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Preparación inmunológica en el grillo *Acheta domesticus*, su relación con la condición individual y control hormonal.

TESIS

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Resumen

Recientemente se ha demostrado la existencia de una respuesta inmunológica adaptativa en invertebrados. Aunque no se saben aún los mecanismos que subyacen a este fenómeno, llamado "priming" inmunológico, algunos estudios sugieren que la defensa celular está involucrada en esta respuesta. A pesar de los diversos estudios en invertebrados que demuestran el priming, existen pocos que han investigado sus costos y mecanismos de control. En mi tesis demostré la existencia del priming inmunológico en machos y hembras del grillo doméstico *Acheta domesticus* hacia la bacteria *Serratia marcescens*, un patógeno común de esta especie. Además, estudié los costos relacionados con la actividad reproductiva y la condición alimenticia de los individuos e investigué el control que tiene la hormona juvenil (HJ) en su respuesta celular (cantidad de hemocitos). No observé costos del priming directamente relacionados con la reproducción pero sí observé costos relacionados con la condición de los individuos De esto último, aquellos cuya ingesta fue principalmente de proteínas, tuvieran una mayor supervivencia después de un segundo encuentro con la bacteria *S. marcescens* en comparación con aquellos cuya ingesta fue principalmente de carbohidratos. No encontré efectos de la HJ en la cantidad de hemocitos. Estos resultados sugieren que el priming inmunológico tiene ventajas adaptativas en esta especie y que puede variar entre individuos, es decir, que es costos.

Abstract

It has been recently demonstrated the existence of an adaptive immune response in invertebrates. Although the mechanisms underlying this phenomenon, called immune priming are unknown, some studies suggest that cellular defense is involved. Despite several studies that demonstrate priming in invertebrates, there are few who have investigated its costs. In this dissertation I demonstrated the existence of immune priming in males and females of the house cricket *Acheta domesticus* against *Serratia marcescens* bacteria, a common pathogen of this species. In addition, I studied the costs associated with reproductive activity and the dietary-mediated condition of individuals and also investigated the control of juvenile hormone (JH) on immune cellular response (number of hemocytes). I did not find costs of priming directly related to reproduction but I observed costs related to the condition of individuals. In relation to the latter, those animals whose intake was mainly protein had a higher survival after a second encounter with the bacterium *S. marcescens* compared to those whose intake was mainly carbohydrates. I did not find effects of JH on hemocyte number. These results suggest that the adaptive immune priming has advantages in this species and may vary between individuals, which means that priming is costly.

Introducción

El tema de la defensa inmune dentro del campo de la ecología evolutiva ha sido de gran interés e importancia para explicar la gran variedad de estrategias adaptativas de los hospederos ante la presencia de patógenos (Sheldon y Verhulst 1996, Rolff y Siva-Jothy 2003, Schulenburg et al. 2009, Schmid-Hempel 2011). Este campo de investigación estudia los factores ecológicos (bióticos y abióticos) que determinan la evolución del sistema inmune (Schulenburg et al. 2009, Brock et al. 2014). Dentro de los principales factores ecológicos que afectan la defensa inmune del hospedero están la gran abundancia y diversidad de patógenos que interactúan con el hospedero y sus limitaciones intraespecíficas (Schulenburg et al. 2009, Hawler y Altizer 2011, Brock et al. 2014). Estas limitaciones se refieren a los costos de la defensa inmune que se manifiestan en disyuntivas en la asignación de recursos entre la respuesta inmunológica y otras características relacionadas con la adecuación (Schulenburg et al. 2009, Hawler y Altizer 2011). La interacción constante con diversos patógenos es una presión selectiva que obliga al hospedero a generar nuevas estrategias para lidiar con ellos y una estrategia es la memoria inmunológica, la cual es característica de la respuesta inmune adaptativa de los vertebrados (Schmid-Hempel 2011). Esta respuesta actúa a través de la recombinación somática de los linfocitos B y T y es específica (Boehm y Swann 2013). Debido a que los organismos invertebrados carecen de estas inmunoglobulinas (Kurtz y Armitage 2006), se tenía la idea de que respondían de igual manera hacia subsecuentes encuentros con patógenos (Kurtz 2004). Sin embargo, desde hace más de dos décadas se ha demostrado que los invertebrados pueden tener una respuesta inmune con características adaptativas análogas a la de vertebrados.

Respuesta inmunológica en invertebrados

Aunque el sistema inmune de los invertebrados carece del sistema adaptativo de los vertebrados, es complejo. Para evitar una infección microbiana, los invertebrados, tienen como primera defensa, barreras físicas y químicas que evitan que los patógenos entren al interior (Tzou et al. 2002). Una vez que atraviesan estas barreras, son reconocidos por el hospedero a través de patrones moleculares

asociados a patógenos (PAMPs), los cuales incluyen lipopolisacáridos, peptidoglucanos y mananos. El reconocimiento de estos patrones activa una serie de cascadas que inducen diferentes respuestas dependiendo del patógeno. Por ejemplo, la vía Toll es activada por hongos y bacterias gram-positivas la cual regula la síntesis de péptidos antimicrobianos (AMPs) y la proliferación de hemocitos, la vía Imd es activada por bacterias gram-negativas y también regula la producción de AMPs (Tzou et al. 2002, Lemaitre y Hoffman 2007) Una vez que se rompe la cutícula del hospedero, se induce la coagulación y la melanización en el sitio afectado para evitar la dispersión del patógeno. Después de una infección sistémica, se activan ya sea una respuesta celular mediada por hemocitos, que involucra la fagocitosis de bacterias, hongos y protozoarios o la encapsulación de patógenos más grandes como nematodos y parásitos (Tzou et al. 2002) También se puede activar una respuesta humoral (AMPs) o la cascada de fenoloxisdasa (FO) cuyo producto final es la melanina y oxígeno y nitrógeno reactivos como subproductos (Tzou et al. 2002, González-Santoyo y Córdoba-Aguilar 2011)

Priming inmunológico

Ante la presencia constante de un patógeno, una estrategia que ha sido favorecida por la selección natural es la memoria inmunológica en vertebrados. La memoria inmunológica es la habilidad de mostrar una respuesta inmune más robusta y/o más rápida después de haber tenido contacto previo con un patógeno (Pham y Schneider 2008). Una característica importante de la memoria inmunológica es que la respuesta al segundo encuentro es específica, es decir, que solo es provocada por el mismo patógeno del primer encuentro (Schmid-Hempel 2011).

En organismos donde es probable que segundos encuentros con patógenos ocurran y éstos afecten su adecuación, debería de haber selección hacia mecanismos que reduzcan dicha afectación (Little y Kraaijeveld 2004). En invertebrados, a pesar de que carecen de inmunoglobulinas, existe evidencia de memoria inmunológica y de respuesta inmune específica (Kurtz 2005, Pham et al.2007, Roth et al. 2008, Rodrigues et al., 2010, Schmid-Hempel 2011). Para distinguir que los mecanismos que subyacen la memoria inmune en vertebrados es diferente a la de los invertebrados, se ha ocupado el término “priming inmunológico” cuando se refiere a invertebrados (Little y Kraajeveld 2004). Un ejemplo de este fenómeno se documentó con el abejorro *Bombus terrestris*. Cuando este animal se expuso a tres tipos de bacterias y luego fue retado con dosis más altas de las mismas bacterias, la supervivencia y la eliminación de la bacteria fue mayor en abejas con retos homólogos en comparación con retos heterólogos y en abejas sin reto previo (Sadd y Schmid-Hempel, 2006).

No se conocen los mecanismos del priming inmunológico en invertebrados, sin embargo, se ha sugerido que la respuesta celular y en particular la fagocitosis podrían ser un mecanismo potencial en el priming inmunológico y la especificidad (Pham et al. 2007, Roth y Kurtz 2009, Zhang et al. 2014). En un estudio realizado con ostras del pacifico (*Crassostrea gigas)*, Zhang y colaboradores encontraron que los individuos a los que se les inoculó la bacteria muerta *Vibrio splendidus*, aumentaron en número total de hemocitos y tuvieron una mayor actividad de fagocitosis en respuesta a un segundo encuentro con la misma bacteria (Zhang et al. 2014). Además, observaron un aumento en los niveles de expresión de RNAm relacionados con la actividad de fagocitosis y la hematopoyesis de éstos individuos (Zhang et al.2014)

Además de conocer los componentes que están involucrados en el priming, es interesante investigar los costos en adecuación que tiene este fenómeno en el hospedero. Se han realizado estudios acerca de los costos del priming trans-generacional. Por ejemplo, en la especie *Tribolium castaneum* los efectos paternos y maternos reducen la fecundidad en su descendencia (Roth et al., 2010). En las abejas *Bombus terrestris* la descendencia de madres retadas con bacteria, muestran una mayor actividad antimicrobiana, sin embargo, se vuelven más susceptibles a una infección con el tripanosoma *Crithidia bombi* (Sadd y Schmid-Hempel 2009). En otro ejemplo muy interesante, se realizó un estudio que analiza los costos del priming en la reproducción del mosquito *Anopheles albimanus* (Contreras-Garduño et al. 2014). Se demostró que individuos expuestos previamente con *Plasmodium berghei* tienen una mayor supervivencia y eliminan mejor al parásito en comparación con los individuos no expuestos. Además, observaron que las hembras con priming producen menos huevos y tienen una menor tasa de eclosión que en las hembras control (Contreras-Garduño et al. 2014).

A pesar de los estudios de costos transgeneracionales del priming, existen pocos estudios en donde se investiguen los costos del priming inmunológico en el mismo individuo.

