UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

ESCUELA NACIONAL DE ESTUDIOS SUPERIORES

UNAM CAMPUS MORELIA

IMPORTANCIA DE LOS PARÁSITOS Y PATÓGENOS SOBRE LOS MECANISMOS DE LA MEMORIA INMUNE INNATA DE SU INSECTO HOSPEDERO

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTOR EN CIENCIAS

PRESENTA:

TEXCA TATEVARI MÉNDEZ LÓPEZ

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Morelia, Michoacán. **Febrero, 2023.**

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COORDINACIÓN DEL POSGRADO EN CIENCIAS BIOLÓGICAS

ENTIDAD ESCUELA NACIONAL DE ESTUDIOS SUPERIORES UNIDAD MORELIA

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ASUNTO: Oficio de Jurado

M. en C. Ivonne Ramírez Wence
Directora General de Administración Escolar, UNAM
P r e s e n t e

Me permito informar a usted que en la reunión ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día **07 de noviembre de 2022** se aprobó el siguiente jurado para el examen de grado de **DOCTOR EN CIENCIAS** del estudiante **MÉNDEZ LÓPEZ TEXCA TATEVARI** con número de cuenta **518009157** con la tesis titulada **"Importancia de los parásitos y patógenos sobre los mecanismos de la memoria inmune innata de su insecto hospedero",** realizada bajo la dirección del **DR. JORGE ALBERTO CONTRERAS GARDUÑO** quedando integrado de la siguiente manera:

Presidente:	DR. JORGE MORALES MONTOR
Vocal:	DR. ROGELIO ENRIQUE HERNÁNDEZ PANDO
Vocal:	DR. LUIS EDUARDO SERVÍN GARCIDUEÑAS
Vocal:	DR. ATILANO CONTRERAS RAMOS
Secretario:	DR. JULIO CÉSAR CARRERO SÁNCHEZ

A T E N T A M E N T E
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Ciudad Universitaria, Cd. Mx., a 13 de enero de 2023

COORDINADOR DEL PROGRAMA

DR. ADOLFO GERARDO NAVARRO SIGÜENZA

POSGRADO EN CIENCIAS BIOLOGICAS
COORDINACIÓN

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"*El miedo y el pánico innecesarios por la enfermedad y la desgracia que rara vez se materializan, son simplemente malos hábitos. Mediante la adecuada ventilación e iluminación de la mente es posible cultivar la tolerancia, el aplomo y el verdadero coraje*"

Elie Metchnikoff

Dedicatoria

Con todo mi corazón a mis amados esposa e hijo, Grecia y Luca. Son mi inspiración, energía y motivación. De ustedes, aprendo la verdad más trascendental del universo: el amor.

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Índice de contenido

Resumen

La memoria inmunitaria innata es un nuevo paradigma en el campo de la inmunología, y ha permitido ampliar el entendimiento de las relaciones parásito-hospedero y su papel en la ecología y evolución de los animales. Por ejemplo, se ha propuesto que la memoria innata puede o no ocurrir, dependiendo de dos factores: la virulencia de los patógenos y los costos evolutivos. El primer punto se ha analizado contra bacterias, pero no contra organismos grandes como nematodos entomopatógenos. Los nemátodos entomopatógenos imponen un reto para la memoria innata de los hospederos porque no solamente inducirían la memoria contra el nematodo, sino también, contra las bacterias que usan estos nematodos para matar al insecto. Además, no se sabe si la virulencia de los nemátodos afectaría el alcance de la memoria. Respecto al segundo punto, aunque la memoria parece ser costa en términos de desarrollo y en producción de huevos, no se sabe si es costosa a corto, mediano y largo plazo, de tal manera que exista una amenaza real a la adecuación de los hospederos que lleven a cabo memoria innata. Hasta donde sabemos, esto no se ha demostrado con la memoria innata de invertebrados, ni con la memoria adaptativa de los vertebrados. En esta tesis usamos al insecto *Tenebrio molitor* para saber si el alcance de su memoria innata se veía afectado por los nemátodos entomopatógenos y si la memoria era costosa a corto (producción de CO_2), mediano (desarrollo) y largo plazo (elección de pareja). Nuestros resultados no mostraron evidencia de memoria innata contra el nemátodo entomopatógeno *Rhabditis regina* (artículo 1). Además, contra el hongo entomopatógeno *Metarhizium brunneum*, la memoria fue costosa con respecto a los grupos control sin memoria, a corto (aumentó la demanda de CO_2), mediano (disminuyó el desarrollo) y largo plazo (los organismos con memoria atrajeron menos parejas) (artículo 2). Finalmente, durante la investigación, notamos dos puntos importantes que se describen en los apéndices: a) no existe información de divulgación sobre el tema de memoria innata y b) no se han indagado los mecanismos de los costos de la memoria. Por ello, en el apéndice 1 revisamos la memoria innata en una revista de divulgación y en el apéndice 2 proponemos que el estudio de la memoria innata desde el enfoque del inmunometabolismo es clave para entender sus mecanismos, y los costos evolutivos. Concluimos que, los nemátodos entomopatógenos no impiden ni favorecen la memoria innata de los hospederos, pero se debe analizar esta pregunta con diversas especies

de nemátodos para saber si se restringe a la especie que utilizamos. Además, mostramos que la memoria innata provee de beneficios a los hospederos en términos de supervivencia, pero también impone costos evolutivos. Tal vez, el balance entre costos y beneficios sea un atributo que restrinja o favorezca la memoria innata, pero se debería analizar a lo largo de la filogenia. Finalmente, proponemos que el enfoque inmunometabólico es crucial para entender los costos de la memoria innata.

Palabras clave: *Memoria inmunitaria innata, inmunología evolutiva, ecoinmunología, virulencia, parásito, patógeno, endociclo, inmunometabolismo, epigenetica, histonas,* CO_2*, costos, elección de pareja.*

Abstract

Innate immune memory is a new paradigm in the field of immunology. It has allowed us to broaden our understanding of parasite-host relationships and their role in the ecology and evolution of animals. For example, it has been proposed that innate memory may or may not occur, depending on the virulence of pathogens and evolutionary costs. The first point has been tested against bacteria but not against large organisms such as entomopathogenic nematodes. Entomopathogenic nematodes pose a challenge to the innate memory of hosts because they would not only induce memory against the nematode but also against the bacteria that use these nematodes to kill the insect. Furthermore, it is not known whether the virulence of nematodes would affect memory span. Regarding the second point, although memory seems to be costly in terms of development and egg production, it is not known if it is costly in the short, medium, and long term, in such a way that there is a real threat to host fitness that carries out innate memory. To the best of our knowledge, this has not been demonstrated with invertebrate innate memory nor with vertebrate adaptive memory. In this thesis, we used the insect *Tenebrio molitor* to find out if the extent of its innate memory was affected by entomopathogenic nematodes and if the memory was costly in the short (CO_2 production), medium (development), and long-term (mate choice). Our results did not show evidence of innate memory against the entomopathogenic nematode *Rhabditis regina* (paper 1). Furthermore, against the entomopathogenic fungus *Metarhizium brunneum*, memory was costly compared to the control groups without memory in short-term (CO_2 demand increased), medium (development decreased), and long-term (organisms with memory attracted fewer mates) (paper 2). Finally, during the research investigation, we noticed two crucial points: a) there is no popular information about innate memory, and b) the mechanisms of memory costs have yet to be investigated. For this reason, in appendix 1 we review innate memory in a popular journal. In appendix 2 we propose that studying innate memory from the immunometabolism approach is key to understanding its mechanisms and evolutionary costs. We conclude that entomopathogenic nematodes do not prevent or favor the innate memory of the hosts. Still, this question should be analyzed with different species of nematodes to see if it is restricted to the species we used. Furthermore, we show that innate memory benefit hosts in terms of survival but also imposes evolutionary costs. The balance

between costs and benefits is an attribute that restricts or favors innate memory, but it should be analyzed throughout the phylogeny. Finally, we propose that the immunometabolic approach is crucial to understanding the evolutionary costs of innate memory.

Keywords: Innate immune memory, evolutionary immunology, ecoimmunology, virulence, parasite pathogen, endocycle, immunometabolism, epigenetics, histones, CO_2, costs, mate choice.

INTRODUCCIÓN

La presión selectiva que imponen los parásitos y patógenos sobre sus hospederos ha favorecido la evolución de la respuesta inmunitaria (Schmid-Hempel, 2021). Una parte conservada de la respuesta inmunitaria es su capacidad de establecer memoria, permitiendo a los hospederos eliminar diversos parásitos y patógenos (Cooper y Alder, 2006). Hasta hace poco se pensaba que los vertebrados eran los únicos animales con memoria inmunitaria. Sin embargo, también ocurre en invertebrados, por ejemplo, ctenóforos, esponjas, nemátodos, insectos, crustáceos y moluscos (Kurtz y Franz, 2003; Milutinovic y Kurtz, 2016; Contreras-Garduño *et al*., 2016). Ese cambio de paradigma se dio en el año 2003 cuando los investigadores alemanes Joachim Kurtz y Karolyne Franz demostraron de manera contundente la capacidad del copépodo *Macrocyclops albidus* de establecer protección específica contra de la infección con el parásito *Schistocephalus solidus*. Ellos observaron una menor tasa de reinfección y una menor carga parasitaria, con respecto al grupo control (sin estimulo) o con respecto a aquellos retados con parásitos diferentes. Ahora, sabemos que la memoria innata ocurre dentro y a través de las generaciones (Little *et al*., 2003; Milutinovic y Kurtz, 2016; Contreras-Garduño *et al*., 2016), y que la memoria inmunitaria innata proteje ante reencuentros con los parásitos en términos de respuesta inmunitaria, eliminación de parásitos y supervivencia (Lanz-Mendoza y Contreras-Garduño, 2022).

Un aspecto importante que se aborda desde el enfoque de la ecología evolutiva es saber por qué no siempre ocurre la memoria innata. Una hipótesis es que los patógenos y sus factores de virulencia afectan el establecimiento de esa memoria inmunitaria (Contreras-Garduño *et al*., 2016; Tate, 2016; Wu *et al*., 2016; Medina-Gómez *et al*., 2018a). Además, se propone que los patógenos imponen costos evolutivos, derivados de la memoria

inmunitaria innata (Contreras-Garduño *et al*., 2015, 2016; Milutinovic y Kurtz, 2016). En esta tesis usamos al escarabajo *Tenebrio molitor* para saber el papel de los nematodos entomopatógenos sobre la memoria innata, y si la memoria es costosa a corto, mediano y largo plazo.

El uso de diversos entomopatógenos para investigar la relación hospedero-patógeno exige una continua busqueda de entomopatógemos para evaluar la resistencia de los hospederos. En caso interesante son los Nemátodos Entomopatógenos (NEPs) porque poseen una relación muy estrecha con diversas bacterias que, además de ser su alimento, pueden matar a los hospederos del nematodo en menos de 72 horas (Brown *et al*., 2012, Dillman *et al*., 2012). El nemátodo *R. regina* fue aislado de larvas infectadas del insecto gallina ciega *Phyllophaga, Paranomala* y *Cyclocephala* en su ambiente natural (Schulte y Poinar, 1991) y de manera experimental, se ha reportado que mata a *T. molitor*, *Ceratitis capitata* y *Galleria mellonella* (Schulte y Poinar, 1991; Jiménez-Cortés *et al*., 2016). La muerte de los hospederos por parte de estos nemátodos ocurre por bacterias como, *Serratia* sp. y *Klebsiella* sp. (Jiménez-Cortés *et al.,* 2016). Sin embargo, *R. regina* es una especie plástica en su modo de alimentación y se desarrolla también en medio saprófito (materia orgánica en descomposición), lo que le confiere variación en su virulencia (Lara-Reyes *et al*., 2020; Trejo-Meléndez *et al*., en prensa). En esta especie y otras de nematodos, se han realizado trabajos de selección experimental de la virulencia, cuya selección se asocia con sus bacterias entomopatógenas (Brown *et al*., 2012; Rafaluk *et al*., 2015). Esto demuestra el modo de vida versátil de *R. regina* y sería interesante saber si su versatilidad está implicada en el alcance de la memoria innata de los insectos. Además, los nematodos entomopatógenos imponen un

reto para la memoria innata de los hospederos porque tanto ellos como sus bacterias asociadas, podrían impedir la memoria innata, al igual que impiden la respuesta inmunitaria (Lara-Reyes *et al*., 2020). Además, no se sabe si la virulencia de los nematodos afectaría el alcance de la memoria.

