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PREPARACIÓN INMUNOLÓGICA Y ASPECTOS DE LA GENÉTICA CUANTITATIVA DE LA RESPUESTA INMUNE DE *AEDES AEGYPTI*

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A mis padres

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A la Universidad Nacional Autónoma de México

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Capítulo I. Introducción General

Teoría Inmunoecológica

El sistema inmune de los animales es un medio de defensa que ha evolucionado para proteger al organismo de los efectos ocasionados por agentes infecciosos. Estudios recientes (ver Schmid-Hempel 2005) han revelado las células y moléculas que participan en el reconocimiento, eliminación y/o tolerancia a los agentes patógenos y sus efectos. Actualmente se sabe que estas células y moléculas actúan de manera conjunta entre sí, y con otros sistemas (ej. nervioso, reproductivo; Tu, Flat & Tatat 2005). Por esta razón se ha propuesto que la actividad inmune está íntimamente relacionada con las estrategias de ciclo de vida de los organismos involucradas con la supervivencia y reproducción diferencial (i.e. adecuación) en un ambiente dado. A este conjunto de estrategias se le conoce como la historia de vida de un organismo, y en conjunto maximizan la probabilidad de supervivencia y reproducción (Stearns, 1992). Estas estrategias están asociadas a características como el tamaño, edad a la maduración, tasa de crecimiento, desarrollo y reproducción. Existen también características asociadas a las características de historia de vida, como la coloración corporal, comportamientos de apareamiento, producción hormonal, y que se clasifican comúnmente como características morfológicas, fisiológicas y conductuales que también están relacionadas con la supervivencia y reproducción diferencial del organismo.

Hamilton y Zuk (1982) y Folstad y Karter (1992), fueron los primeros en proponer que la capacidad inmune de los organismos podía estar relacionada con las características de historias de vida (e intermediarias) antes mencionadas. Estos autores propusieron que la resistencia a parásitos en vertebrados está relacionada con la expresión de características sexuales secundarias relacionadas con la elección de pareja. A partir de entonces surge formalmente una nueva rama

de la biología conocida como *inmunoecología* ("ecological immunity"). La inmunoecología trata de explicar cuáles son los procesos microevolutivos (presiones selectivas, flujo de genes entre poblaciones, efectos de deriva génica y mutaciones) y cómo, en combinación con factores ambientales (ej. cantidad y calidad de recursos, humedad, temperatura) y de interacción con otros organismos (ej. competencia, depredación, parasitismo), ha evolucionado la forma en que los organismos montan una respuesta inmune en contra de patógenos.

La evidencia encontrada en un amplio número de insectos, muestra que existe un costo evolutivo de la habilidad de los organismos de montar y mantener una respuesta inmune para minimizar los efectos de una infección, a esta habilidad se le conoce como inmunocompetencia (Owens & Wilson 1999). Este costo es debido a que las características dirigidas a lidiar con los patógenos puedan estar relacionadas negativamente con características de historias de vida. La respuesta inmune al covariar negativamente con otras características de historia de vida puede estar generando la aparición de disyuntivas (o trade-offs en inglés) (la disyuntiva más común es supervivencia vs. reproducción). Las disyuntivas ocurren cuando dos o más características no pueden ser favorecidas por el mecanismo de la selcción natural (Núñez-Farfán 1993), esto a pesar de que las características en disyuntiva puedan estar genéticamente correlacionadas (Cheveraud et al, 1983) y contribuyendo a incrementar el éxito reproductivo del organismo. De forma tal que las disyuntivas podrán limitar la evolución de las características que se encuentran en disyuntiva. La base de las disyuntivas radica en que los recursos requeridos para la generación de la respuesta inmune pueden ser recursos que también son requeridos para la expresión de otras características involucradas en la supervivencia y reproducción (Zuk y Stoehr 2002). A pesar del amplio conocimiento que se ha generado en los últimos años dentro de la rama de la inmunoecología y que demuestra la existencia de disyuntivas, y su repercusión en la evolución de la respuesta inmune, existen tópicos poco examinados. Dentro de estos se encuentran los

efectos que tiene la constante presencia de un patógeno en la respuesta inmune y componentes de adecuación del hospedero; y la variación fenotípica de origen genético y no genético y a la sensibilidad de la respuesta inmune ante condiciones ambientales heterogéneas.

El objetivo general de esta tesis es incrementar el conocimiento de estos dos puntos antes mencionados. Esto mediante (1) la evaluación del efecto que tienen encuentros consecutivos con un patógeno sobre la respuesta inmune, supervivencia y reproducción del mosquito *Aedes aegypti*; y (2) cómo se afecta la respuesta inmune ante la heterogeneidad en los recursos alimenticios. Este último punto, abordando las diferencias que existen entre la hembra y macho del mosquito, y las determinantes genéticas y ambientales de la respuesta inmune. El conocimiento generado a partir de esta tesis podrá ser utilizado para aumentar la comprensión de la biología de esta especie de mosquito vector del virus Dengue y del de la Fiebre amarilla, y en un futuro proponer estrategias para su control. Esto con la finalidad de reducir el impacto que tiene sobre la salud de las poblaciones humanas.

La tesis comienza con un apartado (*capítulo II*) en el cual se revisan las disyuntivas generadas entre efectores de la respuesta inmune y otras características (morfológicas y conductuales). Se exponen las teorías de las razones de dichas disyuntivas. Se abordan temas que han sido poco estudiados como interacciones patógeno-hospedero y de competencia patógeno-patógeno y su influencia en la evolución del hospedero y del patógeno. Se contemplan las diferencias entre sexos, y su consecuencia evolutiva, que sería el dimorfismo sexual en caracteres morfológicos y de respuesta inmune. Brevemente, y para contemplar su relación con factores ecológicos, se mencionan los mecanismos de generación de la respuesta inmune, y se exploran algunas ideas sobre la capacidad que tiene el sistema inmune de insectos para responder de una manera eficaz a los ataques de patógenos. Por último, se exponen hipótesis que podrían explicar la evolución de

la respuesta inmune, esto considerando la coevolución entre el patógeno y su hospedero; a través de la correlación entre componentes del sistema inmune; y limitaciones generadas por estas asociaciones. Se contempla al estudio de la variación fenotípica de características de respuesta inmune para determinar cómo influye el ambiente sobre la expresión de caracteres cuantitativos (controlados por más de un gen). Se examinan los posibles efectos de la estocasticidad ambiental y efectos maternos sobre dichas características.

El grado de especificidad del sistema inmune de insectos no cuenta con un reconocimiento y respuesta contra agentes infecciosos después de un contacto inicial (como ocurre en vertebrados), es posible que tengan la capacidad de mostrar una mejora su respuesta inmune a lo largo de lo ontogenia e inclusive que pueda ser heredable. Existe evidencia (ver Elliot et al, 2003; Kurtz y Franz, 2003; Moret y Siva-Jothy, 2003) que insectos, presentan un fenómeno análogo a la memoria de vertebrados, denominado prevención inmunológica ("immunological priming"; Little y Kraaijeveld, 2004; Little et al, 2005). Los organismos generan una posterior respuesta inmune más eficaz después de haber tenido una experiencia previa con agentes infecciosos. Sin embargo cabe aclarar que para invertebrados el uso del término memoria se limita a una mejora en la respuesta inmune en posteriores retos inmunes. En el *capítulo III* se evalúa la capacidad que tiene el sistema inmune del mosquito para incrementar su supervivencia al ser infectado con patógenos con los que previamente ya ha tenido contacto. De igual forma, se evalúan dos parámetros inmunes (actividad de fenoloxidasa y producción de óxido nítrico) que posiblemente están relacionados con la capacidad de desarrollar un tipo de memoria inmunológica.

Los organismos tienen que enfrentar el problema de cómo maximizar su adecuación (en términos de supervivencia y reproducción) en ambientes heterogéneos y/o estresantes, los individuos con

una constitución genética que permita variaciones fenotípicas para ajustarse a los diferentes cambios ambientales podrían tener una ventaja selectiva en dicho ambiente (Zhivotovsky *et al*, 1996). En algunas ocasiones un solo genotipo puede tener la capacidad de producir varios fenotipos alternativos como resultado de su interacción (y sensibilidad) con diferentes ambientes. A esto se le conoce como plasticidad fenotípica (Roff, 1997; Nylin y Gotthard, 1998). Existe evidencia de que la limitación de alimento afecta negativamente la respuesta inmune en insectos (Fellowes, 1998; Siva-Jothy y Thompson, 2002). Dado que los organismos tienen que enfrentar el problema de cómo maximizar su supervivencia y reproducción en ambientes que están en constante cambio, los individuos con una constitución genética que permita variaciones fenotípicas para ajustarse a los diferentes cambios ambientales podrían tener una ventaja selectiva en un ambiente cambiante (Via et al, 1995). Bajo este contexto, en el *capítulo IV* se estimó el componente genético y ambiental de la respuesta inmune, si existe plasticidad fenotípica y si existe variación genética para la plasticidad (componente ambiental e interacción GxA) de la respuesta inmune de mosquitos en condiciones de alimentación limitadas. Asociado a esto, se evalúa si existen diferencias entre los sexos en la forma de responder al ambiente. Es probable que, en caso de existir, las diferencias inmunológicas entre sexos sean consecuencia de la variación en la disponibilidad y tipo de recursos utilizados distintamente por hembras y machos.

En el capítulo IV se expone una breve discusión resaltando los principales resultados obtenidos en esta tesis. Además se proponen perspectivas de estudio y el potencial de aplicación en un futuro del conocimiento generado. Adicionalmente en el *apéndice I* se explican las metodologías comunmente usadas para la cuantificación de respuesta a parámetros inmunológicos. Se describe el contexto ecológico y evolutivo de cada uno de estos parámetros y se ofrecen hipótesis que explican la posible relación entre efectores de la respuesta, recurso necesarios para esto y la

consecuencia ecológica (disyuntivas) y evolutiva. Se ofrecen sugerencias para lograr una óptima medición de la respuesta inmune.

La especie de estudio: AEDES AEGYPTI

Aedes aegypti (Diptera, Culicidae) tiene una amplia distribución entre los trópicos y zonas subtropicales llegando hasta los 40° Sur y 45° Norte, (Nelson, 1986; Badii et al., 2007). Por lo general habita en áreas geográficas con una temperatura media anual entre 17-30ºC (Ibáñez-Bernal & Gómez-Dantés 1995). Su rango de distribución altitudinal llega a los 2400 m.s.n.m. (Badii et al 2007). En México el rango de distribución estimado abarca 29 estados (Ibáñez-Bernal y Gómez-Dantés 1995).

A. aegypti tiene cuatro estadios en su desarrollo postembrionario: huevo, larva, pupa y mosquito adulto (Fig. 1.). Los *huevos* son puestos en sustratos sólidos ubicados en la interface agua-tierra. Después de que ha ocurrido la oviposición, se da la melanización, lo que les confiere resistencia a la desecación, entrando en diapausa durante periodos secos, permaneciendo viables hasta 2 años (Christophers 1960). Cada hembra produce de 100 a 120 huevos por puesta (Apóstol et al 1994), con un éxito de eclosión muy alto. La *larva* eclosionada es acuática y pasa por cuatro fases. Las tres primeras fases tienen un desarrollo rápido, mientras que la última es más prolongada que es cuando la larva aumenta de tamaño y peso adquiriendo recursos para el periodo de metamorfosis (Nasci 1986). Por lo general, el tiempo de desarrollo de la etapa larval es de una semana (Christophers 1960), pero en condiciones de baja temperatura o escasez de alimento, la cuarta fase larval puede prolongarse por varias semanas antes de transformarse en pupa (Tun-Lin et al 2000). La mortalidad más alta ocurre durante las dos primeras fases larvales. Su fuente de alimentación se compone de microorganismos, particularmente hongos, algas, protozoarios y otros insectos (Merrit *et al*., 1992). La *pupa* es la fase de metamorfosis de larva a

adulto, la cual tiene la cualidad de desplazarse activamente en el medio acuático en respuesta a estímulos externos como vibraciones o cambios en intensidad lumínica. Esta etapa del ciclo de vida dura aproximadamente de dos a tres días (Christophers, 1960).Las larvas y las pupas de los machos se desarrollan más rápido que las de las hembras.

El *mosquito adulto* recién emergido pasa sus primeras 24 horas en reposo, periodo durante el cual se completa el desarrollo (Clements, 1999). El mosquito presenta dimorfismo sexual, las hembras son más grandes que los machos, sus antenas tienen vellos cortos y escasos, y los palpos son de un tercio o menos de longitud que la proboscis (Busvine, 1975), mientras que el macho tiene antenas plumosas con pelos largos y abundantes y palpos con un tamaño similar a la proboscis (Busvine, 1975). Los machos son poligínicos, y las hembras presentan un patrón de apareamiento monándrico. Un apareamiento es suficiente para fecundar sus huevos. La conducta de reproductiva y de alimentación de sangre (en hembras) aparece entre las 24 y 72 horas post-emergencia adulta (Clements, 1999). Los machos se alimentan del néctar de las flores, las hembras de azúcares y de sangre; esta última le confiere nutrientes para la maduración de los huevos (Clements, 1999). Después de cada alimentación sanguínea se desarrolla un lote de huevos, aunque una alimentación reducida no conlleva a la producción de huevos. Después de 48 a 72 horas post alimentación, se realiza la oviposición (Carrada et al., 1984). Terminada la oviposición, la hembra reanuda la conducta de búsqueda de una nueve fuente de sangre para la producción del siguiente grupo de huevos. El adulto, en condiciones naturales sobrevive en promedio de 15 a 30 días (Badii et al., 2007).

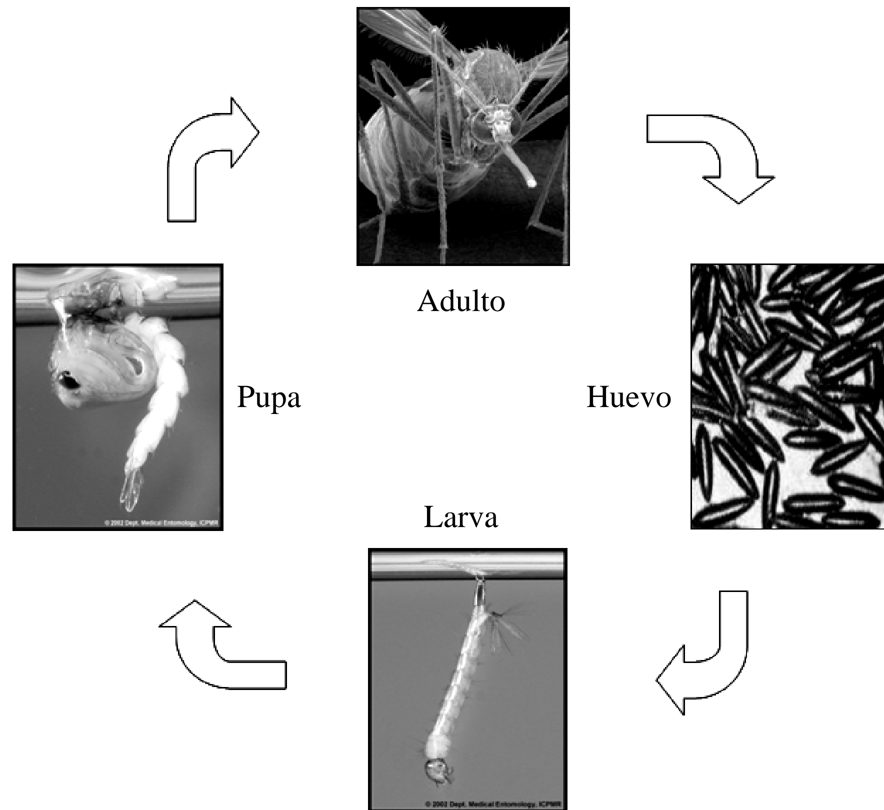

Figura 1. Ciclo de desarrollo de *Aedes aegypti.*

AEDES AEGYPTI y el virus Dengue

El mosquito *Aedes aegypti* es el principal vector del virus dengue, ocasionando fiebre del dengue y fiebre hemorrágica del dengue en humanos (Clarke, 2002). La invasión del virus al mosquito solo ocurre en los casos en que la hembra, al momento de la alimentación sanguínea, pica a un hospedero que porta al virus. Esta hembra ya infectada podrá transmitir el virus a hospederos sanos. La capacidad de diapausa que tienen los huevos del mosquito, rápido ciclo de vida y la preferencia de la hembra por ovipositar en cuerpos de agua relativamente pequeños, son factores que han contribuido en la expansión del rango de distribución del mosquito (Fig. 2). Unido a esto, la preferencia del mosquito a ambientes domésticos en su ciclo de vida y una aclimatación muy rápida a áreas urbanas rurales, y probablemente el cambio climático global, contribuye a que el virus dengue también pueda expandir su rango de distribución (Gubler, 2002), dado que la presencia del vector es indispensable para la transmisión del virus. El control de esta enfermedad

requiere del conocimiento integral del mosquito con una visión holística que genere información para el manejo racional de este vector.

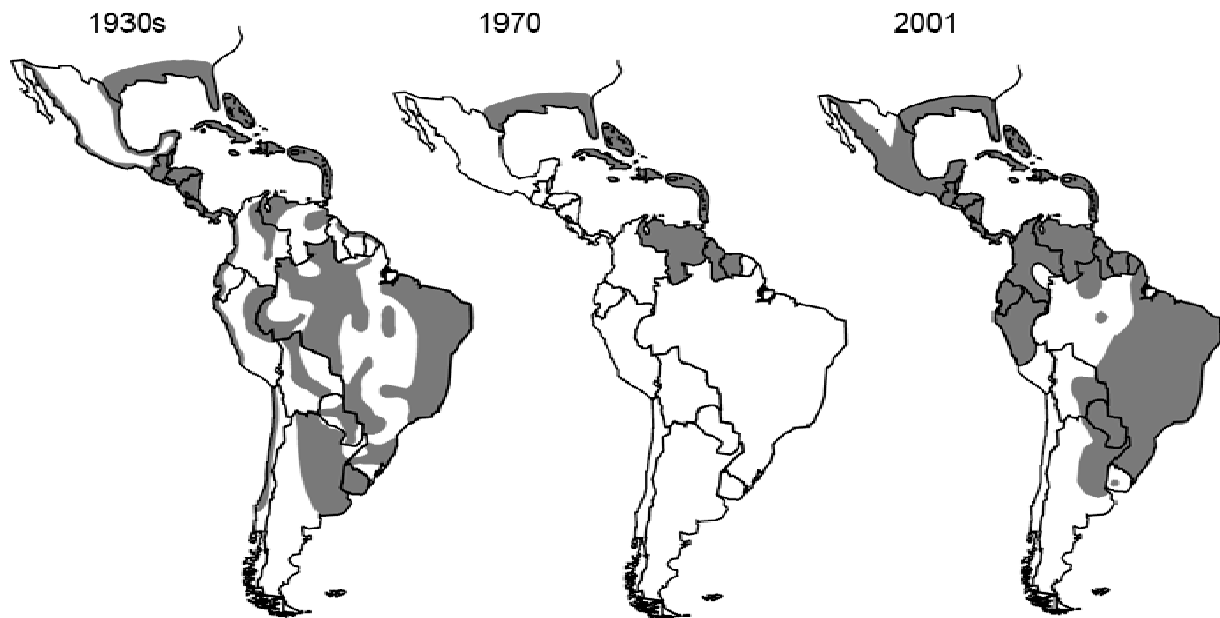

Figura 2. Expansión estimada del rango de distribución de *Aedes aegypti* en América hasta 2001 (tomado de Gubler, 2002).

REFERENCIAS

Apóstol BL, Black WC, Reiter P, Miller BR. 1994. Use of randomly amplified polymorphic DNA amplified by polimerasa chain reaction to estimate the number of *Aedes aegypti* families at oviposition sites in San Juan, Puerto Rico. *Am. J. Trop. Med. Hyg.* **51**: 89-97.

Badii MH, Landeros L, Cerna E, Abreu JL. 2007. Ecología e historia del dengue en las Américas (Ecology and history of dengue in Americas). *Int. J. Good Conscience* **2**: 309-333.

Busvine, J.R. 1975. Arthropod Vectors of Disease. Edward Arnold & Company, London.

Carrada BT, Vázquez VL, García IL. 1984. La ecología del dengue y el *Aedes aegypti*. Investigación Preliminar. Tercera parte. *Sal. Púb. Mex.* **26**: 297-311.

Cheverud JM, Rutledge JJ, Atchley WR. 1983. Quantitative genetics of development: genetic correlations among age-specific trait values and the evolution of ontogeny. *Evolution* **37**: 895-905.

Christophers SR. 1960. *Aedes aegypti*_(L): the Yellowe Fever Mosquito: its Life History, Bionomics and Structure. Cambridge University Press, Cambridge.

Clarke T. 2002. *Dengue virus: break-bone fever. Nature* **416**: 672-674

Clements AN. 1999. The Biology of Mosquitoes. Cabi, Wallingford. UK.

Elliot SL, Blanford S, Horton CM. 2003. Fever and phenotype: transgenerational effect of disease on desert locust phase state. *Ecol. Lett.* **6**: 830-836.

Fellowes MDE, Kraaijeveld AR, Godfray HCJ. 1998. Trade-off associated with selection for increased ability to resist parasitoid attack in *Drosophila melanogaster. Proc. Roy. Soc. Lon.* **265**: 1553-1558.

Folstad I, Karter AJ. 1992. Parasite, bright males and the immunocompetence handicap. *Am. Nat.* **139**: 603-622.

Gubler DJ. 2002. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. *Trends in Microbiol.* **10**:100-103.

Hamilton WD, Zuk M. 1982. Heritable true fitness and bright birds: a role for parasites? *Science* **218**: 384-387.

Ibañez-Bernal S, Gomez-Dantes H. 1995. Los vectores del dengue en méxico: una revision crítica. *Sal. Pub. Méx.* **37**: 53-63.

Kurtz J, Franz K. 2003. Evidence for memory in invertebrate immunity. *Nature* **425**: 37-38.

Little TJ, Kraaijeveld AR. 2004. Ecological and evolutionary implications of immunological priming in invertebrates. *Trends Ecol. Evol.* **19**: 58-60.

Little TJ, Hultmark D, Read AF. 2005. Invertebrate immunity and the limits of mechanistic immunology. *Nat. Immunol.* **6**: 651-654.

Merrit RW, Dadd RH, Walker E D. 1992. Feeding behavior, natural food and nutritional relationship of larval mosquitoes. *Annu. Rev.Entomol.***37:**349-376.

Moret Y, Siva-Jothy MT. 2003. Adaptive innate immunity? Responsive-mode prophylaxis in the mealworm beetle, *Tenebrio molitor. Proc. Roy. Soc. Lond.* **B 270**: 2475-2480.

Nasci RS. 1986. The size of emerging and host-seeking *Aedes aegypti* and the relation of size to blood-feeding success in the field. *J. Am. Mosq. Cont. Assoc.* **2**: 61-62.

Nelson MJ. 1986. Aedes aeqypti: biologia y ecología. OPS, Washingtohn DC.

Nylin S, Gotthard K. 1998. Plasticity in life-history traits. *Annu. Rev. Entomol.* **43**: 63-83.

Núńez-Farfán J. 1993. Selección natural en el campo: revisión de la evidencia reciente. In Núńez-Farfán J, Cordero C, eds. *Tópicos de Biología Evolutiva*. México: Centro de Ecología, UNAM, 19-59.

Owens IDF, Wilson K. 1999. Immunocompetence: a neglected life history trait or conspicuous red herring? *TREE.***14**: 170-172.

Roff DA. 1997. *Evolutionary Quantitative Genetics*. New York: Chapman & Hall.

Schmid-Hempel P. 2005. Evolutionary ecology of insect immune defences. *Annu.Rev. Entomol.* **50**: 529-551.

Siva-Jothy MT, Thompson JJW. 2002. Short-term nutrient deprivation affects immune function. *Physiol. Entomol.* **27**: 206-212.

Stearns SC. 1992. The Evolution of Life Histories. Oxford: Oxford University Press.

Tu MP, Flatt T, Tatar M. 2005. Juvenile and steroid hormones in *Drosophila melanogaster* longevity. In: Masoro EJ, Austad SN, eds. Handbook of the biology of aging. 6th edition. San Diego: Academic Press (Elsevier), 415-448.

Tun-Lin W, Burkot TR, Kay BH. 2000. Effects of temperature and larla diet on development rates and survival of the dengue vector *Aedes aegypti* in north Queensland, Australia. *Med. Vet. Entomol.* **14**: 31-37.

Via S, Gomulkiewicz R, DeJong G, Scheiner SM, Schlichting CD, Van Tienderen PH. 1995. Adaptative phenotypic plasticity: consensus and controversy. *TREE* **10**: 212-217

Zhivotovsky LA, Feldman MW, Bergman A. 1996. On the evolution of phenotypic plasticity in a spatially heterogeneous environment. *Evolution* **50**: 547-558.

Zuk M, Stoehr AM. 2002. Immune defences and host life history. *Am. Nat.* **160**: S9-S22.

CAPÍTULO II

EVOLUTIONARY ECOLOGY OF INSECT IMMUNITY

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ABSTRACT

A large number of recent ecological studies have reported interactions between immunity and traits related to survival and reproduction (i.e. life history traits). In addition, recent molecular/physiological immune mechanisms studies have revealed a wide array of efficient immune responses, which can be enhanced through ontogeny and transferred to the offspring (immunological priming). Here, we review and identify areas where more investigation and integration of information from these different biological fields is needed. We also discuss possible explanations for variation in insect immune response highlighting the ecological and physiological contexts of trade-offs between immunity and other life history traits. We emphasize the analysis of the genetical and environmental bases of variation in immune response, as a way to expand our knowledge of insect immunity evolution and its mechanisms. An interdisciplinary approach will be a key to the advancement of the emerging discipline of immunoecology.

KEYWORDS

Immune response, insects, immunological priming, immunoecology, life history theory, quantitative genetics

INTRODUCTION

The understanding of insect immunity has considerably expanded in recent years. Ecological evidence demonstrates that it is a trait deeply involved in the evolutionary ecology of the reproductive and survival strategies of the organisms. The vast wealth of information on the area, demands integration in order to fully conceptualize the participation and consequences of immune response, and its genetic and phenotypic variation within and among individuals, populations and species. The primary goal of this review is to analyse the current knowledge and ideas related to the adaptive functionality and variation of insect immune response, considering: (1) the ecological context in which immune response is expressed in terms of required resources and related with other fitness-related traits, as well as the evolutionary consequences on life history strategies and the effect of pathogen virulence on the host; (2) the mechanisms responsible for immune response operation with a focus on the possible evolutionary outcome of maladaptive immune responses and individual-primed immune responses through ontogeny and between generations; and (3) the evolutionary features via individual phenotyíc variation (quantitative genetics) of immune response, genetic correlations, and the production of different phenotypes according to distinct environmental conditions, as well as the causes and possible adaptive value of this variation. All these ideas are linked using a standpoint that integrates ecological, physiological and population genetics perspectives. It is hoped that this review will facilitate the reflection on encourage a more unified approach to the study of insect immune responses

LIFE HISTORY THEORY AND IMMUNITY

The majority of empirical research in the field of ecological immunity has been under the life history and trade-offs theory perspective. Life history theory seeks to explain the evolution of traits (e.g. size at birth, growth and mortality rates, size and age at maturity, clutch size and reproductive effort) (modified from Stearns, 1976; Roff, 1992; Stearns, 1992) all related with the

life history of an individual, and the changing environmental conditions to which organisms are exposed. These traits are commonly shaped by intrinsec (genetic, or physiological) restricition called “trade-offs” (Stearns, 2000), which affect both survival and reproduction. Trade-offs occur when natural and/or sexual selection is unable to maximize the expression of two or more traits at the same time (Núñez-Farfán, 1993), due to possible genetic or phenotypic correlation with other traits, and despite their potential contribution to fitness (Cheverud, Rutledge & Atchley, 1983) In insects there is growing empirical evidence of trade-offs between these traits and immune defense. Examples include the reduction in larval competitive ability for dietary resources (Kraaijeveld & Godfray, 1997), an increase in predation vulnerability (Rigby & Jokela, 2000), longer developmental time, reduction in egg viability, lower survival rates (Fellowes, Kraaijeveld & Godfray, 1998; Boots & Begon, 1993; Moret & Schmid-Hempel, 2000; Hoang, 2001), and a decrease in reproductive success (Sandland & Minchella, 2003a; Schmid-Hempel & Schmid-Hempel, 1998). Thus, the ability of an insect to minimize an infection (i.e. immunocompetence, Owens & Wilson, 1999) represents an evolutionary cost given its negative covariation with other fitness components (Schmid-Hempel, 2003).

Ecological basis of trade-offs

Infections affects the optimal allocation of host resources, changing the host’s “internal state” (Agnew, Koella & Michalakis, 2000),in terms of energy reserves and viability (Iwasa, Pomiankowsli & Nee, 1991; Iwasa & Pomiankowski, 1994). In addition, the required resources used for the immune response are also demanded for the expression of other traits linked with survival and reproduction (Sheldon & Verhulst, 1996), resulting in a trade-off. Organisms have limited resources; as a result, they cannot maximize investment on all life history and/or intermediate traits (e.g. body size, colored traits, behavioral traits, etc.) or immunity. Therefore, organisms must physiologically “decide” how to allocate available resources to maximize fitness.

Optimal allocation depends on the strategies used to increase fitness in the particular habitat of the organism (Schlichting & Pigluicci, 1998).

Trade-offs between the immune response and other traits are usually detected in stressing environments (Sandland & Minchela 2003b), particularly with variables involved in the organism's internal state, or condition such as parasite load or prevalence/intensity of infection (McNamara & Houston, 1996), and external variables (e.g. food abundance, predators, population density, etc). Under favorable environmental conditions, trade-offs can be masked, since the value of life history traits are high (de Jong & Van Noordwijk, 1992) or the costs imposed by immune system activation and the consequent trade-offs are not evident (Moret & Schmid-Hempel, 2000). The occurrence of trade-offs may therefore depend on the amount of resources available, acquisition efficiency and their optimal allocation (Van Noordwijk & de Jong, 1986).

Survival, reproduction and immunity

Life history theory predicts that survival and fecundity are not usually maximized simultaneously (Roff, 1992). For example, current reproduction can negatively affect future reproduction due to the prevailing trade-off between reproduction (which uses resources for the expression of sexually selected traits, quantity and quality of eggs produced, etc.) and survival (i.e. resources used to escape from predators, competence or immunity; Reznick, Nunney & Tessier, 2000). The organism can also allocate its resources in relation to chances of future reproduction which is strongly related to life expectancy. Thus, when there is a high probability of future reproduction, an organism must allocate enough resources to current reproduction in such a way that this investment will not affect subsequent reproductive events (Williams, 1966). On the other hand, when the probability of future reproduction is low (i.e. lack of resources, environmental heterogeneity, incidence of infectious agents), the organism must allocate a large portion of its resources to current reproduction (Fessler

et al., 2005). Although this terminal investment theory was originally laid out for iteroparous organisms, it may be applied to semelparous organisms, as long as reproduction occurs in multiple egg laying events (as with insects) where there is sufficient temporal separation in which the organism is expected to vary in investment. This can be expected in social insects, given their generally long life expectancies and high risk of infection due to living in large colonies compared to non-social insects.

Survival and reproductive strategies adopted by an individual also depend on the force and course of infection. Van Baalen (1998) proposed a theoretical host recovery threshold, thus when the intensityof infection is below this threshold (i.e. no significant damage to the host), the organism should not make any investment to immune response at all. Above, but still close to the threshold, it then pays to allocate more into recovery and survival. However, when infection is high, without recovery possibilities, organism must allocate all resources to reproduction because a new infection may occur almost immediately leaving the animal without survival probabilities. Heterogeneity in food availability and kind and burden (and their interaction) of pathogens will have an effect on the evolution of the survival and reproductive strategies. Social insects seem very adequate to test these ideas as the risk of infection is higher in these animals compared to non-social insects.

IMMUNITY AND SEXUAL SELECTION

Sexually selected traits and immune response

The evolution of the immune response has been also examined under the context of sexual selection (competition to leave more offspring; Darwin, 1871), Recent evidence suggests that trade-offs between immunity and sexually selected traits (SST) favored during competition for mates, are common in nature (e.g. Siva-Jothy, 2000; Rantala *et al.*, 2003; Contreras-Garduño, Canales-Lazcano & Córdoba-Aguilar, 2006; Hosken, 2001; McKean & Nunney, 2001). Hamilton

and Zuk (1982) were the first to suggest that in vertebrates pathogen resistance is correlated with the expression of SST, presumably because, androgenic hormones necessary for the expression of sexual traits and behavior can act as immunosuppressors, giving rise to a physiological trade-off (Folstad & Karter, 1992), and rendering males more prone to infection. Thus, SST only could be expressed if males are able to fight-off infections in spite of the immunosuppressive action of hormones, so that the expression of SST becomes an honest indicator of male immunocompetence both to females (during intersexual selection) and/or males (during intrasexual selection) (Folstad & Karter, 1992).

Insects have fundamentally different hormones to those used by vertebrates (Sheridan *et al.*, 2000), but the rationale would be the same as vertebrates. Insects produce juvenile hormone, which has a physiological effect in several traits, for example: sexual maturation, pheromone production, ovaries development, courtship behavior, morphological polyphenisms (Flatt, Tu & Tatar, 2005) and immune function (Tu, Flatt & Tatar, 2005, Rolff & Siva-Jothy, 2002). This suggests that probable, JH induces or prevents allocation of resources to different functions. In beetles the trade-off between immune function and pheromone production seems physiologically mediated by JH (Rantala, Vainikka & Kortet, 2003). As in vertebrates, only males in good condition (i.e. males that can deal successfully with infections,) will be able to produce and maintain SST. These traits could be assessed by females when basing their mating decisions with the presumable indirect benefit of giving birth to resistant offspring.

One essential piece in the immunocompetence hypothesis is that female offspring would obtain disease resistance genes as signaled by SST. However, the relation between SST and the acquisition of genes related to disease resistance is not necessarily direct. Adamo and Spiteri (2005) recently proposed that females may accrue the direct benefit of avoiding infections when mating with males of current good health status (Able, 1996). Furthermore, selection for healthy

males may not only be maintained via female choice; intrasexual competition can also contribute to the maintenance of honest traits, as long as male-male competition implies energetically costly endurance. If males use signals to indicate their fighting ability, it is then likely that these signals may indirectly reveal immune condition. Contreras-Garduño *et al.* (2006) and Serrano-Meneses *et al* (2007) found that in the damselfly *Hetaerina americana*, male wing coloration seemed to indicate territorial fighting ability, in terms of energy reserves. However, unlike other calopterygids of more recent origin (e.g. those of the genus *Calopteryx*), *H. americana* males do no court females but simply grab them and invariably mate with them. Wing coloration areas in species where males court females (e.g. *Calopteryx*) and *Hetaerina* males also positively correlate with immune ability. Given these relationships, in the *Hetaerina* case, it makes more sense to admit that SST indicates energetic condition, rather than immune ability to potential fighting contestants. However, even if courtship does not seem to occur in *Hetaerina* it does not reject the hypothesis that females still obtaining direct benefits for their offspring if they matemales with high fighting abilities (if it has a heritable basis) (Adamo & Spiteri, 2005; Contreras-Garduño, Lanz-Mendoza & Córdoba-Aguilar, 2007).

In some fly and cricket species, male seminal products bear an immunosuppressive effect on the female (McGraw, Gibson & Clark, 2004; Fedorka, Zuk & Mousseau, 2004) diminishing her fitness (Fedorka & Zuk, 2005). Males could increase their fertilization success by reducing female immunocompetence, increasing sperm survival, preventing sperm to be recognized as foreign invading bodies while avoiding female immune response (Fedorka & Zuk, 2005).This sexual conflict perspective for immune response evolution is unique and gives support to this hypothesis, and it should be considered as an alternative to female choice.

Mating systems and differences between the sexes

Sexual selection intensity differs among species depending on their mating system (monogamy vs. polygamy) (Andersson, 1994). This varying intensity should promote differences in the pattern of investment to immune defense by each sex. In polygynous species, males are expected to increase their fitness by reducing their investment on immune defense while investing resources on reproductive effort (for example SST) (Zuk & McKean, 1996; Sadd *et al.*, 2006). On the other hand, natural selection would favor an increase in resource investment to immunity in females, under the assumption that increased longevity could enhance fitness via egg production (Rolff, 2002). In *Acheta domesticus,* females increase egg production when a bacterial infection occurs, compensating the reduced life expectancy and future reproduction due to infection (Adamo 1999). Whereas in another cricket;*Gryllus texensis*, male immunocompetence decreased in the face of an infection, supporting the hypothesis that males trade-off immune response for reproduction (Adamo et al 2001).

Sex biases in immunity may be due to the different cost for SST compared to egg production and laying, and the presumed selection for longer life expectancy for females compared to males (Stoehr & Kokko 2006; Forbes, 2007). One missing piece in the presumed sex bias in immunity is the difference in kinds of pathogens attacking each sex. For example, in mosquitoes, adult females require carbohydrates (sugar) and proteins for vitellogenesis which usually comes in the blood meal. While males, feed on sugar solutions (nectar) (Clements, 1999). This difference does not mean that females are more prone to infections than males, but divergent host-pathogen interactions for each sex are possible and can be reflected in the course of action of individual immune responses. This disparity could lead to wrong conclusions when testing for sex immunity differences, where the same artificial infection (e.g. inoculation of bacteria, yeast, etc.) is employed equally in males as in females. Future studies must consider if males and females share the same pathogens before testing and assuming other reasons for sex immunity biases.

PATHOGEN VIRULENCE, MULTIPLE INFECTIONS AND WITHIN-HOST COMPETITION

In order to explain immunological survival or reproductive differences in the kind of pathogens and its virulence (i.e. harm imposed on a host measured in terms of a reduction in survival or fecundity due to pathogen growth or reproduction) must be considered. Pathogen fitness depends on transmission success to new hosts (May & Anderson, 1983). The transmission increases through enhanced growth or replication rates (Ebert, 1998), but with an associate cost: host fitness. Since this can lead to pathogen dead too, there will be a trade-off between fecundity and longevity for the pathogen (Frank, 1996). Therefore it is expected that pathogens change their virulence depending on the host's natural history. For example, if reproductive adults have a decreased immune response, pathogens should reduce their virulence, although high levels of virulence can be advantageous when infecting non-immune suppressed females (see Pfennig, 2001). Selection could be then favoring the maintenance of sex-or taxon-specific strategies, based on an optimal virulence level understood as that strategy that increases fecundity and longevity of pathogens with low host damage. Different immunological degree responses among hosts are also expected. In order to prove differential effects of pathogens on host, we need measures of differential virulence damage since, currently, variation in virulence is ignored.

An individual can be infected by more than one pathogen lineage (Read & Taylor, 2001). Higher levels of virulence could arise because within-host competition (Frank, 1996). In a mixed infection, low lethal pathogens can be eliminated through competitive exclusion by more virulent pathogens or strains, so selection will favor pathogens or strains less likely to be competitively suppressed (de Roode *et al.*, 2005). Different spatial and temporarily virulence heterogeneity in within-host competition becomes a fluctuating selective pressure altering the expression, variation and evolution of immune mechanisms of insects through generations. How the host's immune ability

varies, according to both virulence and within-host competition, in the context of studies of the evolution of immune response is still an open niche for further investigation.

MECHANISMS OF INSECT IMMUNE RESPONSE

The immune system seeks to maintain a relative homeostatic state under a wide variety of internal and external conditions (e.g. development, infectious agents). The immune responses in insects include cells and products which are interconnected (Hoffmann *et al.*, 1999) (Fig. 1). Here we briefly review general mechanism of insect immunity to build up frameworks of physiological trade-offs, linking immunopathology and immunological priming.

The first lines of defense include the exoskeleton cuticle as a physical barrier, and epidermis, gut epithelium, and male and female reproductive accessory glands (Gillespie *et al*, 1997; Casteels, 1998). These tissues can secrete cytotoxic molecules like lysozymes, reactive oxygen species (ROS, e.g. superoxide anions, peroxides, hydroxyl radicals) (Schmid-Hempel, 2005a), which are transported to the wound or where infection take place (Nappi & Ottovianni, 2000).

Recongnition of pathogens is needed. The membrane of haemocytes comprises proteins called Pattern Recognition Receptors (PRRs). PRRs recognize conserved molecular features of pathogens called Pathogen-AssociatedMolecular Patterns (PAMPs). Lipopolysaccharides (LPS), mannoses, *β*-1, 3 glucans and peptidoglycans are considered the most common PAMPs (Gillespie *et al.*, 1997). Lectins are also involved in the recognition of oligosaccharides and polysaccharides present on the pathogen cell membrane (Wilson, Chen & Ratcliffe, 1999). PRRs include the gram-negative bacteria-binding protein (GNPB) and the peptidoglycan recognition proteins (PGRP); the latter includes molecules of long transmembranal form that are secreted into the haemolymph (Leclerc & Reichhart, 2004).

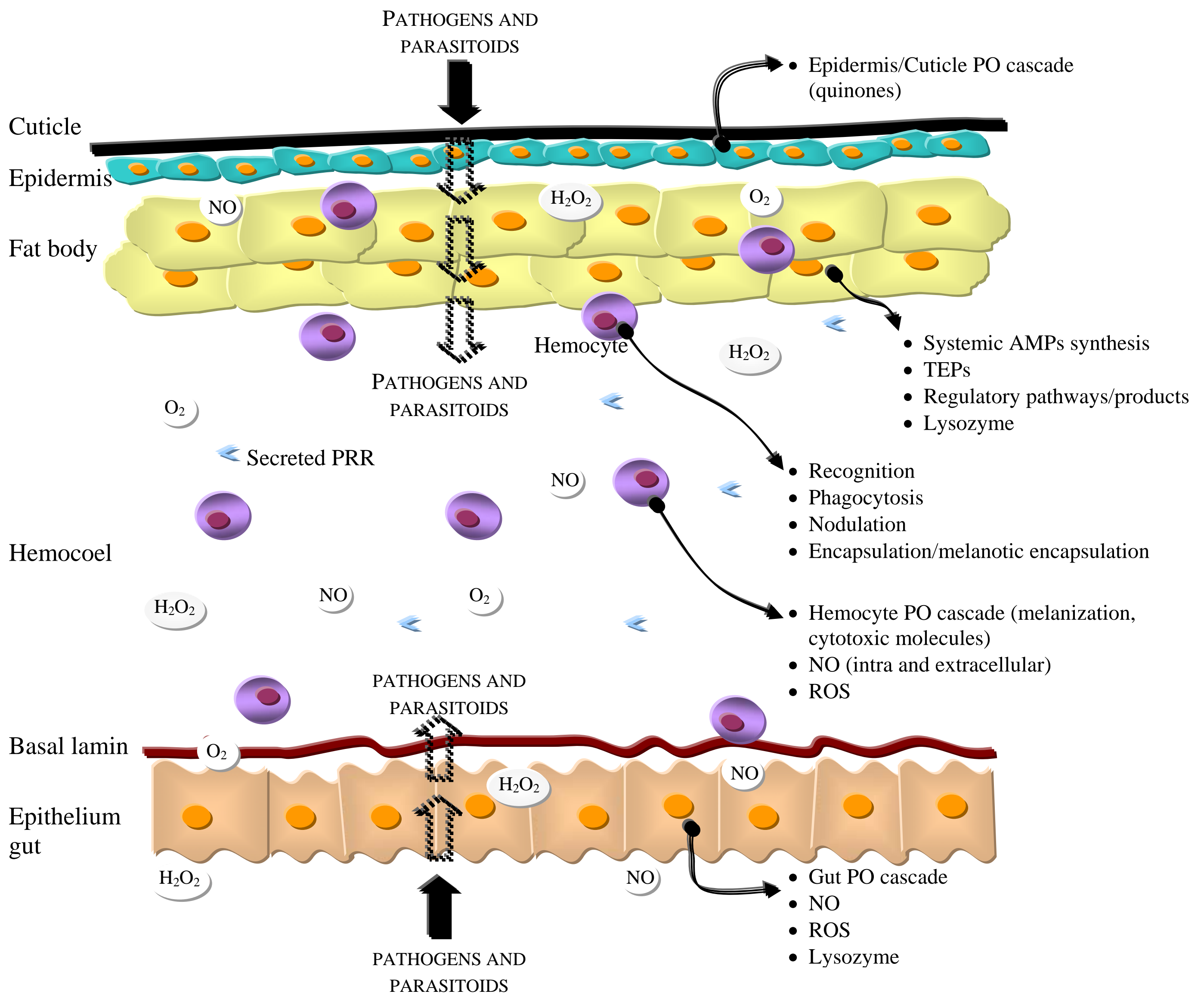

Figure 1. Components of insect immunity.

After recognition, coordinated responses of haemocytes begin. Phagocytosis is a response by which infectious agents become engulfed and destroyed (Gillespie *et al*., 1997). It may also occur that a large number of haemocytes bind to bacterial aggregations to form a nodule (Gillespie *et al.*, 1997). When infectious agents are large in size (for example, parasitoid larvae), the process of encapsulation, which is similar to nodulation (the difference between both process depends on the size of infectious agents), is used. Occasionally, during encapsulation, a melanin layer is produced to cover parasites which die by anoxia, host generation of free radicals or starvation (Nappi *et al*,

2000; Narayanan, 2004). Haemocytes also contribute to clot formation by aggregating at wound sites (Gregoire, 1974). Other immune responses are activated, which includes antimicrobial molecules produced within the reproductive accessory glands, gut cells, fat body and haemocytes (Manetti, Rosetto & Marchini, 1998 1998; Schmid-Hempel, 2005a) which are commonly referred as humoral response. Most of these molecules can be secreted into the haemolymph, epitheliums, Malpighian tubes or near cuticle. These molecules include lysozymes with depolymerizing bacteria (mainly Gram^{-}) cell wall action (Gillespie *et al*, 1997); Thoiester-containing proteins (TEPs), with opsonizination activity that enables phagocytosis (Tzou, De Gregorio & Lemaitre, 2002); nitric oxide (NO), a highly reactive and unstable free radical gas produced during the oxidation of L-arginine to L-citrulline by the nitric oxide synthase (NOS) (Müller, 1997) that crosses cell membranes to act in nearby targets (Müller, 1997), inhibits protein catalytic activity and has protein and pathogen DNA harming effects (Colasanti *et al.*, 2001; Rivero, 2006); ROS, which damages pathogen nucleic acids, proteins and cell membrane (Nappi *et al*, 2000); and antimicrobial peptides (AMPs; e.g. cecropins, atticins, diptericins, drosomycins, metchnikowins, and defensins, all with isoforms; Lemaitre, Reichart & Hoffmann, 1997; Narayanan, 2004) which induce a collapse of the pathogen membrane and/or prevent the synthesis of molecules within the pathogen (Otvos Jr, 2000; Bulet, Charlet & Hetru, 2003).