Costos de la respuesta inmunológica

Un principio básico en el campo de la ecología inmunológica es que el mantenimiento y el uso de la defensa inmune son costosos para el hospedero (Sheldon y Verhulst 1996, Lochmiller y Deerenberg 2000, Rolff y Siva-Jothy 2003). Algunos de estos costos pueden ser energéticos, fisiológicos e incluso

de autoinmunidad (Sheldon y Verhulst 1996, Rolff y Siva-Jothy 2003, Schulenburg et al.2009, Schmid-Hempel 2011). Debido a que en la naturaleza los recursos son limitados, estos costos pueden resultar en disyuntivas en la asignación de recursos entre la defensa inmune y componentes relacionados con la adecuación como el crecimiento, la reproducción y la supervivencia (Stearns 1992). Un ejemplo de costos energéticos y nutricionales de la respuesta inmune se realizó con la larva del gusano africano *Spodoptera exempta* (Povey et al. 2008). Este estudio demostró que las larvas con una dieta rica en proteínas tiene una mayor supervivencia ante una infección con la bacteria *Bacillus subtilis* que las larvas con una ingesta de alimento baja en proteínas (Povey.et al. 2008). Además se documentó una relación positiva entre la ingesta de proteínas y la actividad antibacteriana y de fenoloxidasa (FO) (Povey et al. 2008). Relaionado con la dieta, aunque se han hecho investigaciones de cómo la relación dietética proteínas/carbohidratos puede afectar distintas funciones (Cotter et al. 2011; Ponton et al. 2011) no se ha estudiado sus efectos en el *priming*.

Se ha propuesto que la supresión de la respuesta inmunológica puede estar relacionada con los costos reproductivos (Sheldon y Verhulst 1996), definidos como el decremento de la reproducción futura como consecuencia del esfuerzo reproductivo presente (Stearns 1992, Roff 1992) . En adultos, la asignación óptima de recursos hacia la defensa inmunológica puede diferir entre sexos (McKean y Nunney 2001) En muchas especies, los machos gastan más energía en la competencia por parejas que las hembras (Sheldon & Verhulst 1996; Zuk y McKean 1996). Como consecuencia, los machos pueden invertir menos recursos en el sistema inmune durante su reproducción (Zuk y McKean 1996). En el grillo texano *Gryllus texensis*, se documentó que los machos muestran una disminución en la actividad de FO durante el periodo reproductivo (Adamo et al. 2001). También se ha demostrado que la respuesta inmune puede afectar la fecundidad en las hembras. En la especie *Drosophila nigrospiracula* las hembras con una mejor resistencia hacia un ectoparásito mostraron una disminución en su fecundidad (Luong y Polak 2007).

La defensa inmune también puede estar en disyuntiva con el desarrollo y mantenimiento de características sexuales secundarias. Se ha propuesto que características elaboradas y conspicuas (p.ej. astas, cuernos, plumaje colorido, canto, etc.) pueden indicar la calidad genética y fisiológica del individuo que los produce (Andersson y Simmons 2006). Uno de los aspectos específicos que los ornamentos indicarían es la capacidad de resistencia hacia infecciones patógenas (Hamilton y Zuk 1982). Debido a que los ornamentos y la respuesta inmunológica pueden compartir los mismos componentes o, en general, son relativamente costosos de producir, un organismo podría enfrentar una

disyuntiva (del inglés "trade-off") en la asignación de recursos hacia estas dos funciones (Sheldon y Verhulst 1996, Lochmiller y Deerenberg 2000, Kotiaho 2001). De acuerdo con esta idea, sólo los machos en buena condición serían capaces de mantener una respuesta inmune eficaz y al mismo tiempo producir ornamentos elaborados. El término condición puede ser definido como la cantidad de recursos disponibles hacia características relacionadas con la adecuación (Lorch et al. 2003; Tomkins et al. 2004), asumiendo que estas características son costosas de producir. Por ejemplo, en el grillo de campo *Gryllus bimaculatus* los machos producen cantos que actúan como señales para atraer hembras o para evitar contiendas con otros machos (Simmons 1988). Se sabe además que la producción del canto de cortejo en esta especie tiene una alta demanda energética (Hack 1998). Los componentes del canto de cortejo que son preferidos por las hembras están positivamente relacionados con la tasa de encapsulación, pero negativamente con la actividad lítica (Rantala y Kortet 2003). Esto sugiere una posible disyuntiva entre las respuestas celular y humoral en esta especie (Rantala y Kortet 2003).

Hormona juvenil como mediador de uso de recursos

En los insectos, un regulador fisiológico en la asignación de recursos es la hormona juvenil (HJ). Esta hormona es una molécula con estructura parecida a un lípido que es sintetizada y secretada por la *corpora allata* (Nijhout 1994). Existen cinco estructuras moleculares de la HJ, aunque la forma más común encontrada en insectos es la HJIII (Nijhout 1994). La HJ es una hormona versátil que regula múltiples procesos fisiológicos, de desarrollo y la condición en general de los insectos (Flatt 2005). Por ejemplo, la HJ afecta la formación de caracteres sexuales secundarios (Emlen y Nijhout 1999; Rantala et al. 2003 Fry 2006), regula la actividad reproductiva en las hembras (Dubrovsky et al. 2002; Steigenga et al. 2006; Maeno y Tanaka 2009), favorece el envejecimiento (Herman y Tatar 2001; Heinze y Schrempf 2008) y suprime el sistema inmune (Rantala et al. 2003; Flatt et al. 2008; Contreras-Garduño et al. 2009; González-Tokman et al. 2012). Utilizando estos conocimientos, recientemente se han emprendido una serie de estudios para saber cómo la HJ participa en los dilemas en distribución de recursos. Por ejemplo, un estudio reciente documentó que en la libélula territorial, *Calopteryx virgo,* la HJ favorece la agresión en los machos a expensas de la producción de fenoloxidasa, un factor clave en la respuesta inmune (Contreras-Garduño et al. 2009). Otro estudio realizado con las especies *C .virgo* y *C. haemorrhoidalis* demostró que las larvas en el último estadio que tienen niveles altos de HJ desarrollan como adultos una mayor pigmentación alar (un componente

clave para adquirir más parejas en machos y hembras) y muestran un reducción en las reservas de grasa (Contreras-Garduño et al. 2011). Además, en el mismo estudio se documentó que en las hembras de *C. Splendens*, la HJ disminuye el peso abdominal (un indicador de la fecundidad) (Contreras-Garduño 2011).

De acuerdo con estos antecedentes se esperaría que la HJ esté involucrada en la regulación de recursos hacia la expresión del priming inmunológico y el desarrollo de otras características como por ejemplo el canto en los grillos. Esta acción de la HJ, no se ha estudiado hasta el momento.

El grillo común

El grillo común *Acheta domesticus* es un insecto hemimetábolo perteneciente a la familia Gryllidae. El número de mudas que presenta puede variar de 8 a 12 dependiendo de la dieta y la temperatura a la que se encuentren (Huber et al. 1989). En condiciones de laboratorio puede vivir de 3 a 4 meses (observación personal). Esta especie ha sido modelo de estudio de los costos energéticos de la respuesta inmune y selección sexual, en gran parte por su fácil mantenimiento y reproducción en condiciones de laboratorio (Patton 1978, Huber et al. 1989). A continuación mencionaré algunos ejemplos de estos trabajos. Se ha documentado que los machos de esta especie producen un canto de agresión cuando tienen encuentros con otro macho y un canto de cortejo para atraer a hembras (Huber et al.1989). Otro estudio demostró que existe una relación positiva entre la calidad de aspectos del canto que tienen que ver con atraer pareja en machos y la cantidad de hemocitos en la hemolinfa, siendo este último un determinante en la fagocitosis y encapsulación de patógenos en insectos (Ryder y Siva-Jothy 2000). Además se ha demostrado que la respuesta inmune de encapsulación puede tener costos en el crecimiento de hembras y machos de *A. domesticus* y en las hembras también tiene costos en la supervivencia y en la producción de huevos (Bascuñán-García et al. 2009). Otro trabajo demostró que las hembras incrementan la puesta de huevos ante la presencia de la bacteria *Serratia marcescens* (Adamo 1999). Esta bacteria está distribuida en suelos y cuerpos de agua, y se ha aislado de seis diferentes órdenes de insectos incluyendo los grillos (Steinhaus 1959).

Estructura de la tesis

Considerando la frecuente interacción de los grillos con *S. marcescens* y el efecto en su mortalidad, en mi tesis propuse probar la existencia de priming inmunológico en el grillo *A.domesticus* midiendo como parámetro inmunológico la actividad de fagocitosis además de la supervivencia. Como indiqué antes, la fagocitosis es un componente involucrado en el priming inmunológico (Pham et al. 2007. Pope et al. 2011, Zhang et al. 2014), por lo que resulta idóneo para mi tesis. Para abordar el problema de si el priming es costoso, investigué si el priming es afectado por la dieta, para lo cual manipulé haciéndola rica en proteínas o carbohidratos. Adicionalmente, para saber si la dieta fue costosa o no más allá de la respuesta inmunológica, medí la cantidad de grasa y músculo en los animales bajo experimentación. Para conocer la relación entre la reproducción y el priming, expuse a los individuos a situaciones de competencia sexual para después medir su efecto en el priming. Finalmente, examiné si la HJ tiene un efecto en la respuesta inmune celular de los grillos.

Mi trabajo está compuesto de tres partes, la primera muestra la existencia del priming en hembras y machos adultos en contra de *S. marcescens*. Este primer experimento es la base de mi tesis, demostrando que en esta especie resulta ventajoso tener una respuesta adaptativa en contra un patógeno común y potencialmente mortal como es *S. marcescens*. En esta misma sección analizo el efecto que tiene la actividad reproductiva en este fenómeno. Esta primera parte se mandó a la revista *Acta Ethologica* y está en estatus de revisión. La segunda parte trata de los efectos de la ingesta diferencial de macronutrientes (carbohidratos y proteínas) en la condición de los grillos y los posibles costos en el priming. Esta sección se mandó y se está evaluando a la revista *Ecological Entomology*. En la tercer parte analizo los efectos de la HJ en la cantidad de hemocitos en hembras y machos, y el artículo está publicado en *Neotropical Entomology*. Al final de mi tesis discuto de manera general todos los resultados en el contexto de la biología del sujeto de estudio y de las teorías de historias de vida

Hipótesis y predicciones

Hipótesis. El grillo *A. domesticus* expuesto a un patógeno es capaz de desarrollar priming inmunológico usando la fagocitosis como parámetro inmunológico.

Predicción. Los individuos que hayan desarrollado priming tendrán una mayor actividad fagocítica que los individuos que no se les permitió desarrollar priming.

Hipótesis. La eficacia del priming inmunológico será dependiente de la condición del individuo.

Predicción. Los individuos que se han alimentado con recursos de mejor calidad tendrán mayor actividad de fagocitosis ante un segundo reto, que los individuos que se han alimentado con recursos de menor calidad.