Otro aspecto relevante es que, aunque la memoria innata da beneficios en supervivencia a sus hospederos, también es evolutivamente costosa (Contreras-Garduño *et al*., 2014, 2016). Los costos en la supervivencia y la reproducción son relevantes para entender la memoria innata en los invertebrados desde el abordaje de la ecoinmunología (Armitage *et al*., 2003; Ardia *et al*., 2012; Contreras-Garduño *et al*., 2014). Cabe destacar que la activación y mantenimiento de la respuesta inmunitaria es costosa (Ardia *et al.,* 2012). Este costo puede ser explicado a través de la teoría de historias de vida porque los organismos no pueden invertir sus recursos en mantenimiento, desarrollo, y reproducción al mismo tiempo (Sterans, 2000). Esto se basa en que los organismos enfrentan un compromiso (trade-off en inglés) si deben asignar recursos a dos o más funciones de forma simultánea. Por ejemplo, si deben asignar recursos a la defensa inmunitaria, su asignación a la reproducción se vería comprometida (Lawniczak *et al*., 2007). Esto se debe a que los recursos y la energía son limitados y al ser requeridos para la defensa, es necesario reasignarlos de otra función fisiológica como el desarrollo o la reproducción (Rolff y Reynolds, 2009; Bjørge *et al.,* 2018). Aunque la teoría predice costos asociados con la memoria inmunitaria innata (Contreras-Garduño *et al.,* 2016), hasta el momento solamente existe un artículo que revela que la memoria innata compromete la reproducción en términos de eclosión de huevos (Contreras-Garduño *et al.,* 2014). Sin embargo, no se sabe si es costosa a corto, mediano y largo plazo, de tal manera que exista una amenaza real a la adecuación de los hospederos que

lleven a cabo memoria innata. Por lo tanto, sería interesante saber si la memoria inmunitaria innata impone costos a corto (tasa metabólica), mediano (crecimiento) y largo plazo (reproducción, en términos de elección de pareja).

Por lo anterior, proponemos evaluar la eficiencia de la memoria innata con respecto a los nemátodos entomopatógenos y describir los costos de la memoria a corto, mediano y largo plazo en el escarabajo *T. molitor.* Este escarabajo se ha usado como modelo de estudio para analizar su respuesta inmuniaria innata y la evolución hospedero-patógeno (Grau *et al*., 2017; Canteri de Souza *et al*., 2015). Además, se han identificado diversos mecanismos efectores inmunitarios tanto celulares como humorales (Johnston *et al*., 2014). En los mecanismos humorales se han registrado Péptidos Naturales Antimicrobianos denominados tenecinas (que van de la 1 a la 4) y la cascada de la profenoloxidasa (proPO), que deriva en la producción de melanina (Johnston *et al*., 2014). Además, los factores de crecimiento y del metabólismo celular, son moléculas efectoras en respuesta a la infección y son altamente reguladas por medio del factor nuclear NF-κB (Johnston *et al*., 2014; Haine *et al.,* 2017). Finalmente, en lo que respecta a la respuesta celular, los hemocitos, que conforman la hemolinfa, se componen de prohemocitos (10-15%), granulocitos (fagocitos; 50-60%), plasmocitos (encapsulamiento; 23-28%) y Oenocitoides (secreción de la enzima proPO; 1-2%) (Vigneron *et al.,* 2019).

En *T. molitor* se ha documentado la memoria innata (ver revisión en Lanz-Mendoza y Contreras-Garduño, 2022). Esa memoria se establece en larvas*,* al ser infectadas de manera experimental contra la bacteria Gram (+) *Bacillus thuringiensis* y el hongo *Metarhizium*

brunneum (Castro-Vargas *et al*., 2017; Dhinaut *et al*., 2017; Medina-Gómez *et al*., 2018a), pero de manera muy interesante, no establecieron memoria innata al ser infectadas con la bacteria Gram (-) *Serratia marcescens* (Dhinaut *et al*., 2017; Medina-Gómez *et al*., 2018a). También se conoce que la virulencia de los patógenos afecta el establecimiento de memoria inmunitaria porque hubo memoria contra la cepa silvestre de *M. brunneum,* pero no contra la cepa modificada genéticamente CAT, que sobre-expresa el gen de la catalasa e inhibe la respuesta inmunitaria del insecto, mediada por radicales libres (Medina-Gómez *et al*., 2018b).

Cabe destacar que los hongos entomopatógenos han desarrollado una amplia diversidad de estilos de vida (Yang *et al*., 2018). Son reguladores críticos de las poblaciones de insectos (Wang y Wang, 2017) y por ello, son ampliamente usados para el manejo de plagas en la agricultura (Yang *et al*., 2018). Particularmente, *M. brunneum* comienza su proceso de infección en los insectos adhiriendo sus conidios a la superficie del hospedero y posteriormente penetra a través del integumento (Beys Da Silva *et al*., 2010; Rohlfs y Churchill, 2013). El crecimiento de *M. brunneum* se da tanto de manera vegetativa, como por medio de la producción de micelio, con múltiples hifas y por propágulos infecciosos o conidios. El hongo utiliza presión mecánica y una estrategia de sinergismo en la secreción de enzimas hidrolíticas y lipolíticas para atravesar la cutícula del hospedero (Beys Da Silva *et al*., 2010; Wang y Wang, 2013). Una vez que el hongo penetra la cutícula, secreta proteínas efectoras y metabolitos secundarios para evadir la respuesta del insecto hospedero. Por ejemplo, la catalasa (CAT) neutraliza la producción de peróxido de hidrogeno (H_2O_2), que deriva en la producción de radicales libres que son tóxicos para el hongo (Morales-Hernández *et al*., 2010). Esto explicaría porque la cepa CAT de *M. brunneum* en comparación con la

cepa silvestre, impediría la memoria de *T. molitor*. Algunos efectos de la infección de *M. brunneum* en insectos son la reducción en la supervivencia, la fecundidad y el comportamiento alimenticio (Dubovskiy *et al*., 2013; Cito *et al*., 2014). Sin embargo, en este sistema, no se ha reportado si existen costos evolutivos, generados por la memoria innata.

A continuación, introduciré los temas de esta tesis:

En el **capítulo I** investigué la memoria inmunitaria innata en larvas de *T. molitor* contra el nemátodo entomopatógeno *R. regina*. La pregunta de investigación fue ¿las larvas de *T. molitor* pueden establecer memoria inmunitaria innata contra nemátodos? La hipótesis fue que no debería existir memoria inmunitaria de tenebrio contra los nemátodos entomopatógenos porque la memoria debería realizarse contra el nematodo y contra las bacterias de manera simultánea. Esto predijo que no existirían diferencias significativas en la supervivencia entre los insectos retados de forma homóloga (retados dos veces contra nematodos), con respecto a los insectos con retos heterólogos. No obstante, la memoria también podría depender del grado de virulencia de los nematodos. En este caso, se predijo memoria contra las cepas no virulentas del nemátodo y falta de memoria contra las cepas virulentas. Para esto, usé dos cepas a las que se les sometió a un proceso de selección experimetal de la virulencia basada en su dieta: la cepa AHS y la cepa SS. La primera (AHS) fue criada en insecto, y por ello, estuvo bajo selección del sistema inmunitario, mientras que la segunda (SS), fue criada en carne de res, sin la presión selectiva del sistema inmunitario para quitarle virulencia. Registré la supervivencia para saber si las larvas inmunoestimuladas con la cepa AHS o SS vivían más que las larvas control sin infección. Utilizando este mismo

diseño experimental evalué la tasa metabólica basal (registrando la producción de CO_2), como indicador de un posible costo de la memoria innata.

En el **capítulo II** investigué los costos de la memoria inmunitaria innata en *T. molitor* retado con *M. brunneum*. La pregunta de investigación fue ¿Existe algún costo a corto, mediano y largo plazo por la memoria inmunitaria innata? La hipótesis fue que la memoria inmunitaria innata provee beneficios en términos de supervivencia durante la infección, pero impone costos evolutivos. Predije que las larvas que establezcieran memoria inmunitaria innata, con respecto a las larvas que no usaran su memoria, tendrían una mayor producción de CO_2 (costo a corto plazo), tardarían más en desarrollarse hasta llegar a adultos (costo a mediano plazo) y serían menos efectivos para atraer parejas sexuales, y con ello, no lograrían reproducirse (costo a largo plazo). Para responder la primera predicción usé dos grupos de larvas de *T. molitor*, un grupo control, inyectadas primero con solución Tween y 10 días depues con una dosis letal de *M. brunneum*, y un segundo grupo, el de memoria, a las que primero inyecté con una dosis de 5 conidios de *M. brunneum* y 10 días depués las inyecté con una dosis letal (50 conidias) de *M. brunneum*. El costo a corto plazo se evaluó mediante la tasa metabólica basal (registrando la producción de CO_2)**.** Para responder la segunda predicción, registré el tiempo en el que las larvas de los dos grupos llegaron a convertirse en pupas y el tiempo de pupas a adultos. Para responder la tercera predicción realicé pruebas de elección de pareja en las que evalué las preferencias de los diferentes grupos control y aquellos que establecieron memoria inmunitaria innata.

Mientras realizaba los dos trabajos de investigación anteriores, detecté que el tema de memoria inmunitaria innata se ha revisado a nivel internacional, y goza de difusión entre los

expertos en inmunología, ecología o ciencias biomédicas, pero no encontré trabajos de divulgación para Hispanoparlantes. Por esto, propuse realizar un artículo de divulgación científica que pudiera ser leído por un público más amplio y menos especializado. Este trabajo se puede leer en el apéndice I. Además, revisando la literatura, encontré que no se había abordado el papel del metabolismo durante el establecimiento y la posterior activación de la memoria inmunitaria innata. Esto, no solamente debería revelar mecanismos potenciales detrás de la memoria, sino también, posibles mecanismos que expliquen los costos de la memoria inmunitaria innata. La revisión de este tema derivó en el apéndice II.

Objetivos

Determinar el papel de la virulencia de los nemátodos entomopatógenos sobre la memoria inmunitaria innata y los posibles costos en términos de metabolismo basal.

Determinar si, aunque la memoria inmunitaria innata da beneficios en supervivencia a los hospederos, también impone costos evolutivos.

CAPÍTULO I

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Do entomopathogenic nematodes induce immune priming?