The proteolytic pro-phenoloxidase (proPO) cascade is a key immune component for the synthesis of ROS, cytotoxic molecules and melanin (Iwanaga & Lee, 2005). After pathogen recognition, proPO system activation starts. As a first step, phenylalanine is hydroxilated and converted into tyrosine (Christensen *et al*, 2005). Then proPO is activated by a serin protease to its active form PO (an oxidoreductase enzyme) which catalyzes the reaction where tyrosine is transformed into dopa and then to dopaquinone (Söderhäll & Cerenius, 1998). After non-enzymatic polymerizations, dopaquinone is finally converted to melanin which will be used for wrapping up pathogens and

wound clotting (Nappi & Christensen, 2005). Opsonic factors, ROS, and citotoxins such as quinones and semiquinones are important intermediate molecules, which are highly reactive and toxic to pathogens that greatly amplify immune response (Cerenius & Söderhäll, 2004; Nappi & Ottovianni, 2000).

In *Drosophila*, the immune system is regulated by the products of three signaling pathways: TOLL, Immune Deficiency (Imd) and the Janus kinase/Signal Transducers and Activators of Transcription (JAK/STAT). Regulation is ensured by transcriptional factors produced in the fat body, preventing self tissue damage and keeping haemocyte proliferation and differentiation controlled (Agaisse & Perriomon, 2004). The TOLL pathway bears a key function during antimicrobial peptide production after being activated by fungal and Gram^{+} bacterial infections (Janeway & Medzithov, 2002; Hoffmann & Ligoxygakis, 2004). The Cactus protein, a TOLL transduction pathway product, maintains haemocyte proliferation regulated (Qiu, Pan & Govind, 1998). The Imd pathway gives rise to antimicrobial peptides when activated by Gram^{-} bacteria (Khush, Leulier & Lemaitre, 2002; Hoffmann, 2003). Zaidman-Rémy *et al.* (2006) found that the Imd pathway can be regulated by PGRP-L, preventing host tissue damage as a consequence of extended immune activity. The JAK/STAT pathway regulates the secretion of humoral factors like TEPs (Tzou, De Gregorio & Lemaitre, 2002).This pathway also regulates haemocyte proliferation and differentiation (Agaisse & Perriomon, 2004). Interestingly, not all insects use this differential activation and regulation pathways. For example, in the mosquito *Aedes aegypti* phagocytic and melanization responses are independent of bacterial Gram type (see Hillyer, Schmidt & Christensen, 2004).

Physiology of immune responses and trade-offs

In ecological studies, trade-offs between immune responses and other traits are usually treated as a black box, with very little understanding of their underlying physiological mechanism.This kind of knowledge must be used as a starting point to understand some general aspects about the basis of the trade-offs generated and those that should be looked for. Tyrosine is responsible for coloring the egg chorion in some insects (Li & Christensen, 1993) and other metabolic pathways. Arginine, another essential amino acid, its necessary for NO generation used in immune response (Rivero, 2006), but it is also important for sperm maturation (Osanai & Chen, 1993), egg production (Uchida, 1993), long term memory, chemosensory (antennal lobes, olfaction) and visual information processing (Müller, 1997). Other efectors are the AMPs, which contains fewer than 150–200 amino acids (Bulet *et al.*, 1999). The overall AMPs concentration, in *Drosophila* haemolymph, can reach up to 200 μM (Otvos Jr, 2000), at this concentration, antimicrobial peptides seem costly to produce. Resources (proteins) gathered through ontogeny are indispensable for antimicrobial peptide generation as well as for the synthesis of other molecules. In addition most of the AMPs are cationic, due to their higher content in arginine (Bulet *et al.*, 1999). Consequently arginine could be a limited resource for NO production and AMPs synthesis leading to negative correlations between immune responses. During the PO cascade, tyrosine is used as substrate for the formation of melanin and highly reactive and toxic intermediate molecules. For these reasons, it is likely that tyrosine and arginine are restrictive resources in insects and may lead to trade-offs between immune response and other traits. Experimental manipulation of doses of these amino acids could be used to investigate the insect's response to these changes and trade offs.

Immunopathology: evolutionary and ecological implications

As mentioned before, several molecules (quinones, reactive oxygen and nitrogen molecules, peroxides, etc.) are produced during insect immune response which can be harmful to their own

tissues and cells, a phenomenon referred as immunopathology or autoreactivity. This process has been frequently ignored as a possible constraint for the insect immune system (Schmid-Hempel, 2005a).

It can be argued that natural selection may favor high responsiveness to ensure control of pathogens despite the risk of immunopathology (Graham, Allen & Read, 2005). Nonetheless, activation and upholding of immune response depend on the kind and burden of pathogens, and a novel pathogen could elicit responses beyond immunological control with potentially harmful effects. Mechanisms and molecules produced to kill pathogens could be over expressed or overproduced if monitoring, regulatory, and recognizing systems are not synchronized. On the other hand, if virulence of pathogens elicits autoreactive host responses, low virulence could be favored. Only in cases where competition among pathogens occurs, natural selection will favor high levels of virulence. In both cases there must be fitness advantages to the pathogen regardless of autoreactivity and decrease in host survival. In some occasions hosts could kill pathogens, but bears an extreme survival cost. Meanwhile other species may suffer the damage generated by pathogens but, nevertheless they could be able to survive and reproduce, so a large immunological response could not be the best strategy. The strategy adopted via immune mechanisms and their regulation will depend on pathogens (probably not all novel pathogens will elicit the same degree of autoreactivity), differences in host immune response of the hosts (i.e. unstressed individuals with much to invest in immunity) may be more prone to immunopathological costs (Graham *et al.*, 2005), and cost and benefits between autoreactivity and fitness of pathogens and hosts. Furthermore, immune response suppression during stressful and energetically demanding situations (e.g. molting or during display of sexual traits) can be another strategy to avoid autoreactivity (Kotiaho, 2001 and references therein). In this sense, it is not resource limitation the factor that

produces the phenomenon, the assumed trade-off, but a type of "on-off" switch regulation of the immune system during ontogeny.

Immunological Priming

It has been argued that invertebrates are not capable of developing any kind of immunological memory (as vertebrates do). Nevertheless, recent experiments (Faulhaber & Karp, 1992; Moret & Schmid-Hempel, 2001; Kurtz & Franz, 2003; Moret & Siva-Jothy, 2003) have provided evidence of a phenomenon similar to that in with vertebrate acquired immune response which has been called immunological priming (Little & Kraaijeveld, 2004; Little, Hultmark & Read, 2005; Kurtz, 2005; Schmid-Hempel, 2005a, b). This process assumes that previous experience with infectious agents might enhance individual immunity which can be transmitted to subsequent generations (transgenerational priming) (Kurtz & Franz, 2003; Little *et al.*, 2003; Rahman *et al.*, 2004; Sadd *et al.*, 2005; Moret, 2006; Sadd & Schimd-Hempel, 2006; Pham *et al.*, 2007). This novel idea is nowadays being explored in detail by physiologists and immunoecologists, and, given its key importance, has been coined as prompting a new era in the evolutionary and ecological studies of the immune system (for experimental designs see Little & Kraaijeveld, 2004).

The immune invertebrate memory argument has been criticized (Klein, 1997). The basis of this is the lack of mechanisms for specific Ig production, rearrangement of somatical genes and clonal expansion. Then, if an immune response is not specific it makes no sense to refer it as "memory". So, the term "anticipatory response" could be more suitable in a system with an enhanced but unspecific response after secondary immune challenge (Kurtz, 2004). The specificity of molecules with potential memory function, which enable the host to adapt to infectious agents during its lifetime, needs further investigation. Until then, the term memory must be used with caution. That is why we consider adequate to use the term "immunological priming" to refer to any anticipatory

response with or without specific memory in insects (and invertebrates) (see also Schmid-Hempel, 2005b). Below we put forward some general mechanisms proposed as for how this priming could operate, however, these possible mechanisms need further investigation.

Lectin bindings (Kurtz & Franz, 2003), AMPs (Little *et al.*, 2003, Schmid-Hempel, 2005b) and PRRs (Kurtz, 2005) could be the molecules involved in the priming process. Lectins, through their high structural diversity, highly specific microorganism recognition, agglutination action and opsonization role in phagocytosis (Marques & Barraco, 2000), could be mediators for the enhancing of secondary immune responses. AMPs show great structural diversity (more than 170 isoforms have been found in insects; Bulet *et al.*, 1999) and are produced soon after foreigner recognition (1-4 hours) with a pathogen-specific and efficient killing action (Bulet *et al.*, 1999, Otvos Jr., 2000, Schmid-Hempel, 2005b). Although transcription of AMPs became turned off, some AMPs may remain in haemolymph for up to three weeks (Schmid-Hempel, 2005b), which is convenient during subsequent pathogen encounters. PRRs are differentially induced depending on the infectious agents, and particularly the recognition proteins secreted into the haemolymph could be molecules conferring specificity to invertebrate immune response.

It cannot be excluded the possibility of somatic gene alterations of immune molecules to enhance the response to infectious agents (Flajnik, Miller & Du Pasquier, 2003). In fact, current knowledge shows that some invertebrate IgSF proteins called Down syndrome cell adhesion molecule (Dscam; Watson *et al.*, 2005). Dscam proteins are used as opsonization factors that enhance the phagocytic efficiency of haemocytes, increasing host survival (Dong et al. 2006). Also, the challenge with different pathogens induces specific Dscam repertoires with different affinity, showing specificity. In snails, fibrinogen related proteins (Freps) somatically diversify (like Ig of vertebrates) and are produced in the haemolymph in the presence of pathogens (Zhang *et al.*, 2004). The genetic and

molecular mechanism underlying these rearrangements also needs further investigations. However, given the diversity of insect species, it cannot be exclude the presence of a greater diversity of mechanisms behind immunological memory.

Enhanced immunity of offspring from mothers that were immune stimulated, has been also reported. Mother AMPs (or lectins and PRRs), and also mRNA (Huang & Song, 1999), in a transcription factor-like mode, could be promoting transcriptional initiation of immune response genes. So, there can be heritable changes in immune response among generations that cannot be due to DNA sequences changes or genetic variation, i.e. inherited epigenetic variation (Richards, 2006). DNA methylation can modify histones. These modifications alter affinity of proteins that mediate transcription and affect interaction between nucelosomes and chromatin. As a consequence gene expression and phenotype could be affected (Richards, 2006). As a result heritable changes in gene expression could affect immune response outcome.Therefore, these molecules could be working as elicitors that could interact with the offspring embryonic cells inducing defense; so when immature stages start fighting against infectious agents, they are already primed, increasing survival chances (Rahman *et al.*, 2004).

This idea is not so radical, in distant organisms such as plants. Molinier *et al*. (2006) found that immune elicitors increased epigenetic somatic homologous recombination of a transgenic reporter which can persist in subsequent generations, which could potentially enhance offspring defenses. Although we are not proposing homologous recombination epigenetic change as the general mechanism of immune transgenerational priming, the fact is that transgenerational memory is not an exclusive property of vertebrates and therefore, can take place in different plant and animal groups.

Other relevant issue of priming is the high energetic cost of prolonged activation or synthesis of immune molecules at a higer level. Since pathogens can act as a factor that changes the host's internal environment, an optimal resource allocation is likely to change over time, partly because infections are unpredictable and their impact is not usually immediate. One can expect that the first contact with a pathogen not only enhances immune response, but changes the host's life history strategies. For example, reproductive events, before a secondary infection occurs. It has been widely documented that survival is impaired when hosts devote more resources to immune defense (Schmid-Hempel, 2005a). However, the direction of change in reproductive strategies under priming is unclear. If immune priming occurs, not only survival or immune response need to be measured, but other important trait intimately linked with reproduction (e.g. egg production).

QUANTITATIVE GENETICS OF THE IMMUNE RESPONSE

Traits closely related to fitness are commonly under natural or sexual selective pressures. Traits in ecological and evolutionary analyses are continuous variables and their variation is thought to be polygenic and intimately affected by the biotic and abiotic environment. These traits must be analyzed using quantitative genetics, by partitioning and estimation of variances and covariances into causal components (Falconer & Mackay, 1996) to understand how they respond to selection. Immune response occurs via a set of physiological traits and is supposed to show low additive variance and hence low heritability values (like other physiological traits, see Mousseau & Roff, 1987). Nevertheless, recent studies have shown that different immune components such as haemocyte load (Ryder & Siva-Jothy, 2001; Cotter, Kruuk & Wilson, 2004a; Rolff, Armitage & Coltman, 2005; Simmons & Roberts, 2005), antibacterial activity (Kurtz & Sauer, 1999; Cotter *et al*., 2004; Simmons & Roberts, 2005), PO activity (Hosken, 2001; Cotter & Wilson, 2002; Rolff *et al*., 2005; Schwarzenbach, Hosken & Ward, 2005) and melanization (Fellowes *et al*., 1998; Simmons & Roberts, 2005) tend to have high heritability (from 0.24 to 0.91), indicating high

genetic variance. There are at least three non-mutually excluding hypotheses to explain variance of immune response: host-pathogen co-adaptation cycles, genetic basis of trade-offs (genetic correlations), and phenotypic plasticity.

Host-pathogen co-adaptation cycles

Genes involved in immune resistance always show significant variance because of host-pathogen co-adaptation cycles (Hamilton & Zuk, 1982). The pathogen's short generational cycles may provide enough time to adapt to the host's immune response and given the number of infectious agents, there will be grounds for high genetic variability in immune responses. Variation will allow the host fighting against all varieties of infections produced by different pathogens. This has been used as the explanation for the maintenance of variation in SSTs which are under intense sexual selection (for example female choice; Kirkpatrick & Ryan, 1991). According to a perspective of sexual selection, if genetic variation of immune response is maintained, females (if female choice is the selective filter, but the same may apply for male-male competition and sexual conflict) will be able to continue exerting mate choice over SSTs (as these covariate with immune ability, reflecting male genetic quality), which will passed on to offspring (Rolff *et al.*, 2005).

Correlation among immune effectors

In an individual, different traits are frequently found to be genetic or phenotypically correlated. Genetic correlation arises because a single gene can influence multiple traits in a positive and negative fashion (pleiotropy and antagonistic pleiotropy, respectively) or because of linkage disequilibrium between genes affecting different characters (Falconer & Mackay, 1996). Meanwhile phenotypic correlations include the genetic causes and the positive or negative influences of environmental factors between traits (Roff, 1992). Negative correlations between immune responses and other life history traits (or intermediated traits) have been found and are

clearly detected under stressful conditions (usually resource limitation). However, trade-offs within immune system parameters are also possible. A number of studies have examined genetic and phenotypic correlations among encapsulation, lytic activity, cuticular darkness, PO activity and haemocyte load, showing an unclear pattern with positive and negative correlations (see Rantala & Kortet, 2003; Cotter *et al.*, 2004b; Fedorka *et al.*, 2004; Ryder & Siva-Jothy, 2004; Rantala & Roff, 2005; Roff *et al.*, 2005).

It must be considered that not all pathogens will induce the same reaction, or at least the magnitude of response can differ depending on the host-pathogen interaction type (coevolutionary history). So, genetic correlations, type and kind of pathogens could be closely related with the type and the intensity of correlations. The evolutionary trajectories of different immune responses can be difficult to predict when hosts are exposed to changing pathogens. Pathogens could differ spatially and temporarily in occurrence, burden and virulence so genetic/phenotypic correlations could change among generations. Therefore, pathogens to which different organisms have been exposed to may have played a role in the evolution of immune responses. Natural or sexual selection will be acting simultaneously on the different immune responses constraining their independent evolution, but probably with a fitness advantage for the host.

Phenotypic plasticity

As long as the organism has to deal with the problem of maximizing fitness in changing or stressful environments, a genetic background which can express changes in phenotype expression could have a selective advantage in that environment (Zhivotovsky, Feldman & Bergman, 1996). Occasionally, a single genotype can have the ability to produce distinct phenotypes when exposed to different environments, a phenomenon known as phenotypic plasticity (Schlichting, 1986; Roff, 1997; Nylin & Gotthard, 1998). Phenotypic plasticity could explain the maintenance of genetic

variance because plasticity uncouples the phenotype from genotype, buffering the impact of natural or sexual selection in the gene pool (Stearns, 1992), leading to a slow depletion of genetic variance. Phenotypic plasticity in quantitative traits therefore represents a genetic response to environmental heterogeneity. The quantitative and qualitative differences in immune response could be used as an indicator of the strategies followed by an organism in relation to environment stochasticity, and this is one reason why the evolution of immune response must be intimately related to the adaptive evolution to environmental heterogeneity. Despite the evidence that immune response can be strongly affected by the environment, really only a handful of studies have approached immune response using a phenotypic plasticity perspective. Related to this, Barnes & Siva-Jothy (2000) in beetles, Cotter *et al.* (2004b) in butterflies (reared at different population densities) and Mucklow & Ebert (2003) in water fleas, found phenotypic plasticity for mounting an immune response, concluding that investment in immunity depends on the infection probability based on the number of conspecifics, and that re-distribution of resources is adaptive. However, at the same time that some immune effectors are elevated at high population densities, while others are not. Since immune response is costly to express, this flexibility to cope with fluctuating environments could give fitness advantages.

The adaptive value of phenotypic plasticity depends on the ecological context in which it is expressed. Other ecological studies arguing the adaptive value of phenotypic plasticity (see Stirling, Roff & Fairbairn, 1999; Gebhardt & Stearns, 1993; David, Capy & Gauthier, 1990) have concluded that alternative strategies can be equally adaptive in some environments, but occasionally may not render advantages for the organism in other environments. Even the parental environment could have effects in the offspring behavioral phenotype (e.g. gregarious and solitary), related to immune response enhancing (Elliot, Blandford & Horton, 2003). It is therefore necessary to evaluate if different environments produce different phenotypes and if genotypes

respond differently to these environments (genotype-by-environment interaction, which represents genetic variation for phenotypic plasticity) and if these alternative phenotypes have fitness advantages.

Due to these reactions, phenotypic plasticity should be carefully considered in immunological studies, For example, it may explain biases in immune response between sexes (Joop & Rolff, 2004; McKean & Nunney, 2005), which may represent different male and female reproductive strategies. Sexual differences may not be genetic but simply a differential phenotypic expression under varying environment regimes. Phenotypic plasticity should be carefully analyzed in current immunocompetence hypotheses of male quality. It can be argued that a possible sexual selection cost of the expression of phenotypic plasticity is that the phenotypic traits may not be good indicators of male immunocompetence ability, because there is not a direct concordance between the phenotype, immune response and genotype (for analogous discussions see Adamo & Spiteri, 2005). Nevertheless in a broad sense immunocompetence could not only include the capacity of an individual to resist an infection but also the ability to produce distinct immune response phenotypes when exposed to different environments. This line of thought will enrich our comprehension of what male quality, in immunity terms, may mean and its interpretation in sexual selection studies.

Maternal Effects and Immunological Priming

Maternal effects arise when a mother's phenotype, which is intimately correlated with the environment she experiences, has a phenotypic effect on her offspring (Mousseau and Fox 1998). The local maternal environment might influence offspring phenotype, as maternal experiences may provide an indicator of the environmental conditions that offspring will face (Fox & Mousseau, 1998; Rossiter; 1996). if mothers have been exposed to infectious agents, information could be inherited (via maternal lectins, AMPs, PRRs ormRNA; already discussed previously), inducing

offspring phenotypic variation in immune defense (a kind of transgenerational phenotypic plasticity). Threre are four ecological conditions for transgenerational phenotypic plasticity in immune response to evolve: (1) infectious agents are variable and unpredictable on time, (2) a direct relation between cue and response exists, (3) induced defense is effective and (4) immune response is costly to express (adapted from Harvell, 1990; Harvell & Tollrain, 1999). For these reasons, maternally inherited effects have been proposed as the main explanation for transgenerational priming (Little *et al.*, 2003; Grindstaff, Brodie III & Ketterson, 2003; Sadd *et al.*, 2005; Moret, 2006). Again little evidence has been forwarded to support this.

Little & Kraaijeveld (2004) have proposed immunological priming is expected to evolve under high longevity and clonal reproduction. However, nearly any organism (clonal or sexual, short or long-lived) may be attacked repeatedly by a disease as this is temporally and spatially unpredictable. If, an infectious agent appears in the population, there is a high probability of being reinfected by the same agent and it is the same for the offspring; this is because as prevalence increases, the infectious agent also can increase in frequency (Moret & Siva-Jothy, 2003). Furthermore, immunological (transgenerational) priming can evolve by natural selection in gregarious or social organisms with low mobility and overlapping generations. Additionally, maternal inheritance can produce time lags in the response to selection, hence low depletion of genetic variance. The response to selection will depend on the kind of pathogens (and their virulence) and the evolutionary response for those in the previous generation, as well as the co-occurrence of the same pathogens in the current generation.

Heritability measures and artificial selection experiments (see Kraaijeveld & Godfray, 1997; Fellowes *et al.*, 1998; Rahman *et al.*, 2004), have shown that immune response can evolve. The phenotypic differences between individuals are related with differences in survival and

reproductive success. Natural or sexual selection will act on phenotypic variation, favoring some phenotypes. As long as the phenotypic variation has a genetic background, the population can evolve. The fact that the immune response is enhanced across generations necessarily implies that transgenerational immunological priming not only depends on the offspring's phenotype but on the mother's phenotype too (Kirkpatrick & Lande, 1989). So, variation among mothers in the molecule transmission capacity can also be under selective pressure.

Future studies are essential to understand the mechanisms of intra and intergenerational priming. Until these results are available and similar to what we discussed before with phenotypic plasticity, it is necessary to be careful when interpreting enhanced immunity ability during ontogeny and its heritability basis. As mentioned before, organisms and their offspring are under constant attack by infectious agents, which occasionally lead to infections. However, at other times, the host cannot totally eliminate the pathogen but, just keeps it "under control". This control can be due to the organism's ability to express different survival and reproductive strategies in response to stressful environmental conditions to which is exposed during its lifetime. Previous experience with infectious agents could also generate the expression of behaviors aimed to avoid negative effects of infections (see Loehle, 1995; Moore, 2002). For example behaviors to avoid areas with parasites (Hart, 1990), foraging behaviors for increase resource intake, and also physiological changes for the optimization of resource allocation.

CONCLUSIONS

This review illustrates some evolutionary and ecological principles that can direct the study and understand how the immune system fights infectious agents and how far we are from merging the evolutionary, ecological and physiological disciplines to understand the evolution of immune response. Evolutionary biologists have focused on quantifying phenotypic variation rather than on

understanding its nature this by means, the immune mechanisms, the underlying trait architecture and trade-offs. Ecological insights can be very relevant to understanding immune system dynamics and vice versa. Since immune system is highly complex, several aspects of immune response must be therefore integrated (e.g. immunopathology, immunological priming) to understand the evolutionary ecology of insect immunity. We have attempted to look at the available data on insect immune response pointing out some research avenues and careful considerations. The starting line in insect immunoecology must incorporate the great range of environmental factors involved using a trade-off framework. Resource allocation and investment strategies should be studied by taking into account differences between the sexes, evidence of the real cost in natural condition and their effects in the expression of immune response and generational changes. It must be also considered that host life history traits are evolving in response to selection pressures imposed by the type of pathogens and the interactions among them. Also the mechanisms, the metabolic pathways, synthesis and molecular properties of the products of the immune system cannot be ignored.

It is necessary to include a framework that integrates the host's strategies and the process of coevolution with parasites to understand the host immune response. Finally, it is highly desirable that ecologist and evolutionary biologist alike must incorporate useful molecular techniques (e.g. microassays, proteomics, quantitative Reverse Transcriptase-PCR, etc.) to study individual variation within a framework of population genomics and genetics to identify the mechanisms and the genetic basis of trade-offs between immunity and other life history traits.

REFERENCES

Able D. 1996. The contagion indicator hypothesis for parasite mediated sexual selection. *Proceedings of the National Academy of Sciences USA* **93**: 2229-2233.

Adamo SA. 1999. Evidence for adaptive changes in egg laying in crickets exposed to bacteria and parasites. *Animal Behaviour* **57**: 117-124.

Adamo SA, Jensen M, Younger M. 2001. Changes in lifetime immunocompetence in male and female *Gryllus texensis* (formerly *G. integer*): trade-offs between immunity and reproduction. *Animal Behaviour* **62**: 417-425.

Adamo SA, Spiteri RJ. 2005. Female choice for male immunocompetence: when is it worth it? *Behavioral Ecology* **16**: 871-879.

Agaisse H, Perrimon N. 2004. The roles of JAK/STAT signaling in *Drosophila* immune responses. *Immunological Reviews* **198**: 77-82.

Agnew P, Koella JC, Michalakis J. 2000. Host life history responses to parasitism. *Microbes and Infection* **2**: 891-896.

Andersson M. 1994. *Sexual Selection*. Princeton: Princeton University Press.

Barnes AI, Siva-Jothy MT. 2000. Density-dependent prophylaxis in the mealworm beetle *Tenebrio molitor* L. (Coleoptera: Tenebrionidae): cuticular melanization is an indicator of investment in immunity. *Proceedings of the Royal Society of London* **Ser B 267**: 177-182.

Björklund M. 1996. The importance of evolutionary constraints in ecological time scales. *Evolutionary Ecology* **10**: 423-431.

Boots M, Begon M. 1993. Trade-offs with resistance to a granulosis virus in the indian meal moth, examined by a laboratory evolution experiment. *Functional Ecology* **7**: 528-534.

Bulet P, Charlet M, Hetru C. 2003. Antimicrobial peptides in insect immunity. In Ezekowitz RAB, Hoffmann JA, eds. *Innate Immunity.* Totowa, New Jersey: Humana Press, 89-108.

Bulet P, Hetru C, Dimarq JL, Hoffmann D. 1999. Antimicrobial peptides in insects; structure and function. *Developmental and Comparative Immunology* **23**: 329-344.

Casteels P. 1998. Immune response in Hymenoptera. In Brey PT, Hultmark D, eds. *Molecular Mechanisms of Immune Responses in Insects.* London: Chapman & Hall, 92-110.

Cerenius L, Söderhäll K. 2004. The prophenoloxidase.activating system in invertebrates. *Immunological Reviews* **198**: 116-126.

Cheverud JM, Rutledge JJ, Atchley WR. 1983. Quantitative genetics of development: genetic correlations among age-specific trait values and the evolution of ontogeny. *Evolution* **37**: 895-905.

Christensen BM, Li J, Chen Ch-Ch, Nappi AJ. 2005. Melanization immune responses in mosquito vectors. *Trends in Parasitology* **21**: 192-199.

Clements AN. 1999. *The Biology of Mosquitoes.* Cabi, Wallingford.

Colasanti M, Salvati L, Venturini G, Ascenzi P. 2001. Cysteine protease as a target for nitric oxide in parasitic organisms. *Trends in Parasitology* **17**: 575.

Contreras-Garduño J, Canales-Lazcano J, Lanz-Mendoza H, Córdoba-Aguilar A. 2006. Wing pigmentation, immune ability, fat reserves and territorial status in males of the rubyspot damselfly, *Hetaerina americana. Journal of Ethology* **24**: 165-173.

Contreras-Garduño J, Lanz-Mendoza H, Córdoba-Aguilar A. 2007. The expression of a sexually selected trait correlates with different immune defense components and survival in males of the American rubyspot. *Journal of Insect Physiology* **53**: 612-621.

Cotter SC, Wilson K. 2002. Heritability of immune function in the caterpillar *Spodoptera littoralis. Heredity* **88**: 229-234.

Cotter SC, Kruuk LEB, Wilson K. 2004a. Costs of resistance: genetic correlations and potential trade-offs in an insect immune system. *Journal of Evolutionary Biology* **17**: 421-429.

Cotter SC, Hails RS, Cory JS, Wilson K. 2004b. Density-dependent prophylaxis and condition-dependent immune function in Lepidopteran larvae: a multivariate approach. *Journal of Animal Ecology* **73**: 283-293.

Darwin C. 1871. *The Descent of Man and Selection in Relation to Sex*. John Murray, U. K.

David JR, Capy P, Gauthier JP. 1990. Abdominal pigmentation in growth temperature in *Drosophila melanogaster*: similarities and differences in the norms of reaction of successive segments. *Journal of Evolutionary Biology* **3**: 429-445.

De Jong G, Van Noordwijk AJ. 1992. Acquisition and allocation of resources: genetic (co)variances, selection, and life histories. *American Naturalist* **139**: 749-770.

De Roode JC, Read AF, Chan BHK, Mackinnon MJ. 2003. Infection dynamics and virulence of three-clone infections with the rodent malaria parasite *Plasmodium chabaudi*. *Parasitology* **127**: 411-418.

Dong, Y., H. E. Taylor, and G. Dimopoulos. 2006. AgDscam, a Hypervariable Immunoglobulin Domain-Containing Receptor of the Anopheles gambiae Innate Immune System. PLoS Biol 4:e229.

Ebert D. 1998. Experimental evolution of parasites. *Science* 282: 1432-1435.

Elliot SL, Blanford S, Horton CM. 2003. Fever and phenotype: transgenerational effect of disease on desert locust phase state. *Ecology Letters* **6**: 830-836.

Falconer DS, Mackay TF. 1996. *Introduction to Quantitative Genetics*. New York: Dover.

Faulhaber LM, Karp RD. 1992. A diphasic immune response against bacteria in the American cockroach. *Immunology* **75**: 378-381.

Fedorka KM, Zuk M. 2005. Sexual conflict and female immune suppression in the cricket, *Allonemobious socius. Journal of Evolutionary Biology* **18**: 1515-1522.

Fedorka KM, Zuk M, Mousseau TA. 2004. Immune suppression and the cost of reproduction in the ground cricket, *Allonemobius socius. Evolution* **58**: 2478-2485.

Fellowes MDE, Kraaijeveld AR, Godfray HCJ. 1998. Trade-off associated with selection for increased ability to resist parasitoid attack in *Drosophila melanogaster. Proceedings of the Royal Society of London* **Ser B 265**: 1553-1558.

Fessler DMT, Navarrete CD, Hopkins W, Izard MK. 2005. Examining the terminal investment hypothesis in humans and chimpanzees: associations among maternal age, parity and birth weight. *American Journal of Physical Anthropology* **127**: 95-104.

Flajnik MF, Miller K, Du Pasquier L. 2003. Evolution of the immune system. In Paul WE, ed. *Fundamental Immunology.*US: Lippincott Williams, 519-570.

Flatt T, Tu MP,Tatar M. 2005. Hormonal pleiotropy and the juvenile hormone regulation of *Drosophila* development and life history. *BioEssays* **27**: 999-1010.

Folstad I, Karter AJ. 1992. Parasite, bright males and the immunocompetence handicap. *American Naturalist* **139**: 603-622.

Forbes MR. 2007. On sex differences in optimal immunity. *Trends in Ecology and Evolution* **22**: 111-1113.

Fox CW, Mousseau TA. 1998. Maternal effects as adaptations for transgenerational phenotypic plasticity (TPP).In Mousseau TA, Fox CW, eds. *Maternal Effects as Adaptations*. Oxford: Oxford University Press, 159-177.

Frank SA. 1996. Models of parasite virulence. *Quarterly Review of Biology* **71**: 37-78.

Gebhardt MD, Stearns SC. 1993. Phenotypic plasticity for life history traits in *Drosophila melanogaster.* I. Effect on phenotypic and environment correlations. *Journal Evolutionary Biology* **6**: 1-16.

Gillespie JP, Kanost MR, Trenzeck T. 1997. Biological mediators of insect immunity. *Annual Review of Entomology* **42**: 611-643.

Graham AL, Allen JE, Read AF. 2005. Evolutionary causes and consequences of immunopathology. *Annual Review of Ecology Evolution and Systematics* **36**: 373-397.

Gregoire C. 1974. Hemolymph coagulation. In Rockstein M, ed. *The physiology of Insecta*. Vol. 5. Boca Raton, FL: Academic Press, 309-360.

Grindstaff JL, Brodie III ED, Ketterson ED. 2003. Immune function across generations: integrating mechanism and evolutionary process in maternal antibody transmission. *Proceedings of the Royal Society of London* **Ser B 270**: 2309-2319.

Hamilton WD, Zuk M. 1982. Heritable true fitness and bright birds: a role for parasites? *Science* **218**: 384-387.

Hart BL. 1990. Behavioral adaptation to pathogens and parasites: five strategies. *Neuroscience and Biobehavioral Review* **14**: 273-284.

Harvell CD. 1990. The ecology and evolution of inducible defences. *Quarterly Review of Biology* **65**: 323-340.

Harvell CD, Tollrain R. 1999. Why inducible defences? In Tollrain R, Harvell CD, eds. *The Ecology of Inducible Defences.* Princeton, New Jersey: Princeton University Press, 3-9.

Hillyer H, Schmidt SL, Christensen BM. 2006. The antibacterial innate immune response by the mosquito *Aedes aegypti* is mediated by hemocytes and independent of Gram type and pathogenicity. *Microbes and Infection* **6**: 448-459.

Hoang A. 2001. Immune response to parasitism reduces resistance of *Drosophila melanogaster* to desiccation and starvation. *Evolution* **55**: 2352-2358.

Hoffmann JA. 2003. The immune response of *Drosophila. Nature* **426**: 33-38.

Hoffmann JA, Kafatos FC, Janeway Jr. CA, Ezekowitz RAB. 1999. Phylogenetic perspectives in innate immunity. *Science* **284**: 1313-1318.

Hoffmann JA, Ligoxygakis P. 2004. *Drosophila* response to microbial infection: an overview. In Kaufmann SHE, Medzhitov R, Gordon S, eds. *The Innate Immune Response to Infection.* Washington, D. C.: ASM Press, 31-46.

Hosken DJ. 2001. Sex and death: microevolutionary trade-offs between reproductive and immune investment in dung flies. *Current Biology* **11**: 379-380.

Huang CC, Song YL. 1999. Maternal transmission of immunity to white spot syndrome associated virus (WSSV) in shrimp (*Penaeus monodon*). *Developmental and Comparative Immunology* **23**: 545-552.

Iwanaga S, Lee BL. 2005. Recent advances in the innate immunity of invertebrate animals. *Journal of Biochemistry and Molecular Biology* **38**: 128-150.

Iwasa Y, Pomiankowski A, Nee S. 1991. The evolution of costly mate preference. II. The handicap principle. *Evolution* **45**: 1431-1442.

Iwasa Y, Pomiankowski A. 1994. The evolution of male preference for multiple handicaps. *Evolution* **48**: 853-867.

Janeway Jr. CA, Medzhitov R. 2002. Innate immune recognition. *Annual Review of Immunology* **20**: 197-216.

Joop G, Rolff J. 2004. Plasticity of immune function and condition under the risk of predation and parasitism. *Evolutionary and Ecological Research* **6**: 1051-1062.

Khush RS, Leulier F, Lemaitre B. 2002. Pathogen surveillance- the flies have it. *Science* **296**: 273-275.

Kirkpatrick M, Ryan MJ. 1991. The evolution of mating preferences and the paradox of lek. *Nature* **350**: 33-38.

Kirkpatrick M, Lande R. 1989. The evolution of maternal characters. *Evolution* **43**: 485-503.

Klein J. 1997. Homology between immune responses in vertebrates and invertebrates: does it exist? *Scandinavian Journal Immunology* **46**: 558-564.

Kotiaho J. 2001. Costs of sexual traits: a mismatch between theoretical considerations and empirical evidence. *Biological Reviews* **76**: 365-376.

Kraaijeveld AR, Godfray HCJ. 1997. Trade-off between parasitoid resistance and larval competitive ability in *Drosophila melanogaster. Nature* **389**: 278-280.

Kurtz J. 2004. Memory in the innate and adaptive immune systems. *Microbes and Infection***6**: 1410-1417.

Kurtz J. 2005. Specific memory within innate immune systems. *Trends in Immunology* **26**: 186-192.

Kurtz J, Franz K. 2003. Evidence for memory in invertebrate immunity. *Nature* **425**: 37-38.

Kurtz J, Sauer KP. 1999. The immunocompetence handicap hypothesis: testing the genetic predictions. *Proceedings of the Royal Society of London***Ser B266**: 2515-2522.

Leclerc V, Reichart J-M. 2004. The immune response of *Drosophila melanogaster.Immunological Reviews* **198**: 59-71.

Lemaitre B, Reichdart JM, Hoffmann JA. 1997. *Drosophila* host defense: Differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. *Proceedings of the National Academy of Science USA* **94**: 14614-14619.

Li J, Christensen BM. 1993. Involvement of L-tyrosine and phenoloxidase in the tanning of *Aedes aegypti* eggs. *Insect Biochemistry and Molecular Biology* **23**: 739-748.

Little TJ, Kraaijeveld AR. 2004. Ecological and evolutionary implications of immunological priming in invertebrates. *Trends in Ecology and Evolution* **19**: 58-60.

Little TJ, Hultmark D, Read AF. 2005. Invertebrate immunity and the limits of mechanistic immunology. *Nature Immunology* **6**: 651-654.

Little TJ, O'Connor B, Colegrave N, Watt K, Read AF. 2003. Maternal transfer of strain-specific immunity in an invertebrate. *Current Biology* **13**: 489-492.

Loehle C. 1995. Social barriers to pathogen transmission in wild animal populations. *Ecology* **76**: 326-335.

Manetti AGO, Rosetto M, Marchini D. Antibacterial peptides of the insect reproductive tract. In Brey PT, Hultmark D, eds. *Molecular Mechanisms of Immune Responses in Insects.* London: Chapman & Hall, 67-91.

Marques MRF, Barracco MA. 2000. Lectins, as non-self-recognition factors, in crustaceans. *Aquaculture* **191**: 23-44.

May RM, Anderson RM. 1983. Epidemiology and genetics in the coevolution of parasites and hosts. *Proceedings of the Royal Society of London* **Ser B 219**: 281-313.

McGraw LA, Gibson G, Clark AG. 2004. Genes regulated by mating, sperm, or seminal proteins in mated female *Drosophila melanogaster. Current Biology* **14**: 1509-1514.

McKean KA, Nunney L. 2001. Increased sexual activity reduces male immune function in *Drosophila melanogaster*. *Proceedings of the National Academy of Science USA* **98**: 7904-7909.

McKean KA, Nunney L. 2005. Bateman's principle and immunity: phenotypically plastic reproductive strategies predict changes in immunological sex differences. *Evolution* **59**: 1510-1517.

McNamara JM, Houston AI. 1996. State dependent life histories. *Nature* **380**: 215-221.

Molinier J, Ries G, Zipfel, C, Hohn B. 2006. Transgeneration memory of stress in plants. *Nature* **442**: 1046-1049.

Moore J. 2002. *Parasites and the Behavior of Animals*. New York: Oxford University Press.

Moret Y. 2006. 'Trans-generational immune priming': specific enhancement of the antimicrobial immune response in the mealwormbeetle, *Tenebrio molitor. Proceedings of the Royal Society* **B 273**: 1399-1405.

Moret Y, Siva-Jothy MT. 2003. Adaptive innate immunity? Responsive-mode prophylaxis in the mealworm beetle, *Tenebrio molitor. Proceedings of the Royal Society of London* **Ser B 270**: 2475-2480.

Moret Y, Schmid-Hempel P. 2000. Survival for immunity: the price of immune system activation for bumblebee workers. *Science* **290**: 1166-1168.

Moret Y, Schmid-Hempel P. 2001. Immune defence in bumble-bee offspring. *Nature* **414**: 506.

Mousseau TA, Fox CW. 1998. The adaptive significance of maternal effects. *Trends in Ecology and Evolution* **13**: 403-407.

Mousseau TA, Roff DA. 1987. Natural selection and the heritability of fitness components. *Heredity* **59**: 181-197.

Mucklow PT, Ebert D. 2003. Physiology of immunity in the water flea *Daphnia magna*: environmental and genetics aspects of phenoloxidase activity. *Physiological and Biochemical Zoology* **76**: 836-842.

Müller U. 1997. The nitric oxide system in insects. *Progress in Neurobiology* **51**: 363–381.

Nappi AJ, Christensen BM. 2005. Melanogenesis and associated cytotoxic reactions: applications to insect innate immunity. *Insect Biochemistry and Molecular Biology* **35**: 443-459.

Nappi AJ, Ottaviani E. 2000. Cytotoxicity and cytotoxic molecules in invertebrates. *BioEssays* **22**: 469-480.

Nappi AJ, Vass E, Frey F, Carton Y. 2000. Nitric oxide involvement in *Drosophila* immunity. *Nitric Oxide***4**: 423-430.

Narayanan K. 2004. Insect defence: its impact on microbial control of insect pests. *Current Science* **86**: 800-814.

Núńez-Farfán J. 1993. Selección natural en el campo: revisión de la evidencia reciente. In Núńez-Farfán J, Cordero C, eds. *Tópicos de Biología Evolutiva*. México: Centro de Ecología, UNAM, 19-59.

Nylin S, Gotthard K. 1998. Plasticity in life-history traits. *Annual Review of Entomology* **43**: 63-83.

Osanai M, Chen P. 1993. A comparative study on the arginine degradation cascade for sperm. *Amino Acids* **5**: 341-350.

Otvos Jr. L. 2000. Antibacterial peptides isolated from insects. *Journal of Peptide Science* **6**: 497-511.

Owens IDF, Wilson K. 1999. Immunocompetence: a neglected life history trait or conspicuous red herring? *Trends in Ecology and Evolution* **14**: 170-172.

Pfennig HS. 2001. Evolution of pathogen virulence: the role of variation in host phenotype. *Proceedings of the Royal Society* **Ser B 268**: 755-760.

Pham LN, Dionne MS, Shirasu-Hiza M, Schneider DS. 2007. A specific primed immune response in *Drosophila* is dependent on phagocytes. *PLoS Pathology* **3**: e26.

Qiu P, Pan PC, Govind S. 1998. A role for the *Drosophila* Toll/Cactus pathway in larval hematopoiesis. *Development* **125**: 1909-1920.

Rahman MM, Roberts HLS, Sarjan M, Asgari S, Schmidt O. 2004. Induction and transmission of *Bacillus thuringiensis* tolerance in the flour moth *Ephestia kuehniella*. *Proceedings of the National Academy of Science USA* **101**: 2696-2699.

Rantala MJ, Kortet R. 2003. Courtship song and immune function in the field cricket *Gryllus bimaculatus. Biological Journal of the Linnean Society* **79**: 503-510.

Rantala MJ, Roff DA. 2005. An analysis of trade-offs in immune function, body size, and developmental time in the Mediterranean Field Cricket, *Gryllus bimaculatus. Functional Ecology* **19**: 323-330.

Rantala MJ, Kortet J, Kotiaho J, Vainikka A, Suhonen J. 2003. Condition dependence of pheromones and immune function in the grain beetle *Tenebrio molitor. Functional Ecology* **17**: 534-540.

Rantala MJ, Vainikka A, Kortet R. 2003. The role of juvenile hormone in immune function and pheromone production trade-offs: a test of the immunocompetence handicap principle. *Proceedings of the Royal Society of London* **Ser B 270**: 2257–2261.

Read AF, Taylor LH. 2001. The ecology of genetically diverse infections. *Science* **292**: 1099-1102.

Reznick D, Nunney L, Tessier A. 2000. Big houses, big cars, superfleas and the costs of reproduction. *Trends in Ecology and Evolution* **15**: 421-425.

Richards EJ. 2006. Inherited epigenetic variation: revisiting soft inheritance. *Nature Reviews Genetics* **7**: 395-401.

Rigby M, Jokela J. 2000. Predator avoidance and immune defence: costs and trade-offs in snails. *Proceedings of the Royal Society of London* **B267**: 171-176.

Rivero A. 2006. Nitric oxide: an antiparasitic molecule of invertebrates. *Trends in Parasitology* **22**: 219-225.

Roff DA. 1992. *The Evolution of Life Histories.* New York: Chapman & Hall.

Roff DA. 1997. *Evolutionary Quantitative Genetics*. New York: Chapman & Hall.

Rolff J. 2002. Bateman's principle and immunity. *Proceedings of the Royal Society of London* **Ser B 269**: 867-872.

Rolff J, Siva-Jothy M. 2002. Copulation corrupts immunity: a mechanism for a cost of mating in insects. *Proceedings of the National Academy of ScienceUSA***99**: 9916-9918.

Rolff J, Armitage SAO, Coltman DW. 2005. Genetic constraints and sexual dimorphism in immune defence. *Evolution* **59**: 1844-1850.

Rossiter M. 1996. Incidence and consequences of inherited environmental effects. *Annual Review of Ecology and Systematics* **27**: 451-476.

Ryder JJ, Siva-Jothy MT. 2001. Quantitative genetics of immune function and body size in the house cricket, *Acheta domesticus. Journal of Evolutionary Biology* **14**: 646-653.

Ryder JJ, Siva-Jothy MT. 2000. Male calling song provides a reliable signal of immune function in a cricket. *Proceedings of the Royal Society of London* **Ser B 267**: 1171-1175.