Hipótesis. El priming inmunológico y la reproducción son costosos para los individuos de *A.domesticus,* por lo tanto, un individuo no puede maximizar ambas funciones

Predicción. Los individuos con actividad sexual tendrán menor actividad de fagocitosis vía priming que los individuos con ausencia de actividad sexual.

Hipótesis. La HJ tiene efectos en la producción de hemocitos en los individuos y en la fecundidad en las hembras.

Predicción. Los individuos con altos niveles de HJ tendrán una menor producción de hemocitos que aquellos individuos con niveles bajos de HJ. Las hembras con altos niveles de HJ tendrán una mayor fecundidad que las hembras con niveles bajos de HJ.

Objetivos

Investigar si existe priming inmunológico en los machos de *A.domesticus.*

Determinar si el priming inmunologico depende de la ingesta de alimento en individuos de *A.domesticus*

Determinar si existe una disyuntiva entre el priming inmunológico y la reproducción en el grillo *A.domesticus.*

Investigar el papel de la HJ en la respuesta inmunológica celular y en la fecundidad en individuos de *A.domesticus.*

CAPITULO 1

Does mating activity impair phagocytosis-mediated priming immune response? A test using the house cricket, *Acheta domesticus*

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Abstract

Immune priming provides protection to repeated encounters against pathogens. Recent studies indicate that invertebrates are capable of immune priming (an adaptive immune response functionally similar to that of vertebrates). These studies have also revealed individual variation in immune priming, and one explanation is that this response has associated energetic costs. Life history traits such as reproduction could influence an organism's ability to utilize immune priming based on available energy reserves. According to theory, costs of immune priming would impact life history traits of the challenged animal. We investigated whether mating activity impairs immune priming ability using the house cricket, *Acheta domesticus*. We allowed adults to mate or not, and each group was further divided into two groups: those induced to produce immune priming (using a Lethal Dose 10 (LD10) of the bacteria *Serratia marcescens*, followed by a LD75 of the same pathogen) and those not induced to produce immune priming (challenged with a LD75 of *S. marcescens*). Immune priming response was determined by measuring phagocytic activity levels. As supportive of priming, we found that priming elicited higher phagocytic activity. Also, non-mated individuals showed higher phagocytic rates than mated individuals. However, a priming by mating interaction showed similarly intense phagocytic rates among groups. This implies that resources used for biological functions elicited during (e.g. sperm transfer) and after mating (e.g. egg production), are not costly enough to impair immune priming ability based on phagocytic activity.

Key words: Immune priming, mating activity, trade off, house cricket

Introduction

Reproduction is costly as it implies sharing limited resources with other functions linked to fitness (Stearns 1992; Wolfner 2002; Lawrence and Zera 2006). One negative effect is on the maintenance and use of the immune response due to the high energetic cost of immunity (Hamilton and Zuk 1982; Folstad and Karter 1992; Sheldon and Verhulst 1996). In fact, several studies have shown that reproductive activity increases susceptibility to parasites and diseases via impairment of immune response (reviewed by Lawniczak et al. 2007). For example, an augmented reproductive success is negatively coupled with antibody production and immunoglobulin G level leading to a high ectoparasite load (fleas and ticks) in bank vole males (Mills et al. 2009).

Up until recently, the immune system of invertebrates was supposed to rely only on innate responses. Notwithstanding, numerous studies have shown that invertebrates have a functionally adaptive response - named immune priming - that are functionally similar to that of vertebrates, as they provide protection to repeated encounters against pathogens (Little and Kraaijeveld 2004; Pham et al. 2007; Roth et al. 2009; Cisarovsky et al. 2012; for no evidence of immune priming, see González-Tokman et al. 2010; Reber and Chapuisat 2012). One potential mechanism underlying this priming response is phagocytosis (Pham et al. 2007; Roth and Kurtz 2009; Pope et al. 2011), where phagocytic cells internalize and destroy small microorganisms such a bacteria, yeast, fungi and protozoans (Strand 2008). Priming immune responses are not fixed and their effects will depend on different factors such as age (Daukšte et al. 2012), sex (Zanchi et al. 2011), parental exposure (Moret 2006; Sadd and Schmid-Hempel 2009), diet (Freitak et al. 2009), and environmental rearing conditions (Cisarovsky et al. 2012). One explanation for this variation is that priming has associated costs and so, similar to other immune components, priming can be involved in trade-offs with life history traits. Interestingly, this line of thought has been scarcely explored. For example, a recent study using mosquito females of *Anopheles albimanus* that were primed with the parasite *Plasmodium berghei*, found reproductive

costs of priming (Contreras-Garduño et al. 2014). Primed females had higher survival than control females. However, primed females produced fewer eggs which had a lower hatching rate compared to control females (Contreras-Garduño et al. 2014)

Here, we investigate a possible trade-off between mating activity and immune priming in the house cricket (*Acheta domesticus* L.). For this, we compared the phagocytic efficiency of individuals that were either primed or not (using the bacteria *Serratia marcescens*), after engaging in mating or non-mating activity. Our predictions were that (1) primed individuals will show higher phagocytic activity after a secondary bacterial challenge than non-primed individuals (which is coherent with a priming response), and (2) priming response will be reduced, or even suppressed, in individuals that have previously engaged in mating activity.

Material and methods

Acheta domesticus, the house cricket, has been widely used as study model in the field of ecological immunology. For example, it is known that both sexes experience trade-offs between immune response, growth and reproduction (Ryder and Siva-Jothy 2000; Bascuñán-García et al. 2010).

Animals were derived from the vivarium of the Facultad de Estudios Superiores Iztacala (Universidad Nacional Autónoma de México). Eggs were kept in a humid substrate (peat moss, commercial substrate) at 27 ± 2 ° C with a natural day/night photoperiod. After hatching, crickets were maintained in the same conditions in an aquarium (20 cm x 40 cm x 25 cm) with ample food (fish food) and water. A few days prior to reaching the adult stage, males and females were separated. This separation is facilitated by the fact that the ovipositor and wings are clearly distinguished a few days before reaching the adult molting.

Lethal dose (LD) 50 test

To uncover the susceptibility of crickets to the bacterium *Serratia marcescens*, the LD50 (dose at which 50% of individuals die) was calculated. Using a 10-µl Hamilton syringe, 3µl of diluted *S. marcescens* in Grace media was injected to 3 groups of adult males (n = 18 per group) at the following concentrations: $5x10^5$, $5x10^4$ and $5x10^3$ colony formation units (cfu)/µl. A fourth control group was injected with Grace insect medium (n = 18). Mortality was recorded daily for a week (see suppl. mat. Table 1). We estimated the LD50 from the coefficient of a generalized linear model (GLM) with binomial distribution, as 6.8 x 10^5(cfu)/µl, the LD75 as $9x10^5$(cfu)/µl and the LD10 as $2.4x10^5$(cfu)/µl.

Reproductive costs on immune priming

For assessing reproductive costs according to three different socio-sexual situations and two immune priming treatments, individuals were randomly allocated to the combination of one socio-sexual and one immune priming treatment. The socio-sexual treatments were as follows: isolated males ("non-mated males", n =29), isolated females ("non-mated females", n =15), and male-female paired individuals ("mated individuals"; n =13 males and 13 females). These three different conditions simulated distinct mating histories which would promote differential allocation of energy reserves to immune priming versus reproductive traits. In fact, we did behavioral inspections during the experiment to make sure that all pairs mated at least once. For this, we examined that male genital intromission took place and the couple completed mating. All females had access to a moist substrate (peat moss) to oviposit, and all females laid eggs. In all cases, animals had access to food and water *ad libitum*. Males in each treatment were separated, so that hearing calling songs for all individuals was impeded. With this, males reduced their singing activity so that they do not become energetically depleted, which could affect our results. After a week under these socio-sexual conditions, individuals

from each group received either a priming treatment or a non-primed (control) treatment. In the priming group, each individual was injected with 3µl Grace medium solution containing a LD10 *S. marcescens* 7 days before receiving a second injection with 3µl of *S. marcescens* in Grace media at a dose LD75. The non-primed group was injected with only 3µl of Grace medium before receiving the LD75 bacterial injection. Sample sizes remained as follows: primed/non-mated males = 13, primed/non-mated females = 7, primed/mated individuals = 6 males and 6 females, non-primed (positive control)/non-mated males = 16, non-primed (positive control)/non-mated females = 8, non-primed (positive control)/mated individuals = 7 males and 7 females. One control (positive) group injected with Grace insect medium was used in this experiment as unpublished results (all authors') of phagocytic rate after priming indicated that there was no difference between positive (injected with Grace medium) and negative control (with no manipulation). Except during injections, animals were always in their original containers and socio-sexual situation keeping calling songs inaudible.

Phagocytic activity in the haemolymph was measured one day after the second injection, on the same day for all individuals. For this, we pierced the left leg membrane and extracted 4µl of hemolymph using a 10µl micropipette. The haemolymph was resuspended in 6µl Grace medium and added 2µl of *Escherichia coli* bacteria $6x10^6$/ml with phrodo ™ fluorescent dye. This dye produces fluorescence when the pH becomes more acidic, as it is the case when a particle is phagocytized by cells. The sample was incubated for 30 minutes in a dark room. To ensure hemocyte identification, we added 2µl of DAPI dye (1µg/ml). We observed the samples using a Nikon Optiphot-2 microscope fitted with a digital Nikon Coolpix 4300 camera, through an oil-immersion objective lens [X 100, numerical aperture (NA) = 1.35]. We took pictures of four different fields. Phagocytosis was quantified by counting the number of phagocyting and non-phagocyting hemocytes in each picture using the program ImageJ®.

Statistical analysis

A generalized linear mixed model with binomial distribution was used to assess the effect of sex (males and females), socio-sexual situation (non-mated and mated), and immune situation (primed and control groups) on the rate of phagocytosis. We included a second order interaction for the predictor variables socio-sexual situation and priming situation, to see whether primed, mated individuals showed a reduced phagocytic rate when compared to primed, non-mated individuals. The model used individuals as a random variable since four pictures per individual were included, and an individual random variable was entered to account for overdispersion.

All statistical analyses were performed in R using the package lme4, specific for the analysis of mixed models (R Core Development Team 2012; Bates et al. 2012). To assess the effect of fixed predictor variables, analyses of deviance tests were performed. For each model, predicted effects and 95 % confidence intervals were estimated.