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ABSTRACT

Although the study of immune priming in insects is a growing area of research, its occurrence in various biological models has not been evaluated, and its mechanisms are poorly understood. Whether entomopathogenic nematodes (EPNs) can induce immune priming and what role their virulence might play in it has not been assessed. Here, we tested for the first time: 1) whether a nematode is capable of eliciting immune priming, and 2) whether nematode virulence affects immune priming. Host larvae of *Tenebrio molitor* were first exposed to one of two EPN strains (low or high virulence). They were then exposed again to a challenge (high) dose of their respective strain, and their survival was recorded. Based on current literature, we expected that host larvae primed with a low-virulence strain would not show immune priming but that those exposed to a high-virulence strain would. Instead, we found that host larvae primed with either strain did not exhibit immune priming. Further, the survival of the hosts primed with the highly virulent strain was significantly reduced relative to the control group, and no measurable immune priming was found, as also indicated by resting metabolic rate (production of CO_2). Future research is needed to determine whether virulence-associated bacteria underlie this lowered survival and/or whether another factor, such as immune evasion strategies, is related to these results.

1. Introduction

Immune priming in invertebrates refers to an increase in the host's immune response and survival after a second specific encounter (referred to as a second challenge) with parasites or pathogens [1–3]. Immune priming contributes to an increase in survival [4] via a mechanism related to cellular and humoral changes [5–9].

Previously, it has been well documented that the mealworm beetle *Tenebrio molitor* (Coleoptera: Tenebrionidae) can exhibit immune priming across and within generations [10–12]. For example, across generations, *T. molitor* offspring from mothers challenged with bacterial lipopolysaccharides (LPS) exhibited an enhanced immunity against gram-negative bacteria that have these antigens, relative to offspring from mothers that were not challenged with the LPS [10]. Within generations, *T. molitor* larvae exhibited immune priming against *Bacillus thuringiensis* [13], *Micrococcus lysodeikticus* [14], and the fungus *Metarhizium brunneum* [12,14–16]. However, not all microorganisms elicit immune priming in *T. molitor* [13,15,16]. For instance, larvae of *T. molitor* challenged with *Serratia marcescens* bacteria did not exhibit immune priming [15]. To date, there has been no attempt to assess whether nematodes can induce immune priming in insects.

The reasons for successful immune priming against some parasites or pathogens, but not against others (hereafter referred to as 'differential effectiveness of immune priming'), remain poorly understood [4,16]. One possible explanation of this differential effectiveness of the immune priming is related to the degree of threat or virulence posed by the parasite or pathogen, with both threat and virulence understood as a reduction in host survival and/or reproduction in a short period [17,18]. With a more significant threat or virulence, it is expected greater morbidity and mortality of the host. If true, we would expect immune priming in the case of a threatening or highly virulent pathogen or parasite, but not against a non-threatening or mildly virulent pathogen [15]. However, recent findings reported a more virulent strain of *Metarhizium anisopliae* overexpressing catalase, inducing lower immune priming (survival) than a less virulent strain [16].

Suggesting that elements related to the pathogen's virulence could

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modify the expectation above; specifically, a less virulent strain may induce better survival via immune priming than a more highly virulent strain [16]. Another possible explanation is that the occurrence or absence of immune-priming can be related to some parasites or pathogens' ability to evade the host immune system [4]. According to this hypothesis, parasites with the ability to avoid the immune system would not be expected to elicit immune priming.

Entomopathogenic nematodes (EPNs) are endoparasitic worms that can infect and kill insects [19]. EPNs exhibit different degrees of virulence [20–22] and different evasion strategies of the host immune system [23–26], affecting the immune priming outcome. Besides, previous studies have reported immune priming in *Galleria mellonella* (Lepidoptera: Pyralidae) exposed to LPS of *Photorhabdus luminescens* (a symbiotic bacteria of the EPN *Heterorhabditis bacteriophora*) or to *P. luminescens* itself [27–29]. However, it has not been studied whether exposure to whole EPNs elicits immune priming in insects as far as we know. Therefore, the first objective of this study was to assess whether the infection of a host by individual EPNs induces immune priming. This aim will enhance our understanding of the host-EPN interaction.

Also, EPNs offer an excellent opportunity to study in greater depth the roles that virulence and EPN evasion strategies play concerning the differential effectiveness of immune priming in insect hosts. In the present study, the second objective was to assess whether an EPN with different levels of virulence would induce differential effectiveness of immune priming in *T. molitor*, a host species known to exhibit immune priming within and across generations [10–12,14–16], but not against some other pathogens [13,15,16].

Rhabditis regina [Nematoda: Rhabditidae], an EPN originally isolated from June beetle larvae in Guatemala [30], has been shown to harbor diverse symbiotic bacteria, some strains of which are associated with greater virulence than others against an insect host [22]. Moreover, Trejo-Meléndez et al. [31] found that the immune response to *R. regina* by the beetle host *T. molitor* yielded two main findings. First, the virulence of *R. regina* in *T. molitor* was high or low depending upon the *R. regina* strain. Second, *T. molitor* exhibited an immune response when infected with the highly virulent strain, as shown by an increase in phenoloxidase (PO) levels, a key component of the immune response in insects [32,33].

Based on the above, we first expected that the exposure of *T. molitor* to whole EPN individuals of *R. regina* would elicit an immune-priming response. Second, we hypothesized that the differential effectiveness of immune priming, previously documented for *T. molitor*, can be related to the virulence level of the nematode *R. regina*. Then, we expected that *T. molitor* larvae infected with the high-virulence strain of *R. regina* would exhibit an immune-priming response but that those infected with the low-virulence strain of *R. regina* would not.

To test this hypothesis, we used *R. regina* to infect larvae of the mealworm beetle *T. molitor* as a host. The *T. molitor* were challenged by exposure to whole *R. regina* individuals. This EPN is known to naturally attack larvae of *Phyllophaga*, *Paranomala,* and *Cyclocephala* (Coleoptera: Scarabaeidae) in the field [22,30], and *T. molitor*, *G. mellonella,* and *Ceratitis capitata* (Diptera: Tephritidae) in the laboratory [22,30]. To modify the levels of virulence of *R. regina*, we analyzed the effect of the strain of origin of the nematode [22]. A higher level of virulence is expected with a more highly virulent strain [31]. We also examined whether there was a dose-dependent effect on immune priming [27]. For this, we analyzed the initial number of nematodes injected into the host as the factor of interest. Similar to virulence, it is expected that there will be a better immune priming response with a higher dose [27]. Finally, individuals of *T. mollitor* primed with *M. brunneum* that exhibit immune priming also show an increased resting metabolic rate (CO_2 production) relative to individuals that do not exhibit immune priming [12]. To improve robustness, we tested whether the potential immune priming in *T. molitor* against *R. regina* could also be energetically costly in terms of the resting metabolic rate (measured as CO_2 production) [34].

2. Materials and methods

2.1. Nematodes

Wild nematodes were obtained from *Phyllophaga* spp., new world scarab beetles in the subfamily Melolonthinae parasitized by nematodes, including *Rhabditis regina.* The *Phyllophaga* were collected in cornfields [22] and then monitored in the laboratory for 40 days to detect signs of sickness by nematodes. A laboratory colony was initiated with the nematodes isolated from the infected *Phyllophaga,* and maintenance was continued with pupae of *T. molitor* or cow beef. The beef-fed strain was designated as the saprophytic strain (SS) [35]. The *T. molitor*-fed strain was named as the alternative host strain (AHS) [22]. The new generations of nematodes were moved weekly to a clean container with fresh *T. molitor* pupae or beef. Each strain was bred in these conditions for five years inside environmental chambers (Lumistell) at 25 ± 1 °C and 75% humidity. We used infective-stage dauer larvae (DL) of *R. regina* in all experiments. To ensure that we used the DL stage, we immersed them in 1% sodium dodecyl sulfate (SDS; SIGMA) for 60 min, and we only used larvae that survived the SDS treatment for one hour. After this time, we checked the nematodes' morphology; only those with the entire body surface were designated as DL and used in the immune priming experiments [36].

2.2. Insects

Tenebrio molitor larvae of 1.5–1.7 cm (about 12th instar) [37] were used as nematode hosts. All larvae were fed *ad libitum* with bran and cornmeal (1:1) with fresh apple slices added every other day. *T. molitor* infected with nematodes were maintained in environmental chambers (Lumistell) at 25 ± 1 °C and 75% humidity.

2.3. Immune priming: survival

To assess the effectiveness of immune priming according to the *R. regina* virulence and dose, we used an experimental procedure previously used to demonstrate immune priming in *T. molitor* [14,15]. We used seven groups of *T. molitor* larvae in three replicates each (Table 1). Four of the groups were injected with nematodes during an initial challenge for immune priming and then challenged ten days later via injection with ten nematodes to test for the presence of immune priming. These four groups were: *T. molitor* larvae injected with either 1 or 10 AHS-strain DL diluted in 3 μL of Ringer solution and then injected in a second challenge with 10 AHS-strain DL (Groups AHS1-AHS10 and AHS10-AHS10; n = 151 and 124, respectively); and *T. molitor* larvae injected with either 1 or 10 SS-strain DL diluted in 3 μL of Ringer solution and then injected in a second challenge with 10 SS-strain DL (Groups SS1-SS10 and SS10-SS10; n = 169 and 165, respectively). The other three groups were controls. The first control (Group R-R) consisted of *T. molitor* larvae injected with Ringer solution only, for both the first and the second challenge (n = 180). The other two control groups consisted of *T. molitor* larvae injected with 3 μL Ringer solution only for the first challenge and 10 DL of either the AHS strain (Group R-AHS10) or the SS strain (Group R-SS10) for the second challenge (n = 180 for each group). Across all seven groups, a total of 1149 *T. molitor* larvae were tested (Table 1).

All insects were placed individually inside sterile 24-well plates (125 × 85 × 25 mm) with food provided *ad libitum* [14]. All plates were deposited inside environmental chambers (Lumistell) at 26 ± 1 °C [22]. Survival was recorded every day after the first challenge and ten days after the second challenge. All experiments were carried out in triplicate.

2.4. Resting metabolic rate

According to Li et al. [38], we recorded the resting metabolic rate in

Table 1
Experimental groups and conditions to test whether the nematode *R. regina* and its virulence are factors eliciting immune priming in the host *T. molitor*.

Group	Virulence of strain used for immune priming	DL per *T. molitor* host used at first challenge	DL per *T. molitor* host used at second challenge	Replicates	Total number of *T. molitor* used
R-R (control group Ringer only)	none (control)	0 (Ringer solution only)	0 (Ringer solution only)	3	180
R-SS10 (control group Ringer only, then challenged with SS)	none (control)	0 (Ringer solution only)	10	3	180
R-AHS10 (control group Ringer only, then challenged with AHS)	none (control)	0 (Ringer solution only)	10	3	180
SS1-SS10	low	1	10	3	169
SS10-SS10	low	10	10	3	165
AHS1-AHS10	high	1	10	3	151
AHS10-AHS10	high	10	10	3	124

DL: *Rhabditis regina* dauer larvae; SS: saprophytic strain (low virulence); AHS: alternative host strain (high virulence).

groups of 10 infected larvae per treatment (n = 5; two replicates) by measuring CO_2 production (VCO_2 ppm) with a Q-Box RP1 LP Low-Range Respirometry Package (Qubit Systems Inc), using a cylindrical G115 flow-through chamber (3.8 cm diameter X 20 cm tall, 226.82 mL). Because stress and manipulation may affect the metabolic rate [34,39], each larvae group in each treatment was introduced to the G115 chamber in darkness for 5min for habituation. Although other physiological processes can influence CO_2 production, we decided to use CO_2 to estimate the resting metabolic rate due to the small size of the larvae and the fact that they were resting [47]. Thus, we assumed that the CO_2 generated was of metabolic origin [47]. Additionally, the measurement of CO_2 as a measure of the resting metabolic rate is regular in the literature [12,34,48,49]. The chamber was connected to the respirometer and a computer. The production of CO_2 was recorded every 30 s for 5min, and we reported the mean value per group of larvae [38].