Sadd BM, Schmid-Hempel P. 2006. Insect immunity shows specificity in protection on secondary pathogen exposure. *Current Biology* **16**: 1206-1210.

Sadd B, Holman L, Armitage H, Lock F, Marland R, Siva-Jothy MT. 2006.Modulation of sexual signalling by immune challenged male mealworm beetles (*Tenebrio molitor*, L.): evidence for terminal investment and dishonesty. *Journal of Evolutionary Biology* **19**: 321-325.

Sadd BM, Kleinlogel Y, Schmid-Hempel R, Schmid-Hempel P. 2005. Trans-generational immune priming in a social insect. *Biology Letters* **1**: 386-388.

Sandland GJ, Minchella DJ. 2003a. Effects of diet and *Echinostoma revolutum* infection on energy allocation patterns in juvenile *Lymnaea elodes* snails. *Oecologia* **134**: 479-486.

Sandland GJ, Minchella DJ. 2003b. Costs of immune defence: an enigma wrapped in an environmental cloak? *Trends in Parasitology* **19**: 571-574.

Schlichting CD. 1986. The evolution of phenotypic plasticity in plants. *Annual Review of Ecology and Systematics* **17**: 667-693.

Schlichting CD, Pigliucci M. 1998. *Phenotypic Evolution: A Reaction Norm Perspective*. Sunderland, Massachusetts: Sinauer Associates.

Schmid-Hempel P. 2003. Variation in immune defence as a question of evolutionary ecology. *Proceedings of the Royal Society of London* Ser **B 270:** 357-366.

Schmid-Hempel P. 2005a. Evolutionary ecology of insect immune defences. *Annual Review of Entomology***50**: 529-551.

Schmid-Hempel P. 2005b. Natural insect host-parasite systems show immune priming and specificity: puzzles to be solved. *BioEssays* **27**: 1026-1034.

Schmid-Hempel R, Schmid-Hempel P. 1998. Colony performance and immunocompetence of a social insect, *Bombus terrestris*, in poor and variable environments. *Functional Ecology* **12**: 22-30.

Schwarzenbach GA, Hosken DJ, Ward PI. 2005. Sex and immunity in the yellow dung fly *Scathophaga stercoraria. Journal of Evolutionary Biology* **18**: 455-463.

Serrano-Meneses MA, Sánchez-Rojas G, Córdoba-Aguilar A. 2007. Sexual selection as the possibel underlying force in the calopterygid wing pigmentation: comparative evidence with *Hetaerina* and *Calopteryx* genera. *Odonatologica* **in press**

Sheldon BC, Verhulst S. 1996. Ecological immunology: costly parasite defences and trade offs in evolutionary ecology. *Trends in Ecology and Evolution***11**: 317-321.

Sheridan LAD, Poulin R, Ward DF, Zuk M. 2000. Sex differences in parasitic infections among arthropod hosts: is there a male bias? *Oikos* **88**: 327-334.

Simmons LW, Roberts B. 2005. Bacterial immunity traded for sperm viability in male crickets. *Science* **309**: 2031.

Siva-Jothy MT. 2000. A mechanistic link between parasite resistance and expression of a sexually selected trait in a damselfly. *Proceedings of the Royal Society of London* **Ser B 267**: 2523-2527.

Söderhäll K, Cerenius L. 1998. Role of the prophenoloxidase-activating system in invertebrate immunity. *Current Opinion in Immunology* **10**: 23-28.

Stearns SC. 1976.Life-history tactics: a review of the ideas.*The Quarterly Review of Biology* **51**: 3-47.

Stearns SC. 1992. *The Evolution of Life Histories*. Oxford: Oxford University Press.

Stearns SC. 2000. Life history evolution: successes, limitations, and prospects. *Naturwissenschaften* **87**: 476-486.

Stirling G, Roff D, Fairbairn D. 1999. Four characters in a trade-off: dissecting their phenotypic and genetic correlations. *Oecologia* **120**: 492-498.

Stoehr AM, Kokko H. 2006. Sexual dimorphism in immunocompetence: what does life-history theory predict? *Behavioral Ecology* **17**: 751-756.

Tu MP, Flatt T, Tatar M. 2005. Juvenile and steroid hormones in *Drosophila melanogaster* longevity. In: Masoro EJ, Austad SN, eds. *Handbook of the biology of aging*. 6th edition. San Diego: Academic Press (Elsevier), 415-448.

Tzou P, De Gregorio E, Lemaitre B. 2002. How *Drosophila* combats microbial infection: a model to study innate immunity and host-pathogen interactions. *Current Opinion in Microbiology* **5**: 102-110.

Uchida K. 1993. Balanced amino acid composition essential for infusion-induced egg development in the mosquito (*Culex pipiens pallens*). *Journal of Insect Physiology* **39**: 615-621.

Van Baalen M. 1998. Coevolution of recovery ability and virulence. *Proceedings of the Royal Society of London* **Ser B 265**: 317-325.

Van Noordwijk AJ, de Jong G. 1986. Acquisition and allocation of resources: their influence on variation in life history tactics. *American Naturalist* **128**: 137-142.

Watson FL, Püttmann-Holgado R, Thomas F, Lamar DL, Hughes M, Kondo M, Rebel VI, Schmucker D. 2005. Extensive diversity of Ig-Superfamily proteins in the immune system of insects. *Science* **309**: 1874-1878.

Williams GC. 1966. Natural selection, the cost of reproduction and a refinement of Lack's principle. *American Naturalist* **100**: 687-690.

Wilson R, Chen CW, Ratcliffe NA. 1999. Innate immunity in insects: the role of multiple, endogenous serum lectins in the recognition of foreign invaders in the cockroach, *Blaberus discoidalis*. *Journal of Immunology* **162**: 1590-1596.

Zaidman-Rémy A, Hervé M, Poidevin M, Pili-Floury S, Kim MS, Blanot D, Oh BH,Ueda R, Mengin-Lecreulx D, Lemaitre B. 2006. The *Drosophila* amidase PGRP-LB modulates the immune response to bacterial infection. *Immunity* **24**: 463-473.

Zhang SM, Adema CM, Kepler TB, Loker ES. 2004. Diversification of Ig superfamily genes in an invertebrate. *Science* **305**: 251-254.

Zhivotovsky LA, Feldman MW, Bergman A. 1996. On the evolution of phenotypic plasticity in a spatially heterogeneous environment. *Evolution* **50**: 547-558.

Zuk M, McKean KA. 1996. Sex differences in parasite infections: patterns and processes. *International Journal of Parasitology* **26**: 1009-1024.

Running head: Anticipatory immune response in *Aedes aegypti*

Anticipatory immune response in *Aedes aegypti* against bacterial challenges and its effects on female reproduction

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26 **Abstract**

27 In insects exposure, to pathogens induces an anticipatory immune response, a phenomenon
28 called immune priming. However, little is known of whether immune effectors improve their
29 efficiency with time; the temporal dynamics of immune enhancement; and, its reproduction
30 costs. In this study, we demonstrate that priming (using live bacteria) protects the mosquito
31 *Aedes aegypti* against a lethal second challenge. We experimentally reveal the temporal
32 dynamics of phenoloxidase (a key immune effector) measurement which suggests a
33 deactivation rather than activation; this may be due to host use of the enzyme to control
34 recurrent infection with live bacteria. Meanwhile, nitric oxide (another key immune effector)
35 increased to some extent after second challenge. Finally, that egg production was not affected
36 during the priming dose and second challenge; however, the number of egg laying females
37 with two recurrent infections (i.e. priming dose+second challenge) was lower compared to the
38 unprimed groups. Our study not only corroborates the presence of insect immune priming, but
39 also documents the point that not all immune parameters can be enhanced when hosts have had
40 previous contact with pathogens. Immune priming may be considered not only as an enhanced
41 response, but an optimal response to protect the organisms after a previous experience with an
42 elicitor.

43

44 **Keywords**: immunological priming, phenoloxidase, nitric oxide, *Aedes aegypti*

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52 **Introduction**

53 It is widely acknowledged that, unlike vertebrates, insects do not have mechanisms for specific
54 Ig production, rearrangement of somatic genes and clonal expansion (Janeway, 2005). Also,
55 there are no molecules with potential specific immune memory function (but see Zhang *et al.*,
56 2004; Watson *et al.*, 2005), which enable the host to adapt to infectious agents during its
57 lifetime. However, it can be safely assumed that a vast majority of insects are attacked
58 repeatedly by the same pathogen because the incidence of the latter is temporally and spatially
59 unpredictable. If, for example, an infectious agent appears in the population, there is a high
60 probability of a host being re-infected by the same agent. This is because prevalence of the
61 infectious agent increases in the host population (Moret & Siva-Jothy, 2003). Thus, the ability
62 of an anticipatory immune response in non-vertebrate organisms could represent an adaptive
63 characteristic to such environmental selective pressure.
64

65 Despite the lack of the physiological and biochemical machinery that might allow for an
66 adaptive immune response in insects, it has been long recognized that immunization with
67 killed or living bacteria can induce an anticipatory immune response in insects (Boman et al,
68 1972). This fact has been recently supported by experimental observations (Faulhaber & Karp,
69 1992; Moret & Schmid-Hempel, 2001; Kurtz & Franz, 2003; Moret & Siva-Jothy, 2003). The
70 phenomenon has been coined as immune priming (Little & Kraaijeveld, 2004; Little, Hultmark
71 & Read, 2005; Kurtz, 2004; Schmid-Hempel, 2005a, b) and assumes that a previous
72 experience with an elicitor might raise an enhance immune response when re-exposed to the
73 same agent (Pham & Schneider, 2008).
74

75 Protective enhancement of immune response due to priming (i.e. prolonged activation or
76 increased levels of previously immune stimulated organisms) has been assessed using different
77 response variable such as survival, behavior, reproductive capacity, and different immune

78 effectors (phagocytosis, antimicrobial peptide load and synthesis, and antimicrobial and
79 phenoloxidase activity) (Deverno et al 1983, Adamo, 1998, Rosengaus et al 1999, Brown et al
80 2003, Little et al 2003, Sadd & Schmid-Hempel 2006, Pham et al 2007). In these studies,
81 immune response became more effective upon repeated exposure to the elicitor, an effect that
82 probably persists during the host's lifetime (see model in Fig. 1). However, there is
83 controversy with respect to whether such efficiency applies to all immune effectors. For
84 example, Pham *et al* (2007) found that hemocytes, through phagocytosis, are responsible for
85 an enhancement effect, which was not the case for antimicrobial peptides or the
86 prophenoloxidase (proPO) system. One potential reason as for why immune effectors
87 apparently vary in effectiveness is that they may be better explained by methodological rather
88 than physiological factors. In particular, the moment of quantification of immune parameters
89 should be controlled, while characterizing the dynamics of immune response after a priming
90 dose. This will detect how fast improvement (if it occurs) is acquired. To our knowledge this
91 information has not been gathered by previous studies.
92
93 Priming has been referred to as either as "short" (hours, or few days) or "long" (weeks or
94 lifetime) in terms of immune protection persistence (Pham & Schneider, 2008). However these
95 definitions have yet to be concretely established, as several studies have reported different
96 results. One explanation for these inconsistencies is the variable period of time elapsed
97 between challenges, as well as the nature of the immune response being evaluated in these
98 cases. For example, in the lepidopteran *Heliothis virescens*, a second re-infection was
99 performed at 48 hrs after a priming dose, but immune enhancement reached only 3% after 12
100 hrs (Ourth & Parker, 2006); thus, these authors concluded that in this species priming is not
101 elicited. In this specific case, however, it is likely that the second challenge overlapped with
102 the response generated by the priming dose, avoiding the results of the prime effect. Related to
103 this explanation, in the moth *Galleria mellonella*, humoral antibacterial activity was noted 5 hr

104 after infection, reaching a maximum level at 18-48 hrs post infection, and decreasing over a
105 few days (Jarosz, 1993; Andrejko et al, 2009). Conversely, in other species, immune
106 parameters such as hemocytes and the PO system are very rapidly activated but quickly turned
107 down (Schmid-Hempel & Ebert 2003). Therefore, if priming occurs in these other species, the
108 second challenge and quantification of immune parameters should be performed after the
109 overall immune recovery period (Fig. 1). In general, all these results mean that the nature of
110 immune response time dynamics needs to be taken into account when looking for immune
111 priming effects.
112
113 Another important, yet unexplored, aspect relevant to the time of inducible protection is the
114 high energetic cost of prolonged activation or synthesis of immune molecules. A high
115 energetic cost is expected, as the required resources used for immune response can also be
116 demanded for the expression of other traits linked with survival and reproduction (Sheldon &
117 Verhulst, 1996, Roff, 1992), as predicted by the resource allocation theory (Stearns, 1992).
118 Modulation of the immune response can be expected after previous challenge in order to an
119 optimal adjustment of resources used in immunity and other traits (see Pham & Schneider
120 2008 for a similar claim) (Fig. 1). Then, a recovery period can be expected for resource
121 acquisition and a suitable enhanced immune response.
122
123 Organisms must physiologically "decide" how to allocate available resources to maximize
124 fitness. An optimal allocation depends on the strategies used to increase fitness in the habitat
125 of the organism (Schlichting & Pigluicci, 1998). Since pathogens can act as a factor that
126 changes the host's internal environment, an optimal resource allocation is likely to change
127 over time, partly because infections are unpredictable and their impact is not usually
128 immediate. One can expect that the first contact with a pathogen not only activates immune
129 response, but changes the host's life history strategies, for example, reproductive events,

130 before a secondary infection occurs. It has been widely documented that survival is impaired
131 when hosts devote more resources to immune defense (reviewed by Schmid-Hempel, 2005a).
132 However, the direction of changes in reproductive strategies under recurrent infections is
133 unclear.
134
135 Using *Aedes aegypti* Linnaeus (Diptera: Culicidae), the principal vector of Dengue and Yellow
136 Fever virus, as our study subject, we have first documented its survival following a challenge
137 with a lethal dose of the bacterium 7 days after being previously primed with a non lethal dose.
138 We consider this period of time long enough to detect an effect (if it exists) between the
139 priming dose and the second challenge. This period was based on information about the
140 activation and synthesis of immune molecules in insects (for example, antimicrobial peptide
141 transcription has a range from 6 hrs to 72 hrs after challenge; PO activation ; (Jarosz, 1993;
142 Lemaitre et al 1997; Uttenweiler-Joseph et al 1998; Schmid-Hempel & Ebert 2003; Andrejko
143 et al, 2009). Second, we have documented the temporal dynamics of two immune parameters
144 during the priming dose period and also after a second challenge. The immune parameters
145 used were phenoloxidase activity (PO) and nitric oxide production (NO), two key components
146 during insect immune defense. In insects, PO (an oxidoreductase enzyme), catalyzes the
147 transformation of tyrosine into dopa and then to dopaquinone (Söderhäll & Cerenius, 1998).
148 After a number of non-enzymatic polymerizations, dopaquinone is finally converted to
149 melanin which is used for wrapping up pathogens and wound clotting (Nappi & Christensen,
150 2005). Also, during the PO cascade highly reactive and toxic molecules are produced (opsonic
151 factors, reactive oxygen species, quinones and semiquinones) (Cerenius & Söderhäll, 2004;
152 Nappi & Ottovianni, 2000). NO is a highly reactive and unstable free radical gas produced
153 during the oxidation of L-arginine to L-citrulline by the nitric oxide synthase (NOS) (Müller,
154 1997). NO crosses cell membranes to act on nearby targets (Müller, 1997), inhibit protein
155 catalytic activity and harms protein and pathogen DNA (Colasanti *et al.*, 2001; Rivero, 2006).

156 Finally, and in terms of changes in reproductive strategies and resource allocation theory, we
157 have documented the number of eggs produced and the proportion of egg laying females with
158 and without a priming dose and with recurrent immune challenges
159
160
161 **Materials and Methods**
162 Mosquitoes were reared under insectary conditions at the Instituto Nacional de Salud
163 Pública(INSP), Cuernavaca, Mexico (the colony stock has been maintained at over 2000
164 individuals per generation with random mating). Three day-old adult female mosquitoes were
165 used at the beginning of each experiment. We used live or heat killed *Serratia marcescens* and
166 *Escherichia coli* (both Gram-negative bacteria). Both bacteria were kindly donated by Lilia
167 González Cerón (Centro de Investigaciones en Paludismo, INSP) and Jesús Silva (Centro de
168 Investigaciones Sobre Enfermedades Infecciosas, INSP) respectively. Bacteria were grown
169 overnight in LB-broth at 37°C while shaking at 200 RPM until they reached stationary phase.
170 To determine bacterial concentrations, 100 µl of a 1/1000, 1/10,000 or 1/100,000 bacterial
171 culture was spread on LB agar plates. Plates were grown overnight at 37°C and the colony
172 forming units (CFU) were counted. LD doses were previously determined by inoculating
173 separate groups of mosquitoes with serial bacterial dilutions and selecting the dose closest to
174 killing 100%, 50% , <10% of the animals. We recorded mosquito mortality during the next 24
175 hours after bacterial injection.
176
177 *Survival experiment*
178 For the survival analyses, two experiments were carried out. In the first experiment, three days
179 old adult female mosquitoes were randomly divided into four groups of 100 individuals each:
180 control and three experimental groups. For the priming dose, mosquitoes of group one (RPMI-
181 G) were refrigerated and then inoculated with RPMI directly into the hemocele. The second

182 group (Live-B) was inoculated with live *S. marcescens* ($LD_{<10}$; 74x10^4 CFU) suspended in
183 RPMI. The third group (Dead-B) was inoculated with dead *S. marcescens* (at 74x10^4 CFU)
184 suspended in RPMI. The Control group (C) was only refrigerated at 4° C for about 10 minutes.
185 At day 7 post priming dose, mosquitoes were re-infected via inoculation in the abdomen (see
186 below). For the second challenge RPMI-G, Live-B and Dead-B, were inoculated with live *S.*
187 *marcescens* at a higher concentration ($LD_{>50}$; 12.3x10^5 CFU). C group was again refrigerated.
188 Survival was quantified until the live mosquito proportion ceased to change.
189
190 For the second experiment we used *E. coli*. Three groups (Live-B, RPMI-G and C) of 100
191 females each were used. Dead-B was not included given that previous observations showed
192 that dead bacteria do not elicit priming condition (unpublished data). For the priming dose,
193 Live-B was inoculated with bacteria at 92.5x10^3 CFU ($LD_{<10}$), RPMI-G was inoculated with
194 RPMI medium, and C (negative control) group was only refrigerated. At day 7 post priming
195 dose, mosquitoes were re-infected via inoculation in the abdomen (see below). RPMI-G and
196 Live-B were inoculated with a higher dose of bacteria ($LD_{>50}$; 10.6x10^4CFU). For this
197 experiment we included a primed RPMI-G challenged again with RPMI medium as positive
198 control. Survival was quantified until live mosquitoes proportion ceased to change.
199
200 All inoculations were made using a pulled glass needle attached to a Drumond microinjector.
201 For manipulation, mosquitoes were previously refrigerated at 4°C for 10 min. Organisms were
202 always injected in the abdomen close to the junction between the ventral and dorsal cuticles.
203 The volume of inoculate was ~1μl. After inoculation, all groups were transferred to an
204 insectary and maintained on a 12:12h light:dark cycle at 25-26°C and allowed to feed ad
205 libitum on cotton soaked with sugar solution.
206
207 *Kinetics of immune response, hemolymph collection and immune measurements*

208 Three groups were formed (Live-B, RPMI-G and C). Live-B group was inoculated with *E. coli*
209 in RPMI ($87x10^3$ CFU; non lethal dose) directly to hemocele. RPMI-G was inoculated with
210 RPMI medium only. At day 7 post priming dose, mosquitoes were re-infected. One half of the
211 individuals in RPMI-G was inoculated with RPMI, while the other RPMI-G half and Live-B
212 groups were inoculated with live *E. coli* at $99x10^3$ CFU ($LD_{<5}$), a higher but minimal lethal
213 dose after priming A minimal but lethal dose was used because mosquitoes needed to survive
214 in order to be enough individuals for daily hemolymph collection. C group was never
215 manipulated. Kinetics experiments were repeated twice. C group was included only in the
216 second repetition. Mosquitoes were inoculated as above. After inoculation, all groups were
217 transferred to an insectary and maintained as above.
218
219 Daily, after the priming dose, mosquitoes were macerated with a biovortexer in 130 µl of PBS
220 buffer and each sample was centrifuged for 10 min at 10,000 rpm (4°C). Supernatant was used
221 to record protein load concentration, PO activity and NO production. Prior to recording PO
222 activity, protein concentration was determinate to control for differences in protein content
223 among samples (see Contreras-Garduño et al. 2007). The BCA™ (Pierce) assay kit was used
224 to determine protein concentration for each sample. Briefly, sample supernatant, 40µl of PBS
225 and 150µl of BCA™ kit reagents mix were added to a 96 microwell plate and incubated for 10
226 min. at 37°C. A known concentration of albumin (5-60µgr) was used as a standard reference
227 curve. The absorbance was recorded at 562nm on a plate reader. After protein load adjustment
228 and to measure PO activity, the sample supernatant plus PBS gauged at 50µl was mixed on a
229 96 microwell plate with 50µl L-DOPA (L-dihydroxyphenylalanine; 4mg/ml) as substrate and
230 incubated for 10 min at room temperature (24°C) . 50µl of buffer mixed with 50µl of L-DOPA
231 was used as blank. Absorbance was recorded at 490nm on a plate reader. An increment in OD
232 after 30 minutes was defined as PO activity. Three mosquitoes were required for a single

233 sample since one individual does not provide enough material for spectrophotometer readings.

234 Three samples per group were quantified every day.

235

236 The Griess reaction was used to determine NO concentration (Eckmann et al. 2000). 50 µl of

237 each sample supernatant were mixed with 50µl of 1% sulfanilamide and 50 µl of 0.1%

238 naphthylethylenediamine on a 96 microwell plate and incubated for 10 min at room

239 temperature (24°C). NO was quantified using a $NaNO_2$ (1-100 µM) standard reference curve

240 for each assay. Absorbance was recorded at 540nm on a plate reader. The highest readings

241 obtained in an interval of 30 min (with measurements every 5 min.) were defined as NO

242 production (expressed as µM).

243

244 Maceration and plate filling (for PO and NO) were done in a cold room (4°C) to exclude room

245 temperature changes that could affect quantifications.

246

247 *Egg production and proportion of egg laying females*

248 To record the number of eggs produced and egg laying females, tree groups were used (C,

249 RPMI-G, and Live-B). Live-B was inoculated with *E. coli* in RPMI (87x10^3 CFU) directly to

250 hemocele. RPMI-G was inoculated with RPMI medium. After inoculation, all groups were

251 maintained on a 12:12h light: dark cycle at 25-26°C and allowed to feed ad libitum on cotton

252 soaked with sugar solution. At day 7 post priming dose, half of the individuals of each

253 treatment were allowed to artificially feed on male sheep blood. In order to ensure that only

254 blood-fed females were used, all mosquitoes were chilled on ice and non-fed-females were

255 removed after blood feeding. For egg laying, single females isolated in plastic glass containers

256 with humid filter paper as the egg laying substrate. Half of mosquitoes were re-infected for

257 which half of which were inoculated with RPMI, and the other half with RPMI-G and Live-B

258 groups were inoculated with live *E. coli* at 99x10^3 CFU. C group was never manipulated. After

259 inoculation mosquitoes were maintained as above. At day 7 post second challenge, females of
260 each treatment were allowed to artificially feed and lay eggs as above. The number of eggs
261 produced by each female and the number of egg laying females were recorded.
262
263 *Statistical analysis*
264 We performed a survival analysis using a Log-rank x^2 to detect differences in survival curves.
265 Analyses were performed using JMP 7.0 (SAS Institute, 2004). For the kinetics of PO and NO,
266 we performed repeated measures ANOVAs with time (time period elapsed since the priming
267 dose) and treatment (C, RPMI-G+RPMI-G, RPMI-G+ Live-B, and Live-B+Live-B) as fixed
268 factors. For kinetics, various transformations did not lead to normal distribution of data.
269 However we still used the repeated measures ANOVA since this test is still appropriate for
270 finding significance when they exists even with non-transformed data (Zar, 1999). Analyses
271 were performed using STATISTICA 7.0 (Statsoft, 2004).
272
273 A one-way ANOVA was conducted to test whether the number of eggs produced at day 7
274 (priming dose effect) was significantly different among treatments (C, RPMI-G, Live-B). To
275 achieve normal error distribution, data were transformed using a Box-Cox transformation (Fit
276 Model module of JMP Ver. 7.0). Since the absolute values of egg production at day 14 (second
277 challenge effect) were not normally distributed, a non-parametric Kruskal-Wallis test was
278 performed to compare among groups. A x^2 test analysis was performed to detect differences in
279 the proportion of egg laying females among groups (C, RPMI+RPMI, RPMI+ Live-B, and
280 Live-B+Live-B) at day 7 (priming dose effect) and at day 14 (second challenge effect)
281
282
283 **Results**
284 *Survival*

285 Previous exposure to a minimal lethal dose of *S. marcescens* provided protection to
286 mosquitoes against a lethal challenge administered 7 days later (Log-Rank x^2= 13.72, *df*=3, *P*=
287 0.0033; notice that removal of C group from the survival model did not change the significant
288 differences, Log-Rank x^2=12.30, *df*= 2, *P*= 0.0021) (Fig. 2). Thus, individuals primed with live
289 bacteria died at a slower rate than the RPMI-G+RPMI-G groups. Dead bacteria did not induce
290 the same magnitude of protection against the second lethal challenge (Fig. 2). At day 4 post
291 second challenge, survival curves between RPMI-G+Live-B and Dead-B+Live-B achieved the
292 same survival probability (Fig. 2).
293
294 In groups inoculated with *E. coli*, similar results were observed. Inoculation with a low dose of
295 bacteria affected mosquito survival. After the second inoculation with a high bacterial dose,
296 the Live-B+Live-B group died slower than the RPMI-G+Live-B group, meanwhile C and
297 RPMI-G+RPMI-G groups exhibited a reduced mortality rate (overall model Log-rank x^2=
298 18.61, *df*= 3, *P*=0.0003). When C and RPMI-G+RPMI-G groups were excluded from the
299 survival model, the differences between the curves of RPMI-G+Live-B and Live-B+Live-B
300 groups were still statistically significant (Log-rank x^2=11.16, *df*= 1, *P*=0.008) until day
301 13,which no longer occurred at day 14 (Log-rank x^2=1.74, *df* =1, *P*= 0.18).
302
303 *Kinetics of PO and NO*
304 For PO activity, there were differences among treatments (Table 1a) and a significant
305 interaction Time by Treatment interaction (only for the second repetition; Table 1b) which
306 indicates that the PO activity changed over time but according to treatment. PO values, in
307 both repetitions, for the Live-B+Live-B group were always below the mean PO of the other
308 groups (Fig. 3a, b). However, on some days, mostly after the second challenge, the RPMI-
309 G+Live-B group showed lower PO values (Fig. 3a: day 7 and 9; Fig. 3b: day 11 and 10).
310 Interestingly, the PO dynamics of this group appears less complex in comparison to those that

311 occurred within the RPMI-G+RPMI-G and RPMI-G+Live-B groups. PO mean peaks and falls
312 for the Live-B+Live-B group were not as intense as compared to the other experimental
313 groups in different days. Bacteria induced differences in the dynamics among groups, this
314 because the groups inoculated with bacteria showed an overall mean PO activity lower than
315 the RPMI-G+RPMI-G group.

316

317 NO production, by contrast, showed a more complex dynamics than PO activity (Fig. 4a, b).
318 There were differences among treatments (Table 2a, b) and a significant interaction Time by
319 Treatment interaction (only for first the repetition; Table 2a) which indicates that NO
320 production changed over time but differential for each treatment. During the first repetition,
321 NO values of Live-B + Live-B were always below the mean NO values of the other
322 treatments. Interestingly, in both repetitions, after the second challenge, mean NO production
323 for priming mosquitoes was above that of the other groups (at day 9 and 10 for the first
324 repetition, and markedly after day 8 for the second repetition). For this immune parameter we
325 also noticed an effect of the inoculation with live bacteria: mean NO values of the bacteria
326 challenged groups were constantly below the mean values of the group inoculated only with
327 RPMI. Intriguingly, in the second repetition, C group after day 7 (without any manipulation)
328 showed lower values compared to the manipulated groups (Fig. 4b).

329

330 *Effects on number of eggs produced and egg laying females*
331 We found differences in eggs production among groups ($F_{2,73}$= 3.58, P= 0.032): the Live-B
332 group fed at day seven (priming dose effect) was significantly larger in comparison to egg
333 production of females injected with RPMI-G (Fig. 5A). Egg production between Live-B and C
334 groups was similar. Females in the Live-B+Live-B group produced more eggs at day 14
335 (second challenge effect) compared with the RPMI-G+RPMI-G and RPMI-G+Live-B groups;
336 however, this difference was not significant (Fig. 5B).

337

338 We did not find differences in the proportion of females (fed at day 7; priming dose effect) that
339 laid eggs among C group (96%), RPMI (80%) and Live-B (80%) (Fig. 6A). After the second
340 challenge, the proportion of egg laying females in the Live-B+Live-B group was smaller
341 (45%) compared with the other three groups (C= 66%; RPMI-G+RPMI-G= 61%; RPMI-
342 G+Live-B=65%) (Fig. 6B), however, the overall model was not statistically significant
343 (x^2=2.88; P=0.40).

344

345 **Discussion**

346 Our results demonstrate that *Ae. aegypti* mosquitoes that were previously exposed to live
347 bacteria are more likely to survive a re-exposure 7 days later to the same bacteria at a higher
348 doses. This supports the idea that insects are capable of developing resistance upon a
349 secondary exposure to a pathogen (Little & Kraaijeveld, 2004; Little, Hultmark & Read, 2005;
350 Kurtz, 2005; Schmid-Hempel, 2005a, b; Moret & Schmid-Hempel, 2001; Kurtz & Franz,
351 2003). However, a previous exposure to *E. coli* does not permanently alter the mosquito'
352 response, as it does with *S. marcescens*. The total experimental time span was 23 days (3 days
353 given age of the mosquitoes + 7 days after priming dose +13 days after the second challenge),
354 which is a considerably long time taking into account the maximum lifespan of 21 days
355 reported in the wild for this animal (Clements, 1999). Despite the fact that E. *coli* does not
356 permanently change mosquito resistance, the time of protection was still long enough to
357 ameliorate the negative impact caused by a second infection. The crucial point in this case is
358 the absolute life span relative to time elapsed between exposures.

359

360 While inoculation with dead bacteria did not induce "long" protection (just a weak 3-4 days
361 potentiation), live bacteria *S. marcescens* did enhance protection. Immunization with E. coli
362 provided protection for 13 days. The reasons for these differences remain unclear. It is

363 possible that dead bacteria, used as an elicitor, do not release molecules that act as the
364 inducing agents. A similar phenomenon has been observed in the tsetse fly, *Glossina*
365 *morsitans* (Kaaya and Darji, 1988): inoculation with dead bacteria did not stimulate
366 antibacterial activity; however lysozyme response was weaker in comparison to the response
367 of live bacteria. The use of non-living molecules (e.g. sephadex beads, LPS vs. bacteria) could
368 be useful to prove if soluble substances from the elicitors are responsible for induction, as
369 occurs in other insects (Söderhäll 1982; Lemaitre *et al.* 1997; Moret & Siva-Jothy, 2003; Pham
370 et al, 2007) (see Wiesner, 1991, for similar discussion).
371
372 After the priming dose, we found overall lower PO values for immune-stimulated mosquitoes
373 when compared to RPMI-G+RPMI-G and RPMI-G+Live-B groups. These results suggest a PO
374 deactivation rather than activation possibly because the organism is using it to control live
375 bacteria. Since bacteria used for the priming dose may cause the conversion of proPO to PO,
376 post- priming dose, it is possible that when we collect hemolymph, we are just detected the
377 remaining PO after being used to defend against bacteria. Groups RPMI-G+RPMI-G and C (in
378 second repetition) were always the groups with highest PO activity (Fig 3a, b). This result
379 indicates the natural dynamics of PO in the mosquito and the effect of the wound post
380 manipulation. Contrary to what it was expected with our model (Fig. 1), no enhancement of PO
381 activity was noticed after a second challenge. Immediately after the second challenge, a
382 decrement in the PO activity in the Live-B+Live-B group was observed, however, at day 10 a
383 slight recovery was detected. The effect of the bacteria used for the second challenge was also
384 detected. As mentioned, the dynamics of PO activity for Live-B+Live-B is not as complex as
385 those in RPMI-G+Live-B. This is probably due to previous contact with bacteria, which induced
386 long-term changes, not enhancing PO activity, but giving place to an efficient use of resources.
387 For example, tyrosine is a PO substrate, and is used for the expression and manufacture of other
388 traits. One of this is coloring the egg chorion in some insects (Li & Christensen 1993). This is

389 why in mosquitoes, melanization responses against worms generate a delay in TYR
390 accumulation in the ovaries, and therefore a decrease in the number of eggs produced after an
391 immune challenge (Li & Christensen, 1993) and a delay in oviposition (Ferdig et al. 1993).
392 Examples like this are suggestive that the substrates of PO and melanin substrates - PHE and
393 TYR - are restrictive resources that may lead to trade-offs among immune response and other
394 key functions including molting, basal metabolic rates and protein synthesis. Also, the PO
395 enzyme is used for different physiological processes (wounding, clotting, cuticle composition,
396 melanotic encapsulation, and probably spermatheca formation (Ilango 2005). Due to these
397 different functions, PO is constantly synthesized; therefore it could be costly to produce for the
398 organism. Also, it must be considered that intermediate molecules produced during the PO
399 cascade serve to amplify immune response (Cerenius & Söderhäll 2004; Nappi & Ottovianni
400 2000).
401
402 We have considered two alternative explanations for the low levels of PO. One is based on
403 resource allocation theory. PO enzyme is used for different immune responses (wounding,
404 clotting, cuticle composition, melanotic encapsulation, production of cytotoxic molecules;
405 (Christensen et al. 2005; Nappi & Christensen 2005) and therefore seems costly for the
406 organisms to produce (Schmid-Hempel, 2005b). It is possible that proPO gets activated in the
407 hemocele, consequently leaving other target tissues with a proPO deficiency. This means that a
408 trade off may arise because of a proPO shortage. Other negative effects may arise when PO
409 cascade is continuously activated; this leads to our second explanation of the low PO levels
410 observed. Several molecules (e.g. quinones, reactive oxygen molecules) are produced during
411 the PO cascade (Nappi & Christensen, 2005). These molecules can be harmful to pathogens
412 but also to host tissues and cells, a phenomenon referred to as immunopathology (Graham,
413 Allen & Read, 2005). It is possible that a controlled activation of PO could be a strategy to

414 avoid this negative effect. In this sense, low PO levels would not be the result of resource
415 limitation but a type of “on-off” switch regulation to avoid self harm.
416
417 NO production was similar to that of PO. During first repetition, after a priming dose, NO
418 levels for Live-B+Live-B were below the other groups. This reveal that the production of NO
419 after an injury induced by manipulation is not similar to the NO response to bacteria.
420 Additionally, an interesting NO production dynamics was observed after the second challenge.
421 NO production for Live-B+Live-B was higher than RPMI-G+Live-B, RPMI-B+RPMI-B and
422 C groups after the second challenge. This result was anticipated by our model (Fig. 1). In *Ae.*
423 *aegypti* NO participates in the control of the dengue virus load (Ramos-Castañeda et al. 2008).
424 It is known that during malaria parasite infection, *Anopheles spp* generate NO to an extent that
425 limits parasite development (Herrera-Ortiz *et al*., 2004; Peterson, Gow & Luckhart, 2007). In
426 *Drosophila*, NO is key for activating both the Immune Deficiency (Imd) (Foley & O’Farrel
427 2003) and upstream Imd pathways. It is also a signaling molecule, so a constant production of
428 NOS is necessary for homeostatic purposes, while inducible NOS is only synthesized after an
429 immune challenge (Nappi et al. 2000). Given the importance of NO for control pathogens, it is
430 therefore expected that production increases after an immune challenge. Despite this,
431 Krishnan, Hyršl & Šimek (2006) found that NO production was similar in non-stimulated and
432 stimulated hemocytes in lepidopteran larvae. In this particular case, the authors call attention
433 to the fact that NO synthesized by an inducible NOS, can persist for long periods of time
434 (hours to days). This means that efficacy of NO in killing pathogens may not only reside on
435 NO concentration, but on the duration of NO activity (see Laurent et al. 1996). However our
436 results show that NO production dynamics were affected differently by the presence of
437 bacteria compared to the effect of inoculation. The higher production of NO in the group Live-
438 B+Live-B after the second challenge can be explained by the previous contact with bacteria
439 inducing priming, causing enhanced NO production.

440

441 We detected differences in the enhancement levels of NO production between repetitions. NO
442 production is probably dependent on animal condition. In a previous study there was no
443 environmental effect on basal (without immune challenge) NO production and PO activity,
444 due to food quality and quantity limitation (Moreno-García et al, 2010). However, the
445 presence of recurrent infections could lead to different levels of immune parameters activation.
446 If there is a difference in nutrition among cohorts, possibly priming may be detected only
447 when the organisms had enough resources to mount an enhanced immune response after
448 continuous infections. Related to this, NO is produced during the oxidation of L-Arginine
449 (Müller 1997). Arginine is an amino acid that must be obtained from the diet (Rivero 2006).
450 Arginine is also important in sperm maturation (Osanai & Chen 1993), egg production
451 (Uchida 1993), long term memory, chemosensory (antennal lobes, olfaction), and visual
452 information processing (Müller 1997).
453
454
455 There is currently a gap in terms of the life history consequences of immune priming. Our data
456 demonstrate that a first immune stimulation results in an increase in egg production. It is
457 probable that when this first infection occurs, the organism is allocating resources to
458 reproduction. It is possible that the egg laying strategy depends on the force and course of
459 infection. After the first challenge, females could be allocating their resources to lay eggs, in
460 an effort to compensate for the reduced life expectancy that would diminish their chances of
461 future reproduction. Related to costs, in *Ae. aegypti* and *D. melanogaster*, up regulation of
462 immune genes reduce the life span of the organism (Libert et al, 2006; Kambris et al, 2009;
463 McMeniman et al, 2009), so, investment in early reproduction could be adaptive. After the
464 second challenge there was no difference in egg production among individuals in the different
465 treatments, however, survival and NO production for Live-B+Live-B was higher than the other

466 groups. It can be argued that survival (and related immune traits) and fecundity are being
467 maximized simultaneously. On the other hand, our results also indicate that the proportion of
468 egg laying females in the Live-B+Live-B group was lower than the other groups, indicating
469 that not all females are disposed to produce eggs, increment survival and enhance immune
470 response. It is possible that the egg laying strategy depends on the force and course of
471 infection, and that the organisms that recover from infection and reduce immune activation
472 costs use its resources in relation to chances of future reproduction which is strongly related to
473 life expectancy.
474
475 In summary, we have provided evidence that PO activity and NO production do not show an
476 overall increased level. However, NO production may be enhanced when the host has had a
477 previous contact with live bacteria. Combined with survival results, the immune levels found
478 here can be explained as the efficacy of organisms in fighting bacteria. Depending on the
479 immune markers measured, immune priming can be considered not only an enhanced
480 response, but an optimal response (in terms of survival and reproduction) to protect the
481 organisms after a previous exposure to an elicitor. Reactions and products of immune response
482 are interconnected and the kind of response is related to the pathogens virulence. It is possible
483 that not all pathogens will induce the same reaction, or at least the magnitude of response can
484 differ depending on the host-pathogen interaction type. Therefore, it is recommended to use
485 more than one immune marker when possible.The mechanisms of immune response in insects
486 include reactions and molecules that are interconnected. It is also important to ascertain the
487 contribution of other immune parameters such as hemocyte activation or antimicrobial
488 synthesis whose activities are enhanced after a second pathogen exposure and have an
489 integrated system of dynamics within immune response. Finally, in terms of reproduction
490 effects, we did not detect an obvious negative result on egg production and number of egg
491 laying females. Reasons for this result granted further studies.

492

493

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502

503 **References**

504 Adamo, S. A. 1998. The specificity of behavioral fever in the cricket *Acheta domesticus*.
505 Journal of Parasitology 84: 529-533.
506 Ahmed, A.H., S.L. Baggott, R. Maingon, and H. Hurd. 2002. The costs of mounting an
507 immune response are reflected in the reproductive fitness of the mosquito *Anopheles*
508 *gambiae*. Oikos 97: 371-377.
509 Andrejko, M., M. Mizerska-Dudka, and T. Jakubowicz. 2009. Antibacterial activity in vivo
510 and in vitro in the hemolymph of *Galleria mellonella* infected with *Pseudomonas*
511 *aeruginosa*. Comparative Biochemistry and Physiology B 152 :118-123
512 Ashida, M., and P.T. Brey. 1997. Recent Advances in research on the insect prophenoloxidase
513 cascade. Pages 135-172 in P.T. Brey, and D. Hultmark, editors. Molecular mechanisms
514 of immune responses in insects. Chapman & Hall, London, UK.
515 Boman, H. G., I. Nilsson, and B. Rasmuson. 1972. Inducible antibacterial defence system in
516 *Drosophila*. *Nature* 237: 232-235.

517 Brandt, S.M., and D.S. Schneider. 2007. Bacterial infection of fly ovaries reduces egg
518 production and induces local hemocyte activation. Developmental and Comparative
519 Immunology 31:1121-1130
520 Brown, M. J., Y.Moret,, and P. Schmid-Hempel. 2003. Activation of host constitutive immune
521 defence by an intestinal trypanosome parasite of bumble bees. *Parasitology* 126: 253-
522 260.
523 Cerenius, L., and K. Söderhäll. 2004. The prophenoloxidase.activating system in invertebrates.
524 Immunological Reviews 198: 116-126.
525 Christensen, B.M., J. Li, Ch-Ch. Chen, and A.J. Nappi. 2005. Melanization immune responses
526 in mosquito vectors. Trends in Parasitology 21: 192-199.
527 Clements, A. N. 1999. The Biology of Mosquitoes. Cabi.
528 Colasanti, M., L. Salvati, G. Venturini, and P. Ascenzi. 2001. Cysteine protease as a target for
529 nitric oxide in parasitic organisms. *Trends in Parasitology* 17: 575.
530 Contreras-Garduño, J., H. Lanz-Mendoza, and A. Córdoba-Aguilar. 2007. The expression of a
531 sexually selected trait correlates with different immune defense components and survival
532 in males of the American rubyspot. Journal of Insect Physiology 53: 612-621.
533 Cotter, S.C., R.S. Hails, J.S. Cory, and K. Wilson. 2004. Density-dependent prophylaxis and
534 condition-dependent immune function in lepidopteran larvae: a multivariate approach.
535 Journal of Animal Ecology 73: 283-293.
536 Deverno, P. J., W. P. Aston, and J.S. Chadwick. 1983. Transfer of immunity against
537 *Pseudomonas aeruginosa* P11-1 in *Galleria mellonella* larvae. Developmental and
538 Comparative Immunology 7: 423-434.
539 Eckmann, L., F. Laurent, T. D. Langford, M. L. Hetsko, J. R. Smith, M. F. Kagnoff, and F. D.
540 Gillin. 2000. Nitric oxide production by human intestinal epithelial cells and competition
541 for arginine as potential determinants of host defense against the lumen-dwelling
542 pathogen *Giardia lamblia*. Journal of Immunology 164: 1478-1487.

543 Faulhaber, L.M., and R.D. Karp. 1992. A diphasic immune response against bacteria in the
544 American cockroach. Immunology 75: 378-381.
545 Foley, E., and P.H. O'Farrell. 2003. Nitric oxide contributes to induction of innate immune
546 responses to gram negative bacteria in *Drosophila.* Genes and Development 17: 115-
547 125.
548 Freitak, D., C.W. Wheat, D.G. Heckel, and H. Vogel. 2007. Immune system responses and
549 fitness costs associated with consumption of bacteria in larvae of *Trichoplusia ni*. BMC
550 Biology 5: 56.
551 Graham, A.L., J.E. Allen, and A.F. Read. 2005. Evolutionary causes and consequences of
552 immunopathology. Annual Review of Ecology Evolution and Systematics 36: 373-397.
553 Herrera-Ortiz, A., H. Lanz-Mendoza, J. Martínez-Barnetche, S. Hernández-Martínez, C.
554 Villarreal-Treviño, L. Aguilar-Marcelino, and M.H. Rodríguez. 2004. *Plasmodium*
555 *bergei* ookinetes induces nitric oxide production in *Anopheles pseudopunctipennis*
556 midguts cultured in vitro. Insect Biochemistry and Molecular Biology 34: 893-901.
557 Janeway, C. 2005. Immunobiology: The immune system in health and disease. Garland
558 Science.
559 Jarosz, J.1993. Induction kinetics of immune antibacterial proteins in pupae of *Galleria*
560 *mellonella* and *Pieris brassicae.* Comparative Biochemistry and Physiology B 106: 415-
561 21.
562 Kaaya, G.P., and N. Darji. 1988. The humoral defense system in tsetse: differences in response
563 due to age, sex and antigen types. Developmental and Comparative Immunology 12:
564 255-268.
565 Kambris Z., P.E. Cook , H.P. Phuc, and S.P. Sinkins. 2009. Immune activation by life-
566 shortening Wolbachia and reduced filarial competence in mosquitoes. Science 326: 134-
567 136

568 Kurtz, J., and K. Franz. 2003. Evidence for memory in invertebrate immunity. Nature 425: 37-
569 38.
570 Kurtz J. 2004. Memory in the innate and adaptive immune systems. Microbes and Infection 6:
571 1410-1417.
572 Krishnan, N., P. Hyršl, and V. Šimek. 2006. Nitric oxide production by hemocytes of larva and
573 pharate prepupa of *Galleria mellonella* in response to bacterial lipopolysaccharide:
574 Cytoprotective or cytotoxic? Comparative Biochemistry and Physiology Part C 142:103-
575 110.
576 Laurent, M., M. Lepoivre, and J.P. Tenu. 1996. Kinetic modeling of the nitric oxide gradient
577 generated in vitro by adherent cells expressing inducible nitric oxide synthase.
578 Biochemistry Journal 314: 109-113.
579 Lemaitre, B., J.M. Reichdart, and J.A. Hoffmann. 1997. *Drosophila* host defense: Differential
580 induction of antimicrobial peptide genes after infection by various classes of
581 microorganisms. Proceedings of the National Academy of Science *USA* 94: 14614-
582 14619.
583 Libert, S., Y. Chao, X. Chu, and S. Pletcher. 2006. Trade-offs between longevity and
584 pathogen resistance in *Drosophila melanogaster* are mediated by NFK-B signaling.
585 Aging cell 5: 533-543.
586 Little, T.J., B. O'Connor, N. Colegrave, K. Watt, and A.F. Read. 2003. Maternal transfer of
587 strain-specific immunity in an invertebrate. Current Biology 13:489-492.
588 Little, T.J., and A.R. Kraaijeveld. 2004. Ecological and evolutionary implications of
589 immunological priming in invertebrates. Trends in Ecology and Evolution 19: 58-60.
590 Little, T.J., D. Hultmark, and A.F. Read. 2005. Invertebrate immunity and the limits of
591 mechanistic immunology. Nature Immunology 6: 651-654.