Results

There was no effect on phagocytic rate according to sex (Table 1; Figure 1). However, there was a difference in phagocytic rate according to immune priming and socio-sexual treatments when these two variables were analyzed separately (Table 1). Primed individuals phagocytized more intensively (mean phagocytic rate: 0.268, CI 95 % = 0.225- 0.315) than non-primed individuals (mean phagocytic rate: 0.206, CI 95 % = 0.175-0.241), while non-mated, solitary individuals did better at phagocytizing (0.261, CI 95 % = 0.226 - 0.299) than mated individuals (0.185, CI 95 % = 0.149 - 0.228). When the interaction between socio-sexual and immune priming treatment was examined, this was non-significant (Table 1), which means that the protective effect of immune priming was equally intense in mated and non-mated individuals.

Discussion

Despite our relatively small sample sizes and unbalanced sample sizes among groups, we have shown that when male and female house crickets suffer an initial bacterial infection, they show an increased phagocytic rate during a second bacterial challenge, which is consistent with a priming response. This result is not at all new as phagocytosis is involved in priming responses in other arthropods (Pham et al. 2007; Cong et al. 2008; Roth and Kurtz 2009) and its inhibition prior to priming has severe consequences on insect survival (Pham et al. 2007). Furthermore, given that insect phagocytosis is a costly response (Moret and Schmid-Hempel 2000), it is then expected to be traded-off with other life history traits such as reproduction. Our results partially support this as mating activity impaired phagocytic rate. Nevertheless, this cost of phagocytosis cannot be extrapolated as, contrary to our prediction, this immune component is not affected in the context of both immune priming and mating history. This suggests that reproductive activity is not traded-off with a functionally adaptive immune response. This is somehow paradoxical given the associated immunity costs that have been linked to processes occurring during (e.g. sperm transfer) and/or after mating (i.e. egg production) (Lawniczak et al. 2007). However, if our results hold for other species, it would mean that the presumed cost of priming is not high enough to influence key biological aspects such as mating.

There are several considerations for our results before a generalization can be made. First, our animals were fed *ad libitum*. Trade-offs are expected to be stronger when resource availability is low (Zera and Harshman 2001; Kotiaho 2001). Thus, it remains to be explored whether immune priming can still have a protective effect under adverse nutritional conditions. In a design like ours, one would speculate that crickets should allocate more resources to reproduction (i.e. mating activity) than to priming as reproduction is a key function during adulthood. Second, phagocytosis is not the only

mechanism proposed for immune priming. Other associated immune components are phenoloxidase activity (e.g. Trauer and Hilker 2013), antimicrobial peptides and lysozyme (reviewed by Rowley and Pope 2012). These components should be also investigated. Third, there is the chance that by being together, mating couples may become infected. This can occur, for example, via feeding from the partner's feces, a situation that did not occur to single animals. This possibility should be controlled during mating/non-mating situations. Fourth, our test should be extended to other taxa.

Finally, we found no differences in priming response between males and females, suggesting that mating has similar costs for both sexes in terms of immune priming. In many species, males spend more energy in competing for mates than females (Sheldon and Verhulst 1996; Zuk and McKean 1996), and as a result, males are expected to invest fewer resources in the immune system during reproduction (Zuk and McKean 1996). Related to this, there is evidence that reproduction in *A. domesticus* is highly demanding for both sexes. Females use male courtship calls to choose males on the basis of song complexity which only males in good, immune condition can produce (i.e. Ryder and Siva-Jothy 2000). On the other hand, female fecundity in this species is negatively affected by resources allocated to immune defense (Bascuñán-García et al. 2010). If these reproductive activities are traded-off with phagocytic rate, our results can be interpreted as a lack of sexual dimorphism in reproductive-related costs. However, sexual dimorphism in immune priming should be tested in other insects to assess if our results also occur in species with other mating systems, or if reproductive costs limit the priming responses against certain pathogens in species where immune priming has not been detected (González-Tokman et al. 2010; Longdon et al. 2013).

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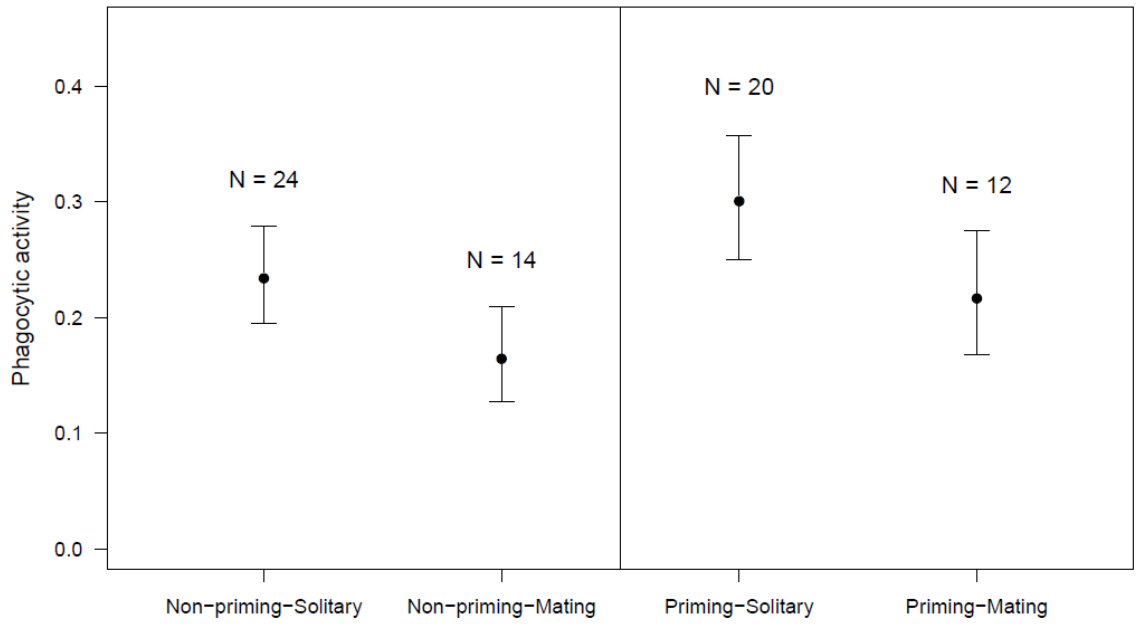
Table 1. Deviance analysis used to compare the effect of sex, socio-sexual situation and immune challenge on phagocytic rate. P values of significant results appear in bold.

Model	Deviance	Δ Deviance	d.f.	p (χ^2)
Null	589.90			
Sex	587.26	2.64	1	0.104
Socio-sexual situation	580.80	6.46	1	**0.011**
Priming situation	*576.33*	4.47	1	**0.034**
Socio-sexual situation: Priming situation	572.14	0.43	1	0.516

Figure legends.

Figure 1. Phagocytosis activity in relation to socio sexual context (mating vs. solitary) and priming treatment (primed with *Serratia marcescens* vs. Grace medium) in both sexes of the house cricket. Error bars depict 95% confidence intervals

Figure 1



CAPITULO II

Proteins play a key role in insect immune priming, fat reserves and muscle mass

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Abstract. 1. Current invertebrate immunity studies have shown that some insect species are capable of producing an enhanced immune response after a first pathogenic encounter, a process called immune priming. However, whether and how such ability is driven by particular diet components (protein/carbohydrate) has not been explored. Such questions are sound given that, in general, immune response is dietary dependent.

2. We have used adults of the house cricket *Acheta domesticus* L. (Orthoptera: Gryllidae) and exposed them to the bacteria *Serratia marcescens*. Having done this, we first addressed whether survival effects after priming and non-priming treatments are dietary-dependent based on access/no access to protein-provided and carbohydrate. In a second experiment, we then investigated how our dietary components affected two key physiological traits in insects: fat reserves and muscle mass. Thus we exposed adult house crickets to either a protein or a carbohydrate diet and measured the two traits.

3. We found that only protein-provided, primed animals survived for longer compared to the other diet treatments. Interestingly, this effect was also sex-dependent with primed males having a higher survival than primed females when protein was supplemented. For the second experiment, protein-fed animals had more fat and muscle mass than carbohydrate-provided animals.

4. All together our results indicate that protein is a key element in insect immune priming and, in general, physiological condition.

Key words: Immunological priming, nutrition, cost, physiological condition, house cricket

Introduction

Adaptive immune response in invertebrates is a controversial topic (e.g. Little & Kraaijeveld, 2004, Schmid-Hempel, 2011) because invertebrates lack B and T lymphocytes that mediate vertebrate adaptive immune responses. Therefore, the traditional scholar opinion is that the invertebrate innate immune system has no memory and responds in a similar fashion to repeated encounters with pathogens (Hauton & Smith, 2007). Despite this, several studies have shown that invertebrates can respond more effectively after a first encounter with a pathogen, a process otherwise known as immune priming (e.g. Cisarovsky *et al.,* 2010, Daukste *et al.,* 2012 but see González-Tokman *et al.,* 2010; Reber & Chapuisat, 2012)

Despite the fact that there is inter-individual variation in immune priming (Daukste *et al.,* 2012) few studies have explored why this is the case. It has been proposed that priming ability can be affected by individual condition (González-Tokman *et al.,* 2010) and age (Daukste *et al.,* 2012), and one underlying explanation for this is that priming is costly. For example, it was recently shown that females of *Anopheles albimanus* Wiedemann (Diptera: Culicidae) pay a reproductive cost via immune priming when infected with the parasite *Plasmodium berghei* (Contreras-Garduño *et al.,* 2013). Authors found a higher egg hatching success in control females than primed females, with primed females that were successful in eliminating parasites becoming unable to produce eggs (Contreras-Garduño *et al.,* 2013). Assuming that dietary resources are generally limited and are shared among several traits, one should expect trade-offs between the immune system and other life history traits (Sheldon & Verhulst, 1996). Therefore, only individuals in good nutritional condition are able to generate effective immune responses. To our knowledge, this hypothesis has not been tested in terms of immune priming.