The CO_2 production (VCO_2 ppm) was recorded in the previously described control and treatment groups (Table 1) 24 and 96 h after the immune challenge. Besides, we performed an additional CO_2 production measure in the R-R control group before the second challenge took place (referred to as R). This lecture allows us a reference point to assess any effect of the second injection in the R-R group on the CO_2 production. Recordings 24 h following the second challenge were performed because this time point coincides with peak activation of the immune response [8,40]. Registrations 96 h following the second challenge were conducted to confirm previous findings indicating that after this period, CO_2 production among control and treatment groups became similar [12], likely due to molecular changes [14]. In general, it is more likely to identify a cost in metabolic rate at 24 h than at 96 h [12].

2.5. Data analysis

Survival comparison among treatments was performed with SPSS Statistics version 25 for Macintosh. Kaplan-Meier log-rank test and Gehan survival analyses were performed [41]. For survival, live insect data were recorded until the end of the study. Then, if the proportional risk assumption was met, the log-rank test was performed. If the proportional risk assumption was not met, Gehan survival analysis was conducted [42]. The Cox model was used to test the relevance of the treatment to the outcomes for different virulence and dose levels. Kaplan-Meier graphs were used for qualitative and quantitative variables. The equality of survival distributions was tested for varying levels of virulence and dose, with a level of significance of $p = 0.05$, to compare survival times at ten days after the first challenge and ten days before the second challenge, with the different treatments between the two doses.

Mean CO_2 production (VCO_2 ppm) after 24 and 96 h was compared among treatments within each period using an analysis of variance after data were log (x+1) transformed [43]. When significant differences were found, multiple comparisons were performed using the contrasts derived from the model [44]. These analyses were performed using R, v3.5.1 [45].

3. Results

3.1. Immune priming: survival

First challenges (priming) did not result in a higher survival probability of primed groups (groups SS1-SS10, SS10-SS10, AHS1-AHS10, and AHS10-AHS10) relative to the control groups (groups R-R, R-SS10, and R-AHS10) (Fig. 1, Table 1). Survival of the group primed with 1 SS nematode (group SS1-SS10) was not different from that of the R-SS10 control group ($X^2 = 1.77$, $p = 0.18$) (Fig. 1). Besides, the survival of the group primed with 10 SS nematodes (i.e., SS10-SS10) was significantly lower than that of the R-SS10 control group ($X^2 = 3.89$, $p = 0.04$). As for the strain AHS, survival of both groups, those primed with 1 and 10 AHS nematodes (groups AHS1-AHS10 and AHS10-AHS10) was significantly lower than survival of the R-AHS10 control group ($X^2 = 9.279$, $p < 0.002$ and $X^2 = 48.607$, $p < 0.001$, respectively).

3.2. Virulence and dose: EPN strain and number of injected nematodes

When survival between nematode strains was compared, we found

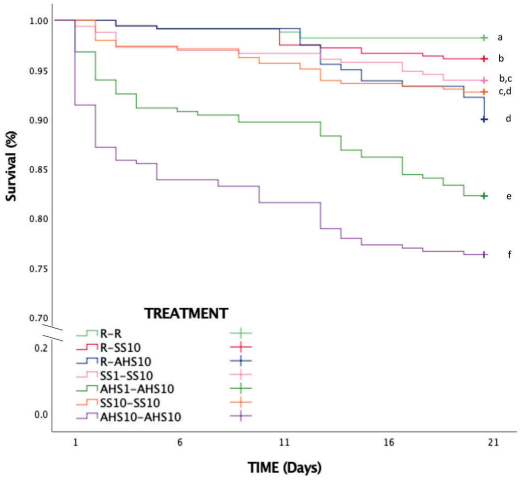

Fig. 1. Kaplan-Meier curves showing the survival of *T. molitor* larvae from day 1 to day 21 of the second challenge, comparing all treatments. SS: nematode Saprophytic Strain (low virulence). AHS: nematode Alternative Host Strain (high virulence). R: larvae injected with Ringer solution. SS1: larvae injected with 1 nematode of the SS strain. SS10: larvae injected with 10 nematodes of the SS strain. AHS1: larvae injected with 1 nematode of the AHS strain. AHS10: larvae injected with 10 nematodes of the AHS strain. Curves with different letters are significantly different (Log-Rank X^2 tests, $P \leq 0.05$).

that *T. molitor* larvae exposed to strain SS showed a significantly higher survival than *T. molitor* larvae exposed to strain AHS (Fig. 1). This indicates a higher virulence for strain AHS relative to strain SS.

When the survival of groups primed with 1 vs. 10 nematodes was compared, we found no significant differences between the strain-SS groups; survival of SS1-SS10 vs. SS10-SS10 was not significantly different ($X^2 = 0.39$, $p = 0.53$). In contrast, when AHS1-AHS10 and AHS10-AHS10 were compared, the survival of AHS1-AHS10 was significantly higher than that of AHS10-AHS10 ($X^2 = 3.56$, $p = 0.05$) (Fig. 1). This indicates that *T. molitor* survival was negatively affected by the higher number of injected nematodes when the AHS strain was used, but not when the SS strain was employed.

3.3. Resting metabolic rate

When mean production of CO_2 was compared among treatments after 24 h of receiving the second challenge, some significant differences were found among treatments ($F_{7,79} = 3.427$; $P = 0.002$) (Fig. 2). The CO_2 produced by the AHS10-AHS10 group was significantly lower than the CO_2 produced in the R-R control group (Fig. 2). However, the CO_2 produced by the other groups was not significantly different from the R-R control group (Fig. 2). Also, no difference was found in the CO_2 production of the R-R group before and after the second injection (comparison R vs. R-R, Fig. 2).

After 96 h following the second challenge injection, our results showed a significantly higher production of CO_2 by individuals in the R-R treatment (Ringer solution only; no nematodes) relative to the other treatments ($F_{7,79} = 3.427$; $P = 0.002$). When the other treatments were compared, the R-AHS10 treatment exhibited a significantly lower production of CO_2 relative to the SS10-SS10 and the AHS10-AHS10 groups (Fig. 3). However, no significant differences among the other treatments were found (Fig. 3). Finally, no difference was found in the CO_2 production of the R-R group before and after the second injection (comparison R vs. R-R, Fig. 3).

4. Discussion

The present study represents the first attempt to test whether nematodes rather than only its associated bacteria can elicit immune priming. We conducted experiments to assess immune priming expression by two methods: survival rate and metabolism, which has been previously shown to increase to carry out the immune priming process [12]. If insect immune priming is activated, primed individuals (those having undergone a first challenge) would be expected to have a higher survival rate after a second challenge than unprimed individuals [1,2].

The mealworm *T. molitor* has been shown to exhibit immune priming when primed with LPS, whole bacteria, and fungus [10,12–15]. For example, *T. molitor* larvae primed with the fungus *M. brunneum* showed higher survival after a second exposure to the fungus, relative to larvae that were not primed [12]. Previous work also showed that a symbiotic bacterium (*P. luminescens*) of the EPN *H. bacteriophora* elicited immune priming in *G. mellonella*. That is, larvae of *G. mellonella* primed with associated EPN bacteria exhibited a higher survival than their unprimed counterparts [27–29].

In a previous study, we had found that the strain AHS showed high virulence against *T. molitor* [31]. In contrast, the strain SS exhibited low virulence against that host, and the AHS strain induced an increased production of PO while the SS strain did not [31]. For these reasons, we expected that *T. molitor* larvae primed with *R. regina* could exhibit immune priming. That is, after a second challenge, we expected a higher survival rate in larvae previously primed with nematodes (i.e., treatments SS1-SS10 and AHS1-AHS10) relative to unprimed individuals (i.e., controls RR-SS10 and RR-AHS10). However, our results did not support this expectation. First, considering the groups primed with a single nematode and challenged with ten nematodes, we found that the survival of the SS control group (R-SS10) was not different from that of the SS primed group (SS1-SS10) (Fig. 1) and the survival of the AHS control group (R-AHS10) was significantly higher than that of the AHS primed group (AHS1-AHS10) (Fig. 1). This indicates that larvae of *T. molitor* primed with one nematode (of both strains SS and AHS) do not exhibit immune priming; their survival was not higher than that of the unprimed groups. It is important to note that while low rates of survivorship (<50%) could have induced a selection or lingering effects of pathology as confounding factors, our tested groups exhibited survival rates higher than 75% (Fig. 1). With these percentages of tested survivors, genetic variability is likely well represented, reducing the probability of selection or the lingering effects of pathology. And, immune priming could have been detected (if present), as shown in previous

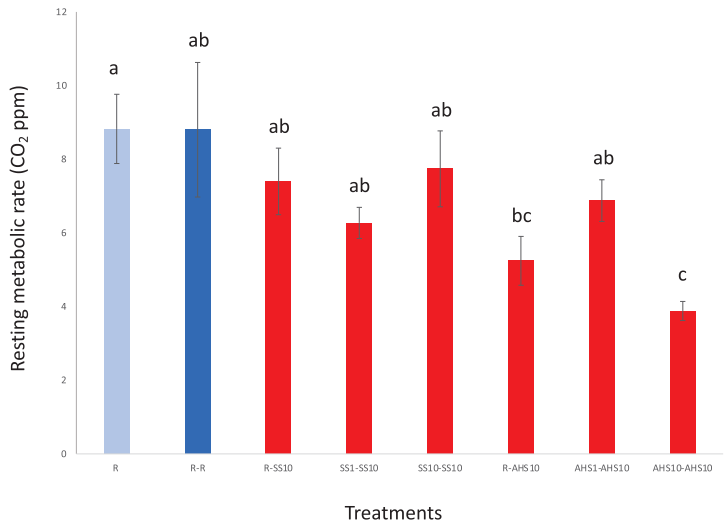

Fig. 2. Resting metabolic rate (CO_2 production) of *T. molitor* larvae 24 h after being injected in a second challenge. SS: nematode Saprophytic Strain (low virulence). AHS: nematode Alternative Host Strain (high virulence). R: larvae injected with Ringer solution. SS1: larvae injected with 1 nematode of the SS strain. SS10: larvae injected with 10 nematodes of the SS strain. AHS1: larvae injected with 1 nematode of the AHS strain. AHS10: larvae injected with 10 nematodes of the AHS strain. Different letters indicate significant differences ($P \leq 0.05$).

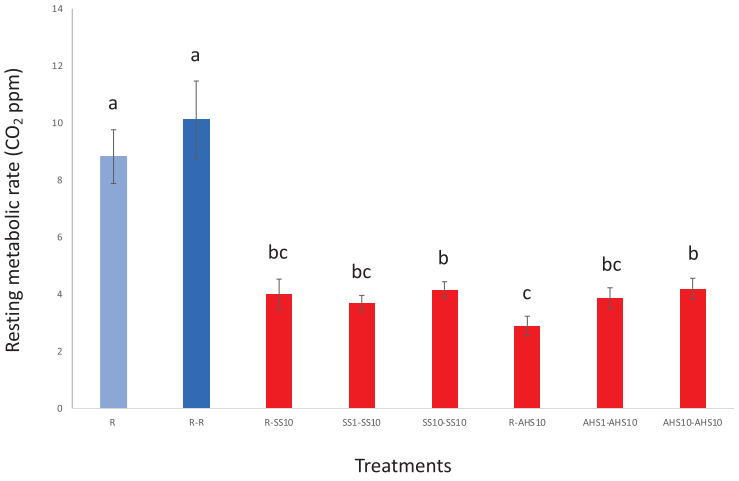

Fig. 3. Resting metabolic rate (CO_2 production) of *T. molitor* larvae 96 h after being injected in a second challenge. SS: nematode Saprophytic Strain (low virulence). AHS: nematode Alternative Host Strain (high virulence). R: larvae injected with Ringer solution. SS1: larvae injected with 1 nematode of the SS strain. SS10: larvae injected with 10 nematodes of the SS strain. AHS1: larvae injected with 1 nematode of the AHS strain. AHS10: larvae injected with 10 nematodes of the AHS strain. Different letters indicate significant differences ($P \leq 0.05$).

studies that reported similar survivorship rates [e.g., 14, 16].