592 Ling, E., and X.Q. Yu. 2006. Cellular encapsulation and melanization are enhanced by
593 immulectins, pattern recognition receptors from the tobacco hornworm *Manduca sexta.*
594 Developmental and Comparative Immunology 30: 289-299.
595 McMeniman, C.J., R.V. Lane, B.U. Cass, A.W.C. Fong, M. Sidhu, Y-F.Wang, and S.L.
596 O'Neil. 2009. Stable introduction of a life shortening *Wolbachia* infection into the
597 mosquito *Aedes aegypti*. Science 323: 141-144.
598 Moreno-García, M., H. Lanz-Mendoza, and A. Córdoba-Aguilar. In press. Genetic variance
599 and genotype-by-environment interaction of immune response in *Aedes aegypti* (Diptera:
600 Culicidae). Journal of Medical Entomology.
601 Moret ,Y., and M.T. Siva-Jothy. 2003. Adaptive innate immunity? Responsive-mode
602 prophylaxis in the mealworm beetle, *Tenebrio molitor.* Proceedings of the Royal Society
603 of London B 270: 2475-2480.
604 Moret, Y., and P. Schmid-Hempel. 2001. Immune defense in bumble-bee offspring. Nature
605 414: 506
606 Müller, U. 1997. The nitric oxide system in insects. Progress in Neurobiology. 51: 363-381.
607 Nappi, A.J., and B.M. Christensen. 2005. Melanogenesis and associated cytotoxic reactions:
608 applications to insect innate immunity. Insect Biochemistry and Molecular Biology 35:
609 443-459.
610 Nappi, A.J., E. Vass, F. Frey, and Y. Carton. 2000. Nitric oxide involvement in *Drosophila*
611 immunity. Nitric Oxide 4: 423-430.
612 Nappi, A.J., and E. Ottaviani. 2000. Cytotoxicity and cytotoxic molecules in invertebrates.
613 BioEssays 22: 469-480.
614 Osanai, M., and P. Chen. 1993. A comparative study on the arginine degradation cascade for
615 sperm. Amino Acids 5: 341-350.
616 Ourth, D.D., and M.S. Parker. 2006. Kinetics of the antibacterial response by *Heliothis*
617 *virescens* larvae. Journal of Entomology 3:95-101.

618 Peterson, T.M.L., A.J. Gow, and L. Luckhart. 2007. Nitric oxide metabolites induced in
619 *Anopheles stephensi* control malaria parasite infection. Free Radical Biology &
620 Medicine 42: 132-142.
621 Pham, L. N., M. S. Dionne, M. Shirasu-Hiza, and D.S. Schneider. 2007. A specific primed
622 immune response in *Drosophila* is dependent on phagocytes. PLoS Pathogens 3: e26.
623 Pham, L.N., and D.S. Schneider. 2008. Evidence for specificity and semory in the insect innate
624 immune response. Pages 97-128 In N. Beckage editor. Insect mmunology. Academic
625 Press, San Diego, USA.
626 Ramos-Castañeda, J., C. González, M.A. Jiménez, J. Duran, S. Hernández-Martínez, M. H.
627 Rodríguez, and H. Lanz-Mendoza. 2008. Effect of nitric oxide on dengue virus
628 replication in *Aedes aegypti* and *Anopheles albimanus.* Intervirology 51: 335-341.
629 Rivero, A. 2006. Nitric oxide: an antiparasitic molecule of invertebrates. Trends in
630 Parasitology 22: 219-225.
631 Roff, D.A. 1992. The evolution of life histories. Chapman & Hall.
632 Rosengaus, R. B., C. Jordan, M.L. Lefebvre, and J.F. Traniello. 1999. Pathogen alarm
633 behavior in a termite: a new form of communication in social insects.
634 Naturwissenschaften 86: 544-548.
635 Sadd, B.M., and P. Schmid-Hempel. 2006. Insect immunity shows specificity in protection
636 on secondary pathogen exposure. Current Biology 16: 1206-1210.
637 SAS Institute Inc. 2007. JMP 7.0. North Carolina, USA.
638 Sheldon, B.C., and S. Verhulst. 1996. Ecological immunology: costly parasite defenses and
639 trade offs in evolutionary ecology. Trends in Ecology and Evolution 11: 317-321.
640 Schlichting, C.D., and M. Pigliucci. 1998. Phenotypic evolution: a reaction norm perspective.
641 Sinauer Associates.
642 Schmid-Hempel, P., and D. Ebert. 2003. On the evolutionary ecology of specific immune
643 defense. Trends in Ecology and Evolution 18: 27-32.

644 Schmid-Hempel, P. 2005a. Evolutionary ecology of insect immune defenses. Annual Review
645 of Entomology 50: 529-551.
646 Schmid-Hempel, P. 2005b. Natural insect host-parasite systems show immune priming and
647 specificity: puzzles to be solved. BioEssays 27: 1026-1034.
648 Söderhäll, K. 1982. Prophenoloxidase activating system and melanization - a recognition
649 mechanism of arthropods? Developmental and Comparative Immunology 6:601-611.
650 Söderhäll, K., and L. Cerenius. 1998. Role of the prophenoloxidase-activating system in
651 invertebrate immunity. Current Opinion in Immunology 10: 23-28.
652 StatSoft, Inc. 2004. Statistica version 7. Tulsa, USA.
653 Stearns, S.C. 1992. The evolution of life histories. Oxford University Press.
654 Uchida, K. 1993. Balanced amino acid composition essential for infusion-induced egg
655 development in the mosquito (*Culex pipiens pallens*). Journal of Insect Physiology 39:
656 615-621.
657 Uttenweiler-Joseph, S., M. Moniatte, M. Lagueux, D.A. Van, J.Hoffmann, and P.Bulet. 1998.
658 Differential display of peptides induced during the immune response of *Drosophila*: a
659 matrix-assisted laser desorption ionization time-of-flight mass spectrometry study.
660 Proceedings of the National Academy of Science 95: 11342-11347.
661 Watson, F.L., R. Püttmann-Holgado, F. Thomas, D.L. Lamar, M. Hughes, M. Kondo, V.I.
662 Rebel, and D. Schmucker. 2005. Extensive diversity of Ig-Superfamily proteins in the
663 immune system of insects. Science 309: 1874-1878.
664 Wiesner, A. 1991. Induction of immunity by latex beads and by hemolymph transfer in
665 *galleria mellonella.* Developmental and Comparative Immunology 15: 241-250,
666 Zar, J.H. 1999. Biostatistical Analysis. Prentice Hall.
667 Zhang S.M., C.M. Adema , T.B. Kepler, and E.S. Loker. 2004. Diversification of ig
668 superfamily genes in an invertebrate. Science 305: 251-254.
669

670 Table 1. Results of repeated measures ANOVA of immune kinetics for (a) NO production and

671 (b) PO activity (first repetition).

NO production	*SS*	*df*	*MS*	*F*	*P*
Treatment	363.3	2	181.6	122.7	0.008
Error	2.9	2	1.4		
Time	1846	10	184.6	5.4	0.000
Time*Treatment	1597.4	20	79.8	2.3	0.032
Error	683.2	20	34.1		

672

673

674

PO activity	*SS*	*df*	*MS*	*F*	*P*
Treatment	0.205	2	0.102	44.6	0.021
Error	0.004	2	0.002		
Time	0.309	10	0.030	3.5	0.007
Time*Treatment	0.225	20	0.011	1.2	0.289
Error	0.175	20	0.008		

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689 Table 2. Results of repeated measures ANOVA of immune kinetics for (a) NO production and

690 (b) PO activity (second repetition).

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NO production	*SS*	*df*	*MS*	*F*	*P*
Treatment	265.2	3	88.4	4.43	0.047
Error	139.5	7	19.9		
Time	1811.2	11	164.6	5.48	0.000
Time*Treatment	1251.7	33	37.9	1.26	0.200
Error	2312.1	77	30		

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PO activity	*SS*	*df*	*MS*	*F*	*P*
Treatment	0.573	3	0.191	50.79	0.000
Error	0.022	6	0.003		
Time	0.085	11	0.007	2.17	0.026
Time*Treatment	0.256	33	0.007	2.18	0.003
Error	0.234	66	0.003		

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708 **Figure 1.** A hypothetical model of the temporal dynamics of the immune response after a
709 priming dose and a second challenge. Dashed line= Prolonged activation.
710 **Figure 2.** Survival curves of mosquitoes after a second exposure with A) *S. marcescens*, and
711 B) *E. coli.*
712 **Figure 3.** In vivo dynamics of (a) PO activity and (b) NO production (first repetition).
713 **Figure 4.** In vivo dynamics of (a) PO activity and (b) NO production (second repetition).
714 **Figure 5.** Egg production in females fed at day 7 after priming dose (a), and in females fed at
715 day 14, after second challenge (b).
716 **Figure 6.** Proportion of egg laying in females fed at day 7 after priming dose (a), and in
717 females fed at day 14, after second challenge (b).
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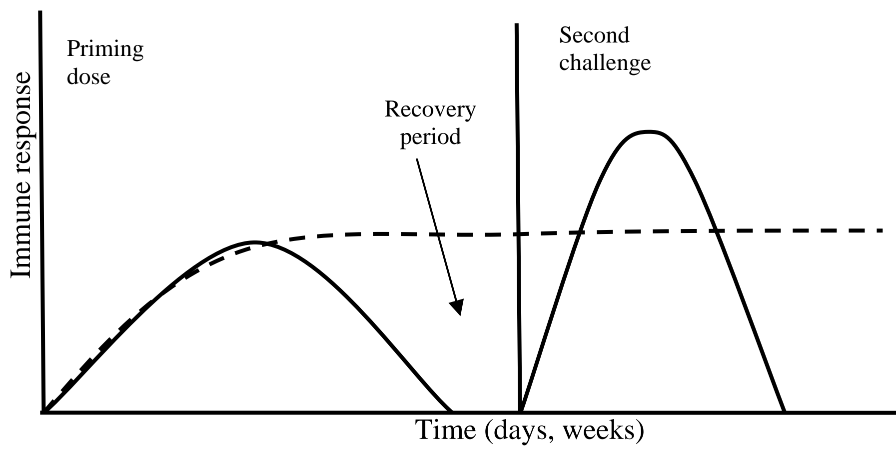

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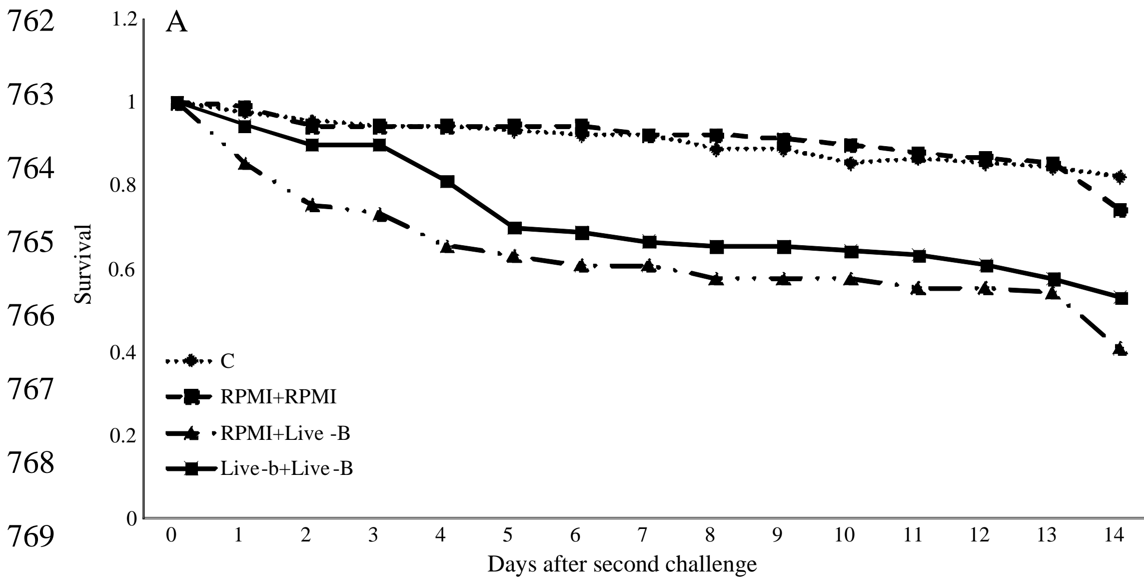

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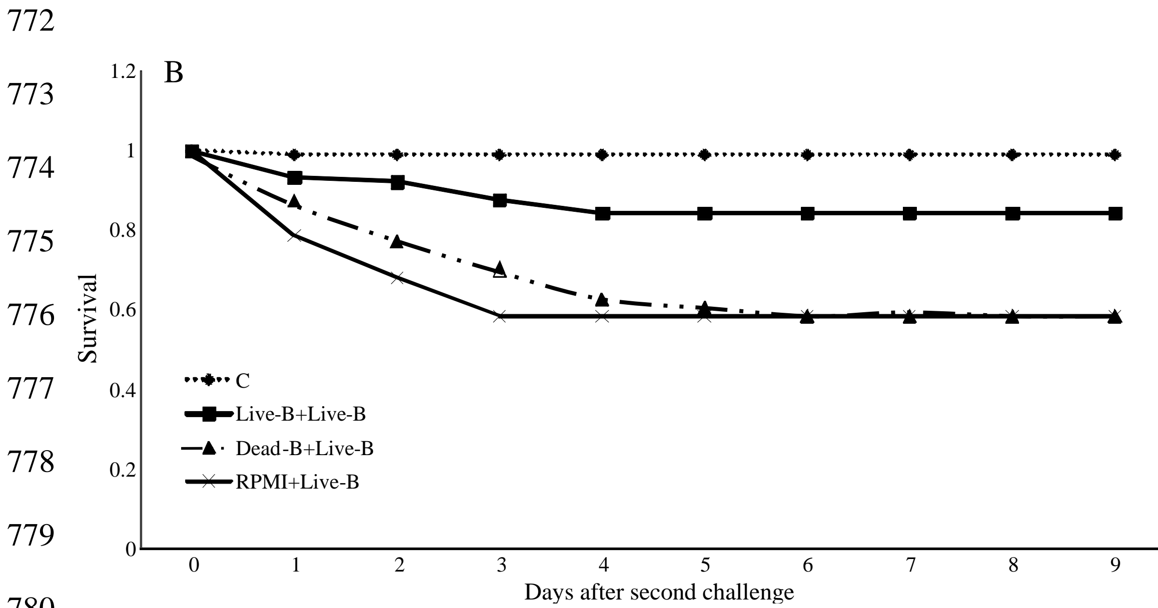

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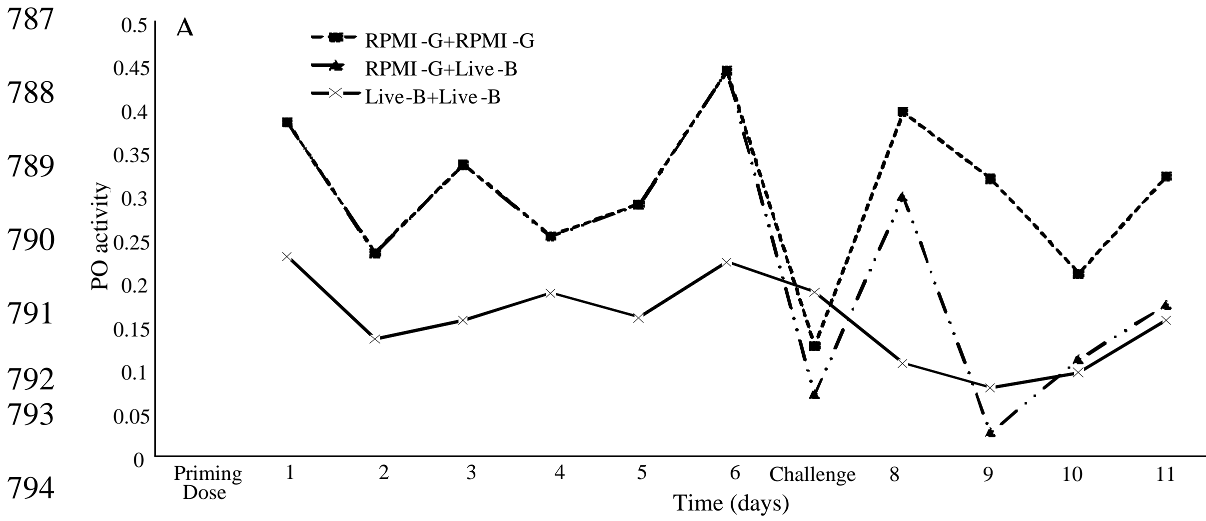

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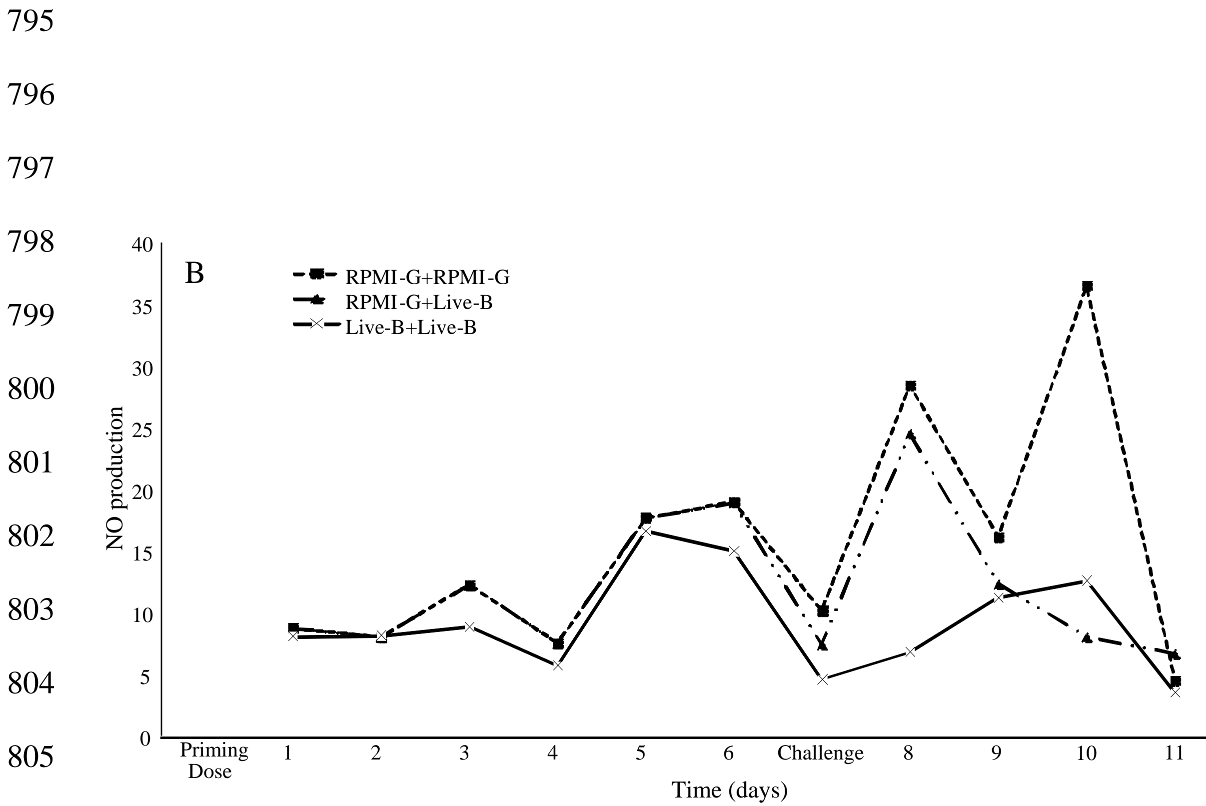

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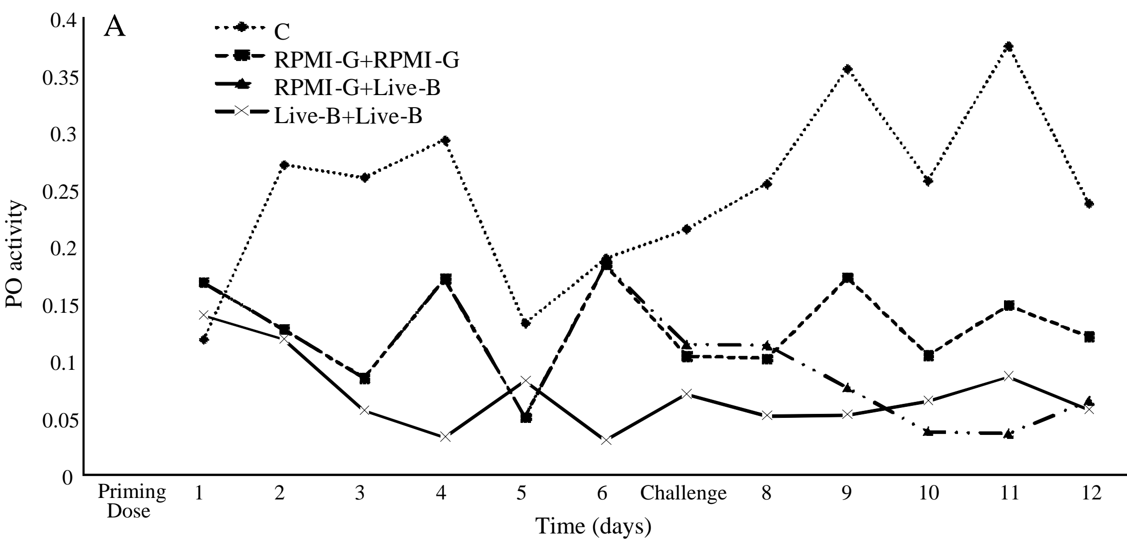

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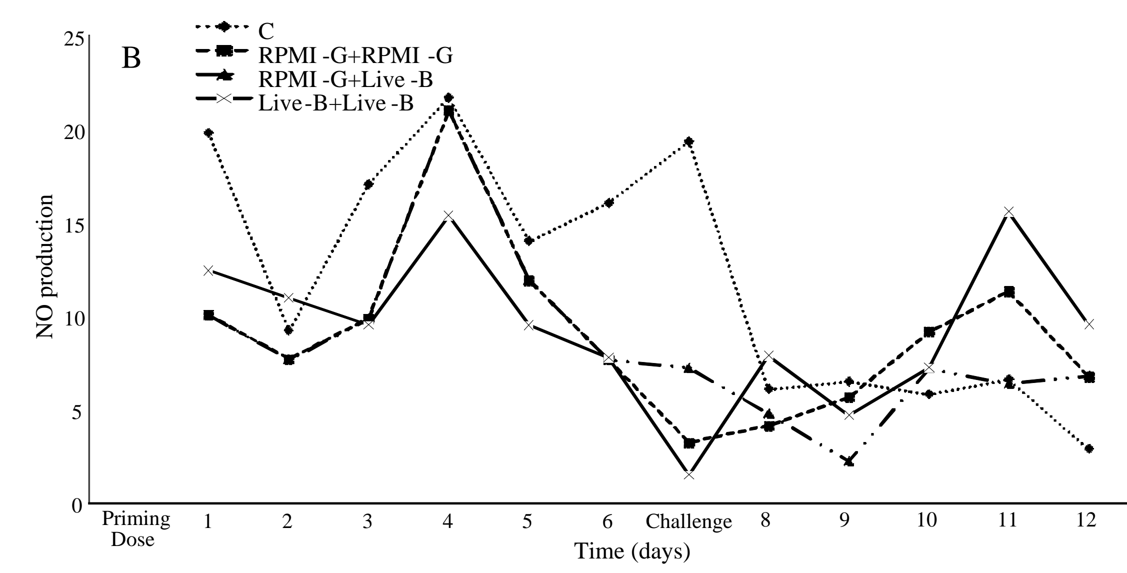

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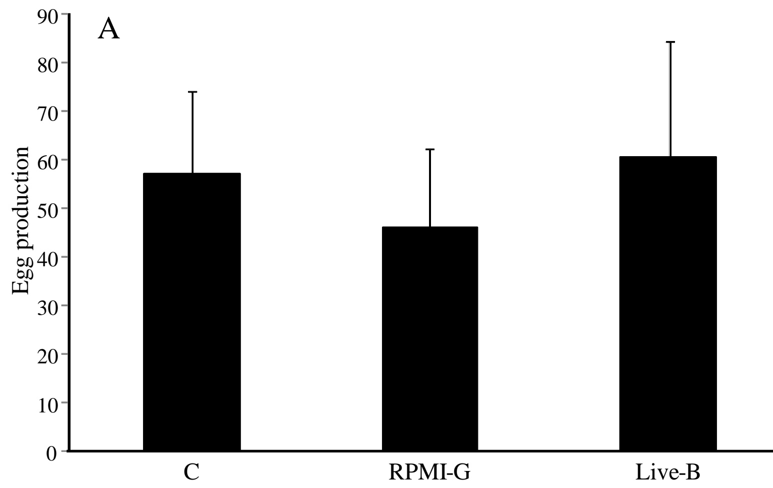

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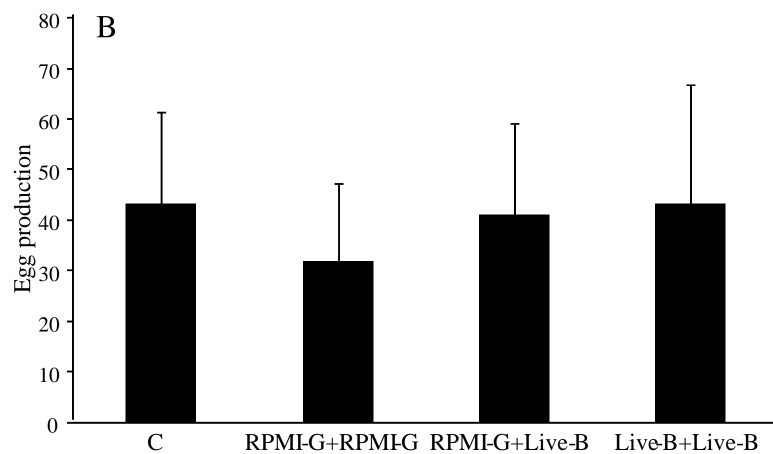

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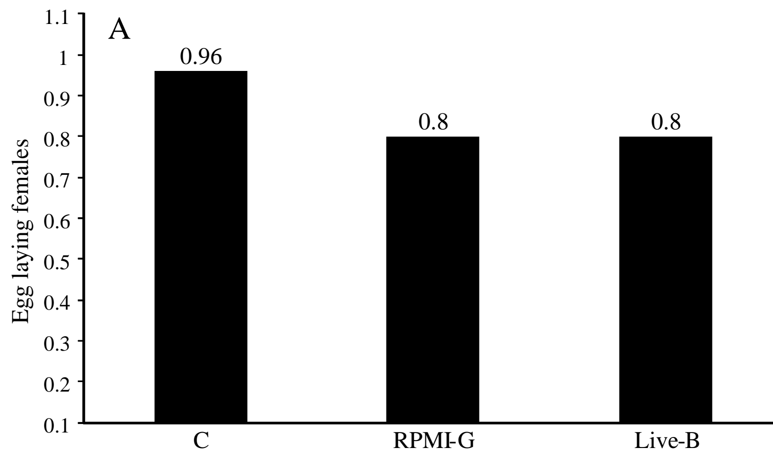

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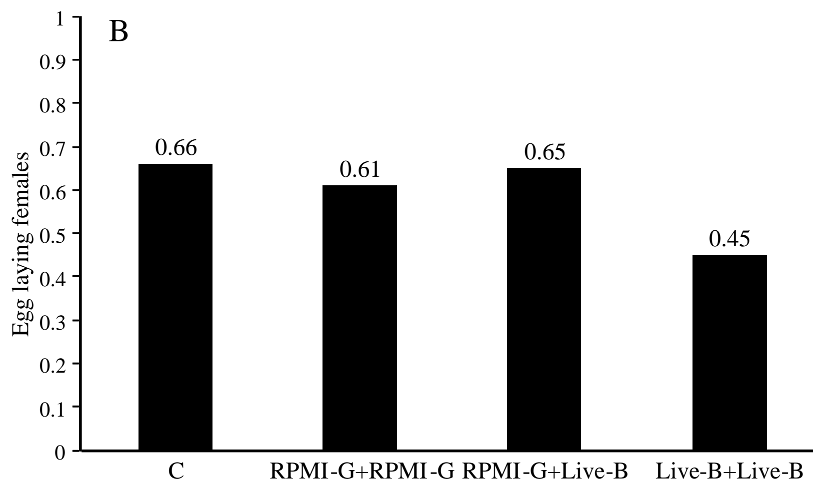

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Genetic Variance and Genotype-by-Environment Interaction of Immune Response in *Aedes aegypti* (Diptera: Culicidae)

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ABSTRACT Immune response can be negatively affected by resource limitation, so it is expected that organisms evolve strategies to minimize the impact of this environmental outcome. Phenotypic plasticity in immune response could represent a genetic response to face such situations. We investigated the effects of high and low quality and quantity of food at the larval stage on two important immune components, phenoloxidase activity (PO) and nitric oxide production (NO) measured in adults of the Dengue vector, *Aedes aegypti*. We reared families to determine the magnitude and pattern of expression of genetic variance, environmental variance and genotype-by-environment interaction (GEI). In addition, we quantified whether there were differences in plastic immune responses in both sexes. Our results indicated additive variance for PO and NO, but rearing environment did not produce differences among individuals. For NO and PO in males, there were large differences among families in plasticity, as indicated by the different slopes produced by each reaction norm. Therefore, there is additive genetic variation in plasticity for NO production and PO activity. One possible interpretation of these results is that different genotypes may be favored to fight pathogens under the different food quality situations. Males and females showed similar overall GEI strategies but there were differences in PO and NO. Males showed a phenotypic correlation between PO and NO, but we did not find genetic correlations between immune parameters in both sexes.

KEY WORDS immune response, quantitative genetics, phenotypic plasticity, *Aedes aegypti*

Immune response is a trait closely linked to survival and reproduction (Schmid-Hempel 2003, Schulenberg et al. 2009). Despite the fact that investment to immunity is adaptive, immune response is strongly impacted by environmental conditions. This occurs in situations of environmental heterogeneity (such as variation in food abundance), in which an overall fitness decrement for a given genotype is observed (e.g., Leclaire and Brandl 1994, Fellowes et al. 1998, Metcalf and Monagham 2001, Siva-Jothy and Thompson 2002). In mosquitoes, for example, larvae that have been reared in crowded or undernourished conditions, give rise to adults with a weak cellular encapsulation (Suwanchaichinda and Paskewitz 1998). On the same line of research, it has been also found that when mosquito adults are stressed with food shortage, encapsulation immune response decreases (Chun et al. 1995, Schwartz and Koella 2002). Therefore, the organisms are expected to deal with the problem of maximizing fitness in changing or stressful environments.

A genetic background that expresses changes in phenotype expression could have a selective advantage in a given environment (Zhivotovsky et al. 1996). In some cases, a single genotype can have the ability to produce distinct phenotypes when exposed to different environments, a phenomenon known as phenotypic plasticity (Roff 1997, Nylin and Gotthard 1998, Schlichting and Pigliucci, 1998). Phenotypic plasticity is shown by many traits and immune response is not an exception (Fordyce 2006). In fact, such immune plasticity could be used as an indicator of the strategies that an organism has followed when dealing with environmental heterogeneity. Paradoxically, given the recent explosion in ecological and evolutionary studies of immunity, only a handful of studies have approached immune response using a phenotypic plasticity perspective (but see Barnes and Siva-Jothy 2000, Mucklow and Ebert 2003, Cotter et al. 2004a, Lazzaro et al. 2008, McKean et al. 2008). To have a better understanding of the evolution of immune responses, more studies of phenotypic plasticity are therefore needed.

To afford the problem of whether plastic immune responses could have an impact in population evolu-

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tion, it is necessary to evaluate if different environments produce different phenotypes and if genotypes respond differently to these environments (genotype-by-environment interaction GEI, which represents genetic variation for phenotypic plasticity). It has been proposed that GEI can allow populations to evolve to an optimum phenotypic mean in different environments, promoting adaptation to heterogeneous environments (Via and Lande 1985). As a result, the amount of genetic variance, as assessed by the slope of reaction norms (i.e., a graphical description of the GEI), may be a strong determinant of the population fitness (Fry 1996). Notwithstanding, a lack of variation may lead to a failure to respond adaptively to environmental changes. There are theoretical advantages of using this approach. For example, phenotypic plasticity could explain the maintenance of genetic variance recently found in different immune components (e.g., see Ryder and Siva-Jothy 2001, Simmons and Roberts 2005, Cotter et al. 2004b, Rolff et al. 2005, Schwarzenbach et al. 2005, Fellowes et al. 1998). The fact that plasticity uncouples the phenotype from genotype and thus releases the gene pool from the immediate impact of natural or sexual selection (Stearns 1992), will lead to slow depletion of genetic variance. However, some reaction norms will not produce the optimal mean trait value in a given environment (Schlichting and Pigliucci 1998, Roff 1997), slowing down the rate of adaptation for immediate generations.

The conventional approach to see how a trait responds to selective pressures is to analyze the trait in question using quantitative genetics by partitioning and estimating variances and covariances into causal components (Falconer and Mackay 1996). In this study we have explored the environmental heterogeneity affecting immune components in the mosquito *Aedes aegypti* Linnaeus (Diptera: Culicidae), the principal vector of Dengue and Yellow Fever virus. We varied levels of food quality and quantity in the larval stage, and as response variables two immune markers were measured in the adult: the basal levels of phenoloxidase (PO) activity and nitric oxide (NO) production. PO is an oxidoreductase enzyme used in insect cellular and humoral response such as cuticle melanization, wound repair, cytotoxin production, and melanotic encapsulation (Söderhäll and Cerenius 1998). NO is a highly reactive and unstable free radical gas that inhibits protein catalytic activity and produces protein and harming effects on pathogens' DNA (Rivero 2006). Recent studies have shown that PO activity is costly to produce (reviewed by Kanost and Gorman 2008). Although there is no direct evidence for such costs for NO, their physiological pathways strongly indicate that it is potentially costly (Rivero 2006, Carton et al. 2008). We evaluated genetic variation, phenotypic plasticity and tested for GEIs of PO and NO using a split family design. We predicted the existence of genetic differences and environmentally sensitive production of alternative phenotypes by given genotypes on these immune markers and a negative effect because of limitation in food quality: mosquito adults whose larval stage had poor quality food would show decreased basal PO activity and NO production while the opposite would be found for mosquito adults whose larval stage had access to high quality food.

Reproductive strategies could promote differences in the pattern of investment to immune defense by each sex. Males are expected to increase their fitness by reducing their investment to immune defense while investing resources to reproductive effort (Zuk and McKean 1996, Sadd et al. 2006). Meanwhile, in females natural selection would favor an increase in resource investment to immunity, under the assumption that increased longevity could enhance fitness via egg production (Rolff 2002). How this presumable sexual dimorphism translates into plastic response differences in both sexes has been little explored. McKean and Nunney (2005) found sex specific plastic responses in relation to the availability of limiting resources. Given this background information, we thus evaluated if males and females express different strategies in their plastic responses in PO activity, NO production, and GEIs strategies.

Materials and Methods

Mosquitoes were obtained from an insectary at the Instituto Nacional de Salud Pública, Cuernavaca, Mexico. The stock population had been held in this place for at least 130 generations. The colony was maintained with a protocol that minimizes inbreeding: fairly high number of individuals per generation (over 2,000 individuals per generation) and random mating. During the study, the colony was kept on a 12L:12D cycle at 25–28°C.

Experimental Design. For PO activity and NO production, we had 44 families by randomly choosing 44 mated and blood fed females. We then split their F1 hatched larvae into one of two rearing environments that differed in food quality and quantity: the high quality food (HQF) environment was based on rat chow, yeast extract and lactoalbumin hydrolisate (1:1:1 mix; 25 g/200 ml), while the low quality food (LQF) environment was based on rat chow only (25 g/200 ml). Preliminary observations have shown these dietary requirements are effective enough to change the individual condition, as reflected by adult size (S. Hernandez-Martinez personal communication), lifespan, and egg clutch and size (see Nasci 1986, Packer and Corbet 1989). Larvae were fed according to the schedule shown in Table 1. Each family of larvae was reared in plastic glasses containing 100 ml of water. Adult females and males of 3 d postemergence from these larvae were used to obtain PO and NO readings.

To minimize degradation of molecules, individuals were collected and frozen at −70°C until they were processed. Given the final large number of individuals and with the aim to avoid sample degradation, maceration, supernatant collection and readings were only done for the exact number of tests supported in the microwell plate (80 wells for test samples plus 16 for standard reference curves, see below) that can be processed and quantified in a single day. A group of

Table 1. Rearing schedule according to food regimes

Day posteclosion	HQF	LQF
1	30 μl	15 μl
2	0 μl	0 μl
3	30 μl	15 μl
4	70 μl	35 μl
5	110 μl	55 μl
6	50 μl	25 μl
7	50 μl	25 μl
8	50 μl	25 μl
9	50 μl	25 μl

HQF, high quality food; LQF, low quality food.

three mosquitoes was required for a single sample because one individual cannot provide enough sample for spectrophotometer readings (MM-G, unpublished data).

All organisms were macerated with a biovortexer in 120 μl of PBS buffer (4°C) and each sample was centrifuged for 10 min at 10,000 rpm (4°C). Supernatant was used to record protein load concentration and PO activity. Before recording PO activity, we determined protein load concentration to control for individual differences in protein content among samples that may bias PO readings (see Contreras-Garduño et al. 2007). The BCA (Pierce) assay kit was used to determine protein concentration for each sample. There were 10 μl of sample supernatant, 40 μl of PBS, and 150 μl of BCA kit reagents mix added to a 96 microwell plate and incubated for 10 min at 37°C. A known concentration of albumin (5–60 μg) was used as a standard reference curve. The absorbance was recorded at 562 nm in a plate reader. For PO activity, the sample supernatant plus PBS gauged at 50 μl (after protein load adjustment) was mixed on a 96 microwell plate with 50 μl L-DOPA (L-dihydroxyphenylalanine; 4 mg/ml) as substrate and incubated for 10 min at room temperature, 50 μl of buffer mixed with 50 μl of L-DOPA was used as blank. The absorbance was recorded at 490 nm in a plate reader. An increment in OD after 30 min was defined as PO activity. PO readings of each microwell plate were obtained as proportional values (highest reading = 100%; lowest reading = 0%).

The Griess reaction was used to determine NO concentration (Eckmann et al. 2000). There were 50 μl of each sample supernatant mixed with 50 μl of 1% sulfanilamide and 50 μl of 0.1% naphthylethylenediamine on a 96 microwell plate and incubated for 10 min at room temperature. NO was quantified using a $NaNO_2$ (1–100 μM) standard reference curve for each assay. Absorbance was recorded at 540 nm in a plate reader. The highest readings obtained in an interval of 30 min (with readings every 5 min) were defined as NO production (expresses as micrometers). NO readings of each microwell plate were obtained as proportional values (highest reading = 100%; lowest reading = 0%).

Plasticity and Genotype-by-Environment Interaction Data Analysis. We first investigated whether there were differences in wing length according to food treatments. For this, a *t*-test was used to compare wing length between adult females raised when larvae in LQF and HQF. We investigated the effects of family, food quality and quantity environment (HQF-LQF) and the interaction (GEI) of PO activity and NO production using a mixed-model ANOVA (type III SS) for unbalanced data using the Variance Components module of STATISTICA 7.0 (StafSoft 2004). Family was entered as a random effect while environment was entered as a fixed effect. This method corresponds to the Scheffé model of Fry's (1992), in which the effect of family is tested using the formula $F = MS_{fam}/MS_{error}$. The MS of the interaction was used as denominator for the fixed effect. To estimate GEIs we first tested for family by rearing environment interactions using the mixed model ANOVA. If a significant interaction was detected, we then evaluated GEI by calculating the cross-environment genetic correlation (r_g; see Fry 1992). r_g is defined as the correlation between the mean of a trait of a genetic group in one environment and the group's mean in another environment. Thus, r_g assumes that a single trait expressed in different environments represents two separate traits (Falconer and Mackay 1996). We used the SAS model of Fry (1992) where $r_g = \mathrm{Cov}(M_{1j}, M_{2j})/\sqrt{[\mathrm{Var}(M_{1j}) \times \mathrm{Var}(M_{2j})]}$, where M_{1j} and M_{2j} are the mean trait values of genetic group *j* under environmental conditions 1(HQF) and 2(LQF) where $\mathrm{Cov}(M_{1j}, M_{2j})$ is the covariance of the mean trait values between conditions HQF and LQF, and where $\mathrm{Var}(M_{1j})$ and $\mathrm{Var}(M_{2j})$ are the variances of the mean trait values under conditions HQF and LQF. Crossover interactions in reaction norms are more feasible when r_g values are $\ll 1$ (Fry 1992). We used families with at least one sample in both rearing environments. ANOVA were performed on untransformed data when non-normal distribution was present. Various transformations did not lead to a normal distribution of data. However, we still performed the ANOVA using the nontransformed as this test is still appropriate as it gives a better chance of finding significances when they exist (Zar 1999). Also, it was necessary to perform the parametric ANOVA to obtain the *MS* given by STATISTICA 7.0 Variance Component module.

Sexual Dimorphism in PO Activity and NO Production. For PO a *t*-test was used to compare the average reaction norm of each sex, using the GEI resultant least-square means of each family reared at HQF and LQF environment. For NO, data has nonnormal distribution, so a Mann–Whitney *U* test was used to compare the average reaction norm of each sex, using the GEI resultant least-square means of each family reared at HQF and LQF environment. We also evaluated if rearing environment induced different plastic responses for PO activity and NO production between the sexes using a Mann–Whitney *U* test for which we used nontransformed data.

Genetic and Phenotypic Correlations. We examined phenotypic and genetic correlations separately for each rearing environment and sex. We estimated genetic correlations between PO activity and NO production by calculating Spearman correlation coeffi-

Table 2. Mixed model ANOVA testing for the effect of family, rearing environment, and GEI on PO activity in adult female and male mosquitoes

Adult mosquitos	df	SS	MS	*F*	*P*
A. Female					
Family	43	2.8752	0.0668	2.0651	<0.0005
Rearing environment (HQF-LQF)	1	0.0093	0.0093	0.2405	>0.25
Family × environment	43	1.666	0.0387	1.1970	0.19
Error	337	10.911	0.0323		
B. Male					
Family	43	3.704	0.0861	3.3089	<0.0005
Rearing environment (HQF-LQF)	1	0.065	0.0657	1.3674	>0.25
Family × environment	43	2.066	0.0480	1.8459	0.0014
Error	396	10.309	0.0260		

HQF, high quality food; LQF, low quality food.

Table 3. Mixed model ANOVA testing for the effect of family, rearing environment, and GEI on NO production activity in adult female and male mosquitoes

Adult mosquitos	df	SS	MS	*F*	*P*
A. Female					
Family	42	2.4284	0.0578	1.6114	<0.025
Rearing environment (HQF-LQF)	1	0.0098	0.0098	0.2751	>0.25
Family × environment	42	1.5019	0.0357	0.9966	0.48
Error	337	12.0920	0.0358		
B. Male					
Family	43	1.0886	0.0253	1.6180	<0.025
Rearing environment (HQF-LQF)	1	0.0024	0.0024	0.1120	>0.25
Family × environment	43	0.9446	0.0219	1.4039	0.052
Error	396	6.1963	0.0156		

HQF, high quality food; LQF, low quality food.

cient for mean trait values for each family. We estimated phenotypic correlations between traits with Spearman correlation coefficient for individual trait values for which we used nontransformed data.

Results

Plasticity and Genotype-by-Environment Interaction. No differences in wing length were observed between diet regimes ($P > 0.05$). The ANOVA revealed a strong family effect on female (Table 2; Fig. 1) and male (Table 3; Fig. 2A) PO activity, which means that there was genetic variation (Family source) for this immune response. There was no significant effect for rearing environment for males and females and no interaction between the effects of family and rearing environment in females. However, our analysis revealed strong effects of family-by-rearing environment on PO activity in males (Table 3; Fig. 2B).