Not all dietary components impact immune ability similarly (Povey *et al.,* 2008; Lee *et al.,* 2006). For instance, although protein and carbohydrate components are key during parasite defense (Povey *et al.,* 2008; Lee *et al.,* 2006) their effects vary: proteins promote phenoloxidase activity, encapsulation response and antimicrobial activity (González-Santoyo & Córdoba-Aguilar, 2012; Lee *et al.,* 2006; Povey *et al.,* 2008; Roth *et al.,* 2010), while carbohydrates increase anti-bacterial activity of lysozyme (Srygley & Lorch, 2013). In terms of immune priming, there are at least two questions in relation to protein and carbohydrate dietary components: 1) how these components independently affect immune priming; and 2) the role of these components on other non-immunological traits that are also play a role in insect fitness, to have more integrated framework of the action of diet on trade-offs between immunological and non-immunological traits.

We know by previous studies that adult crickets *Acheta domesticus* has an enhanced phagocytic activity after a previous encounter with a pathogenic bacteria, *Serratia marcescens* (Nava-Sánchez et al. submitted MS). In the present study we investigated the nutritional cost of protein and carbohydrate components on immune priming and their effects on non-immunological traits. We reared male and female crickets under different dietary conditions - protein- and carbohydrate-based - and measured survival after repeated encounters with the same bacteria. In a second experiment, we measured adult fat reserves and muscle mass after a period of 15 days under these dietary conditions. Our understanding of use of fat reserves and muscle mass indicates that insect fitness depend on these traits for a plethora of activities. Some examples are male-male aggressive contests (e.g. Lailvaux and Irschick, 2006), predator avoidance (e.g. MacLeod *et al.,* 2007), migration (Mc Williams *et al.,* 2004), hibernation (e.g. Humphries *et al.,* 2003), egg production (e.g. Jarvis *et al.,* 2005), among others. We do not have specific predictions in regards to how proteins/carbohydrates will affect immune ability. This is partly because the mechanism of how insect immune priming works is not clear (Hauton & Smith, 2007). Nevertheless, for fat reserves and muscle mass, predictions can be put forward based on

previous studies in insects: while carbohydrate will increase fat reserves, protein diets will positively affect muscle mass (e.g. Chapman, 2012; Roeder & Behmer, 2014).

Material and methods

Acheta domesticus, the house cricket, has been widely used as study model in the field of ecological immunology (e.g. Adamo, 1999; Ryder & Siva-Jothy, 2000). For example, it is known that there are trade-offs between immune response and reproduction in both sexes (Bascuñan-García *et al.,* 2010). Used animals were derived from the vivarium of the Facultad de Estudios Superiores Iztacala (Universidad Nacional Autónoma de México). Eggs were kept in a humid substrate (peat moss, commercial substrate) at 27 ± 2° C with natural photoperiod day/night. After hatching, crickets were kept in aquaria (20 cm x 40 cm x 25 cm). Food was supplied on a cotton placed at the bottom of each container. The cotton was changed for a fresh one every day. When the crickets came to the adult stage, males and females were separated.

Experiment 1. Costs of priming

For assessing diet effects we reared crickets under different food components. A first group of 68 individuals (males and females) in their last larval instar were fed on a protein diet and a second group of 66 individuals of the same instar were fed on a carbohydrate diet (for preparation of both diets, see below). The two groups were maintained on these diets from the last larval instar until 15 days of the adult stage. After this period, each group was divided into priming group (n = 35 protein diet; n = 32 carbohydrate diet) and control group (n = 33 protein diet; n = 34 carbohydrate diet). The primed group was injected with 3 µl Grace medium solution containing a LD10 (2.5×10^5cfu/µl) of *S. marcescens* bacteria while the control group was injected with 3µl of Grace medium. After 7 days the four groups

were injected with 3µl of *S. marcescens* in culture media at a dose LD75 (9 x10^5 cfu/µl). After the second injection, we measured survival every day during a week.

Diet preparation

We prepared the diet based on the methodology used by Simpson *et al*. (2006), which has been used in other studies (Srygley & Lorch, 2011, 2013). According to this methodology, the protein diet consisted of 42% of a 3:1:1 mixture of casein, peptone and albumin; while the carbohydrate diet consisted of 42% of a 1:1 mixture of sucrose and dextrin. The two diets contained 54% cellulose, 2% cholesterol and 2% linoleic acid.

Experiment 2. Effects of diet on muscle mass and fat load

We used 61 adults that had just molted to the adult stage, and divided them in two groups. A first group was fed with the protein diet (n =28) while the second was fed on a carbohydrate diet (n = 33). Such feeding protocol continued for 15 days, time at which we measured fat load and muscle mass.

Quantification of fat reserves and muscle mass

We placed the body in a desiccator (for 48 h) and obtained their dry weight to the nearest 0.001g. Initial dry weight includes basically the exoskeleton, fat and muscle. We then placed samples in chloroform for 24 h to extract the fat. Samples were then re-desiccated and re-weighed. We quantified fat content as the difference between the initial weight and the second weight (Contreras-Garduño *et al.*, 2008). After fat extraction, we submerged the samples in 0.8 M potassium hydroxide for 48 h to extract the muscle. The weight of the body was measured to previous to and after the extraction, and the difference was interpreted as total muscle mass. To account for the effect of body size in fat and muscle quantification, we measured the length of the left femur with a digital micrometer (precision ± 0.01mm; for the use of leg length as an indicator of body size, see Simmons, 1986). After checking

that our measurement error was low (see below), femur length was measured by the same person three times, so that we used an average for subsequent analyses.

Statistical analysis

For experiment 1, we assessed survival of adult individuals using treatment (priming, no priming), diet group (protein, carbohydrate) and gender as predictor variables of individual survival. As the hazard rate was variable, we show parametric models with a Weibull distribution. Global model had an Akaike Information Criteria (AIC) value = 425 while the most supported model had an AIC of 421. We estimated Kaplan-Meier survival curves for each species according to treatment and diet. Analyses were carried out in software R v. 3.02 (R Core Development team, 2013).

For experiment 2, we tested for differences in fat load and muscle mass using two general linear models. In these models, we used either of the variables (priming and diet treatments), and femur length was used as a covariate. Previous to this, we made sure that our measurement error was small enough by using random effects analysis of variance (Bailey & Byrnes, 1990; Yezerinac *et al.*, 1992): error measurement = 3.10%. Fat load data was not normally distributed (Shapiro-Wilk test) and was adjusted by using their $\log_{10}$ transformed values. The interaction term treatment x femur length was tested in both models but it was not significant (fat load: $F = 1.94$, $P = 0.168$; muscle mass: $F = 1.63$, $P = 0.206$). Therefore, we did not consider these terms in further analysis. Analyses were carried out in SPSS (version 20).

Results

Experiment 1. Cost of priming

Survival after a second bacterial challenge was dependent on the interaction diet * treatment (Table 1; Fig. 1): survival was higher in primed male and female treatments reared under the protein diet

compared to the other groups (Table 1; Figs. 1 and 2). Survival was also dependent on gender, with males surviving longer than females (Fig. 2).

Experiment 2. Effects of diet on muscle mass and fat load

Protein fed individuals had a higher fat load than carbohydrate fed individuals (ANCOVA: treatment: $F = 15.815$, $n = 61$, $P < 0.001$; femur length: $F = 5.450$, $n = 61$, $P = 0.023$). Protein fed individuals also had a higher muscle mass than carbohydrate fed individuals (ANCOVA: treatment: $F = 6.137$, $n = 61$, $P = 0.016$; femur length: $F = 21.287$, $n = 61$, $P < 0.001$) (Fig. 3).

Discussion

In the present study we showed that male and female *A. domesticus* crickets that experienced a first encounter with the highly pathogenic bacterium *S. marcescens* gained protection against a second infection with the same pathogen later in life. However, in both males and females, the protective effect of the first encounter took place only when crickets were fed with protein, and not when fed on carbohydrates alone during all adult life. This result confirms our prediction that, just as other components of insect immune response (Schmid-Hempel, 2005), priming response is a costly trait that only individuals in good physiological condition can afford. We also showed that a protein diet improved individual muscle mass and fat load compared to a carbohydrate diet. This despite previous knowledge that indicated that carbohydrates are converted into storage lipids (Arrese & Soulages, 2010). The general salient interpretation of our two experiments is that proteins not only favor immune priming but also physiological condition (Lee *et al.,* 2006; Povey *et al.* 2008).

Different components of insect innate immune response are costly traits because their expression can be genetically and physiologically linked to other immunological or life-history traits (Sheldon & Verhulst, 1996; Schmid-Hempel, 2005; Cotter *et al.,* 2003). As a consequence, immune response is

highly sensitive to food quality (Ponton *et al.,* 2011; Povey *et al.,* 2008; Lee *et al.,* 2006; Srygley *et al.,* 2009, Triggs & Knell, 2011). Our results that priming response against bacteria is sensitive to protein availability, implies that protein deprived insects may become immune depressed. Given that one mechanism by which insect priming takes place is mediated by phagocytes (Pham *et al.,* 2007; Roth & Kurtz, 2009), which are protein-rich haemocytes (Lemaitre & Hoffman, 2007), one explanation for our experiment is that protein limitation led to a reduction in haemocyte number and, consequently, phagocytic activity. Future studies should evaluate experimentally if phagocytosis is reduced upon repeated infections under low protein availability, and if the constant higher survival found in males with respect to females (as previously observed Nowosielski & Patton, 1965) is caused by sexual differences in phagocytic activity.

That protein input is key for enhanced priming responses provides a first robust evidence that immune priming is condition dependent, and therefore opens new questions in insect physiology and evolutionary biology, including, for example, the evolution of adaptive foraging and trans-generational immune ability. For the case of adaptive foraging, since compensatory protein intake shown by infected individuals has consequences on immediate immune responses (Ponton *et al.,* 2011; Lee *et al.,* 2006), one question is whether insects augment their protein consumption if encountering the same pathogen is likely. For the case of trans-generational immune ability, given that parental immunity can be transmitted across generations by both the mother and the father (Zanchi *et al.,* 2011; Little *et al.,* 2003, Roth *et al.,* 2010; also called as cryptic parental care, Jokela, 2010), trans-generational immune priming could be sensitive to parental condition.

Paradoxically, is not a widespread phenomenon in invertebrates. Although mathematical modeling predicts that the benefits of immune priming depend on host lifespan and pathogen virulence (Miller *et al.,* 2007; Best *et al.,* 2012), our results show that priming response also depends on individual

nutritional condition. Thus, not only diet has to be added to mathematical modeling but it may be that not having controlled this may account for those studies where insects were apparently unable to produce priming responses.