We were also interested in testing the hypothesis that the differential effectiveness of immune priming, previously documented for *T. molitor*, could be related to the virulence level and the pathogen or parasite dose [13,15,16,27]. For this, we sought to test different degrees of virulence and injected doses. We compared two different strains: strain SS (mildly virulent) and strain AHS (highly virulent) [31], as well as a different number of injected nematodes (1 vs. 10). With these different levels of virulence and doses, we expected that the primary challenge with the highest virulence and dose would elicit immune priming in *T. molitor* larvae while the primary challenge with the lowest virulence and dose would not [13]. However, our results did not support our hypothesis of differential effectiveness of immune priming due to the virulence level and the dose (Fig. 1). As we expected, neither SS strain groups (mildly virulent) exhibited any evidence of immune priming; the survival of both groups (the SS1-SS10 and SS10-SS10) was not significantly different from the control group R-SS10. The SS strain's lack of immune priming was not unexpected; this result is supported by previous findings showing no immune compounds produced by *T. molitor* when infected with the SS strain [31]. This could be due to several mechanisms, including a possible immune evasion strategy [4] by the SS nematodes. Further experiments looking at the mechanisms underlying this lack of an immune response are needed.

However, in our groups with the highest virulence challenge (individuals primed with the AHS strain), contrary to our expectations, survival was also not increased. Instead, survival was significantly decreased relative to the unprimed group. This decrease is likely due to the virulence of the AHS strain. The mechanism for the enhanced virulence of the AHS strain is unknown, but likely, bacteria associated with AHS strain virulence play a role in impairing the host immune response, as virulence-associated bacteria in *R. regina* have been proven to be highly pathogenic [22]. Impairment of the host immune response has been documented for other nematodes; for example, *Steinernema carpocapsae* can inhibit the host prophenoloxidase system and avoid its encapsulation mechanisms, evading the host immune defenses [25]. Since the AHS-strain *R. regina* elicited an immune response in *T. molitor* in a previous study [31], it is unclear why that strain did not produce immune priming in this study. Regardless of the mechanism, an impairment of the immune response could be responsible for the higher pathogenicity of the AHS strain, resulting in lowered survival in the high-dose initial challenge (AHS10-AHS10) group and the lack of immune priming in the low-dose initial challenge (AHS1-AHS10) group. This possibility is suggested by a previous study reporting weaker immune priming of *T. molitor* exposed to a more highly virulent strain of the fungus *Metarhizium anisopliae,* which overexpresses catalase and probably contributes to or induces the impairment of the host immune response [16]. In addition, since the bacteria *Bacillus thuringiensis* [13] and *Micrococcus lysodeikticus* [14] have been shown to elicit immune priming in *T. molitor*, it could be possible that the presence or absence of immune priming could be related to the presence or absence of endosymbiotic bacteria in the *R. regina* nematodes, which harbor diverse symbiotic bacteria, some strains of which are associated with greater virulence [22]. For example, *R. regina* nematodes from field samplings held different bacteria genera than *R. regina* reared in meat at laboratory conditions [22]. Among those bacteria carried by field *R. regina* but not by laboratory nematodes, *Serratia* sp. exhibited a high virulence against *G. mellonella* [22]. Those reports suggest that our nematode strains (SS and AHS) probably harbor different bacteria genera that could be related to varying levels of virulence and the lack of immune priming. Research into different types of endosymbiotic bacteria and their effects on virulence and immune priming expression in response to *R. regina* infection could shed light on this possibility.

Within the same pathogen species, different mechanisms could be related to the absence of immune priming [13,15,16]. Whether or not this is true for the SS and AHS strains of *R. regina* will require additional experimentation; for example, a comparative analysis of the elements related to the mechanisms of evasion and virulence between both nematode strains [25]. Undoubtedly, it is possible that virulence is not the only attribute that varies between nematode strains, but that there is variation in different characteristics of life histories. Although this topic is under investigation, if there were differences in other parameters of the life histories between the SS and AHS strains, these would not seem relevant for immune memory in this study because we did not find evidence of this in insects infected with the different strains.

Survival results showed that, as expected, the SS strain was less virulent than the AHS strain (Fig. 1). It has been proposed that bacteria associated with each strain are a significant component underlying such differences [22]. However, our results indicate that the initially inoculated dose (i.e., number of nematodes) affected only the AHS and not the SS strain (Fig. 1). The survival of individuals injected with 10 nematodes vs. 1 nematode was only significantly modified when the AHS strain was tested (Fig. 1). On the one hand, these dose-response results suggest that

with the use of nematodes, immune priming does not appear to be dose-dependent. This finding contrasts with the dose-dependent effect observed with EPN-associated bacteria [27].

On the other hand, these results indicate that the initially inoculated dose of nematodes can affect (or not) survival, depending upon the tested strain (Fig. 1). The initial number of nematodes seems to affect the host's survival detrimentally for the virulent strain, but not for the low virulent strain. These results suggest that in the case of nematodes, virulence and dose could, in some cases, show a synergistic effect on survival [46].

Regarding resting metabolic rate, we found that after 24 h following the second challenge, the quantities of CO_2 produced by all our primed groups were similar to those produced by the control group (R-R), except for the AHS10-AHS10 (Fig. 2). This last group exhibited a lower CO_2 production than the control, indicating that the AHS strain infection already induces a reduction of metabolism at this timepoint. Immune priming has been previously associated with a metabolic cost, with immune-primed individuals producing more CO_2 than control groups [12]. Our finding of a lack of metabolic cost is in line with our lack of evidence for immune priming in our groups. After 96 h following the second challenge, the resting metabolic rate (CO_2 production) significantly decreased in all the primed groups relative to the control group (Fig. 3). This result indicates that, 96 h after the second challenge, the metabolism of individuals injected with nematodes was severely reduced compared with the controls, suggesting they may have been near death.

We showed by two approaches, survival and resting metabolic rate, that *R. regina* does not elicit immune priming in *T. molitor*. As our previous research showed evidence of immune response by *R. regina* in *T. molitor* [31], it is of interest why *R. regina* did not elicit an immune-priming response. One clue may be in the complete lack of priming by the AHS strain (group AHS1-AHS10) despite the clear virulence of the strain as evidenced by the lowered survival rate in the AHS10-AHS10 group. A highly pathogenic strain would be expected to elicit some immune priming [13], evidenced by a higher resting metabolic rate (production of more CO_2) [12]. Then, our observed lack of any change in the resting metabolic rate indicates that no immune priming was taking place in response to the infection. Future research is needed to determine whether *R. regina* is employing an immune evasion strategy [4,50] and, if so, what mechanism(s) are being used to do so.

5. Conclusion

Our results showed that larvae of *T. molitor* primed with the EPN *R. regina* do not exhibit immune priming. When two *R. regina* strains were tested (mildly virulent and highly virulent strains), we found that survival of *T. molitor* primed with the mildly virulent strain did not differ from the unprimed group (lack of immune priming). In contrast, the survival of the hosts primed with the highly virulent strain was significantly reduced relative to the control group. While it is clear that the higher virulence of the AHS strain resulted in lower survival, it is not clear what factors could be responsible for our results. Future research will focus on the role of evasion strategies by EPNs and virulence-associated bacteria as factors that impede the expression of host immune priming.

Author statement

M-L TT, C-G J, M-C L, R-R R: Conceptualization, Methodology, Investigation, **C-G J, M-C L :** Resources, Funding, **M-L TT, R-R R:** Formal analysis, **M-L TT, C-G J , R-R R:** Writing-Original Draft, **M-L TT, C-G J, M-C L, R-R R:** Writing-Review & Editing.

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Declaration of competing interest

None.

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CAPÍTULO II

Contreras-Garduño J., Méndez-López T.T., Patiño-Morales A., González-Hernández G.A., Torres-Guzmán J.C., Krams I., Mendoza-Cuenca L.F. & Ruiz-Guzmán G. (2019). The costs of the immune memory within generations. *The Science of Nature*, *106*, 1-9. **F.I. = 2.645.**

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ORIGINAL PAPER

The costs of the immune memory within generations

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Abstract
Immune response is evolutionary costly, but it is not clear whether these costs affect energetic expenditure (short-term cost), growth (medium-term cost), or reproduction (long-term cost). We tested the costs of immune memory in *Tenebrio molitor* against *Metarhizium brunneum*. To do this, we used two groups of *T. molitor* larvae: (a) the control group, which was injected first with Tween solution and 10 days later with *M. brunneum* and (b) the memory group, which was first injected with *M. brunneum* and 10 days later with *M. brunneum*. Compared to controls, larvae of the memory group were more likely to survive, but they also had an increased metabolic rate (CO_2 production), spent a long time before becoming pupae, and had a shorter time from pupae to adulthood. In the adult stage, control females preferred control males, but there was no significant difference in the preference of memory females. Finally, control and memory males preferred control females. These results confirm that immune memory has costs in terms of energetic expenditure, growth, and reproduction. To the best of our knowledge, this is the first experimental demonstration that immune memory in larvae is traded-off with adult sexual selection involving mate choice.

Keywords Immune memory · Life history · Trade-offs · Reproduction · Mate choice · Metabolic rate

Introduction

The immune response is a double-edged sword. On the one hand, immune response is key for animals when combating pathogens, parasitoids, and parasites (here referred to as parasitism) and favors the survival of the host (Schmid-Hempel 2011). On the other hand, the deployment and maintenance of immune responses are costly (Lawniczak et al. 2007). Theory predicts costs associated with immune memory within and across generations (Tate and Rudolf 2012; Best et al. 2013; Tate 2016; Contreras-Garduño et al. 2016). However, most studies have tested these costs across generations (i.e., Roth et al. 2009; Sadd and Schmid-Hempel 2009; Zanchi et al. 2011; Trauer and Hilker 2013; Dhinaut et al. 2018), and only a few have tested them within generations (Lopez 1998; Contreras-Garduño et al. 2014; Khan et al. 2019). The cost of immune memory within generations could be explained through life history theory. This theory proposes that organisms cannot invest their resources in maintenance, development, and reproduction at the same time because they all are costly (Stearns 1989, 1992, 2000). However, as far as we know, the hypothesis that investment in immune memory could be costly in terms of energetic expenditure (short-term cost of maintenance), reduced growth (medium-term cost), and reproduction (long-term cost) remains unexplored.

Evolutionary biologists have challenged the central and unique role of immune memory in vertebrates because immune memory not only occurs in vertebrates but also invertebrates (Kurtz and Franz 2003; Little and Kraaijeveld 2004; Milutinović and Kurtz 2016; Contreras-Garduño et al. 2016;

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conidia/μL of *M. brunneum* (strain Ma10GFP), which killed approximately 50–60% (LD 50–60) of the larvae in the absence of immune priming. We used LD 50–60% to guarantee larval survival until adulthood. We confirmed the dose of the pathogen before each injection.