NO production in females had a significant family effect (Table 3A; Fig. 3A). Females reared in the LQF regime produced more NO than females reared in the HQF regime (Fig. 3B) although such differences were not statistically significant (Table 3A.). For males, rearing environment did not have a significant effect, family revealed a strong effect, and the family-by-rearing environment was marginally nonsignificant

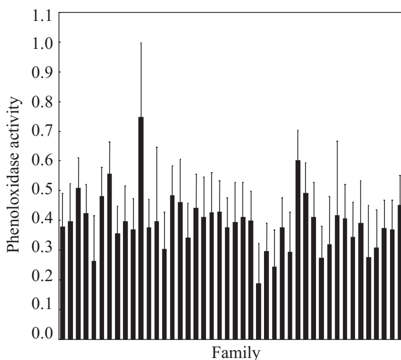

Fig. 1. Genetic differences (means ± SE) of 44 half-sib families for PO activity in adult female mosquitoes.

(Table 3B; Fig. 4A and B). It is possible that genetic variation exists in NO production and genotypes differ in the level or direction of plasticity in this immune component.

The family-by-rearing environment interaction detected for PO activity and NO production in males suggested GEIs. We thus calculated r_g from the variance components of the mixed-model ANOVA (Table 2B; 3B): for PO there was a $r_g = 0.641$; for NO there was a $r_g = 0.535$. These values are consistent with the crossover interactions in reaction norms. Visual inspection of Fig. 2B revealed 19 families (out of 44) that showed higher PO values for individuals reared at LQF compared with his brothers reared at HQF. For NO (Fig. 4B), 16 families (out of 43) showed higher NO production when individuals were reared at LQF contrasting with their brothers reared at HQF. These cross-environmental genetic correlations showed that the PO production and NO activity can be viewed as a different trait whose production depends on the environment.

Sexual Dimorphism in PO Activity and NO Production. PO GEI least-square average means (the average slopes for the reaction norms of each family) produced a sexual difference ($t = 8.862$; df = 1, 172; $P < 0.0001$; Fig. 5A): males showed higher average reaction norm than females. Meanwhile, NO average reaction norm was higher in females ($U = 327$; $P < 0.0001$; Fig. 5B). Concordant with the average reaction norms, males of both rearing environments had higher PO activity than females ($U = 55146$; $P < 0.0001$; Fig. 6A). Females, compared with males, showed higher NO production in both rearing environments ($U = 29392$; $P < 0.0001$; Fig. 6B).

Genetic and Phenotypic Correlations. We only detected a phenotypic correlation between PO and NO in males in both environments (Table 4). This result indicates that variance in PO is related to variance of NO, variation in this immune parameters is not independent. We did not detect any correlation in females.

Discussion

We explored whether there was variance in PO activity and NO production that can be ascribed to

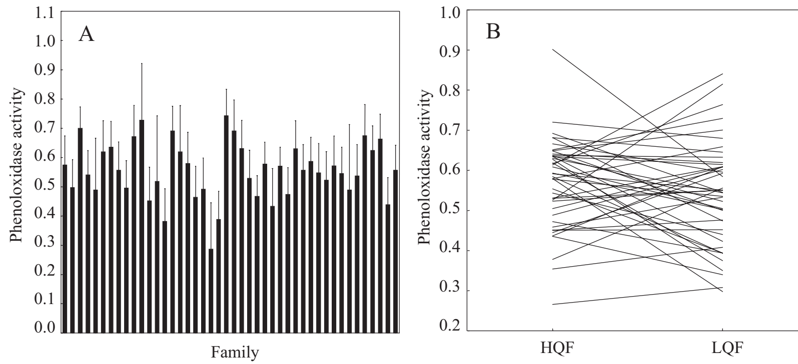

Fig. 2. (A) Genetic differences (means ± SE), and (B) reaction norms of PO according to different food treatments for males of 44 half-sib families. HQF, high quality food; LQF, low quality food.

genetic differences between individuals, as well as the presence of additive genetic variation for plasticity (GEI). PO activity and NO production show genetic variation for both sexes, this fact has been already documented in other insects (e.g., Cotter et al. 2003b; Schwarzenbach and Ward 2006). In these cases, genetic variation has been interpreted as an adaptive response to face pathogen attack: given the large number of infectious agents, there will be ground for high genetic variability in immune response. In the case of our study subject, such variation could allow the male and female mosquitoes to fight against all varieties of infections produced by different pathogens (e.g., the coexistence of different dengue serotypes; Thavaral et al. 2006).

Interestingly, no environmental variance was detected for both overall PO activity and NO production despite contrary evidence suggesting that immunity is affected by, for example, dietary restrictions (e.g., Siva-Jothy and Thompson 2002) among other environmental factors (reviewed by Schmid-Hempel 2003). It may be that the specific dietary components that we varied may not be directly involved in PO activity. One component ascribed to be key as PO substrate is tyrosine, which is derived from phenylalanine hydroxylation (Christensen et al. 2005). Phenylalanine (thus tyrosine) is gathered through larval development in many insects (Kramer and Hopkins 1987). Furthermore, melanin, the final product of the phenoloxidase cascade, is a nitrogen-rich compound. Nitrogen or protein investment is expected to be required for its production (Blois 1978). Poor diet could reduce these compounds destined for melanin production, consequently the production of PO could be synthesized in low quantities. Meanwhile, NO is produced during the oxidation of L-arginine to L-citrulline (Müller 1997); arginine is an essential amino acid which must be obtained from diet (Rivero 2002) mainly through ontogeny. Therefore, suboptimal food quality conditions for larvae could result in a high genetic variability in adult immunocompetence. A situation like this can be found in the wild. It was expected that the beneficial effect of supplementing lactoalbumin and yeast extract (as amino acids donators; see Davis, 1975) on larvae diet may render differences in PO activity and NO production between HQF and LQF. However, rearing effects were not detected in the Mixed ANOVA. It may be that the concentration of proteins mixture in the LQF in this study may not be suboptimal for these immune parameters. However, such explanation does not seem to be entirely correct as the presence of phenotypic correlations in males indicate a degree to which PO

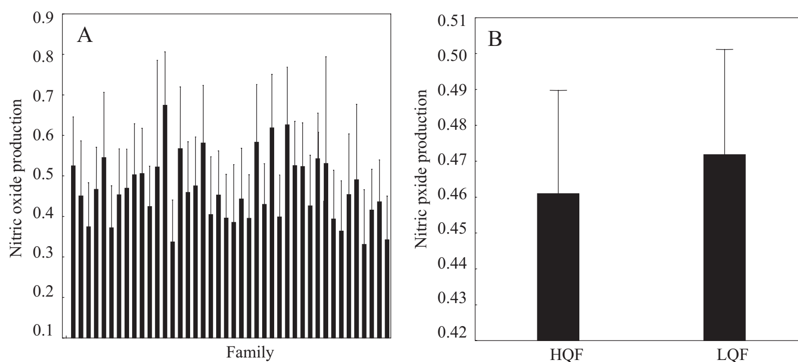

Fig. 3. (A) Genetic differences (means ± SE), and (B) rearing environment effects in NO for adult female mosquitoes (43 families). HQF, high quality food; LQF, low quality food.

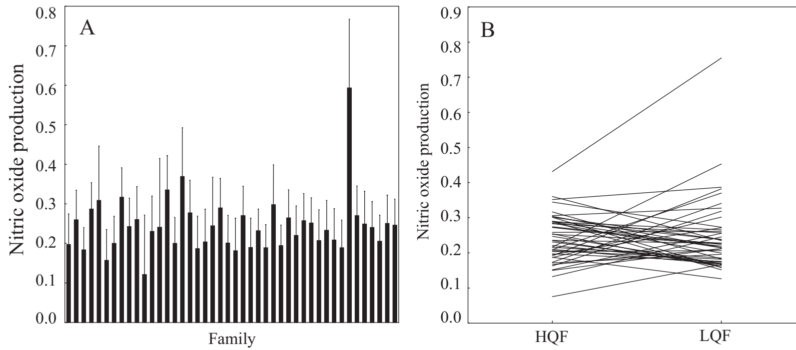

Fig. 4. (A) Genetic differences (means ± SE), and (B) rearing environment effects in NO for adult male mosquitoes (44 families). HQF, high quality food; LQF, low quality food.

and NO respond to variation in the same environmental factors (HQF or LQF).

GEI was not present in females. However, in males there exists some genetic variation in the sensitivity to environmental effects among different mosquito families. The presumed sex-biased genetic-by-environment difference in immunity could be a consequence of the difference in pathogens, quantity of resource acquired during ontogeny, and differences in survival and reproductive strategies. It is possible that females are under the selective pressures of a large variety of pathogens for which, consequently, genetic variance should be expected. However, a female strategy could be to buffer the variation caused by stressing environmental factors (i.e., limited resources), ensuring phenotypic expression within individuals given a specific genotype and environment. The absence of GEI in females for NO and PO could be the result of the ability of females to withstand environmental perturbations and, in combination with the presence of genetic variance, to respond adaptively to changes in the environment and pathogen challenges as a result of stabilizing selection. However, the intrinsic physiological differences between males and females (e.g., fat body reserves, life span and reproductive effort) might also preclude the GEI in females (see below for discussion about sexual dimorphism).

For males, the cross-environmental genetic correlations (r_g) for both immune parameters suggest differences in genetic architecture across the environmental food conditions. It is possible that differences in the amount of reserves accumulated during the larval life affect the immune response of adult mosquitoes that would depend on their genotype. It has been suggested that the presence of GEI may allow adjustment of development that maximizes fitness in a particular environment (Stearns 1992, Fry 1996). For example, under poor food conditions, it may be advantageous to cease growth and allocate the available energy to immunity. However a similar phenotypic response may also occur as consequence of stress, lacking any fitness benefits (Kearsey and Pooni 1996). Although we cannot address the possible adaptive significance of the observed GEI in this study, the genotypes differing in their reaction norms have the potential to ensure immunocompetence during periods of food quality stress and influence the course and rate of evolution in heterogeneous environments (e.g., Gillespie and Turelli 1989). Nevertheless, to see how realistic this situation is in periods of food stress and test that our results are adaptive, further experimental approaches need to be done (for a similar claim see Siva-Jothy et al. 2005).

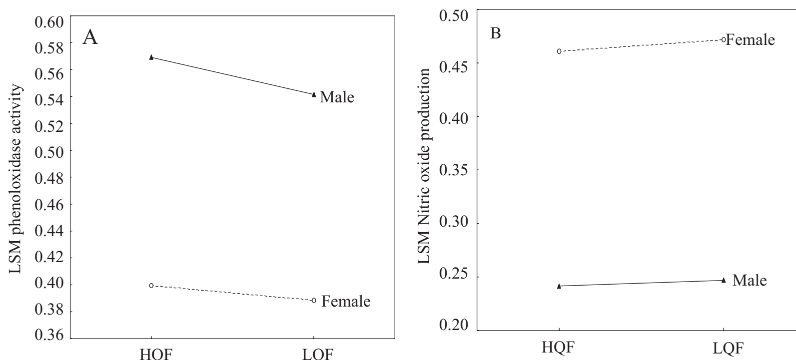

Fig. 5. Means of slopes of each sex's reaction norm for (A) PO activity and (B) NO production based on least-square means. HQF, high quality food; LQF, low quality food.

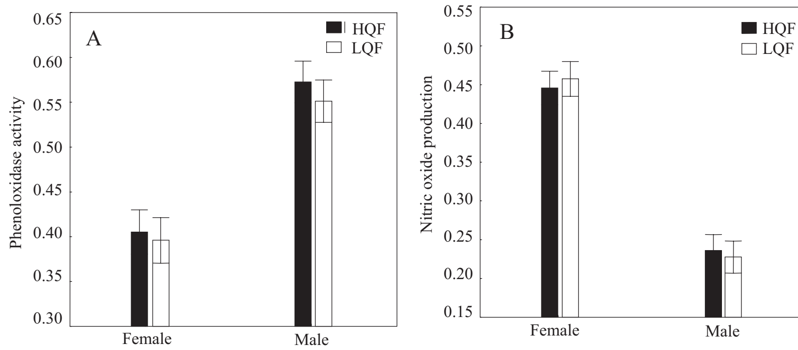

Fig. 6. Sexual dimorphism in (A) PO activity and (B) NO production for adult mosquitoes reared in varying regimes of food quality and quantity. HQF, high quality food; LQF, low quality food.

There were differences between male and female overall values of GEI (Fig. 4A and B), and PO activity and NO production (Fig. 5A and B), which suggests that the two sexes exhibit dissimilar immune strategies. This difference is consistent with the idea that traits involved in immune response do not have the same importance for the adult fitness of the two sexes in terms of condition (McKean and Nunney 2005). PO activity was larger in males than females that can be explained as a consequence of resources being allocated to reproductive activity (host search and oogenesis) rather than to the maintenance of PO cascade. During this cascade, phenylalanine and tyrosine are used as substrates for the formation of melanin and highly reactive and toxic intermediate molecules. Phenylalanine is also involved in cuticle formation and pigmentation, relevant in natural selection functions such as aposematism and crypsis (to avoid predation), thermoregulation, resistance to UV radiation and colored traits that can be under sexual selection (True 2003). Meanwhile, tyrosine is also responsible for coloring the egg chorion in some insects (Li and Christensen, 1993) and other metabolic pathways. It has been shown that mosquitoes undergoing melanization responses against filarial worms after blood-feeding exhibit a delay in tyrosine accumulation in the ovaries, and therefore a delay in egg production and oviposition (Ferdig et al. 1993). For these reasons, it is likely that phenylalanine and tyrosine are restrictive resources in females mosquitoes leading to trade-offs between immune response and other life history traits. In the absence of an immune challenge, females would favor an increase in resource investment to longevity and egg production rather to immunity (see also Rolff 2002).

NO was produced by females in a twofold relation compared with males. It could be argued that a trade-off is also present. Arginine is an important immune molecule, but it is also essential for sperm maturation (Osanai and Chen 1993), egg production (Uchida 1993), long-term memory, chemosensory (antennal lobes, olfaction) and visual information processing (Müller, 1997). However, in *Anopheles stephensi,* NO limits parasite development (Tina et al. 2007), and in *Ae. aegypti* NO participates in the control of the dengue virus load (Ramos-Castañeda et al. 2008). This presumed sex bias in NO production could result from different kinds of pathogens attacking each sex. For example in mosquitoes, adult females require carbohydrates (sugar) and proteins for vitellogenesis that are usually present in the blood meal. Meanwhile, males need sugar solutions (nectar) (Clements 1999). If some pathogens are acquired when feeding (as it is the case of the dengue virus) when each sex occupies or uses different habitats, then both sexes should show extremely different pathogens and, therefore, distinct immune adaptations against pathogens. Also, if adult female mosquitoes that have inherited pathogens from their parents, or become contaminated when mating with infected males (males that have inherited pathogens too) (e.g., Diallo et al. 2000) are expected to enhance survival. Thus, not only there are differences

Table 4. Genetic and phenotypic correlations between PO activity and NO production in adult male and female mosquitoes reared in two different food regimes during larval development

	Males		Females	
	At HQF PO activity	At LQF PO activity	At HQF PO activity	At LQF PO activity
Phenotypic correlations				
NO production	0.191 (**) $N = 244$	0.144 (*) $N = 242$	0.082 (NS) $N = 221$	0.004 (NS) $N = 207$
Genetic correlations				
NO production	0.252 (NS) $N = 44$	0.031 (NS) $N = 44$	0.172 (NS) $N = 44$	0.218 (NS) $N = 44$

Spearman correlation coefficients calculated with untransformed data. P values are indicated between parentheses (*, $P < 0.05$; **, $P < 0.005$). HQF, high quality food; LQF, low quality food; NS, nonsignificant.

in the incidence of different pathogens affecting males and females that explain the sexual dimorphism observed for NO and PO, but different reproductive strategies followed by each sex that could be important. This fact could be reflected in the course of action of individual immune response.

Immune response occurs via a set of physiological traits and is supposed to show low additive variance and hence low heritability values (like other physiological traits, see Mousseau and Roff 1987). Our breeding results of PO activity and NO production confirmed the (additive) genetic variance previously reported for other immune parameters in other insects (e.g., Fellowes et al. 1998, Kurtz and Sauer 1999, Hosken 2001, Rolff et al. 2005, Simmons and Roberts 2005). In male mosquitoes, genetic variation can be maintained if environmental conditions vary and if distinct alleles maximize fitness under each environment. GEIs may contribute to genetic variability in immunocompetence although this consideration has been rarely investigated. Natural or sexual selection will act on phenotypic individual variance, favoring some phenotypes, and as long as the phenotypic variance has a genetic background, the population can evolve. The lack of genetic correlations (pleiotropy) (in males and females) and r_g (for males) indicate that if some genotypes are favored under different environmental conditions, there should be no strong genetic constraints for adaptation to each of the environments for independent PO and NO responses. However, the presence of additive genetic variation could be the result not only of direct selection on the mosquito's immune response, but also because of differences in immune strategies between the sexes, and indirect selection acting on genetically correlated traits (see Koella and Boëte 2002).

It has to be mentioned that our study was conducted using basal immune components (i.e., in the absence of an immune challenge). Moreover, this basal immune response could be adaptive in cases where females have inherited pathogens from their parents, or progeny that do not carry the virus at their emergence but become contaminated while mating with infected partners. Despite our methodological approach, it is likely that the interaction between *Ae. aegypti* genotypes and abiotic environment can affect the evolution of resistance to infection because the performance of different genotypes changed across food quality and quantity environments. In view of this, the effects of environmental variation must not be ignored in future studies of immunocompetence of mosquitoes and other insects. These results may also be important to understand the colonizing success of *Ae. aegypti* to new habitats (in natural or urbanized areas) and/or habitats where resources and pathogens vary seasonally and geographically (Kittayapong et al. 1999, Wearing and Rohani 2006).

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References Cited

Barnes, A. I., and M. T. Siva-Jothy. 2000. Density-dependent prophylaxis in the mealworm beetle *Tenebrio molitor* L. (Coleoptera: Tenebrionidae): cuticular melanization is an indicator of investment in immunity. Proc. Roy. Soc. Lond. B. 267: 177–182.

Blois, M. S. 1978. The melanins: their synthesis and structure, pp. 115–134. *In* K. C. Smith [ed.], Photochemical and Photobiological Reviews, vol. 3, Plenum, New York.

Carton, Y., M. Poirie, and A. J. Nappi. 2008. Insect immune resistance to parasitoids. Insect Sci. 15: 67–87.

Christensen, B. M., L. Jianyong, Ch. Cheng-Chen, and A. J. Nappi. 2005. Melanization immune responses in mosquito vectors. Trends Parasitol. 21: 192–199.

Chun, J., M. Riehle, and S. M. Paskewitz. 1995. Effect of mosquito age and reproductive status on melanization of sephadex beads in *Plasmodium*-refractory and -susceptible strains of *Anopheles gambiae.* J. Invertebr. Pathol. 66: 11–17.

Clements, A. N. 1999. The biology of mosquitoes. Cabi, Wallingford. United Kingdom.

Contreras-Garduño, J., H. Lanz-Mendoza, and A. Córdoba-Aguilar. 2007. The expression of a sexually selected trait correlates with different immune defense components and survival in males of the American rubyspot. J. Insect. Physiol. 53: 612–621.

Cotter, S. C., R. S. Hails, J. S. Cory, and K. Wilson. 2004a. Density-dependent prophylaxis and condition-dependent immune function in *Lepidopteran* larvae: a multivariate approach. J. Anim. Ecol. 73: 283–293.

Cotter, S. C., L.E.B. Kruuk, and K. Wilson. 2004b. Costs of resistance: genetic correlations and potential trade-offs in an insect immune system. J. Evol. Biol. 17: 421–429.

Davis, G.R.F. 1975. Essential dietary amino acids for growth of larvae on the yellow mealworm, *Tenebrio molitor* L. J. Nutr. 105: 1071–1075.

Diallo, W., J. Thonnon, and D. Fontenille. 2000. Vertical transmission of the Yellow Fever virus by *Aedes aegypti* (Diptera: Culicidae): dynamics of infection in F1 adult progeny of orally infected females. Am. J. Trop. Med. Hyg. 62: 151–156.

Eckmann, L., F. Laurent, T. D. Langford, M. L. Hetsko, J. R. Smith, M. F. Kagnoff, and F. D. Gillin. 2000. Nitric oxide production by human intestinal epithelial cells and competition for arginine as potential determinants of host defense against the lumen-dwelling pathogen *Giardia lamblia.* J. Immunol. 164: 1478–1487.

Falconer, D. S., and T.F.C. Mackay. 1996. Introduction to QuantitativeGgenetics, 4th ed. Longman, London, United Kingdom.

Fellowes, M.D.E., A. R. Kraaijeveld, and H.C.J. Godfray. 1998. Trade-off associated with selection for increased ability to resist parasitoid attack in *Drosophila melanogaster.* Proc. R. Soc. Lond. B. 265: 1553–1558.

Ferdig, M., B. Beerntsen, F. Spray, J. Li, and B. Christensen. 1993. Reproductive costs associated with resistance in a mosquito-filarial worm system. Am. J. Trop. Med. Hyg. 49: 756–762.

Fordyce, J. A. 2006. The evolutionary consequences of ecological interactions mediated through phenotypic plasticity. J. Exp. Biol. 209: 2377–2383.

Fry, J. D. 1992. The mixed-model analysis of variance applied to quantitative genetics: biological meaning of the parameters. Evolution 46: 540–550.

Fry, J. D. 1996. The evolution of host specialization: are trade-offs overrated? Am. Nat. 148: S84–S107.

Gillespie, J. H., and M. Turelli. 1989. Genotype-environment interactions and the maintenance of polygenic variation. Genetics 114: 333–343.

Hosken, D. J. 2001. Sex and death: microevolutionary trade-offs between reproductive and immune investment in dung flies. Curr. Biol. 11: 379–380.

Kanost, M. R., and M. J. Gorman. 2008. Phenoloxidases in insect immunity, pp. 69–96. *In* N. E. Beckage [ed.], Insect Immunology. Academic, New York.

Kearsey, M. J., and H. S. Pooni. 1996. The genetical analyses of quantitative traits. Chapman & Hall, London, United Kingdom.

Kittayapong, P., K. J. Baisley, and S. O'Niell. 1999. A mosquito densovirus infecting *Aedes aegypti* and *Aedes albopictus* from Thailand. Am. J. Trop. Med. Hyg. 61: 612–617.

Koella, J. C., and C. Boëte. 2002. A genetic correlation between age at pupation and melanization immune response of the yellow fever mosquito *Aedes aegypti.* Evolution 56: 1074–1079.

Kramer, K. J., and T. L. Hopkins. 1987. Tyrosine metabolism for insect cuticle tanning. Arch. Insect Biochem. Physiol. 6: 279–301.

Kurtz, J., and K. P. Sauer. 1999. The immunocompetence handicap hypothesis: testing the genetic predictions. Proc. R. Soc. Lond. B. 266: 2515–2522.

Lazzaro, B. P., H. A. Flores, J. G. Lorigan, and C. P. Yourth. 2008. Genotype-by-environment interactions and adaptation to local temperature affect immunity and fecundity in *Drosophila melanogaster.* PLoS Pathogens 4: e1000025.

Leclaire, M., and R. Brandl. 1994. Phenotypic plasticity and nutrition in a phytophagus insect: consequences of colonizing a new host. Oecologia 100: 379–385.

Li, J., and B. M. Christensen. 1993. Involvement of L-tyrosine and phenoloxidase in the tanning of *Aedes aegypti* eggs. Insect Biochem. Mol. Biol. 23: 739–748.

McKean, K. A., and L. Nunney. 2005. Bateman's principle and immunity: phenotypically plastic reproductive strategies predict changes in immunological sex differences. Evolution 59: 1510–1517.

McKean, K. A., C. P. Yourth, B. P. Lazzaro, and A. G. Clark. 2008. The evolutionary costs of immunological maintenance and deployment. BMC Evol. Biol. 8: 76.

Metcalf, N. B., and P. Monagham. 2001. Compensation for a bad start: grow now pay later? Trends Ecol. Evol. 16: 254–260.

Mousseau, T. A., and D. A. Roff. 1987. Natural selection and the heritability of fitness components. Heredity 59: 181–197.

Mucklow, P. T., and D. Ebert. 2003. Physiology of immunity in the water flea *Daphnia magna:* environmental and genetics aspects of phenoloxidase activity. Physiol. Biochem. Zool. 76: 836–842.

Müller, U. 1997. The nitric oxide system in insects. Prog. Neurobiol. 51: 363–381.

Nasci, R. S. 1986. Relationship between adult mosquito (Diptera: Culicidae) body size and parity in field populations. Environ. Entomol. 15: 874–876.

Nylin, S., and K. Gotthard. 1998. Plasticity in life-history traits. Annu. Rev. Entomol. 43: 63–83.

Osanai, M., and P. Chen. 1993. A comparative study on the arginine degradation cascade for sperm. Amino Acids 5: 341–350.

Packer, M. J., and P. S. Corbet. 1989. Size variation and reproductive success of female *Aedes punctor* (Diptera: Culicidae). Ecol. Entomol. 14: 297–309.

Ramos-Castañeda, J., C. González, M. A. Jiménez, J. Duran, S. Hernández-Martínez, M. H. Rodríguez, and H. Lanz-Mendoza. 2008. Effect of nitric oxide on dengue virus replication in *Aedes aegypti* and *Anopheles albimanus.* Intervirology 51: 335–341.

Rivero, A. 2006. Nitric oxide: an antiparasitic molecule of invertebrates. Trends Parasitol. 22: 219–225.

Rolff, J. 2002. Bateman's principle and immunity. Proc. R. Soc. Lond. B. 269: 867–872.

Rolff, J., S.A.O. Armitage, and D. W. Coltman. 2005. Genetic constraints and sexual dimorphism in immune defence. Evolution 59: 1844–1850.

Roff, D. A. 1997. Evolutionary quantitative genetics. Chapman & Hall, New York.

Ryder, J. J., and M. T. Siva-Jothy. 2001. Quantitative genetics of immune function and body size in the house cricket, *Acheta domesticus.* J. Evol. Biol. 14: 646–653.

Sadd, B., L. Holman, H. Armitage, F. Lock, R. Marland, and M. T. Siva-Jothy. 2006. Modulation of sexual signaling by immune challenged male mealworm beetles (*Tenebrio molitor*, L.): evidence for terminal investment and dishonesty. J. Evol. Biol. 19: 321–325.

Schlichting, C. D., and M. Pigliucci. 1998. Phenotypic evolution: a reaction norm perspective. Sinauer, Sunderland, MA.

Schmid-Hempel, P. 2003. Variation in immune defence as a question of evolutionary ecology. Proc. R. Soc. Lond. B 270: 357–366.

Schulenberg, H., J. Kurtz, J. Moret, and M. T. Siva-Jothy. 2009. Introduction. Ecological immunology. Phil. Trans. R. Soc. Lond. B. 364: 3–14.

Schwartz, A., and J. C. Koella. 2004. The cost of immunity in the yellow fever mosquito, *Aedes aegypti* depends on immune activation. J. Evol. Biol. 17: 834–840.

Schwarzenbach, G. A., D. J. Hosken, and P. I. Ward. 2005. Sex and immunity in the yellow dung fly *Scathophaga stercoraria.* J. Evol. Biol. 18: 455–463.

Schwarzenbach, G. A., and P. I. Ward. 2006. Response to selection in phenoloxidase activity in yellow dungflies. Evolution 60: 1612–1621.

Simmons, L. W., and B. Roberts. 2005. Bacterial immunity traded for sperm viability in male crickets. Science 309: 2031.

Siva-Jothy, M. T., Y. Moret, and J. Rolff. 2005. Evolutionary ecology of insect immunity. Adv. Insect Physiol. 32: 1–48.

Siva-Jothy, M. T., and J.J.W. Thompson. 2002. Short-term nutrient deprivation affects immune function. Physiol. Entomol. 27: 206–212.

Söderhäll, K., and L. Cerenius. 1998. Role of the prophenoloxidase-activating system in invertebrate immunity. Curr. Op. Immunol. 10: 23–28.

StatSoft, Inc. 2004. Statistica, version 7. StatSoft, Inc., Tulsa, OK.

Stearns, S. C. 1992. The evolution of life histories. Oxford University Press, Oxford, United Kingdom.

Suwanchaichinda, C., and S. M. Paskewitz. 1998. Effects of larval nutrition, adult body size, and adult temperature on the ability of *Anopheles gambiae* (Diptera: Culicidae) to melanize beads. J. Med. Entomol. 35: 157–161.

Thavaral, U., P. Siriyasatien, A. Tawatsin, P. Asavadachanukorn, S. Anantapreecha, R. Wongwanich, and M. S. Mulla. 2006. Double infection of heteroserotypes of dengue viruses in field populations of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) and serological features of dengue virsuses found in patients in Southern Thailand. Southeast Asian J. Trop. Med. Publ. Health 3: 468–476.

Tina, M. L., A. Peterson, A. J. Gow, and S. Luckhart. 2007. Nitric oxide metabolites induced in *Anopheles stephensi* control malaria parasite infection. Free Radical Biol. Med. 42: 132–142.

True, J. R. 2003. Insect melanism: the molecules matter. Trends Ecol. Evol. 18: 640–647.

Uchida, K. 1993. Balanced amino acid composition essential for infusion-induced egg development in the mosquito (*Culex pipiens pallens*). J. Insect Physiol. 39: 615–621.

Via, S., and R. Lande. 1985. Genotype-environment interaction and the evolution of phenotypic plasticity. Evolution 39: 505–522.

Wearing, H. J., and P. Rohani. 2006. Ecological and immunological determinants of dengue epidemics. Proc. Natl. Acad. Sci. U.S.A. 103: 11802–11807.

Zar, J. H. 1999. Biostatistical analysis, 4th ed. Prentice Hall, New Jersey.

Zhivotovsky, L. A., M. W. Feldman, and A. Bergman. 1996. On the evolution of phenotypic plasticity in a spatially heterogeneous environment. Evolution 50: 547–558.

Zuk, M., and K. A. McKean. 1996. Sex differences in parasite infections: patterns and processes. Int. J. Parasitol. 26: 1009–1024.

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CAPÍTULO V. DISCUSIÓN Y CONCLUSIÓN GENERAL

En este trabajo se contribuyó a entender cómo la respuesta inmune afecta y se ve afectada por estrategias y características de historia de vida. En específico, cómo se ve afectada la respuesta inmune ante factores bióticos y abióticos como lo son la presencia recurrente de patógenos, y la heterogeneidad en los recursos alimenticios. Para esto se evaluaron dos aspectos: (1) el efecto de la presencia recurrente de un mismo patógeno en hembras del mosquito *Ae. aegypti*, la forma en que responde inmunológicamente y su consecuencia en la supervivencia y reproducción. (2) El efecto que tiene la variación en la calidad y cantidad de la dieta a lo largo del desarrollo larval en la actividad de fenoloxidasa y producción de óxido nítrico de hembras y machos adultos de *Aedes aegypti.* A continuación se discuten los puntos esenciales de cada una de estas observaciones, y se plantean perspectivas de estudio.

Factores que moldean la respuesta inmune de insectos

A pesar de que los insectos cuentan con un sistema inmune que consta de células especializadas, moléculas efectoras, y mecanismos de reconocimiento y regulación muy eficaces, constantemente sufren infecciones. Además la respuesta inmune varía entre especies. La amplia evidencia generada en diversos estudios de insectos indica que fisiológicamente el individuo tiene que decidir, en base a los recursos energéticos disponibles, la asignación de estos hacia diversas actividades, unas involucradas con la supervivencia y otras con la reproducción (Moret & Schmid-Hempel 2002). La inversión dirigida a montar y mantener la respuesta inmune para combatir patógenos infecciosos en muchas ocasiones limita la reproducción de los organismos (ej. Adamo et al, 2002). El individuo se verá favorecido con la inversión que al final le genere el dejar la mayor descendencia viable. La inversión diferencial de los recursos hacia distintas estrategias afectará directamente la evolución de de la respuesta inmune y otras características (ej. caracteres sexuales secundarios). Además hay que considerar la patogenicidad y virulencia del agente infeccioso (Pfennig, 2001). El daño que un patógeno puede infringir a su hospedero

está en función de estos dos aspectos. El hospedero también tendrá que "decidir" la inversión en energía en función del potencial daño.

Otro aspecto que se considera es que el sistema inmune no es simple, distintos parámetros que lo comprenden pueden estar correlacionados positiva o negativamente. El sistema inmune se activa cuando el organismo reconoce moléculas no propias, sin embargo, se sabe que en ocasiones el tipo de patógeno determinará la activación diferencial de los parámetros inmunes (Janeway & Medzhitov, 2002). Las correlaciones negativas podrán verse favorecidas en el caso en esta correlación tenga como consecuencia la eficaz eliminación del patógeno. Aunque puede darse el caso en que la correlación negativa entre parámetros inmunes sea consecuencia de la limitante en los recursos (de Jong & Van Noordjwik, 1992).

Se debe tener siempre en consideración que la disponibilidad de los recursos y la presencia (y recurrencia) de patógenos son temporal y espacialmente estocásticos. Ambos factores son causas próximas que actúan sobre el sistema inmune de insectos. La expresión de estrategias inmunes estará íntimamente relacionada con estos dos factores ecológicos.

Encuentros recurrentes con patógenos y su efecto en Aedes aegypti

En esta tesis (Capítulo III) se comprobó que el mosquito puede resistir dosis letales de bacterias después de haber tenido un contacto previo con la misma bacteria (viva y a bajas concentraciones). Los datos de supevivencia a constantes retos con patógenos encontrados en este trabajo se une a la evidencia previa que hace constar que mosquitos tengan la capacidad de mostrar un tipo de memoria lo largo de la vida del individuo (i.e. priming inmunológico) (Little & Kraaijeveld, 2004; Little, Hultmark & Read, 2005, Pham & Schneider, 2009). Por primera vez se realizó una cinética midiendo dos parámetros en los que se esperaba que mostraran una mejora en su producción y activación. Sin embargo, ninguno de los efectores (fenoloxidasa y óxido nítrico) cuentificados mostró incremento significativo durante el segundo encuentro con el mismo patógeno. La actividad de fenoloxidasa muestra una continua deactivación. Es

posible que el que la continua activación de la fenoloxidasa evite daño (debido a las moléculas tóxicas producidas durante la cascada) a tejidos propios del mosquito. Lo que indica que el incremento en la supervivencia de los individuos esta relacionada con una regulación de los efectores inmunes, lo que sería vetntajos en caso que se incremente la eficiencia para controlar patógenos. Esposible que la actividad de fenoloxidasa, se vea comprometida con otras características, por ejemplo la producción de huevos. Se necesitan más estudios para poner en prueba esta idea. Así, se tendría un excelente ejemplo de la disyuntiva entre supervivencia y reproducción, y que la memoria inmunológica en el mosquito, a pesar de ser una estrategia adaptativa, también ha sido y es moldeada por factores ecológicos como es la limitante de recursos energéticos.

Como ocurre con frecuencia una pregunta biológica, a parte de responderse, genera más preguntas. En el caso del priming inmune encontrada en *Ae. aegypti* ocurre lo mismo y generan perspectivas de estudio. Por ejemplo, el priming inmune en otros organismos muestra especificidad hacia el patógeno. En insectos es probable que también exista este fenómeno, lo cual sería ventajoso si es que existen vías moleculares inmunes especificas para los distintos tipos de patógenos (bacterias Gram-, Gram+, hongos, protistas, parasitoides o virus). En el mosquito podrían hacerse infecciones cruzadas (ej. Gram- vs Gram+ o viceversa) para establecer si la especificidad también esta presente. Otro aspecto sería evaluar la presencia de un efecto transgeneracional del fenómeno de priming inmune. Este aspecto fue contemplado teóricamente en el Capítulo II. Se presentó evidencia, a partir de diversos estudios llevados a cabo por distintos grupos de investigación, de que padres inmunizados pueden heredar la información a sus hijos, haciéndolos menos susceptibles al ataque de patógenos. Aunque temporalmente es difícil que un mosquito adulto tenga contacto con sus hijos, el ambiente en el que viven sí puede ser compartido. La existencia de priming transgeneracional podría ser una estrategia adaptativa en este grupo de insectos. Por último, no hay que dejar el lado el necesario estudio de los mecanismos genéticos, moleculares y celulares que se dan para que los insectos puedan presentar el fenómeno de priming inmune. En esta tesis se observó la existencia del priming en el mosquito, sin embargo se desconoce el mecanismos de cómo

es que esto ocurre. Actualmente el estudio de los mecanismos inmunes es llevado a cabo por un gran numero de grupos de investigación, diversas herramientas están siendo utilizadas para investigar cuales son los parámetros inmunes que se activan y cómo es que están regulados.

Efecto de la variación en la calidad y cantidad de la dieta en la respuesta inmune del mosquito

Los resultados obtenidos en esta tesis (Capítulo IV) muestran que los individuos tienen una constitución genética que permite variaciones fenotípicas para ajustarse a la heterogeneidad ambiental. En específico, en ocasiones un solo genotipo puede tener la capacidad de producir dos fenotipos alternativos como resultado de su interacción con la limitación de alimento durante el desarrollo (Schlichting 1986). La respuesta inmune se puede ver como la suma de los efectos genéticos y los efectos ambientales que se dan durante el desarrollo larval, dando como resultado variación en la respuesta inmune del adulto. Es posible que la plasticidad pueda estar manteniendo la variación en la población. Las diferencias fenotípicas entre los individuos son, generalmente, las responsables de las diferencias en la adecuación, por lo tanto el cambio evolutivo en la respuesta inmune, debido a selección natural, estará determinado por dicha variación. Relacionado con lo anterior, las diferencias en la respuesta inmune entre hembras y machos pueden ser utilizadas como un indicador de las estrategias que cada sexo sigue dependiendo del ambiente y la forma de respuesta ante éste. Lo que podría estar cambiando entre generaciones no son los genes sino las normas de reacción de los genotipos, los beneficios o costos que la plasticidad traiga va a dar como resultado la evolución de las normas de reacción del fenotipo inmune que se esta expresando.

Al igual que para el priming, los mecanismos moleculares de plasticidad fenotípica son desconocidos. Sin embargo, es un hecho que las señalaes ambientales son moduladores de la actividad transcripcional de genes, alterando su expresión (Kent et al. 2009). La base molecular se desconoce, sin embargo, se ha propuesto que genes con estructura TATA box, son capcaces de de respuesta rápidas y variables (Richards et al. 2006; Liefting et al. 2009). Procesos epigenéticos, como la metilación de ADN, puede modificar el nivel de expresión de caracteres (Bender 2004). Es posible que estos mecanismos sean los

responsbles de la diferencias fenotipicas observadas dentro de los genotipos. Estas diferencias estarían dadas como respuesta a la variación ambiental. El priming inmune también podría estar siendo modulado por fenómenos epigenéticos. La presencia de patógenos puede ser considerada como una señal tanto de ausencia y presencia, como de intensidad (virulencia). La modulación transcripcional a través de epigenesis necesita ser evaluada para lograr entender los mecanismos detrás de la expresión de la respuesta inmune en insectos.

Implicaciones del estudio de la ecología evolutiva del mosquito en salud pública

El mosquito actualmente en zonas en las cuales comúnmente no se encontraba (ver Fig. 2, Capítulo I). Este hecho no solo se debe al incremento de temperatura, que posiblemente contribuya ampliar el rango de distribución del mosquito, sino a la capacidad del mosquito de colonizar y mantenerse en nuevos nichos ecológicos. Esta capacidad inherente del mosquito muy posiblemente este relacionado a la plasticidad del organismo para adaptarse al ambiente. El estudio de la existencia de variación genética, efecto del ambiente y la interacción Genotipo x Ambiente en diversas poblaciones de mosquitos de campo sería valioso para poder concluir si en realidad la plasticidad fenotípica es realmente una característica esencial para el éxito del mosquito.

Todos los organismos están bajo en continuo contacto con agentes infecciosos. Debido a esto es necesario un adecuado y eficiente sistema de defensa para proteger la integridad, y asegurar la supervivencia y reproducción, del organismo. Barreras físicas, células y sobre todo moléculas son comunes entre los distintos taxones (incluyendo plantas y probablemente hongos) (Heine, 2008). En los últimos años la concepción del sistema innato de defensa ha dejado de considerase “sencillo” para ahora tener el lugar que desde un principio le correspondía que es el de “esencial e imprescindible”. El sistema inmune de insectos difiere con mamíferos por la ausencia del llamado sistema adaptativo (linfocitos y anticuerpos). Sin embargo cuentan con un sistema eficaz de defensa. Este sistema, tiene como finalidad conservar un estado de homeostasis entre el organismo y el medio que lo rodea. El estudio de fenómenos

como la memoria inmunológica y aspectos genéticos cuantitativos de la respuesta inmune de los mosquitos son útiles para el entendimiento de los procesos evolutivos yecológicos que la están moldeando. Este conocimiento puede y debe ser aplicado, por ejemplo la memoria inmune de mosquitos puede ser útil para limitar la transmisión del virus Dengue. El esparcir virus Dengue inactivo (inclusive mezclado con insecticida) podría hacer que los mosquitos que sobreviven puedan generar memoria, para que cuando estén en contacto con el virus activo, este tenga una nula o menor tasa de replicación dentro del mosquito y en consecuencia disminuir la incidencia de casos de dengue clásico o hemorrágico en las poblaciones humanas.

Referencias

Adamo SA, Jensen M, Younger M. 2001. Changes in lifetime immunocompetence in male and female *Gryllus texensis* (formerly *G. Integer*): trade-offs between immunity and reproduction. *Animal Behaviour* **62**: 417-425.

Bender J. 2004. DNA methylation and epigenetics. Ann Rev Plant Biol 55:41–68

De Jong G, Van Noordwijk AJ. 1992. Acquisition and allocation of resources: genetic (co)variances, selection, and life histories. *American Naturalist* **139**: 749-770.

Janeway Jr. CA, Medzhitov R. 2002. Innate immune recognition. *Annual Review of Immunology* **20**: 197-216.

Heine H. 2008. Innate immunity of plants, animals and humans. Series: nucleic acids and molecular biology, vol. 21, Springer-Verlag

Kent CF, Daskalchuk T, Cook L, Sokolowski MB, Greenspan RJ. 2009. The Drosophila foraging gene mediates adult plasticity and gene-environment interactions in behaviour, metabolites, and gene expression in response to food deprivation. Plos Genetics 5

Liefting M, Hoffmann AA, Ellers J. 2009. Plasticity versus environmental canalization: population differences in thermal responses along a latitudinal gradient in Drosophila serrata. Evolution 63:1954–1963

Little TJ, Kraaijeveld AR. 2004. Ecological and evolutionary implications of immunological priming in invertebrates. *Trends in Ecology and Evolution* **19**: 58-60.

Little TJ, Hultmark D, Read AF. 2005. Invertebrate immunity and the limits of mechanistic immunology. *Nature Immunology* **6**: 651-654.

Moret Y, Schmid-Hempel P. 2000. Survival for immunity: the price of immune system activation for bumblebee workers. *Science* **290**: 1166-1168.

Pfennig HS. 2001. Evolution of pathogen virulence: the role of variation in host phenotype. *Proceedings of the Royal Society* **ser b 268**: 755-760.

Pham LN, Schneider DS. 2008. Evidence for specificity and semory in the insect innate immune response. Pages 97-128 in N. Beckage editor. Insect mmunology. Academic Press, San Diego,USA.

Richards CL, Bossdorf O, Muth NZ et al (2006) jack of all trades, master of some? On the role of phenotypic plasticity in plant invasions. *Ecology Letters* **9**:981–993

Schlichting CD. 1986. The evolution of phenotypic plasticity in plants. *Annual Review of Ecology and Systematics* **17**: 667-693.

Current immunity markers in insect ecological immunology: assumed trade-offs and methodological issues.

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Keywords:	Evolutionary Biology, Population Genetics
Abstract:	The field of ecological immunology currently relies on using a number of immune effectors or markers. These markers are usually used to infer ecological trade-offs (via conflicts in resource allocation), though physiological nature of these markers remains elusive. Here, we review markers frequently used in insect evolutionary ecology research: cuticle darkening, haemocyte density, nodule/capsule formation, phagocytosis and encapsulation/melanization via use of nylon filaments and beads, phenoloxidase activity, nitric oxide production, lysozyme and antimicrobial peptide production. We also provide physiologically based information that may shed light on the probable trade-offs inferred when these markers are used. In addition, we provide a number of methodological suggestions to improve immune marker assessment.

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Current immunity markers in insect ecological immunology: assumed trade-offs and methodological issues

RUNNING TITLE: INSECT IMMUNO-ECOLOGY MARKERS, MORENO-GARCÍA *et al.*

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20 **Abstract**

21 THE FIELD OF ECOLOGICAL IMMUNOLOGY CURRENTLY RELIES ON USING A NUMBER OF IN

22 EFFECTORS OR MARKERS. THESE MARKERS ARE USUALLY USED TO INFER ECOLOGICAL T

23 CONFLICTS IN RESOURCE ALLOCATION), THOUGH PHYSIOLOGICAL NATURE OF THESE MA

24 ELUSIVE. HERE, WE REVIEW MARKERS FREQUENTLY USED IN INSECT EVOLUTIONARY ECO

25 RESEARCH: CUTICLE DARKENING, HAEMOCYTE DENSITY, NODULE/CAPSULE FORMATION,

26 AND ENCAPSULATION/MELANIZATION VIA USE OF NYLON FILAMENTS AND BEADS, PHENO

27 ACTIVITY, NITRIC OXIDE PRODUCTION, LYSOZYME AND ANTIMICROBIAL PEPTIDE PRODUC

28 PROVIDE PHYSIOLOGICALLY BASED INFORMATION THAT MAY SHED LIGHT ON THE PROBA

29 INFERRED WHEN THESE MARKERS ARE USED. IN ADDITION, WE PROVIDE A NUMBER OF

30 METHODOLOGICAL SUGGESTIONS TO IMPROVE IMMUNE MARKER ASSESSMENT.

31

32 **Keywords**: EVOLUTIONARY ECOLOGY, INSECT IMMUNITY, IMMUNE MARKERS

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Introduction

ECOLOGICAL IMMUNOLOGY IS A RAPIDLY EXPANDING YOUNG DISCIPLINE (ROLFF & REYN 2009). WITHIN THIS FIELD, IMMUNE RESPONSE IS SEEN AS A SET OF TRAITS THAT INVOLVE (SHELDON & VERHULST 1996). RESOURCES MUST BE ALLOCATED BETWEEN AN ORGANISM MAINTENANCE AND PROPER FUNCTIONING AND THE EXPRESSION OF OTHER TRAITS LINK SURVIVAL AND REPRODUCTION, RESULTING IN ECOLOGICAL TRADE-OFFS AMONG TRAIT MOMENT, THERE IS GROWING EMPIRICAL EVIDENCE OF TRADE-OFFS ASSOCIATED BETWE TRAITS (SIZE AT BIRTH, GROWTH AND MORTALITY RATES, SIZE AND AGE AT MATURITY, L SURVIVAL, CLUTCH SIZE AND REPRODUCTIVE EFFORT; REVIEWED IN STEARNS 1992) AND DEFENSE (E.G. KRAAIJEVELD & GODFRAY 1997, RIGBY & JOKELA 2000; FELLOWES *et al.* 1998, BOOTS & BEGON 1993, MORET & SCHMID-HEMPEL 2000, HOANG 2001).