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Figure 1. **Survival after second bacterial challenge.** Kaplan-Meier survival curves according to sex, treatment and diet interaction. a) female, b) male

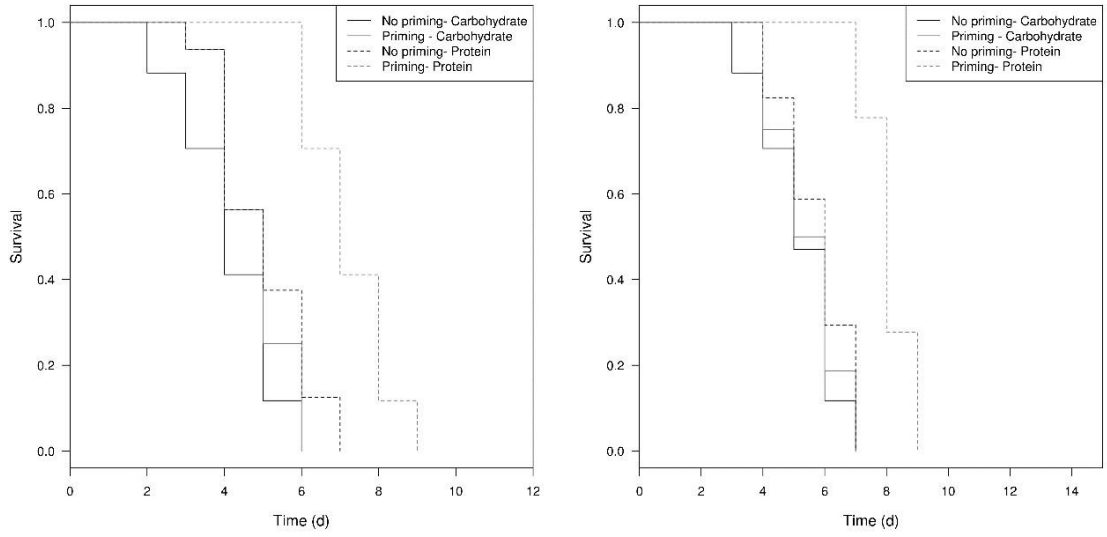

Figure 2. **Expected survival after second bacterial challenge.** Expected median survival time according to sex, treatment and diet in *Acheta domesticus* crickets. Error bars depict 95% confidence intervals

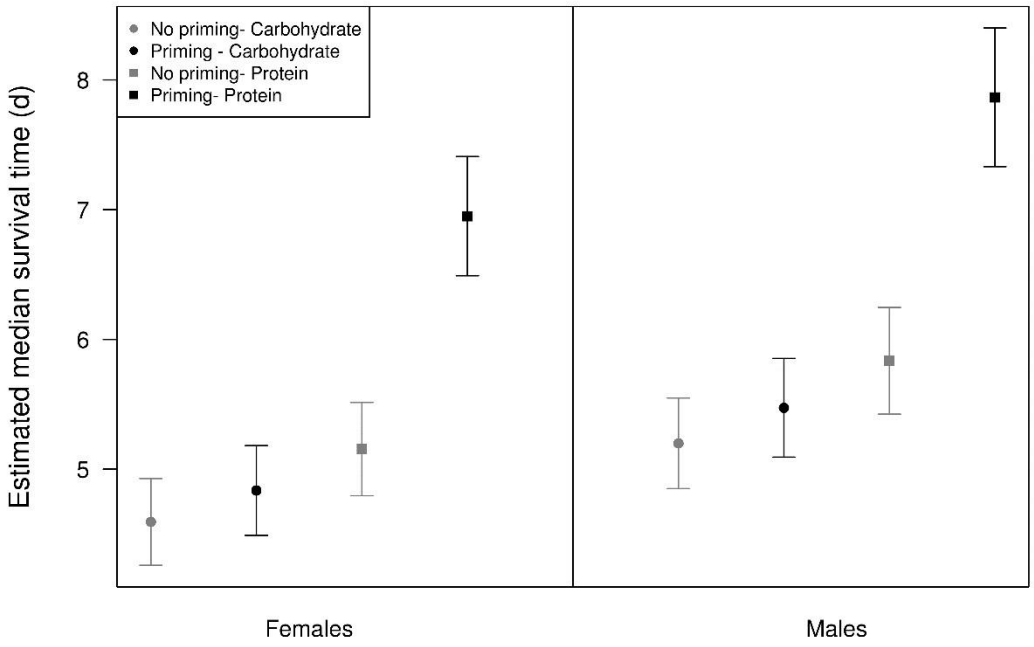

Figure 3. Fat load and muscle mass in relation to a protein and carbohydrate diet treatments.

Fat load and muscle mass (g) after diet treatment. Bars represent standard errors

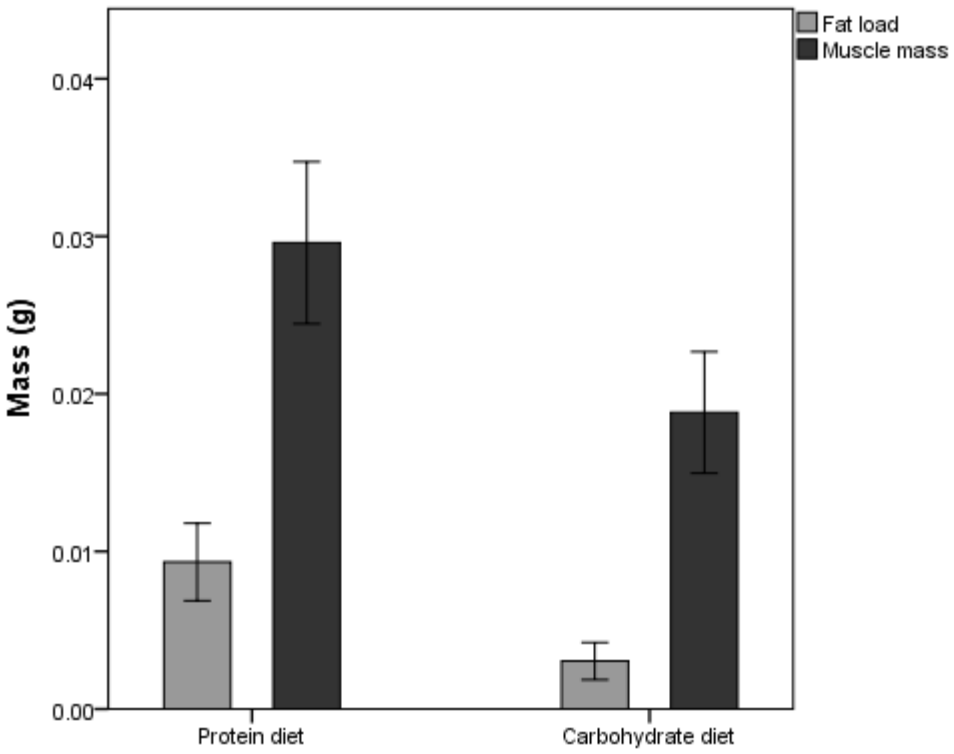

Table 1. **Survival analyisis concerning the effects of treatment (priming and no priming), sex and diet (protein and carbohydrate)**

	Df	Devience	Probability(χ^2)
Null			
Treatment	1	34.80	**<0.001**
Sex	1	9.62	**0.001**
Diet	1	42.08	**<0.001**
Treatment:Diet	1	15.79	**<0.001**

CAPITULO III

No detectable trade-offs among immune function, fecundity and survival via a juvenile hormone analog in the house cricket

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No Detectable Trade-Offs Among Immune Function, Fecundity, and Survival via a Juvenile Hormone Analog in the House Cricket

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Keywords

Acheta domesticus, methoprene, hemocytes, immune response

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Abstract

Hormones are key regulators of resource allocation among functions and thus play an important role in resource-based trade-offs. The juvenile hormone (JH) is an insect hormone that mediates resource allocation between immunity and life history components. Here, we have tested whether this is the case using the house cricket. We investigated whether increased levels of JH (using methoprene, a JH analog) enable an enhanced survival and fecundity (via egg number) at the cost of reduced hemocyte number (a trait that is associated with immune response in insects) in the house cricket, Acheta domesticus L. We had three groups of adult crickets of both sexes: experimental (methoprene and acetone), positive control (methoprene), and negative control (no manipulation). Prior to and after experimental treatments, we counted the number of hemocytes (for the case of both sexes) and recorded the number of eggs laid and survival of females after the manipulation. There was no difference in hemocyte number, egg number, and survival. These results do not support a JH-mediated trade-off among immune ability, survival, and fecundity. We provide arguments to explain the lack of JH-mediated trade-offs in the house cricket.

Introduction

Activation of the immune response requires resources that could otherwise be used for other functions (Sheldon & Verhulst 1996, Lochmiller & Deerenberg 2000, Rolff & Siva-Jothy 2003). Nevertheless, since resources are usually limit-ed, their diversion can result in trade-offs between immune defense traits and life history components such as growth, reproduction, and survival (Stearns 1992). In recent decades, a variety of examples has provided support to these ideas. For example, males of the Texas cricket, Gryllus texensis (Cade & Otte), show a decrease in phenoloxidase activity, a key insect immune component (González-Santoyo & Córdoba-Aguilar 2012), during episodes of increased repro-ductive effort (Adamo et al 2001). Given this, immune func-tion has recently become a cornerstone in understanding trade-offs between life history traits (Rolff & Siva-Jothy 2003, Schulenburg et al 2009, Schmid-Hempel 2011).

The juvenile hormone (JH) is a key player in the mecha-nisms underlying trade-offs between immunity and other functions in insects. The reason is that, similar to vertebrate hormones, JH regulates resource allocation to the different functions in response to the environmental needs. JH con-trols multiple physiological processes, development, and overall condition in insects (reviewed by Flatt et al 2005). For example, JH positively drives the formation of secondary sexual traits (e.g., Emlen & Nijhout 1999, Rantala et al 2003, Fry 2006), female reproductive activity (e.g., Dubrovsky et al 2002, Steingenga et al 2006, Maeno & Tanaka 2009), and aging (e.g., Herman & Tatar 2001), but suppresses the im-mune system (e.g., Rantala et al 2003, Flatt et al 2008). Given this knowledge, JH is considered to be responsible for the resource allocation decisions and resulting trade-offs between immunity and other functions in both sexes (Flatt et al 2005). In males, for example, one study docu-mented that in a territorial damselfly, Calopteryx virgo (L.), JH

promoted male territorial tenure at the expense of phenoloxidase production (Contreras-Garduño et al 2009). In females, high levels of JH increased egg laying rates and early fecundity, but reduced longevity in Bicyclus anynana (Butler) butterflies (Steingenga et al 2006).

