la cutícula de los insectos representa la mejor oportunidad para retardar e incapacitar el desarrollo y penetración de los hongos entomopatógenos. De esta manera, el insecto invertiría en la producción de ácidos grasos fungistáticos para cubrir la epicutícula, en engrosar la cutícula y una menor inversión en una respuesta humoral, ya que esta es más costosa de mantener y sobre todo porque puede generar un daño sobre los tejidos de los insectos (Siva-Jothy *et al.* 2005; Sadd y Siva-Jothy 2006; Bogus *et al.* 2007; Golebiowski *et al.* 2008; Ment *et al.* 2010). A pesar de que se ha reportado que engrosar o tener una cutícula más melanizada para incapacitar el desarrollo de los hongos es costoso (Dubovskiy *et al.* 2013), este sigue siendo mucho menor en comparación con el que representa la defensa inmunitaria (Parker *et al.* 2011).

Los resultados también revelan que podría existir un compromiso en la producción de distintos componentes de la respuesta inmunitaria. La actividad de la FO se observó en las larvas de manera basal, pero, no la actividad lítica y de ON cuando se inyectaron las blasposporas. Esto sugiere que activar varios componentes de la respuesta inmunitaria podría ser costoso para las larvas de *P. polyphylla*. Además, los datos demuestran que la FO puede ser costosa de activar debido a que durante la activación de la cascada de la FO, a menudo va acompañada de la liberación de especies reactivas de oxígeno. Las especies reactivas de oxígeno pueden dañar los tejidos del insecto (Sadd y Siva-Jothy 2006). Por ejemplo, Manalil *et al.* (2009) encontraron que las larvas de gallina ciega activan simultaneamente la respuesta inmunitaria para combatir a los hongos entomopatógenos cuando ingresan al hemocele y los mecanismos antioxidantes para mitigar el daño de las especies reactivas de oxígeno liberadas durante la activación de la

respuesta inmunitaria. Lo anterior, pone de manifiesto que los insectos también necesitan destinar parte de su energía en activar los mecanismos antioxidantes y si estos no son suficientes entonces las larvas pueden morir por una sobre activación de la respuesta inmunitaria.

En general, los hongos entomopatógenos tienen el potencial de regular las poblaciones de las larvas de gallina ciega (Keller 1992; Jackson 1999; Keller *et al.* 1999; Kessler *et al.* 2003,2004). Sin embargo, en campo se han documentado inconsistencias en la eficacia de estos agentes de control biológico para el control de larvas de gallina ciega (Villani *et al.* 1992; Butt *et al.* 2001). Se sabe que factores bióticos y abióticos del suelo influyen en la eficacia de los entomopatógenos (Jaronski 2007), al igual que la resistencia de las larvas de gallina ciega contra sus patógenos (Jackson 1999), lo que ha llevado a concluir a otros investigadores que un solo producto biológico a base de hongos entomopatógenos no tiene la capacidad de controlar las poblaciones de las diferentes especies de larvas de gallina (Morales-Rodriguez *et al.* 2010). La presente investigación aporta evidencias de que las gallinas ciegas tienen un sistema de defensa para combatir las infecciones de los hongos entomopatógenos, y debido a que cada especie de gallina ciega ha estado sometida a diferente presión de selección por sus patógenos y parásitos, tienen sistemas de defensa diferentes. Por ejemplo, las larvas de *P. polyphylla* podrían combatir muy bien a hongos y nematodos debido a que tienen una mayor actividad de PO, mientras que las larvas de *A. cincta* podrían combatir las infecciones por bacterias porque produjeron la mayor cantidad de ON; sin embargo, a pesar de que la activación y producción de estos compuestos es común entre estos insectos, la cantidad que podrían producir es variable aún entre poblaciones de una misma especie de gallina ciega, posiblemente por cambios en factores abióticos como la precipitación. Estas diferencias tienen implicaciones importantes en el manejo de las larvas de gallina ciega con hongos, ya que se podría hipotetizar que larvas inoculadas con hongos en años

con más precipitación, tienen más probabilidad de sobrevivir a pesar de que la condición ambiental más húmeda sugiere un ambiente mas apropiado para el hongo.

Adicionalmente, los resultados de esta investigacion sugieren que para aumentar las probabilidades de éxito en el control biológico de las larvas de gallina ciega será necesario buscar enemigos naturales que logren romper el integumento. Uno de estos enemigos naturales de las gallinas ciegas son los nematodos, por ejemplo, *Heterorhabditis* tiene la capacidad de penetrar directamente el integumento (Bedding y Molyneux 1982). También, podrían hacerse aplicaciones mixtas de patógenos y parásitos para disminuir las poblaciones de gallina ciega combinando hongos y nematodos o nematodos y hongos; no obstante, sería necesario realizar experimentos para estudiar el resultado de estas interacciones en la infección de gallinas ciegas, y su efecto en los mismos patógenos. Otros autores han sugerido el uso de aislamientos de hongos entomopatógenos que sobre expresen proteasas y quitinasas para degradar más rápido los componentes de la cutícula, lo que aseguraría la penetración del integumento en menor tiempo (St. Leger *et al.* 1996; Fang *et al*. 2012).

En conclusión, las barreras no inmunitarias como la cutícula, representan el mecanismo de defensa más importante hacia la infección con hongos entomopatógenos en larvas de *P. polyphylla*. Las larvas de este insecto pueden tener problemas para activar la respuesta inmunitaria por resultarles costosa de montar y mantener, por lo cual han sido seleccionadas para invertir energía engrosando su cutícula para limitar el desarrollo y penetración de los hongos. Se sabe que la mayoría de las especies de insectos habitantes del suelo, como las gallinas ciegas, tienen estrategias defensivas contra patógenos mediante el cambio en comportamiento y estructuras morfológicas que les permiten deshacerse de sus enemigos naturales (Villani *et al.* 1999). Tal vez estas estrategias sean menos costosas en comparación con la activación de la

respuesta inmunitaria, y por tanto común en insectos cuyos estados inmaduros pasan mucho tiempo de manera subterránea.

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