THE ECOLOGICAL IMMUNITY FRAMEWORK HAS BEEN APPLIED TO A NUMBER OF DISCIPLI INCLUDING SEXUAL SELECTION (I.E. DIFFERENTIAL MATING AND FERTILIZATION SUCCES 1871) (E.G. SIVA-JOTHY 2000; MCKEAN & NUNNEY 2001, ROBERTS *et al.* 2004) AND, TO A LESSER EXTENT, SOCIAL EVOLUTION (E.G. CALLERI *et al.* 2007, BOCHER *et al.* 2008), PREDATOR-PREY RELATIONSHIPS (E.G. PACKER *et al.* 2003, ROY & HOLT 2008) AND PARENTAL INVESTMENT (E.G. HOI-LEITNER *et al.* 2001; BRZEK & KONARZEWSKI 2007). THE INFLUENCE OF ECOLOGICAL IMMUNITY HAS ALSO EXTENDED TO BROADER FIELDS SUCH AS LIFE-HISTORY THEORY (SC 2005A), PARASITE-HOST COEVOLUTION (E.G. LADY *et al.* 2007, DUFFY *et al.* 2007), CONSERVATION BIOLOGY (E.G. STEVENSON 2006, TARLOW & BLUMSTEIN 2007) AND LEARNING (E.G. BARNA *al.* 2006), AMONG OTHERS.

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57 ONE ANIMAL GROUP WHERE ECOLOGICAL IMMUNOLOGY HAS BEEN EXTENSIVELY INVEST

58 INSECTS (SEE, FOR EXAMPLE, LAWNICZAK *et al.* 2006). INSECTS HAVE PROPERTIES (E.G. SMALL SIZE,

59 LARGE SAMPLE SIZES, SHORT DEVELOPMENT TIMES) THAT ALLOW A WIDE RANGE OF EXP

60 INSECT STUDIES HAVE MEASURED A NUMBER OF IMMUNE EFFECTORS RELATED TO BOTH

61 HUMORAL DEFENCE REACTIONS (HEREON REFERRED TO AS IMMUNE MARKERS), IN WHIC

62 SUCH IMMUNE RESPONSE, MEASURED THROUGH THESE MARKERS, IS ASSUMED (SEE ALSO

63 2004). THE LOGIC OF CHOOSING SUCH MARKERS HAS BEEN BASED ON THE ASSUMPTION T

64 PROTECT THE INSECT, BUT THEIR PRODUCTION NEGATIVELY CORRELATES WITH OTHER L

65 INTERMEDIATE TRAITS (E.G. COLORED TRAITS, BEHAVIORAL TRAITS, ETC.). MANY FEASIB

66 SENSITIVE TECHNIQUES IN INSECT IMMUNOLOGY (E.G. PROTEOMIC, TRANSCRIPTOMIC, LI

67 CHROMATOGRAPHY /MASS SPECTROMETRY) HAVE BEEN EMPLOYED AT THE INDIVIDUAL-

68 COSTS CONFINE THESE APPROACHES TO EXPLORATORY OR QUALITATIVE STUDIES AT TH

69 LEVEL. THIS TURNS INTO A RESTRICTION WHEN THE OBJECTS OF STUDY ARE PRIMARILY

70 INDIVIDUALS IN NATURAL POPULATIONS; BECAUSE DIFFERENCES AMONG ORGANISMS IN

71 RESPONSES (AND THEIR TRADE-OFFS AMONG OTHER BEHAVIOURAL OR REPRODUCTIVE T

72 AN IMPORTANT CAUSE OF ADAPTIVE CHANGE OF THE POPULATION.

73

74 HERE WE REVIEW EACH OF THE MOST COMMON IMMUNE MARKERS USED IN ECOLOGICAL

75 EVOLUTIONARY STUDIES THAT CAN BE UTILIZED ACROSS LARGE SAMPLE SIZES. OUR FIRS

76 IDENTIFY, WHEN POSSIBLE, THE PHYSIOLOGICAL PROCESSES BEHIND EACH MARKER. THIS

77 THE INFERENCE OF TRADE-OFFS WITH OTHER FUNCTIONS THROUGH INDIVIDUAL DATA. S

78 BRIEFLY OUTLINE THE METHODOLOGICAL INCONVENIENCIES IN THE ASSESSMENT OF SUC

79 SUGGEST SOLUTIONS. OUR AIM IS TO PROVIDE A SET OF MORE ROBUST METHODOLOGICA

80 AN OUTLINE OF THESE TWO AIMS APPEARS IN TABLE 1, WE PRESENT EACH IN GREATER D

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81 BELOW. FIRST, HOWEVER, WE PRESENT A BASIC SHORT REVIEW OF HOW INSECT IMMUNIT

82 WHICH SERVES AS THE FRAMEWORK FOR DISCUSSING THE TRADE-OFFS AND METHODOLO

83

84 **Insect Immune Mechanisms**

85 THE INSECT IMMUNE SYSTEM IS FORMED BY A SET OF CELLS, MOLECULES AND REACTION

86 THESE FEATURES ARE CONTINUOUSLY EVOLVING TO RESIST (ATTACK AND ELIMINATE) P

87 INVASION AND TO LIMIT THE NEGATIVE CONSEQUENCES OF THE INFECTION (HOFFMAN &

88 2002, SCHMID-HEMPEL 2005A, SCHNEIDER 2009). THE FIRST LINES OF DEFENCE INCLUDE TH

89 EXOSKELETON CUTICLE, PHYSICAL AND CHEMICAL PROPERTIES (E.G. PH) OF THE EPIDERM

90 EPITHELIUM, AND MALE AND FEMALE REPRODUCTIVE ACCESSORY GLANDS (GILLESPIE *et al.* 1997;

91 CASTEELS 1998). THESE TISSUES ALSO SECRETE CYTOTOXIC MOLECULES LIKE LYSOZYME

92 REACTIVE OXYGEN SPECIES (ROS: E.G. SUPEROXIDE ANIONS, PEROXIDES, HYDROXYL RAD

93 (SCHMID-HEMPEL 2005A). PATHOGENS ARE MAINLY RECOGNIZED BY THE MEMBRANE OF

94 HAEMOCYTES (FREE IN THE HAEMOLYMPH) OR BY THE MEMBRANES OF EPITHELIAL CELL

95 MEMBRANES BEAR PROTEINS CALLED PATTERN RECOGNITION RECEPTORS (PRRS) THAT R

96 CONSERVED MOLECULAR FEATURES OF PATHOGENS CALLED PATHOGEN-ASSOCIATED MOLECULAR

97 PATTERNS (PAMPS: LPS, MANNOSES, β-1,3-GLUCANS AND PEPTIDOGLYCANS) (GILLESPIE *et al.*

98 1997). ONCE PATHOGENS ARE RECOGNIZED AS NON-SELF, CELLULAR AND HUMORAL IMMU

99 MECHANISMS ARE ACTIVATED. CELLULAR RESPONSES INCLUDE PHAGOCYTOSIS, NODULA

100 ENCAPSULATION. DURING ENCAPSULATION, SMALL AND LARGE PATHOGENS ARE SURRO

101 BOUND BY HAEMOCYTES (GILLESPIE *et al.* 1997). DURING NODULATION AND ENCAPSULATI

102 MELANIN LAYER IS CONSTRUCTED (FREQUENTLY REFERRED TO AS MELANOTIC

103 NODULATION/ENCAPSULATION) TO COVER FOREIGN AGENTS, WHICH ULTIMATELY DIE BY

104 ROS OR STARVATION (NAPPI & OTTOVIANI 2000, NARAYANAN 2004).

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106 MELANIN PRODUCTION IS A CONSEQUENCE OF THE PHENOLOXIDASE (PO) CASCADE (SÖDE
107 CERENIUS 1998). DURING THE CASCADE, OPSONIC FACTORS, ROS, AND CYTOTOXINS SUCH
108 QUINONES AND SEMIQUINONES ARE PRODUCED. THESE IMPORTANT INTERMEDIATE MOL
109 HIGHLY REACTIVE AND TOXIC TO PATHOGENS, AND SERVE TO AMPLIFY THE IMMUNE RES
110 (CERENIUS & SÖDERHÄLL 2004, NAPPI & OTTOVIANNI 2000). PO IS INVOLVED IN VARIOUS
111 PHYSIOLOGICAL PROCESSES INCLUDING: CUTICULAR SCLEROTIZATION, IMMUNE DEFENS
112 MELANOTIC ENCAPSULATION AND WOUND HEALING). THREE KINDS OF PO EXIST (SUGUM
113 KANOST 1993): MONOPHENOL MONOOXIGENASE (ALSO REFERRED TO AS TYROSINASE-TY
114 ASHIDA & BREY 1997), *o*-DIPHENOLOXIDASE (ALSO REFERRED TO AS CATHECOLOXIDASE-T
115 DECKER & JAENICKE 2004) AND *ρ*-DIPHENOLOXIDASE (ALSO REFERRED TO AS LACCASE-TY
116 SUGUMARAN & KANOST 1993). THESE THREE KINDS OF PO AND THEIR ACTIVATING SYSTE
117 STRUCTURALLY ALMOST INDISTINGUISHABLE; HENCE, THE TERM PHENOLOXIDASE IS OF
118 LITERATURE WITHOUT DISTINGUISHING AMONG THEM. PO ASSOCIATED WITH SCLEROTIZ
119 PIGMENTATION OF CUTICLE (MONOPHENOL MONOOXIGENASE AND *ρ*-DIPHENOLOXIDASE)
120 BE UNDER HORMONAL CONTROL (ASHIDA & BREY 1997), WHILE *o*-DIPHENOLOXIDASE IS T
121 BY RECOGNITION OF NON-SELF PARTICLES (ASHIDA & BREY 1997). HAEMOLYMPH PO IS M
122 KEPT INSIDE OR NEAR HAEMOCYTES; NEVERTHELESS, IT CAN BE A FEATURE OF THE HUM
123 THE PRECURSOR OF PO, PRO-PHENOLOXIDASE (PROPO) IS PRIMARILY RELEASED FROM HA
124 INTO THE HAEMOLYMPH AFTER CONTACT WITH NON-SELF PARTICLES (LING & YU 2006). I
125 CONTRIBUTES TO HUMORAL MELANIZATION OF PATHOGENS. THE GENERAL PO ACTIVATI
126 INITIATED WHEN PHENYLALANINE IS HYDROXYLATED AND CONVERTED INTO TYROSINE.
127 INDUCED INTO ACTIVE FORM PO. PO CATALYZES BOTH THE HYDROXYLATION OF TYROSI
128 THE OXIDATION OF DOPA TO DOPAQUINONE. FINALLY, DOPAQUINONE IS CONVERTED TO
129 WHICH IS USED FOR WRAPPING PATHOGENS AND WOUND CLOT. (CHRISTENSEN *et al* 2005,
130 SÖDERHÄLL & CERENIUS 1998, NAPPI & CHRISTENSEN 2005).

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131

132 OTHER HUMORAL RESPONSES INCLUDE ANTIMICROBIAL SYSTEMIC MOLECULES, WHICH A

133 SYNTHESIZED MAINLY IN THE FAT BODY AND REPRODUCTIVE ACCESSORY GLANDS, GUT

134 HAEMOCYTES (MANETTI *et al.* 1998, SCHMID-HEMPEL 2005A). MOST OF THESE MOLECULES ARE

135 SECRETED CLOSE TO THE CUTICLE OR INTO THE HAEMOLYMPH, GUT TRACT, OR MALPIGH

136 ANTIMICROBIAL PEPTIDE (AMP) MOLECULES INDUCE A NUMBER OF NEGATIVE EFFECTS O

137 MEMBRANES INCLUDING MEMBRANE COLLAPSE, PREVENTION OF CELL DIVISION, AND PE

138 DISRUPTION (OTVOS JR 2000, BULET *et al.* 2003). NITRIC OXIDE (NO) IS ANOTHER IMMUNE-

139 RELEVANT MOLECULE: IT IS A HIGHLY REACTIVE AND UNSTABLE FREE RADICAL GAS THA

140 MEMBRANES TO ACT ON NEARBY TARGETS (MÜLLER 1997). NO ALSO INHIBITS PROTEIN CA

141 ACTIVITY AND HAS DAMAGING EFFECTS ON PATHOGEN PROTEIN AND DNA (REVIEWED IN

142 2006). MOLECULES SUCH AS ROS CAN DAMAGE PATHOGEN NUCLEIC ACIDS, PROTEINS AND

143 MEMBRANE (NAPPI *et al.* 2000, HERRERA-ORTIZ *et al.* 2004). LYSOZYMES ARE HYDROLYTIC

144 ENZYMES THAT CLEAVE THE GLYCOSIDIC BOND BETWEEN N-ACETYLMURAMIC ACID AND

145 ACETYLGLUCOSAMINE IN PEPTIDOGLYCAN, A MAJOR COMPONENT OF THE WALL POLYMER GRAM

146 (JOLLÈS 1996).

147

148 *Trade-offs among immune responses*

149 TRADE-OFFS WITHIN IMMUNE SYSTEM EFFECTORS ARE ALSO POSSIBLE. IN AN INDIVIDUA

150 TRAITS ARE FREQUENTLY FOUND TO BE GENETICALLY OR PHENOTYPICALLY CORRELATE

151 CORRELATION ARISES BECAUSE A SINGLE GENE CAN INFLUENCE MULTIPLE TRAITS IN PO

152 NEGATIVE FASHIONS (PLEIOTROPY AND ANTAGONISTIC PLEIOTROPY, RESPECTIVELY) OR

153 LINKAGE DISEQUILIBRIUM BETWEEN GENES AFFECTING DIFFERENT CHARACTERS (FALCO

154 1996). PHENOTYPIC CORRELATIONS INCLUDE THE POSITIVE OR NEGATIVE INFLUENCES OF

155 ENVIRONMENTAL FACTORS ON TRAITS (ROFF 1992). REACTIONS AND PRODUCTS OF IMMU

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156 ARE INTERCONNECTED AND THE KIND OF RESPONSE COULD BE RELATED TO THE TYPE OF

157 PATHOGEN. A NUMBER OF STUDIES HAVE EXAMINED GENETIC AND PHENOTYPIC CORREL

158 AMONG ENCAPSULATION, LYTIC ACTIVITY, CUTICULAR DARKNESS, PO ACTIVITY AND HA

159 IN SUMMARY, THESE STUDIES REPORT POSITIVE AND NEGATIVE CORRELATIONS RESULTI

160 CLEAR PATTERN ACROSS SPECIES OR EVEN POPULATIONS (SEE RANTALA & KORTET 2003,

161 2004, FEDORKA *et al.* 2004, RYDER & SIVA-JOTHY 2001, RANTALA & ROFF 2005, ROFF *et al.*

162 2005, MORENO-GARCÍA *et al.* 2010). THIS POINT WILL BE RECONSIDERED LATER.

163

164

165 **Immune Markers Used in Evolutionary Ecology Research**

166

167 ***Cuticle darkness***

168 THE MAIN COMPONENTS OF INSECT CUTICLE –QUINONES AND MELANIN (SEE PHENOLOXI

169 ACTIVITY) – ARE ALSO USED FOR OTHER FUNCTIONS, THUS STRONGLY SUGGESTING A TR

170 BARNES & SIVA-JOTHY 2000. ARMITAGE & SIVA-JOTHY 2005). QUINONE COMPOUNDS

171 DETERMINE CUTICLE PIGMENTATION (VIA SCLEROTIZATION) BY MAKING COVALENT LINI

172 PROTEINS, RESULTING IN COLOURED PRODUCTS, A PROCESS CALLED QUINONE TANNING

173 HIRUMA 1988). QUINONES ALSO FORM METHIDE DERIVATIVES THAT CROSSLINK CUTICLE

174 THROUGH THE QUINONE SIDE CHAIN (*β*-SCLEROTIZATION REACTION) AND ARE RESPONSIBLE FOR CU

175 HARDENING (SAUL & SUGUMARAN 1988). CUTICLE DARKNESS IS THUS RELATED TO HOW M

176 QUINONE IS DEPOSITED IN EVERY SHED OR WOUND REPAIR SITE.

177

178 *Methodology*

179

For Review Only

180 CUTICULAR COLOUR IS FREQUENTLY ASSESSED UNDER A FLUORESCENT WHITE LIGHT AN

181 COLOUR CLASSES ARE ANALYZED USING A GREYSCALE VALUE (E.G. BARNES & SIVA-JOTH

182 THOMPSON *et al.* 2002, ROLFF *et al. 2005*). HOWEVER, SINCE THE CUTICLE STRUCTURE IS

183 COMPOSED OF HUNDREDS OF PROTEINS (ANDERSEN 1995, SHEN *et al.*) AND LIPIDS (CHAPMAN

184 1998), AND SUCH PROTEIN COMPOSITION GREATLY INFLUENCES CUTICLE MECHANICAL PR

185 (E.G. FLEXIBILITY; GOSLINE *et al.* 2002, HAAS *et al.* 2000), THERE COULD BE A POSSIBLE

186 OVERESTIMATION OF QUININE AND MELANIN TANNING. FURTHERMORE, SINCE CUTICLES

187 TAXA VARY CONSIDERABLY IN STIFFNESS, HARDNESS, AND PIGMENTATION, IT IS POSSIBL

188 ASPECT OF A PARTICULAR TISSUE MAY NOT NECESSARILY REFLECT OR BE RELATED TO A

189 HEIGHTENED IMMUNE ABILITY. IN COMBINATION WITH CUTICULAR COLOUR ANALYSIS, W

190 THE USE OF TRANSVERSE SECTION PREPARATIONS (USING A MICROTOME) FROM DIFFERE

191 REPRESENTATIVE REGIONS OF THE INSECT BODY AND CUTICLE WIDTH MEASUREMENT. T

192 REFLECT HOW MUCH THE INSECT HAS INVESTED IN PRODUCING A THICK CUTICLE AND, P

193 QUININES AND MELANIN. NOTE, HOWEVER, THAT CUTICULAR MELANIZATION IS NOT ALW

194 WITH GREATER IMMUNOGENIC RESPONSE (SEE ROBB *et al* 2003).

195

196

197 ***Cellular Responses***

198

199 *Total number of haemocytes (haemocyte density)*

200

201 HAEMOCYTE COUNTING AND/OR ACTIVITY IS A STRAIGHTFORWARD WAY OF ASSESSING C

202 IMMUNE ABILITY (E.G. RANTALA *et al.* 2000, KRAAIJEVELD *et al.* 2001; COTTER *et al.* 2004).

203 HAEMOCYTES ARE GENERALLY RESPONSIBLE FOR IMMUNE ACTIVITIES SUCH AS ENGULF

204 SURROUNDING AND DESTROYING INFECTIOUS AGENTS. AFTER BACTERIA HAVE PENETRA

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205 HAEMOCOEL, HAEMOCYTES ARE THE FIRST AND MOST EFFICIENT BACTERIA-CLEARING M

206 (HILLYER *et al.* 2003). HOWEVER, THERE ARE DIFFERENT HAEMOCYTE TYPES, EACH PLAYING A

207 DIFFERENT FUNCTION (RECOGNITION, PHAGOCYTOSIS, NODULATION, ENCAPSULATION OF

208 (SEE REVIEWS BY LAVINE & STRAND 2002, RIBEIRO & BREHELÍN 2006). DIFFERENCES IN

209 PATHOGEN TYPE (E.G. BACTERIA, VIRUS, NEMATODES, ETC.) AMONG INDIVIDUALS, SEXES

210 MUST BE CONSIDERED. FOR EXAMPLE, IN *Aedes aegypti*, *Escherichia coli* IS MAINLY

211 PHAGOCYTOSED, MEANWHILE *Micrococcus luteus* IS MELANIZED (HILLYER *et al.* 2003).

212 HOWEVER, THE FACTORS ELICITING PHAGOCYTIC VS. MELANIZATION RESPONSES AGAINS

213 INDEPENDENT OF GRAM TYPE (HILLYER *et al.* 2004).

214

215 *Methodology*

216

217 FOR TOTAL AND VIABLE COUNTS, HAEMOLYMPH IS COLLECTED AND INCUBATED IN HUM

218 THEN COUNTED USING AN HEMOCYTOMETER USING (PHASE CONTRAST) MICROSCOPY (E.G. *et*

219 *al.* 2000; KRAAIJEVELD *et al.* 2001). FOR COLLECTION AND INCUBATION OF HAEMOLYMPH, WE

220 SUGGEST THE USE OF CELL CULTURE MEDIA SUCH AS RPMI, SCHNEIDER OR GRACE RATHE

221 BUFFERS. THIS MEDIA MAY ALLOW A BETTER VIABILITY OF CELLS. TRYPAN BLUE CAN BE

222 EXCLUDE BETWEEN DEAD AND VIABLE CELLS. HAEMOLYMPH EXTRACTION MAY BE ACC

223 PERFUSION-BLEED; A DROPLET CAN BE COLLECTED FROM A PUNCTURE/CUT ON THE THO

224 REMOVED LEG. ALTHOUGH THIS MAY APPEAR AN EASY TASK, HAEMOCYTES CAN ADHERE

225 SUCH AS THE FAT BODY. ONE SOLUTION TO THIS PROBLEM IS TO USE ANTICOAGULANT BU

226 PH (PECH *et al.* 1994). AN ACCURATE EXTRACTION OF THESE CELLS CAN ALSO BE CARRIED O

227 INJECTING A PROTEASE INHIBITOR BLEND (E.G. PMSF, TLCK, LEUPEPTINE, EDTA). HOWEVE

228 OPTIMAL CONDITIONS FOR OBTAINING INSECT HAEMOCYTES CANNOT BE GENERALIZED,

229 PROCEDURE OF EXTRACTION THE USE (OR NON-USE) OF ANTICOAGULANT MUST BE ADJU

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230

231

232 *Phagocytosis*

233

234 PHAGOCYTIC ACTIVITIES REQUIRE THE INTERNALIZATION OF THE SURFACE MEMBRANE A

235 SEVERAL ORGANELLES, INCLUDING ENDOSOMES AND LYSOSOMES, WHICH FUSE WITH TH

236 MEMBRANE AND PROVIDE THE MEMBRANE FOR PHAGOSOME FORMATION (DESJARDINS *et* 2005).

237 IN MOSQUITOES, THE SYNTHESIS OF LIPIDS FOR THE MEMBRANES OF THE PHAGOSOME, PL

238 MEMBRANE AND FOR THE PRODUCTION OF CYTOTOXIC PROTEINS FOR THE DEGRADATIO

239 PHAGOCYTISED MATERIAL DECREASES THEIR REPRODUCTIVE OUTPUT (FERDIG *et al.* 1993, HOGG &

240 HURD 1995, HILLYER *et al.* 2003). FOR THESE REASONS, TRADE-OFFS ARE EXPECTED AMONG

241 PHAGOCYTIC ACTIVITIES AND FITNESS TRAITS. FURTHERMORE, IT IS ALSO PROBABLE THAT HAEMOCYTES

242 UNDERGO APOPTOSIS AFTER PHAGOCYTOSIS, WHICH REDUCES THE NUMBER OF CIRCULA

243 HAEMOCYTES (AS OCCURS IN *A. aegypti*, HILLYER *et al.* 2005). THIS WOULD LEAD TO ANOTHER

244 TRADE-OFF, AS IT WOULD GENERATE A DECREASE IN THE HOMEOSTASIS AND REMODELL

245 AS WELL AS THE CAPACITY FOR FIGHTING PATHOGENS. PHAGOCYTOSIS IS AN IMPORTANT

246 REMOVE DEAD OR DAMAGED CELLS, AS WELL AS TO REMODEL ORGANS DURING METAMO

247 (ABRAMS *et al.* 1993, FRANC ET AL, 1996). THUS, A DELAY IN GROWTH SEEMS NECESSARY FOR

248 PATHOGEN CLEARING, VIA RELEASING HAEMOCYTES FROM THEIR IMMUNE ACTIVITY AN

249 THE WOUND REPAIRING PROCESS BEFORE CUTICLE SHEDDING (LAVINE & STRAND 2002).

250

251 *Methodology*

252

253 PHAGOCYTIC ACTIVITY HAS BEEN MEASURED BY EXPOSING THE HOST TO MICROBIAL PA

254 AS BACTERIA AND YEAST CELLS OR POLYSTYRENE BEADS, AND ASSESSING *in vitro* PHAGO

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255 (KURTZ & SAUER 1999, KURTZ *et al.* 2000, KURTZ 2002). ONE WAY TO ASSESS THIS PROCESS IS BY
256 INOCULATING FLUORESCENT, LABELLED BEADS OR BACTERIA *in vivo* (KURTZ 2002). IN EITHER PROCESS,
257 THE PHAGOCYTISED INTRUDERS ARE COUNTED MANUALLY OR BY USING IMAGE ANALYS
258 CARE MUST BE TAKEN TO ENSURE THE BEADS DO NOT BECOME OBSTRUCTED BY MELANI
259 OF REMAINING BEADS (IF POSSIBLE) FROM THE HAEMOLYMPH IS A USEFUL METHOD TO E
260 PHAGOCYTOSIS ACTIVITY. TRYPAN BLUE CAN ALSO FURTHER ASSESS THE VIABILITY OF I
261 MOLECULES. THE EFFECT OF THIS COLORANT IS THAT ONLY THE INGESTED PARTICLES RE
262 FLUORESCENCE, WHILE NON-INGESTED PARTICLES BECOME COATED WITH TRYPAN BLUE
263 *et al*. 2000). ANOTHER METHOD IS TO USE PHRODO DYE CONJUGATED BACTERIA. BACTERIA
264 FLUORESCENT ONLY IN THE ACIDIC ENVIRONMENT OF THE HAEMOCYTE PHAGOSOME (SE
265 FEDROW *et al.* 2009); WHILE EXTRACELLULAR NON-PHAGOCYTOSED BACTERIA DO NOT FLUO
266 WHEN USING LIVE BACTERIA TO EVALUATE THE EFFICIENCY OF PHAGOCYTOSIS, OR ANY
267 PARAMETERS REFERRED TO IN THIS PAPER, WE RECOMMEND FIRST TO ASSESS THE BACTE
268 OF THE INFECTED HOST. TO DO THIS, SUPERNATANT OF HAEMOLYMPH OR HOMOGENIZED
269 CAN BE SPOTTED IN APPROPRIATE PLATES AND COLONY FORMING UNITS COUNTED (SEE H
270 2007).
271
272
273 *Capacity to attach to non-self surfaces (Nodule/Capsule Formation)*
274
275 QUITE FREQUENTLY, RESEARCHERS HAVE INTRODUCED PATHOGENS OR MICROBIAL CELL
276 COMPONENTS TO INSECT HOSTS TO ASSESS CELLULAR IMMUNE RESPONSE (E.G HOWARD *et al.* 1998)
277 THIS INDUCES THE PRODUCTION OF NODULES/CAPSULES, WHICH ARE THEN COUNTED WH
278 HAEMOLYMPH IS EXTRACTED AFTER THE CHALLENGE OR DIRECTLY IN THE HAEMOCOEL
279 *al.* 1996, GOLDSWORTHY *et al.* 2003). NODULES CAN PERSIST IN INSECTS FOR LONG PERIODS OF T

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280 (CARTON & NAPPI 1997), WHICH MEANS THAT THE HAEMOCYTES USED FOR NODULE FORM

281 REMAIN ATTACHED, THEREBY DECREASING HAEMOCYTE DENSITY DURING INSECT LIFET

282 REDUCTION IN HAEMOCYTE NUMBER COULD IN TURN LIMIT THE AVAILABILITY OF HAEM

283 BECOME RELEVANT DURING MOULTING AND/OR METAMORPHOSIS. NOT ALL ADULT INSE

284 HAEMOCYTES SINCE HAEMATOPOIETIC ORGANS ARE NOT ALWAYS PRESENT AT THIS STA

285 1998). IN LEPIDOPTERANS AND DIPTERANS, HEMATOPOIETIC ORGANS HAVE BEEN IDENTIF

286 EXCLUSIVELY IN THE EMBRYONIC, LARVAL AND PUPAL STAGES (GARDINER & STRAND 20

287 *al*. 2003, HOLZ *et al*. 2003, WILLIAMS 2007). THIS MEANS THAT ANY HAEMOCYTES PRESENT IN

288 THE ADULT STAGE WERE PRODUCED DURING EMBRYOGENESIS, LARVAL STAGES AND ME

289 HOWEVER, THE PRESENCE OF HEMATOPOIETIC ORGANS IN ADULT GRASSHOPPERS HAS BE

290 (HOFFMAN 1973, HOFFMAN *et al*. 1974). NATURAL HISTORY DIFFERENCES AMONG INSECT GROUP

291 (E.G. THE PRESENCE OF METAMORPHOSIS) LIKELY LEADS TO VARIATION IN HAEMOCYTE

292 (CONSEQUENTLY CELL NUMBER).

293

294 *Methodology*

295 PATHOGENS SUCH AS BACTERIA, YEAST CELLS ARE INOCULATED DIRECTLY TO HAEMOC

296 *al*. 1989). ALSO, INJECTED PARASITES CAN BE USED TO ASSESS NODULE/ENCAPSULATION F

297 (SEE ELEFTHERIANOS *et al*. 2008). HAEMOLYMPH IS COLLECTED AND INCUBATED IN HUMIDITY

298 CHAMBER, NODULES ARE OBSERVED USING A MICROSCOPE AND COUNTED. CARE MUST B

299 PATHOGENS CAN BE DESTROYED BEFORE NODULATION TAKES PLACE, FOR EXAMPLE, IF F

300 THE MIDGUT (LUCKHART *et al*. 1998). FURTHERMORE, NODULES MUST BE COUNTED EVEN WHEN T

301 SIZE VARIES, WHICH CAN OCCUR EVEN WITHIN THE SAME INDIVIDUAL (SEE HOWARD *et al*. 1998).

302 ENUMERATION OF DIFFERENT SIZES CAN PROVIDE FURTHER INFORMATION ON DIFFEREN

303 ABILITY AMONG HOSTS. WE RECOMMEND TO DIRECTLY ASSESS THE SIZE OF NODULES EI

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304 QUALITATIVELY (BY CATEGORIZING THE DIFFERENT SIZES AND COUNTING HOW MANY O

305 THE DISTINCT CATEGORIES) OR QUANTITATIVELY WITH THE USE OF IMAGE ANALYSIS SO

306

307

308 *Melanotic nodulation/encapsulation (Nylon Monofilament and Beads Insertion)*

309

310 FOR ENCAPSULATION/MELANOTIC ENCAPSULATION ASSESSMENT, INOCULATION WITH SE

311 POLYSTYRENE BEADS OR INSERTION OF A NYLON MONOFILAMENT INTO THE HAEMOCOE

312 USED. IN THIS METHOD, NYLON AND BEADS ARE THOUGHT TO MIMIC NATURAL INFECTIO

313 A PARASITE/PARASITOID) WHILE AVOIDING THE DAMAGING EFFECTS OF PATHOGENS (E.G

314 2000, KOSKIMÄKI *et al.* 2004, SIMMONS *et al.* 2005, RANTALA & ROFF 2007). CELLULAR AND

315 HUMORAL RESPONSES ARE ACTIVATED, WHICH COULD LEAD TO TRADE-OFFS SIMILAR TO

316 FORMATION AND PHAGOCYTOSIS ARE ELICITED. IN THE MELANIN LAYER – WHICH IS FREQ

317 PRODUCED DURING NODULATION/ENCAPSULATION -- SYNTHESIS OF MELANIN IS REQUIRE

318 COSTLY TO PRODUCE, NOT ONLY BECAUSE OF THE RESOURCES USED (SEE *Phenoloxidase Ac*

319 BELOW), BUT BECAUSE MELANIN IS REQUIRED FOR CUTICLE FORMATION, HUMORAL TOX

320 PRODUCTION, AND COLOURING AND HARDENING OF OTHER STRUCTURAL TRAITS (E.G. W

321 SPERMATHECA; LI & CHRISTENSEN 1993, ILANGO 2005).

322

323 *Methodology*

324

325 AFTER INSERTION, FILAMENT/BEADS ARE THEN RETRIEVED AND THE VOLUME OF MELAN

326 MELANIZED CELL MASSES THAT ENCAPSULATE THE FILAMENT ARE MEASURED (SIVA-JOT *In*

327 *vitro* ENCAPSULATION ASSAYS CAN BE ALSO BE PERFORMED. FOR THIS, HAEMOLYMPH IS C

328 FROM EACH INDIVIDUAL AND MIXED WITH BEADS. ENCAPSULATION AND MELANIZATION

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329 AFTER INCUBATION BY MICROSCOPY. THREE METHODS HAVE BEEN DEVELOPED FOR MEA

330 MASSES. ONE METHOD OBSERVES THE MEAN GRAY SCALE OF THE COVERED AREA USING

331 ANALYSIS SOFTWARE (SIMMONS *et al* 2005), WHILE A SECOND USES IMAGES SOFTWARE FILTERS TO

332 MEASURE THE DENSITY OF THE COVERED AREA (KOSKIMÄKI *et al* 2004). ANOTHER APPROACH

333 MEASURES THE ENCAPSULATED/MELANIZED AREA (CONTRERAS-GARDUÑO *et al* 2006).

334 MEASURES, HOWEVER, ARE UNABLE TO DIFFERENTIATE THE FUNCTIONAL ASPECTS OF HA

335 CELLULAR NODULATION/ENCAPSULATION AND HUMORAL MELANIZATION ARE TWO SEPA

336 PROCESSES (LING & YU 2006). HAEMOCYTES RELEASE OR CONTAIN SURFACE PROPO THAT

337 ACTIVATED TO PO, CAUSES MELANIZATION. THIS CELLULAR ENCAPSULATION ALSO ENHA

338 INTERACTIONS BETWEEN HAEMOLYMPH AND PO. LING & YU (2006) NOTICED THAT MELAN

339 DID NOT OCCUR WHEN ISOLATED HAEMOCYTES WERE USED, IT OCCURRED ONLY IN THE I

340 PLASMA. THIS SUGGESTS THAT FACTORS IN HAEMOLYMPH ARE REQUIRED FOR PO CASCA

341 AND HENCE MELANIZATION. THEREFORE, CELLULAR ENCAPSULATION COULD BE IMPLICA

342 CELLULAR AND HUMORAL IMMUNE RESPONSES.

343

344 TRADE-OFFS AMONG IMMUNITY AND OTHER TRAITS (E.G. DEVELOPMENTAL TIME) USING

345 HAVE BEEN FOUND (SEE RANTALA & ROLFF 2005). THE USE OF NON-NATURAL INFECTIONS

346 BE A SUITABLE INDEX OF HOST IMMUNE ACTIVATION-RECOGNITION AND ITS ECOLOGICA

347 RANTALA & ROFF 2007). FOR THE METHODS ABOVE DISCUSSED, UNFORTUNATELY, THE DI

348 OF MELANIZED/ENCAPSULATED AREAS IS OFTEN SO IRREGULAR THAT IT IMPEDES THORO

349 (I.E. IT DEPENDS ON THE POSITION OF THE ARTIFICIAL OBJECT DURING AREA MEASUREME

350 RECOMMEND SEVERAL MEASURES OF THE SAME IMPLANT. ADDITIONALLY, INSERTING A

351 A DIFFICULT TASK FOR SMALL INSECTS. IN THIS CASE, WE RECOMMEND THE USE OF INOC

352 WE ALSO RECOMMEND USING LIVE PATHOGENS THAT INDUCE MELANOTIC ENCAPSULATI

353 IMMUNE RESPONSES. THIS CAN BE USEFUL, FOR EXAMPLE, WHEN TESTING FOR IMMUNE T

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354 AND BEHAVIOUR (SEE AYRES & SCHNEIDER 2009). TOLERANCE THEORY PREDICTS A POSSI

355 INCREASE IN THE PATHOGEN BURDEN, BUT THE IMMUNE RESPONSE WILL LIMIT THE HEAI

356 CONSEQUENCES OF THIS PATHOGEN LOAD (SCHNEIDER & AYRES 2008, READ *et al.* 2008).

357 ONLY LIVE PATHOGENS CAN REPLICATE AND CONTINUOUSLY DAMAGE A HOST. IT IS ALS

358 LIVE PATHOGENS ACTIVATE MORE IMMUNE PATHWAYS (COMPARED WITH INERT IMPLAN

359 THEREFORE GIVE A MORE COMPLETE PICTURE OF AN IMMUNE RESPONSE. HOWEVER, WHI

360 LIVE PATHOGENS, IT IS IMPORTANT TO FIRST ESTIMATE THE DOSAGE OF PATHOGEN NEED

361 MEASUREMENT OF A PARTICULAR IMMUNE RESPONSE. IT HAS BEEN OBSERVED THAT IN S

362 LOW TO MEDIUM DOSES ELICIT PHAGOCYTOSIS, MODERATE TO HIGH DOSES ELICIT NODU

363 OVERDOSES CAN PROMOTE SEPTIC SHOCK (SALVADOR HERNÁNDEZ-MARTÍNEZ, *personal*

364 *communication*). BEFORE A CHALLENGE, WE RECOMMEND TO DISCOVER THE IMMUNE RESP

365 THAT IS ELICITED IN RELATION TO THE TYPE AND DOSE OF INFECTIOUS AGENT (NATURAL

366 MODEL.

367

368

369 ***Humoral responses***

370

371 *Phenoloxidase Activity*

372

373 PO IS THE MOST WIDELY USED IMMUNE MARKER IN ECOLOGICAL IMMUNOLOGY STUDIES

374 GOLDSWORTHY *et al.* 2003, WILSON *et al.* 2003, JACOT *et al.* 2005). THE PO ENZYME IS USED

375 FOR DIFFERENT PHYSIOLOGICAL PROCESSES (WOUNDING, CLOTTING, CUTICLE COMPOSIT

376 ENCAPSULATION, PRODUCTION OF CYTOTOXIC MOLECULES), AND PROBABLY SPERMATH

377 (ILANGO 2005). DUE TO THESE DIFFERENT FUNCTIONS, PO IS CONSTANTLY SYNTHESIZED, T

378 COULD BE COSTLY TO PRODUCE FOR THE ORGANISM. RECALL THAT PROPO MOLECULES A

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379 MAINLY BY HAEMOCYTES (OTHER TISSUES, SUCH AS FAT BODY AND MIDGUT EPITHELIUM

380 PRODUCE PROPO IN SMALLER QUANTITIES), AND THAT SOME OF THESE MOLECULES REMA

381 TO HAEMOCYTES, WHILE SOME PROPO MIGHT END UP IN SEVERAL OTHER TISSUES (I.E. CU

382 MIDGUT, SALIVARY GLANDS, EGGS; ASHIDA & BREY 1997). IF PATHOGENS ARE DETECTED

383 PROPO TRANSPORTATION FROM HAEMOCYTES TO OTHER TISSUES, IT IS POSSIBLE THAT T

384 ACTIVATED IN THE HAEMOCELE FOR CLEARING PATHOGENS, CONSEQUENTLY LEAVING T

385 WITH A PROPO DEFICIENCY. THIS MEANS THAT A TRADE-OFF MAY ARISE BECAUSE OF A P

386 DEFICIENCY (BUT NOT OF OTHER COMPONENTS, SUCH AS PO SUBSTRATES; SEE BELOW), A

387 ASSUMED IN GENERAL STUDIES WHERE PO AND MELANIN HAVE BEEN INVOLVED (SIVA-JO

388 THIS DIFFERENT PERSPECTIVE OF A POSSIBLE TRADE-OFF WHEN MEASURING PO ACTIVITY

389 BEEN PUT FORWARD NOR EXAMINED PREVIOUSLY AND NEEDS FURTHER EMPIRICAL STUD

390

391 THE PO SUBSTRATE TYROSINE (TYR) AND TYROSINE SUBPRODUCTS ARE USED FOR THE EX

392 AND MANUFACTURE OF OTHER TRAITS. TYR IS OBTAINED THROUGH FOOD AND IS NATUR

393 SYNTHESIZED BY ORGANISMS OR BY THE HYDROXYLATION OF PHENYLALANINE (PHE) (C

394 *al.* 2005). SINCE PHE IS AN ESSENTIAL AMINO ACID FOR PROTEIN SYNTHESIS, ITS IMPORTAN

395 IN THE COST OF ACQUIRING THIS RESOURCE. TYR IS ALSO INVOLVED IN A VARIETY OF FU

396 OF THESE, FOR EXAMPLE, IS THE COLOURING OF THE EGG CHORION IN SOME INSECTS (LI &

397 CHRISTENSEN 1993). IN MOSQUITOES, MELANIZATION RESPONSES AGAINST WORMS GENE

398 DELAY IN TYR ACCUMULATION IN THE OVARIES, AND THEREFORE A DECREASE IN THE NU

399 PRODUCED AFTER AN IMMUNE CHALLENGE (LI & CHRISTENSEN, 1993) AND A DELAY IN OV

400 (FERDIG *et al.* 1993). EXAMPLES LIKE THIS ARE SUGGESTIVE OF SUBSTRATES OF PO AND MEL

401 PHE AND TYR - BEING RESTRICTIVE RESOURCES THAT MAY LEAD TO TRADE-OFFS BETWE

402 IMMUNE RESPONSE AND OTHER KEY FUNCTIONS INCLUDING MOULTING, BASAL METABO

403 PROTEIN SYNTHESIS. ALTHOUGH TYR CAN BE STORED (AS TYROSINE-O-PHOSPHATE, BETA

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404 TYROSINE, TYROSINE GLUCOSIDE; MITCHELL & LUNAN 1964, LEVENBOOK *et al.* 1969, CHEN

405 1978), TRADE-OFFS COULD BE DETECTED IF NOT ENOUGH TYR IS GATHERED IN ORDER TO

406 AMINO ACID TO ALL THE METABOLIC REQUIREMENTS THROUGH THE INSECT LIFETIME. H

407 INSECTS NECESSARILY USE TYR OR PHE IN THE SAME WAY, PO MEASURES NEED TO BE CA

408 INTERPRETED IN EVERY INSECT MODEL. MELANIN, THE FINAL PRODUCT OF THE PO CASCA

409 INVOLVED IN OTHER MORPHOLOGICAL TRAITS SUCH AS CUTICLE FORMATION AND PIGMI

410 MAY AFFECT FUNCTIONS RELATED TO APOSEMATISM AND CRYPSIS, THERMOREGULATIO

411 UV RADIATION AND COLOURED SEXUAL TRAITS. MANY OF THESE FUNCTIONS AND THEIR

412 TRADE-OFFS HAVE NOT BEEN ADEQUATELY INVESTIGATED.

413

414 *Methodology*

415

416 PO ACTIVITY IS ASSAYED BY THAWING HAEMOLYMPH AND *o*-DIPHENOLS (DOPA OR DOPA

417 SUBSTRATE INTO A MICROPLATE WELL. THIS ASSAY WILL NOT DISTINGUISH THE THREE D

418 ACTIVITIES, SINCE IT IS INFERRED THE *o*-DIPHENOLOXIDASE ACTIVITY OF ALL THREE ENZYMES W

419 BE DETECTED (SUGUMARAN & KANOST 1993). CARE MUST BE TAKEN WHEN USING *o*-DIPHENOLS,

420 SINCE THERE COULD ALSO BE A PEROXIDASE-MEDIATED MELANIN FORMATION (CHRISTE

421 2005), WHICH CAN RESULT IN AN OVERESTIMATION OF PO ACTIVITY. WE RECOMMEND THI

422 PEROXIDASE SUPPRESSOR (E.G. HYDROGEN PEROXIDE IN METHANOL OR COMMERCIALLY

423 ANOTHER CURRENT PROBLEM WITH ASSESSING PO IS THAT PROTEIN LOAD STANDARDIZA

424 BEFORE MEASUREMENT, USING A STANDARD PROTEIN ASSAY (CONTRERAS-GARDUÑO *et al.* 2007), A

425 PRACTICE THAT IS RARELY CARRIED OUT AND WITHOUT WHICH MAY RESULT IN DISPARA

426 PO READINGS. SINCE OCCASIONALLY REAL PROTEIN LOAD CAN BE MASKED IF DIFFERENC

427 INDIVIDUALS, (E.G. BIGGER SIZE OF FEMALES THAN MALES; INACCURATE EXTRACT OF TO

428 HAEMOLYMPH); WE RECOMMEND THE PROTEIN LOAD PRACTICE MENTIONED ABOVE. FUI

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429 THERE MIGHT BE A RAPID DEGRADATION OF PO, IF STORED FOR RELATIVELY LONG TIME.