In all the experiments carried out with the memory group, the first challenge was done with mCherry because its red fluorescence allowed us to confirm the infection. Under a fluorescence microscope, it is possible to confirm both the clearance of the first infection before the second challenge with the strain that shows green fluorescence (Ma10GFP) and the death of the animals as a result of the second challenge in the absence of the first challenge (Castro-Vargas et al. 2017). Under a fluorescence microscope (Zeiss Axioscope 5), our pilot experiments showed no signs of infection with mCherry after 9 days of infection ($n = 20$), before inoculation with 50 conidia/μL of Ma10GFP. Ten days after the second challenge, under a fluorescence microscope (Zeiss Axioscope 5), we confirmed that insects died only due to Ma10GFP ($n = 20$).

Given that infection or parts of the parasite persist for a long period inside the host (Freitak et al. 2014; Knorr et al. 2015; Duneau and Lazzaro 2018), in another pilot experiment, we confirmed the absence of the second infection over a long period. Using a fluorescence microscope (Zeiss Axioscope 5), we corroborated infections by the Ma10GFP strain outgrowth detection for 20 days after the second challenge and were not infected with the mCherry strain ($n = 20$).

We recorded survival after the second challenge every day for 10 days to measure the benefit of immune priming (memory) (Castro-Vargas et al. 2017; Medina-Gómez et al. 2018a). Following the treatments, the larvae were maintained individually in 6-well plates (Corning) inside an environmental chamber at 27 ± 0.5 °C and with food (Lumistell ICP-55).

Cost I: metabolic rate

We challenged *T. molitor* with *M. brunneum* according to Castro-Vargas et al. (2017). We recorded the resting metabolic rate in individual larvae by measuring CO_2 production (VCO_2 ppm) with the equipment Q-Box RP1 LP and the low range respirometry package (Qubit Systems Inc). Because stress and manipulation may affect the metabolic rate (Ardia et al. 2012; Krams et al. 2014), each larva was introduced to a container (3.8 cm diameter × 20 cm tall) in darkness for 5 min for habituation. The container was connected to the respirometer and a computer. The production of CO_2 by an individual larva was recorded every 30 s for 5 min, and we reported the mean value per larvae (Li et al. 2016).

To test if immune activation and not immune memory may enhance the CO_2 production, we carried out another experiment. In this second experiment, we recorded and compared the CO_2 production (VCO_2 ppm) in three groups. The first group was challenged with Tween 80 (0.01%) and served as the control group for injection. Another group was injected with 5 conidia of *M. brunneum* (strain mCherry) diluted in 1 μL of Tween 80 (0.01%). The third group was injected with 200 conidia of strain mCherry diluted in 1 μL of Tween 80 (0.01%). The comparison of the CO_2 production after a first challenge with the CO_2 production after the second challenge allowed us to compare the cost of immune activation with the cost of immune priming (i.e., immune memory) in terms of CO_2 production.

We measured CO_2 production (VCO_2 ppm) 24 or 96 h after the immune challenge. The peck of activation of immune response is reached about 24 h after challenge (Haine et al. 2008; Johnston et al. 2014), and it would be more likely to detect a cost in metabolic rate. The time of 96 h was a control for time because molecular changes have been reported during immune priming at 24 but not at 96 h (Castro-Vargas et al. 2017). Hence, it was more likely to identify a cost in metabolic rate at 24 but not 96 h.

Cost II: developmental costs

After the second challenge, we recorded the number of days that it took each larva to reach the pupal stage, and then we recorded the time between pupal stage to adults.

Cost III: reproductive costs

We conducted mate choice trials when the individuals of *T. molitor* had reached their 12th day of life. Adults of this species begin reproduction at approximately 7 days (Tschinkel et al. 1967), but the peak of sexual maturity and pheromone production is reached at approximately 10–12 days (Tschinkel et al. 1967; Cole et al. 2003). We conducted focal observations in an arena that consisted of a central container with two smaller containers attached to the outside walls (Kivleniece et al. 2010; Márquez-García et al. 2016). A 12-day-old virgin female was placed in the central container and one 12-day-old virgin male per treatment (memory or control) was placed into a smaller container. All the containers had small holes to allow the pheromones to pass through the chambers and enable females to receive the males' pheromones (Kivleniece et al. 2010). This procedure was carried out with a focal female from the memory or control experiment or a control or memory male. We left both males and the virgin female in the containers for 5 min to be habituated and to produce and smell the pheromones. Then, we released the females out of their containers to allow them to choose a mate (Fig. 1a). All trials consisted of 10 min of focal observations in which female or male preference was evaluated according to the time spent in the enclosure of the opposite sex: a greater amount of time in the cage indicates greater preference (Rantala et al. 2002; Krams et al. 2015; Márquez-García et al. 2016). In all trials, we controlled for age and size of potential male or female

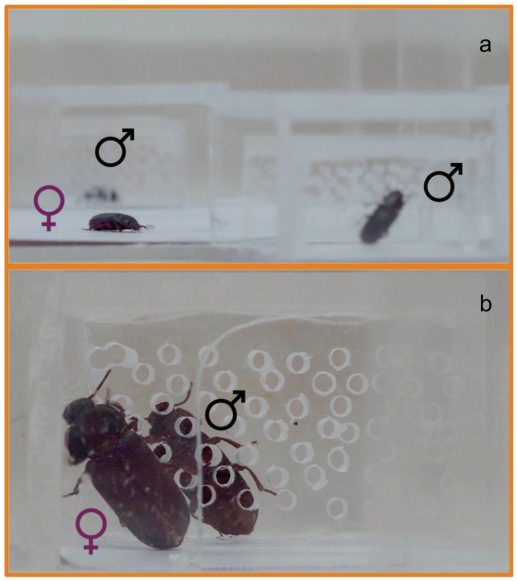

Fig. 1 Behavioral tests in which females of *Tenebrio molitor* evaluated two male options. In **a** is shown a female within the cage of the preferred male. In **b**, we show that cages have holes to allow the insects to smell each other

partners although odor is the only form of communication during mate choice in *T. molitor* because pheromones cause mate aggregation (Fig. 1b; Tanaka et al. 1986; Rantala et al. 2002, 2003a, b; Carazo et al. 2004; Bryning et al. 2005; Vanderwel et al. 2017).

Although our pilot experiments revealed that larvae eliminate *M. brunneum* after the second challenge, it was still possible that some males or females that did not escape from infection could carry out a terminal investment strategy and be more preferred than control males (Rantala et al. 2002, 2003a, b; Sadd et al. 2006; Vainikka et al. 2007; Nielsen and Holman 2012; Krams et al. 2011, 2015). We attempted to rule out this potential problem by testing first whether females preferred control males over males infected with *M. brunneum* (strain mCherry). In this experiment, males were either injected with Tween or with 200 conidia/μL of *M. brunneum* (mCherry). Then, both male types were offered to healthy females, and they chose a mate, and by using a similar procedure of mate choice as that described above, we determined whether females preferred infected over healthy males.

Statistics

Survival after feeding was evaluated with the Kaplan–Meier estimator. To compare the rate of survival between control and priming groups, we first calculated the proportional risk of death and then used the log-rank test. To evaluate the costs of immune memory on CO_2 production after the first challenge, we used ANOVA because the data fit the criteria of variance homogeneity and normality required for the test. To compare the CO_2 production between the control and memory groups, we used Student's *t* test without assuming equal variances. We used Student's *t* tests that do not assume equal variances to compare larval and pupal development because the data fit the criterion of normality. Finally, we used paired *t* tests to analyze mate choice because each sex chooses between a control or experimental (memory) potential partner. All tests were conducted in IBM SPSS ver. 20. For all measured variables, we provide mean ± standard error. We controlled for sample size in all experiments by matching insects of similar sizes.

Results

Benefit of immune priming: survival

Individuals from the memory group (n = 276) were more likely to survive than individuals from the control group (n = 276; log-rank test = 24.06; $p < 0.0001$; Fig. 2).

Cost I: metabolic rate

Given that this is the first time that the metabolic rate has been compared during immune priming, we recorded CO_2 production after the first and second challenge to determine the cost

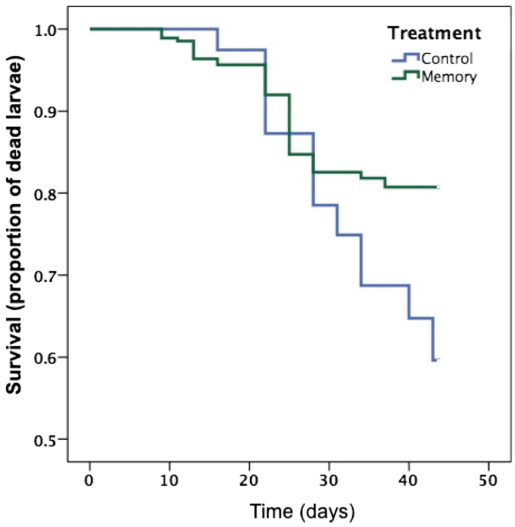

Fig. 2 It was more likely for larvae of *Tenebrio molitor* in the memory group to survive for a greater number of days than larvae of the control group. Sample sizes are provided in the results section

of immune activation (control of injection with Tween), the cost of being infected with *M. brunneum* (200 conidia/μL), and the cost of immune memory (memory *vs* control groups).

Although immune activation (injection with Tween) resulted in the lowest CO_2 production (2.98 ± 0.25, n = 20), no differences were found between the challenges with 5 (6.06 ± 0.51, n = 20) or 200 (6.02 ± 0.52, n = 20) conidia/μL (F = 13.94, d.f. = 2, $p < 0.0001$; Fig. 3a). This shows that the metabolic rate in terms of CO_2 production increased for 51% after 24 h of the immune challenge with *M. brunneum*. However, after 96 h of immune challenge, no differences were found in CO_2 production (Tween: 4.6 ± 0.7, n = 20; 5 conidia/μL: 5.92 ± 0.91, n = 20; 200 conidia/μL: 4.95 ± 0.76, n = 20; F = 0.66, d.f. = 2, p = 0.51). On the other hand, we observed an increase of 18% in metabolic rate in individuals in the memory group (6.78 ± 0.48, n = 20) in comparison to those in the control group (5.56 ± 0.33, n = 20) after 24 h of the second challenge (t = − 2.01, d.f. = 33.62, p = 0.04; Fig. 3b). However, no differences were found 96 h after the second challenge (memory group: 6.43 ± 0.76, n = 20; control group: 6.9 ± 0.74, n = 20; t = 0.52, d.f. = 37.97, p = 0.6).

Cost II: developmental time

Insects in the memory group took more time (35.84 ± 0.2, n = 59) to reach the pupal stage than larvae in the control group (32.75 ± 0.32, n = 59) after the second challenge (t = − 3.99, d.f. = 174; p = 0.001; Fig. 4a). However, insects in the control group remained longer in the pupal stage (10.24 ± 0.20, n = 30) than those in the memory group (8.47 ± 0.14, n = 30; t = 7.24, d.f. = 194; p = 0.001; Fig. 4b).

Cost III: mate choice

Control females preferred control males (202.37 ± 18.88, n = 44) over males of the memory group (132.57 ± 16.90, n = 44; t = 2.38, d.f. = 44; p = 0.02; Fig. 5a). However, females in the memory treatment did not prefer control males (204.89 ± 16.57, n = 44) over memory males (172.42 ± 13.51, n = 44; t = 1.38. d.f. = 44; p = 0.17). Control males preferred control females (215.43 ± 23.96, n = 42) over memory females (101.69 ± 11.91, n = 42; t = 3.87, d.f. = 41; p = 0.001; Fig. 5b). Memory males preferred control females (183.69 ± 18.24, n = 42) over memory females (126.38 ± 17.97, n = 42; t = 2.22, d.f. = 41; p = 0.03; Fig. 5c).