The house cricket, Acheta domesticus (L.), is a model species in the study of trade-offs between immune response and fitness-related components (e.g., Adamo 1999, Ryder & Siva-Jothy 2000, Bascuñan-García et al 2010). In particular, two different instances of trade-offs have been detected. In one case, it was found that the male calling song, a key trait used for mate acquisition, is traded off with hemocyte number (Ryder & Siva-Jothy 2000). Hemocyte number is known to be directly associated with the rate of phagocytosis and encapsulation of pathogens in insects (Strand 2008). In another case, when both sexes were repeatedly exposed to a nylon challenge during ontogeny, animals ended up with a reduced body size, survival, and egg production (Bascuñan-García et al 2010). Given this background information, in the present study, we used the house cricket for testing the presumed trade-offs induced by JH between immunity, survival, and fecundity. First, we tested if increased JH titers negatively affect immune response in both sexes measured as the number of circulating hemocytes in the hemolymph. As explained before, increased JH levels lead to a reduction of resources allocated to hemocyte production (i.e., Sujatha & Dutta-Guptha 1993, Kim et al 2008). Second, we tested if increased JH titers had a positive effect on fecundity and survival in females, since it is known that in this species, JH promotes vitellogenesis and is involved in ovary growth (Strambi et al 1997). To increase JH levels, we used methoprene, a JH analog (JHa) (Nijhout 1994), which has been used in numerous studies to mimic the JH effects in modulating resource allocation to different components (e.g., González-Santoyo and Córdoba-Aguilar 2012, Snell-Rood et al 2011, Contreras-Garduño et al 2009). Our predic-tions for these two aims are, respectively, that individuals treated with methoprene will have fewer hemocytes, an increased number of eggs, and decreased survival. This result would illustrate the expected trade-off between immune function and life history components. Our intention of these two aims is to gather information towards a general principle of how JH serves as the mechanism that mediates resource allocation and, therefore, trade-offs between immune func-tion and life history traits in insects.

Material and Methods

Crickets were obtained from the Facultad de Estudios Superiores Iztacala (Universidad Nacional Autónoma de México). Eggs were kept in a humid substrate (peat moss, commercial substrate) at 27±2°C with a natural day/night photoperiod. After hatching, crickets were kept in the same conditions in a 20-cm-high×40-cm-long×25-cm-wide glass container with food (fish food) and water ad libitum. However, once the experiment began, individuals did not receive any food. When the crickets became adults, males and females were separated and were ready for experimental manipulation.

JHa preparation and experimental groups

We dissolved 5 mg of methoprene acid (Sigma) in 1,000 μL of distilled water. We prepared a dilution of 1:1,000 in acetone and topically applied 7 μL (35 ng of JHa/per organism) on the dorsal area between the head and thorax of adults of the house cricket, close to the corpora allata (Nijhout 1994). As a positive control group, we applied 7 μL of acetone only on the same body area. A last negative control group had no manipulation.

We chose our JHa dose based on the average mass of our animals following the protocol of previous studies (see, for example, Contreras-Garduño et al 2009, 2011). Given that our crickets weighed around 300±18 mg (n=20), the equiv-alent dose is 35 ng/per organism.

Effects of JHa on hemocyte number in males

To investigate whether JH affects hemocyte number, we estimated the number of hemocytes prior to and after JH manipulation. For this, we extracted 4 μL of hemolymph from the left leg of adult males (n=106) and females (n= 92), using a 10μL micropipette to which we added 2 μL of Grace's medium. We observed these samples using a Nikon Optiphot-2 microscope fitted with a digital Nikon Coolpix 4300 camera, through an oil immersion objective lens ×100, numerical aperture=1.35. We took three photographs of different fields. We then counted the number of hemo-cytes in each field using the program ImageJ®. After 10 days, we divided all animals into the three groups as described and treated above (JHa-treated, n=38 males and 30 females; negative control, n=30 males and 32 females; positive con-trol group, n=38 males and 30 females). Twelve hours after manipulation, we measured again the number of hemocytes in each individual of the three groups via the hemolymph-extraction method described earlier. We chose the 12-h interval because this was shown to be the time JHa effects could be more evidently observed (e.g., Contreras-Garduño et al 2012).

Effects of JHa on female fecundity and survival

To determine whether JH has a positive effect on fecundity and survival, we used the same females from the three groups indicated above (JHa-treated, n=30; negative control,

n=32; positive control group, n=30). After manipulation, females were provided with access to a moist substrate for oviposition (Bascuñan-García et al 2010). Two days later, we counted the number of eggs that each female laid and used them as a proxy of lifetime fecundity (again, because JHa effects can be detected more intensively in the short term; Contreras-Garduño et al 2012). We also recorded the survival of these females. We checked whether females were still alive every 24 h by gently moving each individual and assessing whether it still moved or not.

Statistical analysis

For the experiment of JHa effects on hemocyte number, a linear mixed model was implemented (Zuur et al 2009). For this, we used hemocyte number from the three observed fields in each picture, which was compared among the three groups (negative control, positive control, and experimental). The model included individual males as a random variable given the three pictures of each male. For the experiment of JHa effect on female hemocytes, we employed a linear mixed model. As predictor variables, we included the effect of treatment (JHa-treated, negative control, or positive control), time (first or second measure), and number of eggs in a female's hemocyte load. Additionally, a second-order interaction between treatment and time was included to estimate if the amount of hemocytes changed after treat-ment. The model included individual females as a random variable since the three pictures for each female were in-cluded. We employed the F test to assess the significance of predictor variables.

Finally, we performed a survival analysis with Weibull distribution to estimate if survivorship was affected by treat-ment (JHa-treated, negative control, or positive control) or by number of eggs. An analysis of deviance was performed to test these hypotheses.

Results

Effects of JHa on hemocyte number in males

There was no difference in the number of hemocytes among groups ($F_{2,50}$ =0.08, p=0.923, n=53) and between the first and second measurements ($F_{1,50}$ =3.07, p=0.081; Table 1). The interaction treatment and time was also nonsignificant ($F_{2,50}$ =0.34, p=0.712).

Effects of JHa in hemocyte number, fecundity, and survival in females

There was no significant difference in hemocyte number among groups ($F_{2,42}$ =0.523, p=0.596, n=46) or between the first and second measurements ($F_{1,43}$ =1.409, p=0.241; Table 2). The number of hemocytes did not differ with respect to the number of eggs laid between groups ($F_{1,42}$ =7.379, p= 0.095). The interaction between treatment and time was nonsignificant ($F_{2,43}$ =1.868, p=0.166). Finally, we did not find differences in survival across groups (deviance=1.519, df=2, p=0.467; Table 2) nor was this explained by the number of eggs laid (deviance=0.283, df=1, p=0.594; Table 2).

Table 1 Hemocyte number (mean±SE) prior and after JHa manipulation in males.

Treatments	Hemocyte number		F test between times (p)
	Prior	After	
JHa	137.0±67.7	146.6±40.2	3.07 (0.081)
Positive control	125.9±69.7	144.7±64.8	
Negative control	123.6±68.3	156.1±77.9	
F test among groups (p)	0.08 (0.923)		

Discussion

Two previous studies have detected trade-offs in the house cricket. Ryder & Siva-Jothy (2000) found that male cricket song was a good indicator of immunocompetence, assessed as hemocyte load. Both cricket song and hemocyte load are heritable traits, which implied an evolutionary trade-off (Ryder & Siva-Jothy 2001). A resource allocation conflict was later corroborated in both sexes by Bascuñan-García et al (2010): repeated immune investment (in the form of melanization of a nylon filament) over immature develop-ment led to a decreased body size, fecundity, and survival in the adult stage. Given this, it was expected not only that the proposed trade-off between immunity and life history com-ponents would hold true, but that JH would mediate this. But, despite previous studies indicating an immunosuppres-sive role of JH in insects (e.g., Rolff & Siva-Jothy 2002, Rantala et al 2003, Contreras-Garduño et al 2009, 2011, González-Santoyo and Córdoba-Aguilar 2012), we found no

Table 2 Hemocyte number (mean±SE) prior to and after JHa manipulation, egg number (mean±SE), and survival (mean±SE) in females.

Treatments	Hemocyte number		Egg number	Survival
	Prior	After		
JHa	61.4±57.5	48.6.±20.4	16.7±52.9	7.5±3.9
Positive control	51.9±39.3	75.6±61.8	69.6±128	6.1±3.6
Negative control	52.3±44.6	75.1±56.5	22.18±57.5	5.7±3.1
F test (p)	0.523 (0.596)			
Deviance (p)			0.283 (0.594)	1.519 (0.467)

support for this hypothesis. Nevertheless, our results do not fully discard this hypothesis. It may be that the negative effects on immunity do exist, but we could not detect them at the level of the hemocyte number. For example, it has been detected that JH negatively affects humoral compo-nents (Rantala et al 2003, Contreras-Garduño et al 2009, 2011). Possibly, trade-offs between immunity and life history functions may not affect all immune components similarly. Partly, this may be explained by the fact that not all immune components are equally costly (e.g., Adamo 2004). For example, in male Celithemis eponina (Drury) dragonflies, there were no effects of methoprene on melanization rate, albeit individuals with higher levels of methoprene had an increased number of mites compared to control animals (González-Santoyo and Córdoba-Aguilar 2012). Perhaps, further simultaneously measurements of several immune components would clarify this issue (e.g., Matson et al 2006).

We expected at least a trade-off between survival and fecundity, given that JHa would favor egg production at the cost of longevity (e.g., Flatt et al 2005). One reason is that probably methoprene application did not occur when JH is needed for vitellogenin synthesis. In female Byciclus anynana butterflies, a JH analog affected the reproductive output and longevity only when applied at the beginning of the oviposi-tion period (Steingenga et al 2006). A similar claim has been suggested in damselflies, since adults may show a short response window to increased methoprene levels (Contreras-Garduño et al 2012). However, assuming that this was the case for our study, then methoprene-treated ani-mals recovered from an initial, excessive energetic invest-ment on egg production as compared to the other two groups. If this were the case, it would seem strange that such recovery was completely successful (in other words, resulting in no costs).