430 BE RESOLVED BY USING A PROTEASE INHIBITOR COCKTAIL (E.G. PMSF, LEUPEPTIN). ANOT

431 PROBLEM IS THAT SMALL-SIZED INSECTS MAY NOT PROVIDE PO READINGS, SINCE PO QUA

432 SO SMALL. THIS PROBLEM CAN BE SOLVED BY USING MORE THAN ONE INDIVIDUAL FOR A

433 SAMPLE TO OBTAIN PO READINGS. AS WELL, THE TOTAL PO ACTIVITY CAN BE ESTIMATED

434 ENZYME α-CHYMOTRYPSIN TO ACTIVATE ALL PRO-PO PRESENT IN THE HAEMOLYMPH (SEE

435 ZUK 2010).

436

437

438 *Nitric Oxide*

439

440 NO QUANTIFICATION IS A RELATIVELY NEW IMMUNE MARKER IN ECOLOGICAL IMMUNITY

441 FOR EXAMPLE, MORENO-GARCÍA *et al.* 2010). NO DAMAGES PATHOGENS AND IS ALSO USED AS A

442 SIGNALLING MOLECULE. IN *Drosophila*, NO IS INDISPENSABLE FOR ACTIVATION OF THE IMMUNE

443 DEFICIENCY (IMD) PATHWAY (FOLEY & O'FARREL 2003). THIS IS ONE OF THE THREE PATHW

444 (THE OTHER TWO BEING TOLL AND JAK/STAT SEE FERRANDON *et al.* 2007), THAT PRODUCE

445 TRANSCRIPTIONAL FACTORS IN THE FAT BODY, LEADING TO THE SYNTHESIS OF ANTIMIC

446 AND OTHER FACTORS THAT PREVENT SELF TISSUE DAMAGE AND CONTROL HAEMOCYTE

447 DIFFERENTIATION (FOLEY & O'FARRELL 2003; AGAISSE & PERRIOMON 2004). ANOTHER INT

448 POINT IS THE FACT THAT THE NO SYNTHASE (NOS) (AN ENZYME THAT CONVERTS L-ARGIN

449 CITRULLINE AND GENERATES NO) IS CONSTITUTIVE AND INDUCIBLE (MÜLLER 1997). NO IS

450 SIGNALLING MOLECULE, SO A CONSTANT PRODUCTION OF NOS IS NECESSARY FOR HOME

451 PURPOSES, WHILE INDUCIBLE NOS IS ONLY SYNTHESIZED AFTER AN IMMUNE CHALLENGE

452 *al.* 2000). IT IS THEREFORE EXPECTED THAT NO PRODUCTION INCREASES AFTER AN IMMUN

453 CHALLENGE. NEVERTHELESS, KRISHNANA, HYRšL & ŠIMEK (2006) FOUND THAT NO PRODU

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454 SIMILAR IN BOTH NON-STIMULATED AND STIMULATED HAEMOCYTES IN LEPIDOPTERAN L

455 PARTICULAR CASE, THE AUTHORS INDICATE THAT NO CAN BE CONTINUOUSLY SYNTHESIZ

456 INDUCED NOS FOR LONG PERIODS OF TIME (HOURS TO DAYS). THIS MEANS THAT THE EFFI

457 FOR KILLING PATHOGENS WITH NO MAY NOT ONLY RESIDE ON NO CONCENTRATION, BUT

458 DURATION OF NO SYNTHESIS (SEE LAURENT *et al* 1996).

459

460 NO IS PRODUCED DURING THE OXIDATION OF L-ARGININE (MÜLLER 1997). ARGININE IS AN

461 ACID, WHICH MUST BE OBTAINED FROM THE DIET (RIVERO 2006). ARGININE IS ALSO IMPOR

462 SPERM MATURATION (OSANAI & CHEN 1993), EGG PRODUCTION (UCHIDA 1993), LONG TERM

463 MEMORY, CHEMOSENSORY (ANTENNAL LOBES, OLFACTION), AND VISUAL INFORMATION I

464 (MÜLLER 1997). TRADE-OFFS ARE THUS EXPECTED AMONG THESE TRAITS AND THE IMMUN

465

466 *Methodology*

467

468 BASAL LEVELS OF NO ARE NORMALLY USED, WHICH PROVIDES AN INCOMPLETE PICTURE

469 IMMUNE ABILITY. A BETTER ALTERNATIVE IS TO ASSESS NO PRODUCTION AFTER HOST IN

470 INGESTION OF PATHOGENS. THE STANDARD METHOD TO MEASURE NO IS TO USE THE GRI

471 THAT MEASURES NITRITE PRODUCTION – AN INDIRECT MEASURE OF NO (BREDT & SNYDE

472 USE OF CONTROLS SUCH AS AN L-NAME (A NOS-INHIBITORY ARGININE ANALOGUE; SEE RI

473 2006) AND ITS INACTIVE ENANTIOMER D-NAME IS RECOMMENDED. CONTROLS ARE NEEDE

474 BECAUSE NITRITES CAN ALSO BE SUB-PRODUCTS OF OTHER REACTIONS NOT RELATED WI

475 FOR THIS METHOD, HOWEVER, IT IS UNKNOWN WHETHER HAEMOLYMPH PROTEIN LOAD

476 STANDARDIZATION IS NEEDED BEFORE MEASUREMENT. THIS IS A TECHNICAL PROBLEM T

477 RESOLVED IN THE NEAR FUTURE. SIMILAR TO PO, MORE THAN A SINGLE INDIVIDUAL MAY

478 AN INDIVIDUAL HOST SAMPLE IS TOO SMALL.

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479

480

481 *Lysozyme Activity and Antimicrobial Peptides*

482

483 LYSOZYME IS AN ENZYME WITH HYDROLYTIC ACTION MAINLY AGAINST THE PEPTIDOGLY

484 GRAM+ CELL WALLS. IT CAN BE INDUCED, OR CONSTITUTIVELY EXPRESSED IN THE GUT TRA

485 *al*. 1997), HAEMOCYTES AND FAT BODY (GILLESPIE *et al.* 1997). AMPS SHOW GREAT STRUCTURAL

486 DIVERSITY (MORE THAN 170 ISOFORMS HAVE BEEN FOUND IN INSECTS; BULET *et al.* 1999) AND ARE

487 PRODUCED SOON AFTER FOREIGN RECOGNITION (1-4 HOURS) WITH AN EFFICIENT PATHOG

488 KILLING ACTION (SCHMID-HEMPEL 2005B). SOME AMPS MAY REMAIN IN HAEMOLYMPH FO

489 TO THREE WEEKS (SCHMID-HEMPEL 2005B), WHICH IS CONVENIENT DURING SUBSEQUENT

490 ENCOUNTERS. AMPS ARE GENERALLY SHORT PEPTIDES, CONTAINING FEWER THAN 150–20

491 ACIDS (BULET *et al.* 2004), SO THEY ARE CONSIDERED ENERGY EFFICIENT, QUICK AND ECONOM

492 PRODUCE (OTVOS JR 2000). HOWEVER, THE OVERALL AMPS CONCENTRATION IN *Drosophila*

493 HAEMOLYMPH CAN REACH 200 µM (OTVOS JR 2000). AT THIS CONCENTRATION, ANTIMICRO

494 PEPTIDES DO SEEM COSTLY TO PRODUCE. IT IS POSSIBLE THAT RESOURCES (PROTEINS) GA

495 THROUGH ONTOGENY ARE INDISPENSABLE FOR ANTIMICROBIAL PEPTIDE GENERATION A

496 SYNTHESIS OF OTHER MOLECULES. THIS FACT COULD POTENTIALLY LEAD TO TRADE-OFF

497 IMMUNITY AND SYNTHESIS OF OTHER PRODUCTS (E.G. SPERMATOPHALYX PEPTIDES) AND

498 CONFORMATION.

499

500 *Methodology*

501

502 LYSOZYME ACTIVITY IS COMMONLY ASSAYED VIA THE CLEARANCE RATE OF BACTERIAL

503 USING HAEMOLYMPH. THE METHOD IS RELATIVELY SIMPLE: AFTER THE HAEMOLYMPH IS

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504 AND MIXED WITH A BACTERIAL SOLUTION, A TURBIDITY ASSAY IS THEN PERFORMED (E.G

505 2004). SMALL ABSORBANCE VALUES INDICATE HIGH LYTIC ACTIVITY. ANOTHER WAY IS TO

506 DROPS OF HAEMOLYMPH TO BACTERIAL CULTURE, MEASURING THE AREA OF LYTIC ACTI

507 TIME (12 HOURS). THE LYTIC ACTIVITY APPEARS AS CLEAR CIRCULAR ZONES IN THE CULT

508 INDICATES THAT BACTERIA HAVE BEEN CLEARED; THE DIAMETER OF THE HOLE IS MEASU

509 AS THE INDICATOR OF LYTIC EFFECTIVENESS.

510

511 AMPS HAVE BEEN MEASURED BY INJECTION OR INGESTION OF COMPLETE BACTERIA, FUN

512 OR THEIR FRACTIONS (E.G. LPS, PEPTIDOGLYCANE). AMPS ACTIVITY IS ASSESSED IN THREE

513 MEASUREMENT OF THE CLEARANCE RATE OF A BACTERIAL SUSPENSION (VIA A TURBIDIT

514 TO THE LYSOZYME PROTOCOL DESCRIBED ABOVE); 2) MEASUREMENT OF THE RATE OF BA

515 GROWTH ON A PLATE, AFTER THE SAME BACTERIA HAVE BEEN INJECTED INTO THE HOST

516 HAEMOLYMPH HAS BEEN EXTRACTED AFTER SOME TIME; AND, 3) THE ANTIBACTERIAL ZO

517 MEASUREMENT OF THE HOLE AREA, SIMILAR TO THE LYSOZYME PROTOCOL DESCRIBED A

518 PROBLEM WITH THESE DIFFERENT ASSAYS IS THAT ANTIMICROBIAL ACTIVITY CAN BE DU

519 MOLECULES AND NOT NECESSARILY AMPS. SINCE LYSOZYME NEEDS A PH 8 TO 6.5 FOR OP

520 ACTIVITY THIS CAN BE USED TO DISTINGUISH BETWEEN OTHER AMP ACTIVITY (RANTALA

521 2004, AHTIANEN ET AL 2005, BUT SEE DA SILVA ET AL 2000, ADAM & PARSOSN 2006).

522

523

524 **Some concluding remarks**

525

526 ONE INTENTION OF THIS REVIEW IS TO HIGHLIGHT THE ECOLOGICAL AND PHYSIOLOGICA

527 INSECT IMMUNE MARKERS AND THEIR VALUE FOR ESTIMATING TRADE-OFFS BETWEEN IM

528 OTHER TRAITS. THUS, CURRENT (AND NEW) KNOWLEDGE OF IMMUNE PHYSIOLOGICAL M

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529 MUST BE INCORPORATED INTO ECOLOGICAL DATA TO ALLOW FOR A DEEPER UNDERSTAN
530 REASONS FOR EVOLUTIONARY CORRELATIONS AND CONSTRAINTS. THE USE OF EACH MET
531 HERE MUST BE DETERMINED BY EACH RESEARCHER IN TERMS OF ITS OVERALL COST (IN T
532 CONSUMPTION OR PRICE), DIFFERENCES IN THE KIND OF PATHOGENS OR ELICITORS USED
533 IMMUNE RESPONSE, AND THE IMMUNE MARKER THAT CAN ADDRESS QUESTIONS VIA AN A
534 EXPERIMENTAL DESIGN. ANOTHER IMPORTANT CONSIDERATION FOR EACH RESEARCHER
535 TO MAKE REPEATED IMMUNE MEASUREMENTS ON THE SAME SAMPLE. UNFORTUNATELY
536 MEASUREMENT METHODS REFERRED HERE ARE NOT SUITABLE TO BE REPEATED MORE TI
537 EXAMPLE, PO AND NO CONTAINED IN HAEMOLYMPH EXTRACTION GRADUALLY DEGRADE
538 IN AN ULTRAFREEZER (-70 °C). ALSO, UNFREEZING AND RE-FREEZING SAMPLES ACCELERATE
539 DEGRADATION. OCCASIONALLY, HAEMOCYTE MEMBRANES CAN BECOME DISRUPTED EVE
540 SOLUTION OR CULTURE MEDIUM IF STORED FOR LONG PERIODS OF TIME (E.G. WEEKS TO M
541 RECOMMEND EXTRACTING ENOUGH SAMPLE TO DO ALL MEASUREMENTS REQUIRED FOR
542 EXPERIMENTAL DESIGN. IF A LARGE NUMBER OF INDIVIDUALS ARE REQUIRED, THEY (OR
543 CAN BE STORED AND MEASUREMENTS SHOULD BE ONLY DONE FOR THE EXACT NUMBER
544 CAN BE PROCESSED AND QUANTIFIED IN A SINGLE DAY.
545
546 FINALLY, THERE ARE TWO RELATED POINTS THAT WE WOULD LIKE TO HIGHLIGHT: (1) TH
547 IMMUNE MARKERS MENTIONED HERE ARE USUALLY SEEN AS RESPONSES TO ATTACK AN
548 PATHOGENS; AND (2) THE CORRELATIONS AMONG IMMUNE RESPONSES. AS MENTIONED B
549 NEGATIVE AND POSITIVE CORRELATIONS HAVE BEEN FOUND AMONG IMMUNE TRAITS, BU
550 SEEM TO BE HOST-SPECIFIC. COMMONLY, RESOURCES LIMITATION HAS BEEN USED TO EX
551 NEGATIVE CORRELATIONS, HOWEVER, THE NON-EXPRESSION OF AN IMMUNE EFFECTOR T
552 NECESSARILY MEANS THAT THERE IS NO RESPONSE. REACTIONS AND PRODUCTS OF IMMU
553 ARE INTERCONNECTED AND THE KIND OF RESPONSE IS RELATED TO THE PATHOGENS VIR

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554 MENTIONED BEFORE, NOT ALL PATHOGENS WILL INDUCE THE SAME REACTION, OR AT LE
555 MAGNITUDE OF RESPONSE CAN DIFFER DEPENDING ON THE HOST-PATHOGEN INTERACTIO
556 OCCASIONALLY TOLERANCE COULD BE THE BEST STRATEGY TO COPE WITH INFECTIONS.
557 OR TOLERANCE STRATEGIES ADOPTED BY AN INDIVIDUAL WILL DEPEND ON THE FORCE A
558 INFECTION. THERE COULD BE A CRITICAL HOST DAMAGE THRESHOLD, THUS WHEN THE IN
559 INFECTION IS BELOW THIS THRESHOLD (I.E. NO SIGNIFICANT DAMAGE TO THE HOST), THE
560 TOLERATE. ABOVE THE THRESHOLD, IT THEN PAYS TO ALLOCATE MORE INTO RESIST THE
561 EXAMPLE, PHAGOCYTIC ACTIVITY COULD BE TURNED ON, MEANWHILE PO ACTIVITY COU
562 DOWN REGULATED; IN THIS CASE PROBABLY THE HOST IS AVOIDING AUTOREACTIVITY M
563 (GENERATED THROUGH THE PO CASCADE). IN THIS EXAMPLE, THE IMMUNE STRATEGY IS
564 ORDER TO LIMIT PATHOGEN AND SELF DAMAGE, NEVERTHELESS THE PATHOGEN IS NOT E
565 SINCE IMMUNE SYSTEM IS HIGHLY COMPLEX, SEVERAL ASPECTS OF IMMUNE RESPONSE M
566 THEREFORE MEASURED TO COMPREHEND THE INSECT IMMUNITY STRATEGIES. ALSO, OCC
567 SOME IMMUNE RESPONSES ARE NOT SUITABLE TO TEST SOME HYPOTHESIS, FOR EXAMPLE
568 CASCADE AND PO ACTIVITY OCCASIONALLY IS NOT AFFECTED BY THE NUTRITIONAL CON
569 RESPONSES, SUCH AS LYSOZYME, ARE AFFECTED (SEE JACOT *et al.* 2005, MORENO-GARCÍA
570 2010). THEREFORE, IT IS RECOMMENDED TO USE MORE THAN ONE IMMUNE MARKER WHEN
571 POSSIBLE. FINALLY, THE STARTING LINE IN INSECT IMMUNOECOLOGY IS THAT IT MUST IN
572 GREAT RANGE OF ENVIRONMENTAL FACTORS INVOLVED USING A TRADE-OFF FRAMEWOR
573 ALLOCATION AND INVESTMENT STRATEGIES SHOULD BE STUDIED BY TAKING INTO ACCO
574 THE REAL COST IN NATURAL CONDITION AND THEIR EFFECTS IN THE EXPRESSION OF IMM
575 AND GENERATIONAL CHANGES.
576
577 IDEALLY, AN ECOLOGICAL STUDY OF INSECT IMMUNITY SHOULD START WITH THOUGHTF
578 REGARDING APPROPRIATE IMMUNE MARKERS. REGARDING METHODOLOGICAL ISSUES, IN

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579 METHODOLOGICAL INFORMATION IS AVAILABLE BUT IT IS POSSIBLE THAT THE *status quo* O

580 EVOLUTIONARY ECOLOGY RESEARCHERS HAVE USED SUCH MARKERS LEADS OTHERS NO

581 ABOUT THE BEST WAY TO PROCEED. LASTLY, WE ENCOURAGE ECOLOGICAL AND EVOLUT

582 BIOLOGISTS ALIKE TO INCORPORATE USEFUL MOLECULAR TECHNIQUES (E.G. MICROASSA

583 QUANTITATIVE REVERSE TRANSCRIPTASE-PCR, ETC.) TO IDENTIFY THE POSSIBLE MECHAN

584 THE GENETIC BASIS UNDERLYING THE TRADE-OFFS BETWEEN IMMUNITY AND LIFE HISTO

585 HOPED THAT THIS INFORMATION PROVES CONSTRUCTIVE IN PAVING THE WAY TO A MORE

586 OF ECOLOGICAL IMMUNOLOGY.

587

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597

598

599 **References**

600 ABRAMS, J.M., WHITE, K., FESSLER, L.I. & STELLER, H. (1993) PROGRAMMED CELL DEATH DU

601 *Drosophila* EMBRYOGENESIS. *Development*, **117**, 29-43.

602 ADAMO, S.A. (1999) EVIDENCE FOR ADAPTIVE CHANGES IN EGG LAYING IN CRICKETS EXPO

603 BACTERIA AND PARASITES. *Animal Behaviour*, **57**, 117-124.

604 ADAMO, S.A. (2004) ESTIMATING DISEASE RESISTANCE IN INSECTS: PHENOLOXIDASE AND

605 LYSOZYME-LIKE ACTIVITY AND DISEASE RESISTANCE IN THE CRICKET *Gryllus texensis*. *Journal*

606 *of Insect Physiology*, **50**, 209-216.

607 ADAMO, S. A., & PARSONS, N. M. (2006) THE EMERGENCY LIFE-HISTORY STAGE AND IMMUN

608 IN THE CRICKET, *Gryllus texensis. Animal Behaviour,* **72,** 235-244.

609 AGAISSE, H. & PERRIMON, N. (2004) THE ROLES OF JAK/STAT SIGNALING IN *Drosophila*

610 IMMUNE RESPONSES. *Immunological Reviews*, 198, 77-82.

611 AHTIAINEN, J J., ALATALO, R.V., KORTET, R., & RANTALA, M.J. (2005) A TRADE-OFF BETWE

612 SEXUAL SIGNALLING AND IMMUNE FUNCTION IN A NATURAL POPULATION OF THE D

613 WOLF SPIDER *Hygrolycosa rubrofasciata. Journal of Evolutionary Biology,* **18,** 985-

614 991.

615 ANDERSEN, S. O., HOJRUP, P. & ROEPSTORFF, P. (1995) INSECT CUTICULAR PROTEINS. *Insect*

616 *Biochemistry and Molecular Biology*, 25, 153-176.

617 ARMITAGE, S.A.O. & SIVA-JOTHY, M.T. (2005) IMMUNE FUNCTION RESPONDS TO SELECTION

618 CUTICULAR COLOUR IN TENEBRIO MOLITOR. *Heredity*, **94**, 650-656.

619 ASHIDA, M. & BREY, P.T. (1997) RECENT ADVANCES IN RESEARCH ON THE INSECT

620 PROPHENOLOXIDASE CASCADE. *Molecular Mechanisms of Immune Responses in Insects*

621 (EDS P.T. BREY & D. HULTMARK), PP. 135-172. CHAPMAN & HALL, NEW YORK.

622 AYRES, J. S. & SCHNEIDER, D. S. (2009) THE ROLE OF ANOREXIA IN RESISTANCE AND

623 TOLERANCE TO INFECTIONS IN *Drosophila. PLoS Biology*, **7**, E1000150.

624 BAILEY, N.W. & ZUK, M. (2010). CHANGES IN IMMUNE EFFORT OF MALE FIELD CRICKETS IN

625 WITH MOBILE PARASITOID LARVAE. *Journal of Insect Physiology,* **54**, 96-104.

626 BARNARD, C.J., COLLINS, S.A., DAISLEY, J.N. & BEHNKE, J.M. (2006) ODOUR LEARNING AND

627 IMMUNITY COSTS IN MICE. *Behavioural Processes*, **72**, 74-83.

For Review Only

628 BARNES, A.I. & SIVA-JOTHY, M.T. (2000) DENSITY-DEPENDENT PROPHYLAXIS IN THE MEALW

629 BEETLE *Tenebrio molitor* (COLEOPTERA: TENEBRIONIDAE): CUTICULAR MELANIZATION I

630 INDICATOR OF INVESTMENT IN IMMUNITY. *Proceedings of the Royal Society of London*

631 *Ser B*, **267**, 177-182.

632 BOCHER, A., DOUMS, C., MILLOT, L. & TIRARD, C. (2008) REPRODUCTIVE CONFLICTS AFFECT

633 AND IMMUNE DEFENSE IN THE QUEENLESS ANT DIACAMMA SP. *Evolution*, **62**, 123-

634 134.

635 BOOTS, M. & BEGON, M. (1993) TRADE-OFFS WITH RESISTANCE TO A GRANULOSIS VIRUS IN

636 INDIAN MEAL MOTH, EXAMINED BY A LABORATORY EVOLUTION EXPERIMENT. *Functi*

637 *Ecology,* **7,** 528-534.

638 BREDT, D.S. & SNYDER, S.H. (1994) NITRIC OXIDE: A PHYSIOLOGICAL MESSENGER MOLECUL

639 *Annual Review of Biochemistry*, **63**, 175-195.

640 BROOKMAN, J.L., ROWLEY, A.F. & RATCLIFFE, N.A. (1989) STUDIES ON NODULE FORMATION

641 LOCUSTS FOLLOWING INJECTION OF MICROBIAL PRODUCTS. *Journal of Invertebrate*

642 *Pathology,* **53**, 315-323.

643 BRZEK, P. & KONARZEWSKI, M. (2007) RELATIONSHIP BETWEEN AVIAN GROWTH RATE AND

644 IMMUNE RESPONSE DEPENDS ON FOOD AVAILABILITY. *Journal of Experimental Biology*,

645 **210**, 2361-2367.

646 BULET, P., CHARLET, M. & HETRU, C. (2003) ANTIMICROBIAL PEPTIDES IN INSECT IMMUNITY

647 *Innate Immunity* (EDS R.A.B. EZEKOWITZ RAB & J.A. HOFFMANN), PP. 89-108.

648 HUMANA PRESS, NEW JERSEY.

649 BULET, P., STÖCKLIN, R. & MENIN, L. (2004) ANTI-MICROBIAL PEPTIDES: FROM INVERTEBRA

650 VERTEBRATES. *Immunological Review*, **198**, 169-184.

For Review Only

651 BULET, P., HETRU, C., DIMARQ, J.L. & HOFFMANN, D. (1999) ANTIMICROBIAL PEPTIDES IN
652 INSECTS; STRUCTURE AND FUNCTION. *Developmental and Comparative Immunology*, **23**,
653 329-344.
654 CALLERI, D.V., ROSENGARS, R.B. & TRANIELLO, J.F. A. (2007) IMMUNITY AND REPRODUCTIO
655 DURING COLONY FOUNDATION IN THE DAMPWOOD TERMITE, *Zootermopsis angusticollis*.
656 *Physiological Entomology*, **32**, 136-142.
657 CARTON, Y. & NAPPI, A.J. (1997) DROSOPHILA CELLULAR IMMUNITY AGAINST PARASITOIDS
658 *Parasitology Today*, **13**, 218-227.
659 CASTEELS, P. (1998) IMMUNE RESPONSE IN HYMENOPTERA. *Molecular Mechanisms of*
660 *Immune Responses in Insect* (EDS P.T. BREY & D. HULTMARK), PP. 92-110. CHAPMAN
661 & HALL, NEWYORK.
662 CERENIUS, L. & SÖDERHÄLL, K. (2004) THE PROPHENOLOXIDASE.ACTIVATING SYSTEM IN
663 INVERTEBRATES. *Immunological Review,* **198**, 116-126.
664 CHAPMAN, R.F. (1998) *The Insects. Structure and Function*. CAMBRIDGE UNIVERSITY PRESS,
665 CAMBRIDGE.
666 CHEN, P.S., MITCHELL, H K. & NEUWEG, M. (1978) TYROSINE GLUCOSIDE IN *Drosophila*
667 *busckii. Insect Biochemistry, 8,* 279-286.
668 CHRISTENSEN, B.M., LI, J., CHEN, CH-CH. & NAPPI, A.J. (2005) MELANIZATION IMMUNE
669 RESPONSES IN MOSQUITO VECTORS. *Trends in Parasitology*, **21**, 192-199.
670 CONTRERAS-GARDUÑO, J., CANALES-LAZCANO, J. & CÓRDOBA-AGUILAR, A. (2006) WING
671 PIGMENTATION, IMMUNE ABILITY, FAT RESERVES AND TERRITORIAL STATUS IN MAL
672 RUBYSPOT DAMSELFLY, *Hetaerina americana*. *Journal of Ethology*, **24**, 165-173.
673 CONTRERAS-GARDUÑO, J., LANZ-MENDOZA, H. & CÓRDOBA-AGUILAR, A. (2007) THE EXPRE
674 OF A SEXUALLY SELECTED TRAIT CORRELATES WITH DIFFERENT IMMUNE DEFENSE C

For Review Only

675 AND SURVIVAL IN MALES OF THE AMERICAN RUBYSPOT. *Journal of Insect Physiology*, **53**,

676 612-621.

677 COTTER, S.C., HAILS, R.S., CORY, J.S. &WILSON, K. (2004) DENSITY-DEPENDENT PROPHYLAX

678 AND CONDITION-DEPENDENT IMMUNE FUNCTION IN LEPIDOPTERAN LARVAE: A MUL

679 APPROACH. *Journal of Animal Ecology*, **73**, 283-293.

680 DA SILVA, C.C.A., DUNPHY, G.B. & RAU, M. E. (2000) INTERACTION OF XENORHABDUS

681 NEMATOPHILUS (ENTEROBACTERIACEAE) WITH THE ANTIMICROBIAL DEFENSES OF T

682 CRICKET, ACHETA DOMESTICUS. *Journal of Invertebrate Pathology*, **76**, 285-292.

683 DARWIN, C. (1871) *The Descent of Man and Selection in Relation to Sex*. JOHN MURRAY,

684 LONDON.

685 DAY, T., GRAHAM, A.L. & READ, A.F. (2007) EVOLUTION OF PARASITE VIRULENCE WHEN HO

686 RESPONSES CAUSE DISEASE. *Proceedings of the Royal Society of London* SER B, **274**,

687 2685-2692.

688 DECKER, H. & JAENICKE, E. (2004) RECENT FINDINGS ON PHENOLOXIDASE ACTIVITY AND

689 AINTIMICROBIAL ACTIVITY OF HEMOCYANINS. *Developmental and Comparative*

690 *Immunology*, **28**, 673-687.

691 DESJARDINS, M., HOUDE, M. & GAGNON, E. (2005) PHAGOCYTOSIS: THE CONVOLUTED WAY

692 NUTRITION TO ADAPTIVE IMMUNITY. *Immunological Reviews*, **207**, 158-165.

693 DUFFY, M.A. & SIVARS-BECKER L. (2007) RAPID EVOLUTION AND ECOLOGICAL HOST-PARA

694 DYNAMICS. *Ecology Letters*, **10**, 44-53.

695 ELEFTHERIANOS, I., BALDWIN, H., FRENCH-CONSTANT, R.H. & REYNOLDS, S.E.(2008)

696 DEVELOPMENTAL MODULATION OF IMMUNITY: CHANGES WITHIN THE FEEDING PERI

697 FIFTH LARVAL STAGE IN THE DEFENCE REACTIONS OF *Manduca sexta* TO INFECTION BY

698 *Photorhabdus*. *Journal of Insect Physiology*, **54**, 309-318.

For Review Only

699 FALCONER, D.S. & MACKEY, T.F. (1996) *Introduction to Quantitative Genetics.* DOVER,

700 NEW YORK.

701 FARALDO, A. C., SÁ-NUNES, A., DEL BEL, E. A., FACCIOLI, L. H.& LELLO, E. (2005) NITRIC

702 OXIDE PRODUCTION IN BLOWFLY HEMOLYMPH AFTER YEAST INOCULATION. *Nitric Ox*

703 240-246.

704 FEDORKA, K.M., ZUK, M, & MOUSSEAU, T.A. (2004) IMMUNE SUPRESSION AND THE COST OF

705 REPRODUCTION IN THE GROUND CRICKET, *Allonemobius socius. Evolution,* **58,** 2478-2485.

706 FELLOWES, M.D.E., KRAAIJEVELD, A.R. & GODFRAY, H.C.J. (1998) TRADE-OFF ASSOCIATED W

707 SELECTION FOR INCREASED ABILITY TO RESIST PARASITOID ATTACK IN *Drosophila*

708 *melanogaster. Proceedings of the Royal Society of London Ser B,* **265,** 1553-1558.

709 FERDIG, M., BEERNTSEN, B., SPRAY, F., LI, J. & CHRISTENSEN, B. (1993) REPRODUCTIVE COST

710 ASSOCIATED WITH RESISTANCE IN A MOSQUITO-FILARIAL WORM SYSTEM. *American Jo*

711 *Tropical Medicine & Hygiene*, **49**, 756-762.

712 FERRANDON, D., IMLER, J.L., HETRU, C. & HOFFMAN, J.A. (2007) THE *Drosophila* SYSTEMIC

713 IMMUNE RESPONSE: A SENSING AND SIGNALING DURING BACTERIAL AND FUNGAL IN

714 *Nature Reviews in Immunology*, **7**, 863-874.

715 FOLEY, E. & O'FARRELL, P.H. (2003) NITRIC OXIDE CONTRIBUTES TO INDUCTION OF INNATE

716 RESPONSES TO GRAM NEGATIVE BACTERIA IN *Drosophila. Genes & Development*, **17**, 115-

717 125.

718 FRANC, N.C., DIMARCQ, J.-L., LAGUEUX, M., HOFFMANN, J. & EZEKOWITZ, R.A.B. (1996)

719 CROQUEMORT, A NOVEL DROSOPHILA HEMOCYTE/MACROPHAGE RECEPTOR THAT RE

720 APOPTOTIC CELLS. *Immunity,* **4,** 431-443.

721 GARDINER, E.M. & STRAND, M.R. (2000) HEMATOPOIESIS IN LARVAL *Pseudoplusia includens*

722 AND *Spodoptera frugiperda. Archives of Insect Biochemistry and Physiology*, **43**,

723 147-164.

For Review Only

724 GILLESPIE, J.P., KANOST, M.R. & TRENZECK, T. (1997) BIOLOGICAL MEDIATORS OF INSECT
725 IMMUNITY. *Annual Review of Entomology*, **42**, 611-643.
726 GOLDSWORTHY, G., MULLEN, L., OPOKU-WARE, K. & CHANDRAKANT, S. (2003) INTERACTIO
727 BETWEEN THE ENDOCRINE AND IMMUNE SYSTEMS IN LOCUSTS. *Physiological Entomolog*
728 **28**, 54-61.
729 GOSLINE, J., LILLIE, M., CARRINGTON, E., GUERETTE, P., ORTLEPP, C. & SAVAGE, K. (2002)
730 ELASTIC PROTEINS: BIOLOGICAL ROLES AND MECHANICAL PROPERTIES. *Philosophical*
731 *Transactions of the Royal Society of London Ser B,* **357**, 121-132.
732 HAAS, F., GORB, S. & BLICKHAN, R. (2000) THE FUNCTION OF RESILIN IN BEETLE WINGS.
733 *Proceedings of the Royal Society of London Ser B*, **267**, 1375-1381.
734 HERRERA-ORTIZ, A., LANZ-MENDOZA, H., MARTÍNEZ-BARNETCHE, J., HERNÁNDEZ-MARTÍNE
735 VILLARREAL-TREVIÑO, C., AGUILAR-MARCELINO, L. & RODRÍGUEZ, M.H. (2004)
736 *Plasmodium berghei* OOKINETES INDUCES NITRIC OXIDE PRODUCTION IN *Anopheles*
737 *pseudopunctipennis* MIDGUTS CULTURED IN VITRO. *Insect Biochemistry and Molecular*
738 *Biology*, **34**, 893-901.
739 HETRU, C., HOFFMAN, D. & BULET, P. (1997) ANTIMICROBIAL PEPTIDES FROM INSECTS.
740 *Molecular Mechanisms of Immune Responses in Insects* (EDS P.T. BREY & D.
741 HULTMARK), PP. 40-66. CHAPMAN & HALL, NEW YORK.
742 HILLYER, H., SCHMIDT, S.L. & CHRISTENSEN, B.M. (2003) RAPID PHAGOCYTOSIS AND
743 MELANIZATION OF BACTERIA AND PLASMODIUM SPOROZOITES BY HEMOCYTES OF T
744 *Aedes aegypti*. *Journal of Parasitology*, **89**, 62-69.
745 HILLYER, J.F., SCHMIDT, S.L. & CHRISTENSEN, B. M. (2004) THE ANTIBACTERIAL INNATE IM
746 RESPONSE BY THE MOSQUITO *Aedes aegypti* IS MEDIATED BY HEMOCYTES AND INDEPENDENT
747 OF GRAM TYPE AND PATHOGENICITY. *Microbes and Infection,* **6,** 448-459.

For Review Only

748 HILLYER, H., SCHMIDT, S.L., FUCHS, J.F., BOYLE, J.P. & CHRISTENSEN, B.M. (2005) AGE-
749 ASSOCIATED MORTALITY IN IMMUNE CHALLENGED MOSQUITOES (*Aedes aegypti*) CORRELATES
750 WITH A DECREASE IN HAEMOCYTE NUMBERS. *Cellular Microbiology*, **7**, 39-51.
751 HOANG, A. (2001) IMMUNE RESPONSE TO PARASITISM REDUCES RESISTANCE OF *Drosophila*
752 *melanogaster* TO DESICCATION AND STARVATION. *Evolution,* **55,** 2352-2358.
753 HOFFMANN, J. A. (1973) BLOOD-FORMING TISSUES IN ORTHOPTERAN INSECTS: AN ANALOG
754 VERTEBRATE HEMOPOIETIC ORGANS. *Cellular and Molecular Life Sciences*, **29**, 50-51.
755 HOFFMANN, D., BREHELIN, M. & HOFFMANN, J. A. (1974) MODIFICATIONS OF THE HEMOGRA
756 AND OF THE HEMOCYTOPOIETIC TISSUE OF MALE ADULTS OF *Locusta migratoria* (ORTHOPTERA)
757 AFTER INJECTION OF *Bacillus thuringiensis*. *Journal of Invertebrate Pathology*, **24**, 238-
758 247.
759 HOFFMANN, J. A., & REICHHART, J.-M. (2002) *Drosophila* INNATE IMMUNITY: AN EVOLUTIONA
760 PERSPECTIVE. *Nature Immunology*, **3**, 121-126.
761 HOGG, J.C. & HURD, H. (1995) MALARIA-INDUCED REDUCTION OF FECUNDITY DURING THE
762 GONOTROPHIC CYCLE OF *Anopheles stephensi* MOSQUITOES. *Medical and Veterinary*
763 *Entomology*, **9**, 176-180.
764 HOI-LEITNER, M., ROMERO-PUJANTE, M., HOY, H. & PAVLOVA, A. (2001) FOOD AVAILABILIT
765 AND IMMUNE CAPACITY IN SERIN (*Serinus serinus*) NESTLINGS. *Behavioral Ecology and*
766 *Sociobiology*, **49**, 333-339.
767 HOLZ, A., BOSSINGER, B., STRASSER, T., JANNING, W. & KLAPPER, R. (2003) THE TWO ORIGIN
768 OF HEMOCYTES IN *Drosophila*. *Development*, **130**, 4955-4962.
769 HOWARD, R.W., MILLER, J.S. & STANLEY, D.W. (1998) THE INFLUENCE OF BACTERIAL SPECI
770 AND INTENSITY OF INFECTIONS ON NODULE FORMATION IN INSECTS. *Journal of Insect*
771 *Physiology*, **44**, 157-164.

For Review Only

772 ILANGO, K. (2005) STRUCTURE AND FUNCTION OF THE SPERMATHECAL COMPLEX IN THE
773 PHLEBOTOMINE SANDFLY PHLEBOTOMUS PAPATASI SCOPOLI (DIPTERA: PSYCHODIDA
774 ULTRASTRUCTURE AND HISTOLOGY. *Journal of Bioscience*, **30**, 101-131.
775 JACOT, A., SCHEUBER, H., KURTZ, J. & BRINKHOF, M.W.G. (2005) JUVENILE IMMUNE SYSTEM
776 ACTIVATION INDUCES A COSTLY UPREGULATION OF ADULT IMMUNITY IN FIELD CRIC
777 *campestris*. *Proceedings of the Royal Society of London Ser B*, **272**, 63-69.
778 JOLLÈS, P. (1996) LYSOZYMES: MODEL ENZYMES IN BIOCHEMISTRY AND BIOLOGY. BIRKHÄ
779 VERLAG, SWITZERLAND.
780 KOSKIMAKI, J., RANTALA, M.J., TASKINEN, J., TYNKKYNEN, K. & SUHONEN, J. (2004)
781 IMMUNOCOMPETENCE AND RESOURCE HOLDING POTENTIAL IN THE DAMSELFLY, *Cal*
782 *virgo* L. *Behavioral Ecology*, **15**, 169-173.
783 KRISHNANA, N., HYRšL, P. & ŠIMEK V. (2006) NITRIC OXIDE PRODUCTION BY HEMOCYTES O
784 LARVA AND PHARATE PREPUPA OF *Galleria mellonella* IN RESPONSE TO BACTERIAL
785 LIPOPOLYSACCHARIDE: CYTOPROTECTIVE OR CYTOTOXIC? *Comparative Biochemistry and*
786 *Physiology Part C*, **142**, 103-110.
787 KRAAIJEVELD, A.R. & GODFRAY, H.C.J. (1997) TRADE-OFF BETWEEN PARASITOID RESISTAN
788 LARVAL COMPETITIVE ABILITY IN *Drosophila melanogaster*. *Nature*, **389**, 278-280.
789 KRAAIJEVELD, A.R., LIMENTANI, E.C. & GODFRAY, H.C.J. (2001) BASIS OF THE TRADE-OFF
790 BETWEEN PARASITOID RESISTANCE AND LARVAL COMPETITIVE ABILITY IN *Drosophila*
791 *melanogaster*. *Proceedings of the Royal Society of London Ser B*, **268**, 259-261.
792 KUMAR, S., CHRISTOPHIDES, G.K., CANTERA, R., CHARLES, B., SOO HAN, Y., MEISTER, S.,
793 DIMOPOULOS, G., KAFATOS, F.C. & BARILLAS-MURY, C. (2003) THE ROLE OF REACTIVE
794 OXYGEN SPECIES ON *Plasmodium* MELANOTIC ENCAPSULATION IN *Anopheles gambiae*.
795 *Proceedings of the National Academy of Science USA*, **100**, 14139-14144.

For Review Only

796 KURTZ, J. (2002) PHAGOCYTOSIS BY INVERTEBRATE HEMOCYTES: CAUSES OF INDIVIDUAL

797 IN *Panorpa vulgaris* SCORPIONFLIES. *Microscopy Research and Technique*, **57**, 456-

798 468.

799 KURTZ, J. & SAUER, K.P. (1999) THE IMMUNOCOMPETENCE HANDICAP HYPOTHESIS: TESTIN

800 GENETIC PREDICTIONS. *Proceedings of the Royal Society of London Ser. B*, **266**, 2515-

801 2522.

802 KURTZ, J., WIESNER, A., GÖTZ, P. & SAUER, K.P. (2000) GENDER DIFFERENCES AND INDIVIDU

803 VARIATION IN THE IMMUNE SYSTEM OF THE SCORPIONFLY *Panorpa vulgaris* (INSECTA:

804 MECOPTERA). *Developmental and Comparative Immunology*, **24**, 1-12.

805 LACKIE, A.M. (1988) HEMOCYTE BEHAVIOUR. *Advances in Insect Physiology*, **21**, 85-178.

806 LAURENT, M., LEPOIVRE, M. & TENU, J.P. (1996) KINETIC MODELLING OF THE NITRIC OXIDE

807 GRADIENT GENERATED IN VITRO BY ADHERENT CELLS EXPRESSING INDUCIBLE NITR

808 SYNTHASE. *Biochemistry Journal*, **314**, 109-113.

809 LAVINE, M.D. & STRAND, M.R. (2002) INSECT HEMOCYTES AND THEIR ROLE IN IMMUNITY. *Insect*

810 *Biochemistry and Molecular Biology*, **32**, 1295-1309.

811 LAWNICZAK, M.K.N., BARNES, A.I., LINKLATER, J.R., BOONE, J.M., WIGBY, S. & CHAPMAN, T.

812 (2006) MATING AND IMMUNITY IN INVERTEBRATES. *Trends in Ecology and Evolution*, **22**,

813 48-55.

814 LEVENBOOK, L., BODNARYK, R. P. & SPANDE, T. F. (1969) P-ALANYL-L-TYROSINE: CHEMICA

815 SYNTHESIS, PROPERTIES AND OCCURRENCE IN LARVAE OF THE FLESHFLY *Sarcophaga*

816 PARKER. *Biochemical Journal*, **113**, 837-841.

817 LI, J. & CHRISTENSEN, B.M. (1993) INVOLVEMENT OF L-TYROSINE AND PHENOLOXIDASE IN

818 TANNING OF *Aedes aegypti* EGGS. *Insect Biochemistry and Molecular Biology*, **23**,

819 739-748.

For Review Only

820 LING, E. & YU, X.Q. (2006) CELLULAR ENCAPSULATION AND MELANIZATION ARE ENHANCE

821 IMMULECTINS, PATTERN RECOGNITION RECEPTORS FROM THE TOBACCO HORNWORM

822 *sexta*. *Developmental and Comparative Immunology*, **30**, 289-299.

823 LUCE-FEDROW, A., VON OHLEN, T, & CHAPES, S.K. (2009) *Ehrlichia chaffeensis* INFECTIONS

824 IN *Drosophila melanogaster*. *Infection and Immunity*, **77**, 4815-4826.

825 LUCKHART, S., VODOVOTZ, Y., CUI, L. & ROSENBERG, R. (1998) THE MOSQUITO *Anopheles*

826 *stephensi* LIMITS MALARIA PARASITE DEVELOPMENT WITH INDUCIBLE SYNTHESIS OF N

827 OXIDE. *Proceedings of the National Academy of Science USA*, **95**, 5700-5705.

828 MANETTI, A.G.O., ROSETTO, M., & MARCHINI, D. (1997) ANTIBACTERIAL PEPTIDES OF THE IN

829 REPRODUCTIVE TRACT. *Molecular Mechanisms of Immune Responses in Insects* (EDS

830 P.T. BREY & D. HULTMARK), PP. 67-91. CHAPMAN & HALL, NEW YORK.

831 MAVROULI, M.D., TSAKAS, S., THEODOROU, G.L., LAMPROPOULOU, M. & MARMARAS, V. J.

832 (2005) MAP KINASES MEDIATE PHAGOCYTOSIS AND MELANIZATION VIA PROPHENOLO

833 ACTIVATION IN MEDFLY HEMOCYTES. *Biochimica et Biophysica Acta - Molecular Cell*

834 *Research*, **1744,** 145-156.

835 MCKEAN, K.A. & NUNNEY, L. (2001) INCREASED SEXUAL ACTIVITY REDUCES MALE IMMUN

836 FUNCTION IN *Drosophila melanogaster*. *Proceedings of the National Academy of*

837 *Science USA*, **98**, 7904-7909.

838 MILLER, J.S., HOWARD, R.W., NGUYEN, A., ROSARIO, R.M.T. & STANLEY-SAMUELSON, D.W.

839 (1996) EICOSANOIDS MEDIATE NODULATION RESPONSES TO BACTERIAL INFECTIONS I

840 THE TENEBRIONID BEETLE, *Zophobas atratus*. *Journal of Insect Physiology*, **42**, 3-12.

841 MITCHELL, H. K.& LUNAN, K. D. (1964) TYROSINE-O-PHOSPHATE IN *Drosophila*. *Archives of*

842 *Biochemistry and Biophysics,* **106**, *219-222.*

For Review Only

843 MORENO-GARCÍA, M., LANZ-MENDOZA, H. & CÓRDOBA-AGUILAR, A. (2010) GENETIC VARIA

844 AND GENOTYPE-BY-ENVIRONMENT INTERACTION OF IMMUNE RESPONSE IN *Aedes aeg*

845 (DIPTERA: CULICIDAE). *Journal of Medical Entomology*, **47**, 111-120.

846 MORET, Y. & SCHMID-HEMPEL, P. (2000) SURVIVAL FOR IMMUNITY: THE PRICE OF IMMUNE

847 SYSTEM ACTIVATION FOR BUMBLEBEE WORKERS. *Science,* **290**, 1166-1168.

848 MÜLLER, U. (1997). THE NITRIC OXIDE SYSTEM IN INSECTS. *Progress in Neurobiology*, **51**, 363-

849 381.

850 NAPPI, A.J. & CHRISTENSEN, B.M. (2005) MELANOGENESIS AND ASSOCIATED CYTOTOXIC

851 REACTIONS: APPLICATIONS TO INSECT INNATE IMMUNITY. *Insect Biochemistry and*

852 *Molecular Biology*, **35**, 443-459.