Finally, results showed no significant difference in mate choice between healthy males (193.40 ± 19.68; n = 60) and males infected with mCherry (216.40 ± 19.91 s; n = 60; t = 0.82, p = 0.41). This suggests that immune challenge with *M. brunneum* (200 conidia/μL) 3 h after challenge alone does

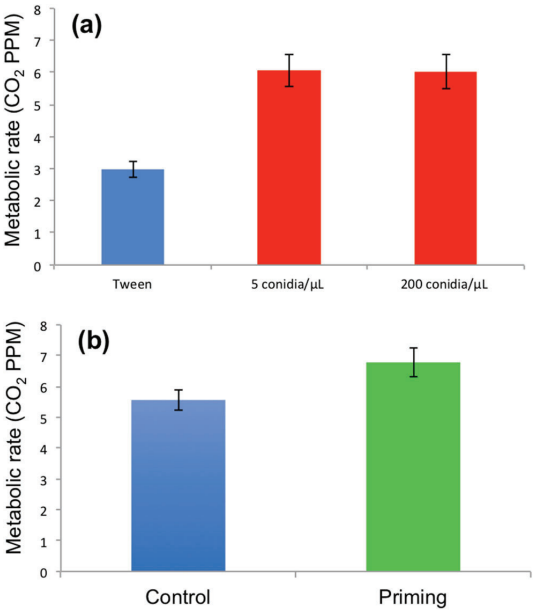

Fig. 3 CO_2 production in larvae of *Tenebrio molitor* 24 h after a first challenge with Tween or 5 or 200 conidia/μL of *Metarhizium brunneum* (**a**) or after a second challenge in the memory and control groups (**b**). Sample sizes, mean, and standard error are provided in the results section

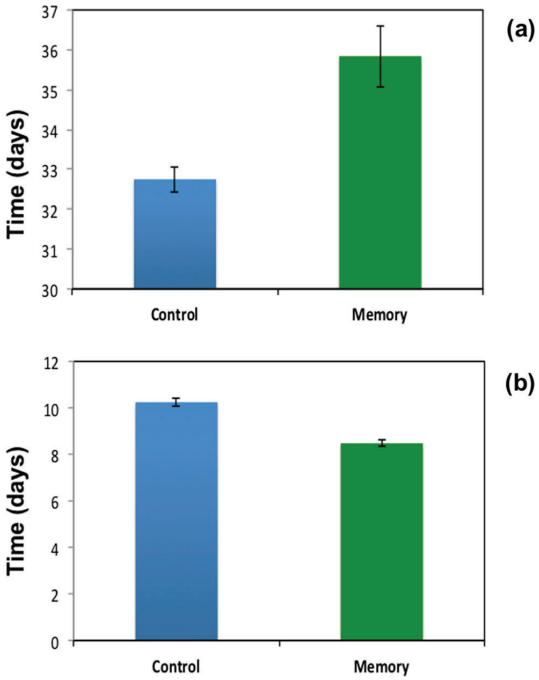

Fig. 4 Larvae of *Tenebrio molitor* in the memory group took more days to reach the pupal stage (**a**) but fewer days to reach adulthood (**b**) than larvae in the control group. Sample sizes, mean, and standard error are provided in the results section

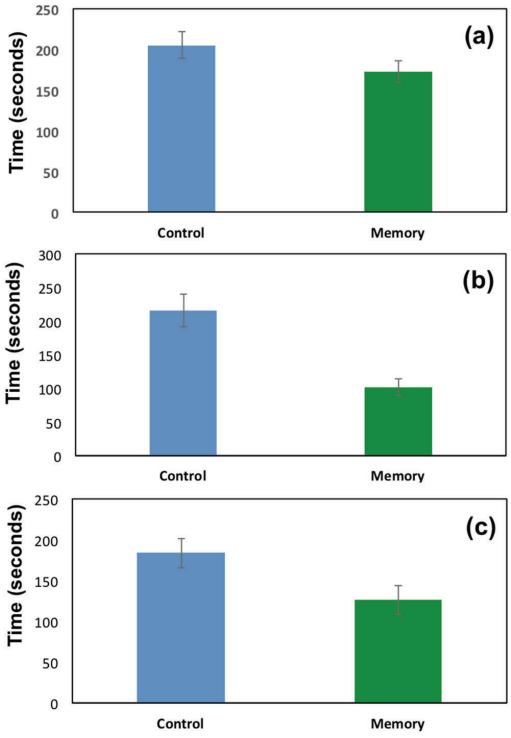

Fig. 5. On the one hand, control females of *Tenebrio molitor* preferred the control males more often than the memory males (**a**). On the other hand, control (**b**) and memory (**c**) males preferred the control females more often than the Memory females. Sample sizes, mean, and standard error are provided in the results section.

not affect mate preference, so the potential differences in our experimental setup could be due to immune priming (memory) and not infection alone.

Discussion

We found that immune priming (immune memory) is a double-edged sword. On the one hand, it favors survival against pathogens. On the other hand, it reduces larvae developmental rate, increases resting metabolic rate, and reduces the probability of accessing a mating partner. Our results correspond with other studies that found that immune priming favors survival (Contreras-Garduño et al. 2016; Milutinović and Kurtz 2016) and with the few studies reporting costs of immune priming within generations (Contreras-Garduño et al. 2014; Khan et al. 2019).

Immune priming studies carried out across generations have shown that offspring of primed parents developed slower than those of unprimed parents (Roth et al. 2009; Sadd and Schmid-Hempel 2009; Zanchi et al. 2011; Trauer and Hilker 2013; Dhinaut et al. 2018), but here, we report that larvae having undergone immune priming developed more slowly than unprimed larvae. We propose that the consequence of a slow developmental rate is that it can lead to a smaller size in larvae, which may affect food competition (Trauer and Hilker 2013) and prompt larvae to cannibalism (Ichikawa and Kurauchi 2009). Indeed, cannibalism affects the reproduction of the colonies for *T. molitor* (Deruytter et al. 2019). In addition, if small larvae become small adults, they experience costs in reproduction compared with larger adults (Koella and Boëte 2002). Hence, *T. molitor* could try to compensate reduced mating opportunities by accelerating development in the pupal stage to reach the reproductive stage (Metcalfe and Monaghan 2001). Such a compensatory strategy would explain why the pupae in the memory group developed into the adult stage more rapidly than those in the control group. Therefore, our findings suggest that insects undergoing immune priming during the larval stage may show flexible developmental strategies, including a prolonged larval stage but rapid pupal development to reduce the cost of low developmental rate in the larval stage.

A previous study found that adults that invested fewer resources in maintaining the immune response as larvae had increased mating success in comparison to larvae that invested more resources in maintaining the immune response (Valtonen and Rantala 2012). Such investment during the juvenile period might also lead to severe repercussions in adulthood (Metcalfe and Monaghan 2001). However, regarding immune memory, studies have focused on costs within the youthful or adult periods. For example, in children vaccinated against meningitis, the second vaccine (7 months after the first) induced the number of B cells and antibodies almost threefold (Rohner et al. 2008). Adult males of the fish *Poecilia reticulata* with immune memory (dual challenges against *Gyrodactylus*) proved to be more attractive to females and court them more often than males in the control (sham) group (Lopez 1998). This suggests that males that show immune memory receive benefits rather than bear costs in terms of mate choice. Other possible explanations are that (a) those males are genetically more resistant to infection and are in better condition than control males (Lopez 1998), (b) they carry out terminal investment (Krams et al. 2015), or (c) they invested in the short term at the cost of a second mating (Stearns 1992). These hypotheses warrant further research. However, to the best of our knowledge, here, we show for the first time evidence that insects having developed immune memory at the juvenile stage (due to exposure to a pathogen) are less attractive to potential mates in the adult stage and that this cost seems not to be due just to infection with *M. brunneum* (see Materials and methods). We recognize that more control or experimental groups are needed to disentangle the costs of immune priming. For example, the use of naïve

and Tween groups will show baseline development, CO_2 production, and mate choice to know how these attributes differ in the control and memory groups. In addition, here, we did not test the role of nonspecific memory *vs.* that of specificity. For example, we should compare the cost in CO_2 production and mate choice using dual infections with *M. brunneum* with another dual infection but with *Serratia marcescens* in the first challenge and *M. brunneum* in the second challenge or with Tween and then *M. brunneum* (*i.e.*, Medina-Gómez et al. 2018a).

In addition, highlighting the cost of immune memory in terms of sexual reproduction here, we assume that the resources that an individual has are limited. If a male or female, in the larval state, had to invest more resources into immune response, then as an adult, that individual will have fewer resources to invest into characteristics that make it more attractive to the opposite sex, such as the production of pheromones. We assume that immune response undergoes a trade-off with sexual selection due to limited resources, but (a) another attribute not related to sexual selection should also be measured to ensure that the trade-off is only involved with sexual selection (as in the heightened condition hypothesis; Contreras-Garduño et al. 2008) and (b) the exact molecular and physiological mechanisms should be disentangled.

The physiological basis of the cost of immune memory is known. However, here, we show costs in terms of metabolic rate. Costs in terms of metabolic rate in insects due to immune activation with nylon monofilament have already been described (Ardia et al. 2012), and our results are consistent with these findings because the injection of Tween resulted in less CO_2 production than the injection of Tween plus the fungus. This evidence suggests that immune challenge increases the insects' metabolic rate, but we also show that when controlling for the infection in the second challenge, the insects in the immune priming group increased their metabolic rate by approximately 18%. We can only speculate that this increase in addition to the increase in metabolic rate due to infection alone could prompt insects to face a long-term cost. Also, it is interesting to note that after 96 h of infection, there was no difference in metabolic rate according to the first challenge and immune priming. This difference in response between 24 and 96 h is very interesting because a previous study showed differential methylation of ARN at 24 h but not at 96 h (Castro-Vargas et al. 2017). An in-depth molecular analysis will help to understand what molecular processes are occurring during this period and indicate whether these changes affect the costs and benefits of immune priming over medium and long periods.

A novel result is that we show for the first time mutual mate choice in *T. molitor* and that although sexual dimorphism in immune priming has not been found in adults of *T. molitor* (Castro-Vargas et al. 2017), we detected sexual dimorphism in regard to mate choice as a function of the activation of immune memory during the larval stage: females seemed to suffer from higher costs than males because primed females seemed to be less choosy than unprimed females.

We recognize that although we attempted to control for potential behavioral differences, an in-depth study should be carried out to know the involved molecules in such chemical communication. Moreover, it would be interesting to study mechanisms by which immune priming compromises mate choice and, perhaps, the competition for mates.

Finally, we propose to examine whether the constant use of immune memory during the juvenile stages (as a benefit of natural selection) affects attributes of sexual selection in adults (in vertebrates and invertebrates), because a reduction in reproduction could be the price that animals may pay for having immune memory.

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Compliance with ethical standards The work has been carried out under the ethical standards of the Ethical commission in research of the ENES, UNAM, Morelia, México.

Conflict of interest The authors declare that they have no conflict of interest.

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DISCUSIÓN GENERAL

En este trabajo pusimos a prueba el efecto de los parásitos y patógenos sobre la respuesta inmunitaria, así como sus costos evolutivos asociados.