Finally, although it has been claimed that JH is the connecting mechanism between immunity and life history traits that explain resource allocation trade-offs in insects (e.g., Emlen & Nijhout 1999, Rantala et al 2003, Flatt et al 2008), our results do not support this principle. Although we have discussed several potential methodological pitfalls, the fact that we followed a standard protocol implies that the presumed role of JH in resource allocation trade-offs does not apply in the house cricket. At best, our results suppose that the hypothesized extended role of JH in resource allo-cation conflicts should be taken with caution.

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Discusión General

En mi tesis doctoral puse a prueba la existencia de priming inmunológico en el grillo *A. domesticus* contra la bacteria *S. marcescens* un patógeno común y potencialmente mortal en esta especie. Además probé si este priming variaba entre individuos, es decir, si tenía un costo en el contexto reproductivo y en la condición del individuo con respecto a la dieta. Finalmente analicé si la HJ tenía efectos en la fecundidad y supervivencia de las hembras y en el número de hemocitos de machos y hembras de esta especie. Retomé los resultados de estos objetivos y los discutiré en un contexto general, más allá de lo abordado en cada uno de los capítulos.

Los individuos de *A. domesticus* que tuvieron un primer encuentro con la bacteria *S. marcescens* muestran una mayor actividad fagocítica después de un segundo encuentro en comparación a los que no habían estado expuestos previamente con la bacteria. Por un lado, estos resultados son consistentes con estudios previos que muestran que los invertebrados pueden responder de forma adaptativa a repetidos encuentros con agentes patógenos (por ejemplo, Kurtz 2005; Schmid-Hempel 2011). Para los organismos que ocupan un tiempo y un lugar donde los encuentros secundarios con patógenos son probables y pueden tener un impacto en la adecuación, deberían seleccionarse mecanismos que reduzcan los costos de tales encuentros (Little y Kraajeveld 2004). Para el caso de mi objeto de estudio y el patógeno, se sabe que *S. marcescens* se encuentra en el suelo y el agua y es en realidad un patógeno muy común en ortópteros (Walker y Masaki 1989). De hecho, los insectos infectados con *S. marcescens* mueren entre uno y tres días y frecuentemente presentan una coloración roja (Boucias y Pendland 1998). Sin embargo, no todas las cepas de *Serratia* producen este color. Dado que *A. domesticus* puede vivir hasta 3 meses como adulto, el riesgo de las exposiciones repetidas es alto. Sería interesante exponer a los grillos a segundos encuentros en tiempos diferentes para conocer la duración del efecto del priming. Esto daría una mejor idea de las ventajas adaptativas del primimg en esta especie. Se esperaría que el efecto fuera de larga duración con respecto a la vida total de los individuos ya que la exposición a la bacteria es principalmente a través del forrajeo.

La respuesta adquirida que medí en este estudio no es específica ya que utilicé dos bacterias diferentes para retar a los individuos y medir la fagocitosis. Una de las características de la memoria inmunológica en vertebrados es que es específica, y es un componente importante en la memoria inmunológica análoga en invertebrados. Sin embargo, como lo han reportado diversos estudios, una forma de respuesta adquirida que se ha observado en invertebrados actúa de manera inespecífica (Brown et al. 2003, Jacot et al. 2005, Eleftherianos et al. 2006, Schmid-Hempel 2011). De esta manera, mi diseño no invalida la puesta a prueba de priming.

En el tema de los costos del priming no encontré evidencias relacionadas con la actividad reproductiva. En un estudio reciente se demostró que los mosquitos *Anopheles albimanus* expuestos al parásito *Plasmodium berghei* tienen una mayor supervivencia y eliminan mejor al parásito, en comparación con los individuos que no estuvieron expuestos (Contreras-Garduño et al. 2014). Además observaron costos reproductivos en las hembras con priming, las cuales tuvieron una menor tasa de eclosión y menor producción de huevos (Contreras-Garduño et al. 2014). De acuerdo con esta evidencia, la defensa inmunológica de las hembras de *A. albimanus* hace la reproducción extremadamente costosa, lo que puede tener consecuencias en la adecuación (Contreras-Garduño et al. 2014). En mi búsqueda de pruebas directas en relación con los costos del priming, no encontré diferencias en la tasa de fagocitosis entre los individuos con priming expuestos a la actividad de apareamiento y los individuos con priming sin apareamiento. Esto no va en contra de la hipótesis de que el priming es costoso, sino simplemente que si existe tal costo, éste no surge durante y después de las actividades de apareamiento. Uno de los procesos fisiológicos que subyacen las disyuntivas o *trade-offs* es la ingesta de nutrientes (Zera y Harshman 2001): si los recursos internos son limitados e insuficientes para el mantenimiento de dos caracteres o más relacionados entre sí entonces el incremento en la asignación de recursos hacia un carácter resultará en la disminución de recursos a otro carácter (Zera y Harsman 2001). Durante mi experimento los grillos recibieron comida y agua ad libitum, por lo que es probable que los costos de la actividad reproductiva fueron enmascarados debido a la abundancia de recursos.

Por el lado de los costos del priming en el contexto de los macronutrientes en la dieta, encontré que los machos y hembras tienen una mayor actividad de fagocitosis a un segundo encuentro con *S. marcescens* en el grupo donde fueron alimentados con proteína y no en aquellos que se alimentaron de carbohidratos. Este resultado confirma la predicción de que, al igual que otros componentes de la respuesta inmune en insectos (Schimd-Hempel 2005), el priming es una respuesta costosa que sólo los individuos en buen estado fisiológico pueden presentar. A pesar de que los carbohidratos se convierten el lípidos, y se sabe que éstos son importantes en la respuesta inmune de los invertebrados, la importancia de las proteínas podría estar en la respuesta inmune celular, ya que los hemocitos son ricos en proteínas y su deficiencia podría tener efectos en el número de hemocitos y como consecuencia en la fagocitosis. En el grillo mormon *Anabrus simplex* se ha demostrado que los individuos forrajean preferencialmente proteínas y sales y la deficiencia de éstos puede provocar la migración y el canibalismo (Simpson et al. 2006). Sería muy interesante explorar si en el grillo común los individuos busquen más fuentes de proteína cuando el riesgo de encontrarse de forma repetida con los mismos patógenos es mayor.

Finalmente, uno de mis objetivos era conocer el papel de la HJ como regulador fisiológico entre el sistema inmune y otros componentes importantes en la adecuación, sin embargo, contrario a diversos estudios que demuestran su efecto en la supresión del sistema inmunológico, no encontré evidencias que apoyaran esta idea. Puede ser que existe el efecto negativo sobre la inmunidad, pero no puede ser detectado a nivel de número de hemocitos. Por ejemplo, se ha detectado que JH afecta negativamente componentes humorales (Rantala 2003; Contreras-Garduño et al. 2009; 2011) que puede no ser necesariamente el caso para los componentes celulares como, por ejemplo, hemocitos. Posiblemente, las compensaciones entre las funciones de inmunidad y de historias de vida, no afectan a todos los componentes inmunológicos de manera similar. En parte, esto puede explicarse por el hecho de que no todos los componentes inmunológicos son igualmente costosos (Adamo 2004). Por ejemplo, en machos de las libélulas *Celithemis eponina*, no hubo efectos del metopreno en la tasa de melanización, aunque los individuos con niveles más altos de metopreno, un análogo de la HJ, tenían un mayor número de ácaros en comparación con los individuos control (González-Tokman et al. 2012). Otra de las razones por las que tal vez no encontré un efecto es por la cantidad de metopreno que aplique a los

grillos. Esto es paradójico Ya que calculé de manera indirecta la concentración de metopreno tal como se ha hecho en trabajos previos. El tiempo del efecto del metopreno es otro factor que pudo afectar los resultados. Aunque usé como referencia el tiempo de efecto observado en un trabajo previo (Contreras-Garduño et al. 2012), este tiempo puede variar entre especies. Ciertamente, mis resultados abren nuevas incógnitas sobre el papel potenciador de la HJ en los caracteres de historias de vida

Perspectivas

El grillo domestico *A. domesticus* es un buen modelo para seguir explorando el priming inmunológico. Algunas preguntas pendientes y que emanan directamente de mi tesis es probar si el priming es específico (Kurtz 2005), utilizando patógenos naturales de la especie. Otro aspecto importante es saber la duración del priming (Kurtz 2004). Esto porque, el tiempo entre el primer y segundo encuentro puede ser un indicador de que la respuesta inmunológica no es inducida, si no constitutiva (Kurtz 2005), pero para saberlo se necesita medir la respuesta entre el primer y segundo encuentro. Una respuesta análoga a la de los vertebrado debería ser bifásica (Schimd-Hempel 2011).

Por otra parte, aunque dado que el parámetro inmunológico que medí fue la fagocitosis, una respuesta celular, el potencial para abordar la parte de desarrollo inmunitario es enorme. Por ejemplo, para conocer mejor los mecanismos del priming, se podría medir la diferenciación de hemocitos antes y después del primer encuentro con la bacteria. Se esperaría encontrar una mayor cantidad de plasmatocitos después del primer encuentro. Dentro del contexto de las disyuntivas en historias de vida, se debería intentar asociar si el efecto de mayor desarrollo de plasmatocitos afecta la supervivencia, el crecimiento y/o la reproducción y si el mediador es la HJ (aunque mi estudio no provea pistas en este sentido). Relacionado con las disyuntivas, sería interesante saber si otros componentes no relacionados con el priming se "apagan" para solventar el problema de un uso desmedido de recursos por los plasmatocitos.

Finalmente, vislumbro que mi tesis tenga salidas en temas aplicados. El uso de entomopatógenos en el control de plagas ha sido una alternativa para evitar la contaminación por plaguicidas (Kaur et al. 2014, Lacey y Georgis 2012). Así quizás el

priming podría ser relevante para elegir el patógeno, las concentraciones y el tiempo entre cada exposición adecuado para que sea efectivo el control. Hasta donde conozco, los principios del priming no se han llevado al terreno agronómico.

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