853 NAPPI, A.J. & OTTAVIANI, E. (2000) CYTOTOXICITY AND CYTOTOXIC MOLECULES IN INVERT

854 *BioEssays*, **22**, 469-480.

855 NAPPI, A.J., VASS, E., FREY, F. & CARTON, Y. (2000) NITRIC OXIDE INVOLVEMENT IN

856 *Drosophila* IMMUNITY. *Nitric Oxide*, **4**, 423-430.

857 NARAYANAN, K. (2004) INSECT DEFENCE: ITS IMPACT ON MICROBIAL CONTROL OF INSECT

858 *Current Science*, **86**, 800-814.

859 NARDI, J.B., PILAS, B., UJHELYI, E., GARSHA, K. & KANOST, M.R. (2003) HEMATOPOIETIC

860 ORGANS OF *Manduca sexta* AND HEMOCYTE LINEAGES. *Development, Genes and*

861 *Evolution,* **213**, 477-491.

862 OHNISHI, A., HAYAKAWA, Y., MATSUDA, Y., KWON, K.W., TAKAHASHI, T.A. & SEKIGUCHI, S.

863 (1995) GROWTH-BLOCKING PEPTIDE TITER DURING LARVAL DEVELOPMENT OF PARAS

864 COLD-STRESSED ARMYWORM. *Insect Biochemistry and Molecular Biology*, **25**, 1121-

865 112.

For Review Only

866 OSANAI, M. & CHEN, P. (1993) A COMPARATIVE STUDY ON THE ARGININE DEGRADATION CA

867 FOR SPERM MATURATION OF *Bombyx mori* AND *Drosophila melanogaster*. *Amino Acids*,

868 **5**, 341-350.

869 OTVOS JR, L. (2000) ANTIBACTERIAL PEPTIDES ISOLATED FROM INSECTS. *Journal of Peptide*

870 *Science*, **6**, 497-511.

871 PACKER, C., HOLT, R.D., HUDSON, P.J., LAFFERTY, K.D. & DOBSON, A.P. (2003) KEEPING THE

872 HERDS HEALTHY AND ALERT: IMPLICATIONS OF PREDATOR CONTROL FOR INFECTIOU

873 *Ecology Letters*, **6**, 797-802.

874 PECH, L.L., TRUDEAU, D. & STRAND, M.R. (1994) SEPARATION AND BEHAVIOR IN VITRO OF

875 HEMOCYTES FROM THE MOTH, *Pseudoplusia includens*. *Cell Tissue Research*, **277**, 159-

876 167.

877 PHAM, L,N., DIONNE, M.S., SHIRASU-HIZA, M. & SCHNEIDER, D.S. (2007) A SPECIFIC PRIMED

878 IMMUNE RESPONSE IN *Drosophila* IS DEPENDENT ON PHAGOCYTES. *PLoS Pathogens*, **3**,

879 E26.

880 RANTALA, M.J. & KORTET, R. (2003) COURTSHIP SONG AND IMMUNE FUNCTION IN THE FIEL

881 CRICKET *Gryllus bimaculatus*. *Biological Journal of the Linnean Society,* **79,** 503-

882 510.

883 RANTALA, M. J. & KORTET, R. (2004) MALE DOMINANCE AND IMMUNOCOMPETENCE IN A FI

884 CRICKET. *Behavioral Ecology*, **15,** 187-191.

885 RANTALA, M.J. & ROFF, D.A. (2005) AN ANALYSIS OF TRADE-OFFS IN IMMUNE FUNCTION, BO

886 SIZE, AND DEVELOPMENTAL TIME IN THE MEDITERRANEAN FIELD CRICKET, *Gryllus*

887 *bimaculatus*. *Functional Ecology,* **19**, 323-330.

888 RANTALA, M.J. & ROFF, D.A. (2007) INBREEDING AND EXTREME OUTBREEDING CAUSE SEX

889 DIFFERENCES IN IMMUNE DEFENCE AND LIFE HISTORY TRAITS IN *Epirrita autumnata*.

890 *Heredity*, **98**, 329-336.

For Review Only

891 RANTALA, M.J., KOSKIMÄKI, J., TASKINEN, J., TYNKKYNEN, K. & SUHONEN, J. (2000)

892 IMMUNOCOMPETENCE, DEVELOPMENTAL STABILITY AND WINGSPOT SIZE IN THE DA

893 *Calopteryx splendens* L. *Proceedings of the Royal Society of London Ser B*, **267**,

894 2453-2457.

895 READ, A. F., GRAHAM, A. L. & RÅBERG, L. (2008) ANIMAL DEFENSES AGAINST INFECTIOUS

896 AGENTS: IS DAMAGE CONTROL MORE IMPORTANT THAN PATHOGEN CONTROL. *PLoS B*, **6**,

897 E1000004.

898 RIDDIFORD, L.M. & HIRUMA, K. (1988) REGULATION OF MELANIZATION IN INSECT CUTICLE

899 *Program of Clinical Biological Research*, **256**, 423-436.

900 RIBEIRO, C., & BREHÉLIN, M. (2006) INSECT HAEMOCYTES: WHAT TYPE OF CELL IS THAT? *Jo*

901 *of Insect Physiology*, **52**, 417-429.

902 RIGBY, M. & JOKELA, J. (2000) PREDATOR AVOIDANCE AND IMMUNE DEFENCE: COSTS AND

903 OFFS IN SNAILS. *Proceedings of the Royal Society of London B*, **267**, 171-176.

904 RIVERO, A. (2006) NITRIC OXIDE: AN ANTIPARASITIC MOLECULE OF INVERTEBRATES. *Trends in*

905 *Parasitology*, **22**, 219-225.

906 ROBB, T., FORBES, M.R. & JAMIESON, I.G. (2003) GREATER CUTICULAR MELANISM IS NOT

907 ASSOCIATED WITH GREATER IMMUNOGENIC RESPONSE IN ADULTS OF THE POLYMOR

908 MOUNTAIN STONE WETA, *Hemideina maori*. *Ecological Entomology*, **28**, 738-746.

909 ROBERTS, M.L., BUCHANAN, K.L. & EVANS, M.R. (2004) TESTING THE IMMUNOCOMPETENCE

910 HYPOTHESIS: A REVIEW OF THE EVIDENCE. *Animal Behaviour*, **68**, 227-239.

911 ROFF, D.A. (1992) *The Evolution of Life Histories.* CHAPMAN & HALL, NEW YORK.

912 ROLFF, J., ARMITAGE, S.A.O. & COLTMAN, D.W. (2005) GENETIC CONSTRAINTS AND SEXUAL

913 DIMORPHISM IN IMMUNE DEFENCE. *Evolution,* **59,** 1844-1850.

914 ROLFF, J. & REYNOLDS, S (EDS). (2009) *Insect Infection and Immunity. Evolution, Ecology*

915 *and Mechanisms*. OXFORD UNIVERSITY PRESS, OXFORD.

For Review Only

916 ROY, M. & HOLT, R.D. (2008) EFFECTS OF PREDATION ON HOST-PATHOGEN DYNAMICS IN SI

917 MODELS. *Theoretical Population Biology*, **73**, 319-331.

918 RYDER, J.J. & SIVA-JOTHY, M.T. (2001) QUANTITATIVE GENETICS OF IMMUNE FUNCTION AN

919 BODY SIZE IN THE HOUSE CRICKET, *Acheta domesticus. Journal of Evolutionary Biology,*

920 **14**, 646-653.

921 SAUL, S. & SUGUMARAN, M. (1988) A NOVEL QUINONE: QUINONE METHIDE ISOMERASE GEN

922 QUINONE METHIDES IN INSECT CUTICLE. *FEBS Letter,* **237**, 155-158.

923 SCHMID-HEMPEL, P. (2005A) EVOLUTIONARY ECOLOGY OF INSECT IMMUNE DEFENCES. *Ann*

924 *Review of Entomology*, **50**, 529-551.

925 SCHMID-HEMPEL, P. (2005B) NATURAL INSECT HOST-PARASITE SYSTEMS SHOW IMMUNE PI

926 AND SPECIFICITY: PUZZLES TO BE SOLVED. *BioEssays*, **27**, 1026-1034.

927 SCHNEIDER, D. S. & AYRES, J.S. (2008) TWO WAYS TO SURVIVE INFECTION: WHAT RESISTAN

928 TOLERANCE CAN TEACH US ABOUT TREATING INFECTIOUS DISEASES. *Nature Reviews*

929 *Immunology*, **8,** 889-895.

930 SCHWARTZ, A. & KOELLA, J.C. (2004) THE COST OF IMMUNITY IN THE YELLOW FEVER MOSQ

931 *Aedes aegypti* DEPENDS ON IMMUNE ACTIVATION. *Journal of Evolutionary Biology*, **17**,

932 834-840.

933 SHELDON, B.C. & VERHULST, S. (1996) ECOLOGICAL IMMUNOLOGY: COSTLY PARASITE DEF

934 AND TRADE OFFS IN EVOLUTIONARY ECOLOGY. *Trends in Ecology and Evolution*, **11**, 317-

935 321.

936 SIMMONS, L.W., ZUK, M. & ROTENBERRY, J.T. (2005) IMMUNE FUNCTION REFLECTED IN CAL

937 SONG CHARACTERISTICS IN A NATURAL POPULATION OF THE CRICKET *Teleogryllus com*

938 *Animal Behaviour*, **69**, 1235-1241.

For Review Only

939 SIVA-JOTHY, M.T. (2000) A MECHANISTIC LINK BETWEEN PARASITE RESISTANCE AND EXPR

940 A SEXUALLY SELECTED TRAIT IN A DAMSELFLY. *Proceedings of the Royal Society of Londo*

941 *Ser B*, **267**, 2523-2527.

942 SÖDERHÄLL, K. & CERENIUS, L. (1998) ROLE OF THE PROPHENOLOXIDASE-ACTIVATING SYST

943 INVERTEBRATE IMMUNITY. *Current Opinion in Immunology*, **10**, 23-28.

944 STEARNS, S. C. (1992) *The Evolution of Life Histories.* OXFORD UNIVERSITY PRESS, OXFORD.

945 STEVENSON, R.D. (2006) ECOPHYSIOLOGY AND CONSERVATION: THE CONTRIBUTION TO EN

946 INTRODUCTION TO THE SYMPOSIUM. *Integrative and Comparative Biology*, **46**, 1088-

947 1092.

948 STRAND, M.R. & PECH, L.L. (1995) IMMUNOLOGICAL BASIS FOR COMPATIBILITY IN PARASIT

949 HOST RELATIONSHIPS. *Annual Review of Entomology*, **40**, 31-56.

950 STRAND, M.R., HAYAKAWA, Y. & CLARK, K.D. 2000. PLASMATOCYTE SPREADING PEPTIDE (

951 AND GROWTH BLOCKING PEPTIDE (GBP) ARE MULTIFUNCTIONAL HOMOLOGS. *Journal*

952 *Physiology*, **46**, 817-824.

953 SUGUMARAN, M. & KANOST, M.R. 1993. REGULATION OF INSECT HAEMOLYMPH PHENOLOX

954 *Parasites and Pathogens of Insects. Volume I. Parasites* (EDS N.E BECKAGE, S.N

955 THOMPSON & B.A FEDERICO), PP. 317-342. ACADEMIC PRESS, SAN DIEGO.

956 TARLOW, E.M. & BLUMSTEID, D.T. (2007) EVALUATING METHODS TO QUANTIFY ANTHROPO

957 STRESSORS ON WILD ANIMALS. *Applied Animal Behaviour Science*, **102**, 429-451.

958 THOMPSON, J.J.W., ARMITAGE, S.A.O & SIVA-JOTHY, M.T. (2002) CUTICULAR COLOUR CHAN

959 AFTER IMAGINAL ECLOSION IS TIME CONSTRAINED: BLACKER BEETLES DARKEN FAS

960 *Physiological Entomology*, **27**, 136-141.

961 UCHIDA, K. (1993) BALANCED AMINO ACID COMPOSITION ESSENTIAL FOR INFUSION-INDUC

962 DEVELOPMENT IN THE MOSQUITO (*Culex pipiens pallens*). *Journal of Insect Physiology*,

963 **39**, 615-621.

For Review Only

964 WILLIAMS, M.J. (2007) *Drosophila* HEMOPOIESIS AND CELLULAR IMMUNITY. *Journal of*
965 *Immunology*, **178**, 4711-4715.
966 WILSON, K., KNELL, R., BOOTS, M. & KOCH-OSBORNE, J. (2003) GROUP LIVING AND INVESTMENT
967 IN IMMUNE DEFENCE: AN INTERSPECIFIC ANALYSIS. *Journal of Animal Ecology*, **72**, 133-
968 143.
969

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TABLE1. IMMUNE MARKERS USED IN INSECT ECOLOGICAL IMMUNITY STUDIES (SEE TEXT FOR FULL DISCUSSION)

IMMUNE MARKER	IMMUNE FUNCTION	METHODOLOGY	RECOMMENDATIONS	EXAMPLE REFERENCES
CUTICLE	ONE OF THE FIRST LINES OF DEFENSE AGAINST PATHOGENS; IT SENSES INJURIES AND THE PRESENCE OF BACTERIA AND FUNGI. USED FOR STRUCTURAL EXTERNAL BARRIER QUANTIFICATION.	CUTICULAR COLOR IS ASSESSED UNDER A FLUORESCENT WHITE LIGHT. COLOR CLASSES ARE ANALYZED THROUGH DIGITAL IMAGES USING A GRAYSCALE VALUE.	CUTICULAR TRANSVERSE SECTION PREPARATIONS (USING MICROTOME TECHNIQUES) FROM LEGS, THORAX OR ABDOMEN COULD DETERMINE CUTICLE RESISTANCE.	BARNES & SIVA-JOTHY 2000 THOMPSON *et al.* 2002 ROLFF *et al.* 2005
HAEMOCYTE DENSITY	HAEMOCYTES MEDIATE PHAGOCYTOSIS, NODULATION, ENCAPSULATION/MELANIZATION, CLOTTING AND RECOGNITION OF FOREIGNNESS. USED FOR CELLULAR IMMUNE RESPONSE QUANTIFICATION.	HAEMOLYMPH EXTRACTION FOR CELL COUNTING. HAEMOCYTES (SOMETIMES STAINED) ARE COUNTED USING A NEUBAUER CHAMBER.	THE ADHESIVE PROPERTIES OF HAEMOCYTES ARE REDUCED BY USING LOWER PH, ANTICOAGULANT BUFFERS. FOR ACCURATE EXTRACTION OF HAEMOCYTES (AS MOST OF THEM REMAIN ATTACHED TO TISSUES), AN INJECTED PROTEASE INHIBITOR BLEND (E.G. PMSF, TLCK, LEUPEPTINE, EDTA) COULD BE USED.	RANTALA *et al.* 2000 SIVA-JOTHY *et al.* 2001 PECH *et al.* 1994
PHAGOCYTOSIS	THE HAEMOCYTE PROCESS UTILIZED TO INGEST AND KILL MICROBIAL PATHOGENS. USED FOR CELLULAR IMMUNE RESPONSE QUANTIFICATION.	*In vitro* PHAGOCYTOSIS. FLUORESCENT LATEX BEADS, BACTERIA OR YEAST CELLS ARE ATTACHED TO HAEMOCYTES *in vitro*. LABELED CELLS OR BEADS ARE THEN QUENCHED WITH TRYPAN BLUE AND PHAGOCYTE CELLS OR BEADS REMAIN FLUORESCENT. INGESTED CELLS OR BEADS ARE COUNTED MANUALLY OR USING IMAGE ANALYSIS SOFTWARE. PHAGOCYTIC ACTIVITY IS GIVEN AS THE NUMBER/PROPORTION OF CELLS OR BEADS INGESTED PER µL HAEMOLYMPH	THE USE OF SMALL PATHOGENS (BACTERIA) IS ALSO RECOMMENDED. PATHOGENS INTERMEDIATE IN SIZE MAY INDUCE HIGHEST NODULE FORMATION AND MELANIZATION. BACTERIAL AGGREGATIONS MAY ALSO INDUCE SOME NODULE FORMATION. ASSESSMENT OF CELL VIABILITY USING TRYPAN BLUE IS ALSO RECOMMENDED.	MILLER *et al.* 1996 HOWARD *et al.* 1998 MAVROULI *et al.* 2005
NODULE/CAPSULE FORMATION	THIS RESULTS WHEN MULTIPLE HAEMOCYTES ATTACH TO AGGREGATIONS OF PATHOGENS. USED FOR CELLULAR IMMUNE RESPONSE QUANTIFICATION	AN INJECTION OF PATHOGENS* OR MICROBIAL CELL WALL COMPONENTS (E.G. LPS, PEPTIDOGLYCAN) IS NEEDED. AFTER SOME TIME, HEMOLYMPH IS EXTRACTED OR HAEMOCELE EXPOSED AND THE NUMBER OF NODULES IS COUNTED.	CATEGORICAL MEASURES (SMALL, MEDIUM, LARGE), OR QUANTITATIVE MEASURES (WITH STAGE AND OCULAR MICROSCOPE MICROMETERS), IN ADDITION TO THE NUMBER OF NODULES, COULD BE SUITABLE FOR NODULE QUANTIFICATION.	BROOKMAN *et al.* 1989 KURTZ & SAUER 1999 ELEFTHERIANOS *et al.* 2008

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NYLON MONOFILAMENTS	MIMIC PARASITE/PARASITOID INFECTIONS. USED FOR CELLULAR ENCAPSULATION AND MELANIZATION QUANTIFICATION.	NYLON IS MANUALLY INSERTED, USUALLY THROUGH AN ABDOMINAL PLEURA OR SOFT BODY PART. QUALITATIVE MEASURE: PRESENCE/ABSENCE OF VISIBLE MELANIZATION QUANTITATIVE MEASURES: ENCAPSULATED-MELANIZED AREA; MELANIN COVERAGE OR INTENSITY PERCENTAGES; MEAN GRAY SCALES USING IMAGE ANALYSIS SOFTWARE; DENSITOMETRY (USING RED+GREEN+BLUE FILTERS).	THE USE OF DEAD PATHOGENS (E.G. YEAST) MAY BE USEFUL SINCE THEY ALSO INDUCE MELANOTIC ENCAPSULATION AND ARE NATURALLY RECOGNIZED BY THE IMMUNE SYSTEM. NYLON IMPLANTS IS NOT RECOMMENDED FOR SMALL INSECTS.	SIVA-JOTHY 2000 KOSKIMÄKI *et al.* 2004 SIMMONS *et al.* 2005
NYLON/SILICA BEADS	MIMIC SMALL PATHOGEN INFECTION. USED FOR CELLULAR NODULATION, ENCAPSULATION AND MELANIZATION QUANTIFICATION.	INOCULATION WITH MICROSYRINGE. MEASUREMENT METHODOLOGY SIMILAR TO THAT OF NYLON MONOFILAMENTS.	THE USE OF DEAD, MEDIUM-SIZED PATHOGENS (E.G. YEAST) COULD BE USEFUL SINCE THEY ALSO INDUCE MELANOTIC ENCAPSULATION AND ARE NATURALLY RECOGNIZED BY THE IMMUNE SYSTEM.	ADAMO 1999 KUMAR *et al.* 2003 SCHWARTZ & KOELLA 2004
PHENOLOXIDASE (PO)	A CONSTITUTIVE OXIDOREDUCTASE ENZYME USED DURING THE EARLY STEPS OF MELANIN FORMATION; HIGHLY REACTIVE AND TOXIC INTERMEDIATE MOLECULES ARE PRODUCED. USED FOR HUMORAL RESPONSE QUANTIFICATION.	HAEMOLYMPH EXTRACTION. PO ACTIVITY IS ASSAYED BY THAWING HAEMOLYMPH AND L-DOPA OR DOPAMINE (AS SUBSTRATE) INTO A MICROPLATE WELL. SEVERAL READINGS ARE TAKEN ≈ 490NM IN A MICROPLATE READER FOR VARYING PERIODS (20-120MIN). HEMOLYMPH PROTEIN LOAD MUST BE STANDARDIZED BEFORE MEASUREMENT (E.G. STANDARD PROTEIN ASSAY). α-CHYMOTRYPSIN CAN BE USE TO ACTIVATE ALL PRO-PO PRESENT IN THE HAEMOLYMPH	THE USE OF A PROTEASE INHIBITOR COCKTAIL (E.G. PMSF, LEUPEPTIN) CAN INHIBIT PRO-PO SPONTANEOUS ACTIVATION OF STORED HEMOLYMPH. THE USE OF PEROXIDE SUPPRESSORS MAY INHIBIT PEROXIDASES THAT OXIDIZE CATECOHLS. PO WILL EXHIBIT DEGRADATION/SPONTANEOUS ACTIVATION WHEN STORED FOR A LONG PERIOD OF TIME.	GOLDSWORTHY *et al.* 2003 WILSON *et al.* 2003 JACOT *et al.* 2005

For Review Only

NITRIC OXIDE (NO)	AN INDUCIBLE REACTIVE OXYGEN MOLECULE WITH AN IMPORTANT ROLE IN DESTROYING INFECTIOUS AGENTS. USED FOR HUMORAL RESPONSE QUANTIFICATION. IN SOME CASES, NO GENERATION IS ACTIVATED BY INGESTION/INJECTION OF BACTERIA (OR CELL WALL COMPONENTS, E.G. LPS, PEPTIDOGLYCAN), VIRUS, FUNGI OR PARASITE INOCULATION.	INDIRECT QUANTIFICATION USING NITRITE PRODUCTION VIA THE GRIESS REACTION. HAEMOLYMPH, SULFANILAMIDE AND NAPHTHYLETHYLENEDIAMINE ARE THAWED INTO A MICROPLATE WELL. ABSORBANCE IS RECORDED AT 540NM. COMMERCIAL KITS ARE AVAILABLE FOR NITRATE AND NITRITE MEASUREMENT. THE USE OF L-NAME NOS INHIBITOR AS A CONTROL VERIFIES THAT QUANTIFIED NITRITES ARE A SUB PRODUCT OF ARGININE DEGRADATION BY NOS.	INOCULATION WITH DEAD PATHOGENS ACTIVATES THE IMMUNE SYSTEM WITHOUT FURTHER HOST DAMAGE.	NAPPI *et al.* 2000 HERRERA-ORTIZ *et al.* 2004 FARALDO *et al.* 2005
LYSOZYME ACTIVITY	A CONSTITUTIVE AND INDUCIBLE NON-SPECIFIC ENZYME WITH HYDROLYTIC ACTION AGAINST THE PEPTIDOGLYCAN IN GRAM+ CELL WALLS AND ANTIFUNGAL ACTIVITY. USED FOR HUMORAL RESPONSE QUANTIFICATION.	ASSAYED BY THE CLEARANCE RATE OF BACTERIAL SUSPENSION USING HAEMOLYMPH. EXTRACTED HAEMOLYMPH IS MIXED WITH A BACTERIAL SOLUTION AND CONTINUOUSLY MEASURED AT ≈ 492NM FOR A PERIOD OF TIME. SMALL ABSORBANCE VALUES INDICATE HIGH LYTIC ACTIVITY. IN THE LYTIC ZONE ASSAY, IN WHICH A BACTERIAL CULTURE IN SOLID AGAR MEDIA IS PINCHED, HOLES ARE FILLED WITH HAEMOLYMPH SAMPLE. DIAMETER OF CLEAR LYTIC ZONES IS THEN MEASURED.	OCCASIONALLY, THE PH OF BUFFER SOLUTIONS IS AN IMPORTANT FACTOR AS PH IS COMMONLY USED TO DISCRIMINATE AMONG LYTIC ACTIVITY WITH OTHER ANTIMICROBIAL (PEPTIDE) ACTIVITY.	KURTZ *et al.* 2000 RANTALA & KORTET 2003 ADAMO 2004

For Review Only

ANTIMICROBIAL PEPTIDES	INDUCIBLE SMALL PEPTIDES. THEY DESTABILIZE BACTERIA (GRAM+, GRAM-) AND FUNGI MEMBRANES. USED FOR HUMORAL RESPONSE QUANTIFICATION. ACTIVITY IS INDUCED WITH INGESTION/INJECTIONS OF BACTERIA, FUNGI OR PARASITES (OR CELL WALL COMPONENTS E.G. LPS, PEPTIDOGLYCAN).	(1) ASSAYED VIA THE CLEARANCE RATE OF BACTERIAL SUSPENSION, USING HAEMOLYMPH (AS USED IN LYZOZYME ACTIVITY PROTOCOL). (2) ASSAYED VIA THE CLEARANCE RATE OF AN INJECTION OF BACTERIA. A STANDARD BACTERIAL DOSE IS INJECTED INTO THE HOST. EXTRACTED HAEMOLYMPH IS PLATED IN MEDIA AGAR AND CULTIVATED; PLATES ARE THEN SCORED FOR THE NUMBER OF BACTERIAL COLONIES FORMED BY USING A COLONY COUNTER. THIS MAY ALSO BE DONE VIA AN ANTIBACTERIAL ZONE ASSAY (AS USED IN LYZOZYME ACTIVITY PROTOCOL).	LYSOZYME GENERIC INHIBITOR (E.G. TRI-N-ACETYLGLUYCOSAMINE) CAN BE USED TO DISTINGUISH AMONG ANTIMICROBIAL MOLECULES.	MORET & SCHMID-HEMPEL 2000 MCKEAN & NUNNEY 2001

* BEFORE INOCULATION, MINIMAL LETHAL AND/OR SUB-LETHAL DOSES MUST BE DETERMINED. OVERDOSES CAN PROMOTE SEPTIC SHOCK, RATHER THAN A ME
** BECAUSE ANTIMICROBIAL ACTIVITY CAN BE DUE TO LYSOZYMES, REACTIVE OXYGEN SPECIES AND/OR ANTIMICROBIAL PEPTIDES, IMMUNE RESPONSE MEAS
AS HEMOLYTIC, LYZOZYME-LIKE OR ANTIBACTERIAL ACTIVITY.

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For Review Only

DR. ROBERT FRECKLETON
EDITOR IN CHIEF
METHODS IN ECOLOGY AND EVOLUTION

DEAR DR. FRECKLETON:

THANKS VERY MUCH FOR YOUR COMMUNICATION REGARDING THE REVIEWERS' COMMENTS TO O *Current immunity markers in insect ecological immunology: assumed trade-offs and methodological issues*. WE ARE HAPPY TO KNOW THAT SUCH COMMENTS WERE FAIRLY POSITIVE. WE GRATEFUL WITH THE REVIEWERS AS THEIR COMMENTS HAVE BEEN VERY ENRICHING. WE HAVE PR VERSION ACCORDING TO THESE COMMENTS.

YOU WILL FIND IN DETAIL HOW WE HAVE ADDRESSED SUCH CHANGES. SUCH CHANGES CAN BE TRA DIRECTLY TO THE LINE NUMBERS THAT ARE ALSO INDICATED BELOW.

For Review Only

Reviewer 1

1- ONE FIRST GENERAL COMMENT IS RELATED TO IMMUNITY EVOLUTION. WE AGREE THAT IMMUNE IS EVOLVING. HOWEVER, THE INSECT IMMUNE SYSTEM IS A SET OF CELLS, MOLECULES AND REACTIO FEATURES NOT ONLY RESIST PATHOGEN INVASION, BUT ALSO LIMITS THE DAMAGE CONSEQUENCE INFECTION (THIS IDEA IS NOW MENTIONED IN LINE 85-88). ALL THIS FEATURES CHARACTERIZE THE SYSTEM OF INSECTS AND CAN CHANGE AMONG GENERATIONS. IN CONSEQUENCE, WE CAN STAT IMMUNE SYSTEM IS CONTINUOUSLY EVOLVING. REVIEWER IS ALSO CONCERNED ABOUT THE HOW TH MARKERS ARE RELATED WITH RESISTANCE AGAINST PATHOGENS. AT THE INSECT IMMUNE MECH WE MENTIONED THE EFFECT OVER THE PATHOGEN OF EVERY IMMUNE RESPONSE (WHICH ARE RE IMMUNE MARKERS) MENTIONED HERE. ALSO, ALONG THE TEXT WE MENTIONED THE EFFECTS OF E ON THE PATHOGEN (E.G. SEE LINES 104-105, 109-110, 136-146) OR ITS RELATION WITH HOST DEFENC (PLEASE SEE LINES 175-176, 203-206).

2- A SECOND GENERAL COMMENT IS RELATED TO THE EMPHASIS OF THE ECOLOGY AND THE RATI OFFS. THE OCCURRENCE OF THIS TRADE-OFFS HAVE BEEN PREVIOUSLY AND ACCURATELY PROVEN RESEARCHES. OUR INTENTION IS NOT TO GIVE A RESUME OF THESE PAPERS, WHICH IS THE REASO GIVE DETAILS OF EACH ONE OF THIS STUDIES. OUR MAIN INTENTION IN THIS REVIEW IS TO PROPOS BE TESTED) ABOUT THE PHYSIOLOGICAL RELATIONSHIP BETWEEN IMMUNITY AND OTHER LIFE HIST INTERMEDIATE CHARACTERS, THAT ARISE TRADE-OFFS. HOWEVER, WE COMPLETELY AGREE WIT THAT IS NECESSARILY TO DISTINGUISH THE LEVEL OF THE TRADE-OFFS DETECTED. FOR THIS REAS PARAGRAPH ENTITLE *Trade-offs among immune responses* HAS BEEN INCLUDED IN THE INSECT IMMUNE MECHANISMS SECTION (SEE LINES 148-162).

3- WE DELETE THESE LINES IN ORDER TO AVOID CONFUSION.

4- THE REVIEWER IS CONCERNED ABOUT THE METHODS REPEATABILITY. FOR THIS REASON A NEV WAS INCLUDED IN THE CONCLUSION (SEE LINES 534-544). WE APPRECIATE THIS COMMENT.

5- THE REVIEWER NOTICE THAT SOME METHODS (E.G. GENETICS, PROTEOMICS) ARE NOT DISCUSS EXPLANATION FOR THIS HAS BEEN INCLUDED IN THE INTRODUCTION (SEE LINES 65-72).

DETAILS

6- SEXUAL SELECTION IS NOW DEFINED AS THE "DIFFERENTIAL MATING AND FERTILIZATION SUCCE

7- WE REFER AS SHORT DEVELOPMENTAL TIMES NOW (LINE 59).

8- PARENTHESIS WAS CHECKED

9- WE DIFFER FROM THE REVIEWER'S OPINION RESOLVING THAT MELANIN IS A POLYMER NON-TOX MOLECULES PRODUCED THROUGH THE PO CASCADE ARE (LINES 108-110).

For Review Only

Reviewer 2

1- WE HAVE CHANGED THE LINES OF THE ABSTRACT, IN ORDER TO AVOID CONFUSION WHEN WE R USE OF IMMUNE MARKERS. SEE NOW LINES 27-30.

2- WE AGREE THAT THE IMMUNE MARKER DOES NOT HAVE A COST, BUT THE IMMUNE FUNCTION R MARKER HAS. THIS PARAGRAPH WAS RE-WRITTEN TO AVOID CONFUSION (PLEASE SEE LINES 60-

3- WE NOW GIVE EXAMPLES AND REASONS OF THE IMMUNE ASSAYS THAT CAN NOT BE SUITABLE F NOW SEE LINES 65-72.

4- THE REVIEWER IS COMPLETELY CORRECT. WE REWRITE THIS LINE (SEE LINES 80-81).

5- INSECT PO PATHWAY WAS CHECKED. WE AGREE THAT CATECHOLAMINES ARE BETTER PO SUBS HOWEVER PO ALSO HYDROXILATE TYROSINE, WHICH IS A PRECURSOR OF THE PO CASCADE IN INVERTEBRATES. SOME MORE DETAILS OF THIS CASCADE ARE GIVEN IN LINES 125-130.

6- WE REFER AS CUTICLE DARKNESS NOW. SEE LINE 167.

7- ROBB ET AL (2003) REFERENCE HAS BEEN INCLUDED TO EXPLAIN THAT IN SOME SPECIES CUTICLE IS NOT RELATED TO DISEASE RESISTANCE (SEE LINE 193-194)

8- THE REVIEWER IS ENTIRELY CORRECT. WOUND REPAIR CONSIDERED AS AN IMMUNE RESPONSE SEE LINE 111-112.

9- WES HOW NOW EXAMPLES AND REFERENCES ABOUT NON-IMMUNE FUNCTIONS OF HAEMOCYTE 206-208). THIS SECTION WAS ALSO RE-WRITTEN TO AVOID CONFUSION (SEE LINES 203-213).

10- WE EXPLAIN METHODS TO OBTAIN HAEMOCYTES. HOWEVER, WE STATE THAT THE METHOD US AND THE USE OF ANTICOAGULANTS MUST BE CONSIDERED PROPERLY BY EACH RESEARCHER (I 226-229). THE REVIEWER IS CONCERNED ABOUT THE PROBLEMS GENERATED BY THE USE OF ANTICOAGULANTS AND THE DILUTION FACTOR. BEFORE EXTRACTION EACH RESEARCHER COM

ESTABLISHES THE AMOUNT OF MEDIUM IN WHICH SAMPLE (WITH OR WITHOUT ANTICOAGULANT
OBTAINED AND DILUTION FACTOR IS DETERMINED BEFORE EXTRACTION. AS WE CONSIDER TH
PRACTICE WE DID NOT INCLUDE THIS ISSUE IN THE TEXT.

11- AS PERFECTLY SUGGESTED BY THE REVIEWER, WE REFER NOW THE EXISTENCE OF HAEMATOP
IN SOME INSECTS (PLEASE SEE LINES 289-290).

12- WE DETAILED THE ENHANCING INTERACTION BETWEEN HAEMOLYMPH AND PO ACTIVITY (SEE
342).

13- WE HAVE RE-WRITTEN THIS LINE IN ORDER TO MAKE IT CONSISTENT WITH THE WHOLE SENTENCE
347).

14- WE STATE THAT THE USE OF IMPLANTS ARE A SUITABLE METHOD TO TEST FOR TRADE-OFFS (PL
345-347). AS ACCURATELY SUGGESTED BY THE REVIEWER, WE DISCUSS NOW THE PROBABLE DIF
WHEN USING DEAD OR LIVE PATHOGENS. ALSO WE GIVE AN EXAMPLE TO ILLUSTRATE THIS DIFFER
LINES 352-359).

15- THE REVIEWER WAS COMPLETELY RIGHT. WE WERE WRONG WHEN WE STATE THAT THERE CA
DEFICIENCY WITHOUT A MELANIN DEFICIENCY. THIS LINE IS CORRECTED NOW. SEE LINES 385-3

16- WE AGREE WITH THE REVIEWER THAT PO CAN BE PRODUCED IN OTHER TISSUES, AND THIS IDE
WRITTEN IN THE TEXT (SEE LINES 378-382). NEVERTHELESS, WE ATTEMPTED TO MAKE A HYPOTHESIS
RELATED TO THE PO TRANSPORTATION TO OTHER TISSUES AND THE POSSIBLE BASE OF A TRA
NOT HAVE ANY EVIDENCE IF THIS IDEA IS SPECIES-SPECIFIC. HOWEVER, WE ARE AWARE THAT
HYPOTHESIS NEEDS FURTHER EXPLORATION; THIS HAS BEEN STATED IN THE TEXT (SEE LINES 3

17- THE REVIEWER IS CORRECT; TYROSINE (TYR) CAN BE STORED AS MULTIPLE INTERMEDIATE MO
IS NOW STATED IN THE TEXT (SEE LINES 403-405). HOWEVER, TRADE-OFFS CAN BE DETECTED IF
ENOUGH TYR (AND ITS SUBPRODUCTS) IS GATHERED THROUGH ONTOGENY (PLEASE SEE LINES 4
AS SUGGESTED BY THE REVIEWER, NOW WE STATE THAT PO MEASURES NEED TO BE INTERPRE
CONTEXT OF THE SPECIE'S TYROSINE METABOLISM, PLEASE SEE LINES 406-407.

18- THE PEROXIDE SUPPRESSOR HAS BEEN STATED IN THE TEXT (SEE LINE 422).

19- DETAILS OF WHY CONTROLLING TOTAL PROTEIN LOAD IS NOW EXPLAINED. PLEASE SEE LINES 426-428.

20- REVIEWER IS COMPLETELY CORRECT. WE NOW STATE THAT NO CAN BE CONTINUOUSLY SYNTHESIZE
INDUCED NOS FOR LONG PERIODS OF TIME (SEE LINES 454-456).

21- THE REVIEWER IS CONCERNED ABOUT THE USE OF MODERN METHODS (E.G. GENE EXPRESSION
EXPLANATION FOR THIS HAS BEEN INCLUDED IN THE INTRODUCTION (SEE LINES 65-72). ALSO, A
RECOMMENDATION WAS ALSO INCLUDED IN THE CONCLUSION SECTION (SEE LINES 581-586).

22- WE APPRECIATE THE REVIEWER ADVICE. HOWEVER, IN THE INTRODUCTION WE POINT OUT THA
METHODS MENTIONED IN THE MANUSCRIPT ARE SUMMARIZED IN TABLE 1. WE CONSIDER REDU
REFER TABLE 1 IN EVERY IMMUNE MARKER MENTIONED IN THE TEXT.

For Review Only

23- WE AGREE THAT THE JH CAN BE A COMPLICATE MODEL TO EXEMPLIFY THE HANDICAP HYPOTH
DEMONSTRATE THE CORRECT USE OF AN IMMUNE ASSAY. AS PROPERLY SUGGESTED BY THE R
WHOLE SECTION WAS REMOVED. REVIEWER ASKED FOR AN EXAMPLE WHERE AN IMMUNE MARK
NOT USEFUL TO ANSWER A RESEARCH QUESTION. WE DID NOT REFER TO ANY PAPER SINCE OUR
WAS NOT UNDERESTIMATE THE CURRENT RESEARCH IN INSECT ECOLOGICAL IMMUNITY. THE S
PAPER IS TO HIGHLIGHT IMMUNE MARKERS THAT HAVE BEEN CAREFULLY AND PROPERLY USED I
WORKS. FOR THESE REASONS WE DELETED OR RE-WROTE LINES WHERE THIS CONFUSING OR N
WAS USED (SEE LINES 566-572). NEEDLESS TO SAY, WE ARE VERY GRATEFUL WITH THESE THOUGH
COMMENTS.

24- THE SCHNEIDER´S WORK HAS NOW BEEN USED TO EXPLAIN NOT ONLY THE USE OF IMMUNE MAR
RESIST PATHOGENS, BUT ALSO TO TOLERATE AND AVOID DAMAGE (PLEASE SEE LINES: 354-359).
THIS, WE ALSO MENTION THE NEED OF MULTIPLE MEASURES OF IMMUNE RESPONSE NOT ONLY T
TRADE-OFFS WITH OTHER TRAITS, BUT TRADE-OFFS AMONG IMMUNE RESPONSES (SEE LINES: 5
THANK THE REVIEWER FOR THIS EXCELLENT SUGGESTION.

For Review Only

Reviewer 3

1- THE REVIEWER IS CONCERNED ABOUT THE NEGATIVE TONE OF THE ARTICLE. OUR AIM IS TO NO
UNDERESTIMATE THE CURRENT METHODOLOGIES IN INSECT ECOLOGICAL IMMUNITY. THE SCOP
PAPER IS TO HIGHLIGHT IMMUNE MARKERS THAT HAVE BEEN CAREFULLY AND PROPERLY USED IN
WORKS. FOR THESE REASONS WE DELETED OR RE-WROTE LINES WHERE THIS NEGATIVE TONE W
EXAMPLE, SEE LINES: 310-314, 344-347, 526-530). WE AGREE WITH THE REVIEWER THAT THE IMMU
MARKER IS NO THE OBJECT OF STUDY ITSELF. COMMONLY THE ECOLOGICAL AND EVOLUTIONARY
CONSEQUENCE OF THE TRADE-OFFS ARE DISCUSSED, BUT THE POSSIBLE PHYSIOLOGICAL LINK AM
IMMUNITY AND OTHER TRAITS ARE NOT CONSIDERED. WE BELIEVE THAT THIS LINK CAN BE USE
DISCUSSIONS IN ECOLOGICAL IMMUNITY RESEARCHES. FOR THESE REASONS, WE ATTEMPTED TO MAKE
(WITH THE AVAILABLE INFORMATION BUT IMMUNOLOGICAL AND ECOLOGICAL) HYPOTHESES RELA
POSSIBLE BASE OF A TRADE-OFF AMONG IMMUNITY AND OTHER TRAITS. HOWEVER, WE ARE AV
THESE HYPOTHESES NEED FURTHER EXPLORATION; THIS HAS BEEN STATED IN THE TEXT (SEE LI

2- AS RECOMMENDED BY THE REVIEWER, WE CAREFULLY REVISED THE IMMUNOLOGY LITERATURE
HERE. WE WERE WRONG WHEN WE STATED THAT USING THE PHRODO ASSAY IS BETTER THAN
ASSAY, THIS LINE WAS RE-WRITTEN TO AVOID CONFUSIONS (SEE LINES 263-265). WE THANK THE
FOR POINTED OUT THIS MISTAKE. HOWEVER, IT SEEMS THAT THE REVIEWER FOUND OTHER PLAC
FURTHER IMPROVEMENT CAN BE MADE. WE ARE OPEN TO SOLVE THESE ISSUES IF THEY ARE OUT
REVIEWER

3- WE AGREE THAT IS NOT RELEVANT TO TEST AMP SPECIFICITY, FOR THIS REASONS WE DELETED
THE TEXT AS WELL IN TABLE 1. WE HAVE DONE SOME RECOMMENDATION BASED IN OUR OWN EX
FOR EXAMPLE, IN ONE OF OUR INSECT MODELS, CUTICULAR THICKNESS, BUT NOT COLOUR, WAS RE

AN IMMUNE CHALLENGE (UNPUBLISHED DATA). NONETHELESS, MEASUREMENTS COULD BE NOT AI
TASK, IT IS POSSIBLE TO GATHER A GREAT NUMBER OF INDIVIDUAL MEASUREMENTS, AND ITS CO
ELEVATED. OF COURSE, THE USE OF EVERY METHOD MENTIONED HERE MUST BE DETERMINED B
RESEARCHER NOT ONLY IN TERMS OF ITS OVERALL COST, BUT ALSO IN THE QUESTION TO BE ANSV
IDEA HAS BEEN INCLUDED IN TEXT, PLEASE SEE LINES 530-534).

4- ONE OF OUR AIMS IN THIS REVIEW IS TO GIVE AN OVERVIEW OF THE MOST FREQUENTLY-USED IMMUNOLOGICAL MARKERS APPLIED IN THE FIELD OF EVOLUTIONARY ECOLOGY OF INSECTS. AS M BEFORE, WE HAVE DONE SOME RECOMMENDATION NOT ONLY TRYING TO IMPROVE TECHNIQUE GAVE SOME POSSIBLE SOLUTIONS; ALL BASED IN THE EXISTENT LITERATURE, AND OUR OWN EXPER CONSIDER THAT WE HAVE PROPOSED THE USE OF NEW AND FEASIBLE TECHNIQUES, FOR EXAMP THICKNESS, PHRODO ASSAYS, NO MEASUREMENTS.WE AGREE WITH THE REVIEWER IN THE SEN OF PATHOGEN MUST BE CONSIDERED, THIS IDEA HAS BEEN INCLUDED IN THE TEXT (PLEASE SEE 213, 364-366, 553-555). ALSO, THE REVIEWER IS COMPLETELY CORRECT, MAXIMAL IMMUNE RESPON IS NOT NECESSARILY RELATED WITH MAXIMUM FITNESS, EVEN SOMETIMES, THE ABSENCE OF IM RESPONSE CAN BE ADVANTAGEOUS. THIS IDEA IS MENTIONED IN THE CONCLUSION (LINES 560-56

5- THE REVIEWER IS CONCERNED ABOUT THE USE OF NON PATHOGENS TO INDUCE AN IMMUNE RE INTENTION WAS TO MAKE SOME RECOMMENDATIONS NOT TO CONSIDER THEM AS INAPPROPR STUDIES IN THE FIELD OF ECOLOGICAL IMMUNOLOGY. WE DELETED WORDS OR RE-WROTE LINES T CONFUSING CONNOTATION, FOR EXAMPLE PLEASE SEE NOW LINES 253-260, 310-314, 344-347. WE COMPLETELY AGREE WITH THE REVIEWER THAT THE USE OR NOT USE OF NON LIVING PARTICLES EXCELLENT ISSUE TO DISCUSS. HOWEVER, OUR PRINCIPAL AIM IN THIS PAPER WAS TO DISCUSS ME AND PROPOSED THEORIES (SOME OF THEM TO BE TESTED) ABOUT THE PHYSIOLOGICAL LINK WIT (MEASURED WITH THE MARKERS MENTIONED IN THE TEXT) AND OTHER LIFE HISTORY TRAITS. WE DO NOT INCLUDE THIS SUBJECT IN THE MS. WE DO NOT WANT TO MAKE FEEL THE REVIEWER DISAPPOINTED TO SEE THAT THE NEW MANUSCRIPT DOES NOT INCLUDE THIS SUGGESTION, BUT THAT THIS ISSUE COULD BE PROPERLY FOR AN EXTEND DISCUSSION (EVEN A REVIEW FOR PUBLICA

6- AN ADDITIONAL COLUMN WITH REFERENCES HAS BEEN INCLUDED IN TABLE 1. SOME SHORTENIN TEXT WAS DONE WHEN POSSIBLE. WE APPRECIATE THIS USEFUL SUGGESTION.

THE PAPER HAS BEEN REVISED BY TWO ENGLISH SPEAKING PERSONS

FINALLY, SOME NEW REFERENCES HAVE BEEN ADDED.

PLEASE, DO NOT HESITATE TO CONTACT ME IF THERE ARE FURTHER ISSUES TO DISCUSS.

SINCERELY,

MIGUEL MORENO-GARCÍA

For Review Only