En el **capítulo I,** utilizamos al parásito *Rhabditis regina* y su virulencia para poner a prueba la capacidad de las larvas de *T. molitor* para establecer memoria innata, y también registramos la producción del CO_2 como un indicador del costo de la respuesta inmunitaria (Ardia *et al*., 2012). La hipótesis no se cumplió debido a que no se observó memoria inmunitaria y no se encontró correlación entre la producción de CO_2 con la activación de la respuesta inmunitaria en las larvas infectadas. Las larvas de *T. molitor* retadas con la dosis subletal de *R. regina* no mostraron evidencia de activación de la respuesta inmunitaria. Sin embargo, cuando se probaron dos cepas: SS y AHS (Trejo-Meléndez *et al*. En prensa), la supervivencia de *T. molitor* retada con la cepa SS no difirió del grupo control. Esto podría deberse a estrategias de inmunosupresión y/o estrategia de evasión inmunitaria por parte del nematodo (Lara-Reyes *et al*., 2020). Por el contrario, las larvas de *T. molitor* retadas con la cepa AHS vivieron menos con relación al grupo control. Este último hallazgo indicó que la virulencia de la cepa AHS es muy alta y/o que algún componente relacionado con su virulencia alteró la respuesta inmunitaria del hospedero. Los resultados apoyan los trabajos de Medina-Gómez *et al.* (2018 a, b), dado que la virulencia del patógeno tiene un efecto importante sobre el establecimiento de la memoria innata en *T. molitor*.

En el **capítulo II** se evaluaron larvas de *T. molitor* retadas con *M. brunneum* como un modelo ya probado de memoria inmunitaria (Médina-Gómez *et al.,* 2018a). Así, pusimos a aprueba la hipótesis de que la memoria innata es evolutivamente costosa (Contreras-Garduño *et al*., 2014; 2016). Estos costos se evaluaron a: a) corto (producción de CO_2 por la activación

de la respuesta inmunitaria), b) mediano (tiempo que tarda una larva en llegar a su etapa adulta) y c) largo plazo (costo reproductivo en elección de pareja). Los resultados mostraron que la activación de la memoria inmunitaria tuvo costos evolutivos. El costo a corto plazo indicó que la activación de la respuesta inmunitaria aumentó la producción de CO_2, y se cumplió la hipótesis, ya que este resultado corresponde con los trabajos de Ardia *et al*. (2012). Además, corresponden con los tiempos de activación máxima de la respuesta inmunitaria en *T. molitor,* como lo muestran los trabajos de Haine *et al*. (2008). Cabe destacar que la dosis subletal inicial y el grupo control no mostraron diferencias significativas en CO_2, por lo que podemos concluir que la activación de la respuesta no es costosa, pero sí lo es, la memoria inmunitaria. Por el contrario, al analizar la memoria inmunitaria con respecto al control sin memoria, los resultados sugieren que la memoria innata es una espada de doble filo: por un lado, favorece la supervivencia, pero por el otro, incrementa la tasa metabólica basal (corto plazo), reduce el desarrollo (mediano plazo) y reduce la probabilidad de acceder a una pareja reproductiva (largo plazo). Nuestros resultados apoyan la hipótesis de que la memoria innata favorece la supervivencia, pero impone un costo reproductivo (Contreras-Garduño *et al.,* 2014, 2016). Sin embargo, la importancia de esta tesis es que muestra un costo de la memoria en la fase adulta por tener memoria en la etapa juvenil. Hasta donde sé, estos costos no se han documentado en la memoria adaptativa de vertebrados.

En el **apéndice I**, se realizó una revisión de divulgación sobre el tema general de memoria innata. Ahí, me pareció importante hacer notar que el tema es relevante y novedoso por su aporte teórico a la inmunología, pero, sobre todo por su potencial aplicación en el campo de las “vacunas” para invertebrados. Por ejemplo, se ha logrado favorecer la

resistencia de camarones contra el virus de la mancha blanca (Amatul-Samahah *et al*., 2020). Este tema, sin duda garantiza información a futuro.

En el **apéndice II**, se revisó el estado del arte del inmunometabolismo y la memoria porque es un campo de estudio reciente que ayudará a entender la estrecha relación entre la respuesta inmunitaria, la tasa metabólica, y el impacto de esta relación sobre la supervivencia o la reproducción (Fok *et al*., 2019; Domínguez *et al*., 2021). El abordaje del inmunometabolismo (Wang *et al*., 2019) en el estudio de la memoria inmunitaria innata en invertebrados es un campo relativamente nuevo, pero ha permitido entender la conexión entre el hospedero, el uso de sus recursos y la energía, para conocer la activación de la memoria inmunitaria innata y cómo algunas rutas metabólicas se relacionan con las modificaciones epigenéticas (Vilcinskas, 2021). Además, un aspecto importante de la memoria inmunitaria innata es su relación con la activación del endocilo (Edgar *et al*., 2014; Contreras-Garduño *et al*., 2015). Estos mecanismos de respuesta fisiológica de las células permiten al hospedero enfrentar retos de alta demanda de energía y utilizar recursos, al sintetizar una gran cantidad de DNA, sin que la célula entre en mitosis y parecen relacionarse con el inmunometabolismo (Maya-Maldonado *et al*., 2021). En el apéndice II, propongo líneas de investigación centradas en el inmunometabolismo, la epigenética, y el endociclo para entender la memoria innata. Además, el inmunometabolismo es clave en la activación de la memoria inmunitaria innata (Arts *et al*., 2016; Samaddar *et al*., 2020) y mi revisión muestra que esta conexión entre la respuesta metabólica y la memoria innata afecta la disposición de los recursos y energía y podría explicar los costos evolutivos. Esto podría explicar los costos de la memoria reportamos en el capítulo 3 de la tesis y garantiza investigación a futuro. Sería relevante

indagar este tema, tomando en cuenta la capacidad de los hospederos para establecer memoria innata en contra diferentes tipos de parásitos y patógenos.

CONCLUSIONES

Se utilizó el escarabajo *T. molitor* para poner a prueba el efecto de los nematodos entomopatógenos sobre la memoria inmunitaria innata, así como los costos evolutivos de la memoria. Los resultados de esta tesis y otras publicaciones sugieren que la memoria inmunitaria innata puede o no ocurrir dependiendo del patógeno y su virulencia. Dado que esta tesis sugiere que la memoria innata es costosa, sería interesante conocer los costos de la memoria adaptativa.

Hasta el momento no se sabe cómo se almacena y se reclama la memoria inmunitaria. Es importante indagar el papel de las histonas y sus modificaciones epigenéticas, en particular en la histona 3. De igual manera, el estudio del inmunometabolismo tiene mucho que aportar al estudio de la memoria innata y la regulación de las rutas metabólicas, como lo es el factor de transcripción HIF-1 α/β. Finalmente, el estudio del endociclo sigue siendo un tema de importancia para entender la memoria innata, por lo que hace falta saber su papel en la regulación postranscripcional de histonas. Los estudios de memoria innata podrían tener aplicaciones en diferentes campos, como el control de plagas, la acuacultura y la biomedicina.

En conjunto, esta tesis sugiere nuevas líneas de investigación de la memoria innata a nivel ecológico-evolutivo y también, a nivel de mecanismos.

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Descripción general de los apéndices

La historia de cómo se logró la identificación de la memoria inmunológica y sus generalidades se abordaron en el **apéndice I**. Se definió a la memoria inmunitaria innata como el nuevo paradigma de la inmunología. Esto se escribió en formato de divulgación de la ciencia, con el objetivo de hacerlo llegar a un público a partir de bachillerato. Se describió a detalle el trabajo de los investigadores que demostraron de manera irrefutable el establecimiento de la memoria inmunitaria en los invertebrados, seguido de las caracteristicas que definen a la memoria innata, los mecanismos inmunitarios efectores que se le han relacionados, para luego indagar sus implicaciones ecológicas. Finalmente, se ahondó la memoria en las potenciales aplicaciones biotecnológicas, médicas y en producción acuícola.

En el **apéndice II** desarrollamos un artículo de revisión del estado actual del tema del inmunometabolismo de las células inmunocompetentes en los invertebrados durante el establecimiento y activación de la respuesta inmunitaria innata. Realicé una bísqueda exhaustiva de la literatura, abarcado los temas de la conexión de la fisiología metabólicade las células, durante la activación de la respuesta inmunitaria en los invertebrados. Analizamos las evidencias inmunometabólicas de la activación y del establecimiento de la memoria inmunitaria innata en invertebrados, después indagué la relación entre los costos energéticos, el metabólismo, la activación del endociclo. Finalmente se muestran las modificaciones epigenéticas relacionadas.

APÉNDICE I

Méndez-López T. T. & Contreras-Garduño J. 2021. Memoria inmunitaria innata en invertebrados: Un cambio de paradigma en biología con distintas aplicaciones. *Saber Más*. Año 10/Septiembre -Octubre/ No. 59.

ARTÍCULO DE PORTADA

Memoria inmunitaria innata en invertebrados: Un cambio de paradigma

Texca Tatevari Méndez-López y Jorge Contreras-Garduño

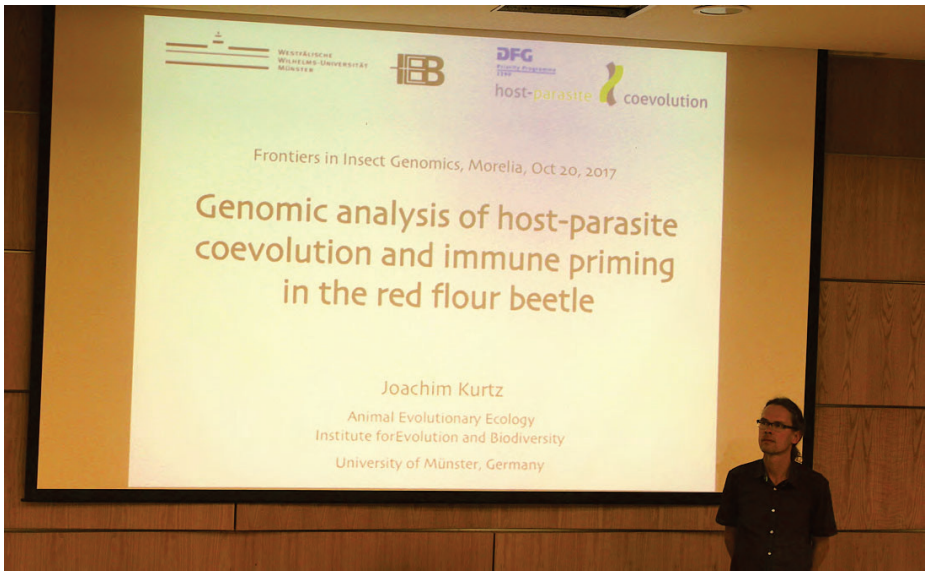
Hasta hace poco se pensaba que los vertebrados eran los únicos animales con memoria inmunitaria. Sin embargo, **un nuevo paradigma en biología** sugiere que la memoria también ocurre en invertebrados, por ejemplo, en ctenóforos, cnidarios, nemátodos, insectos, crustáceos y moluscos. Se ha demostrado que esta memoria innata ocurre dentro y a través de las generaciones, pero aún no se sabe el papel de los patógenos sobre el alcance de la memoria innata, sus posibles mecanismos, cómo los parásitos y patógenos afectan la plasticidad de los mecanismos de la memoria, y demuestra una compleja estrategia que ha evolucionado en los hospederos del reino animal contra sus parásitos y patógenos.

Memoria inmunitaria innata

Según el filósofo Thomas Kuhn, en ciencia, los paradigmas se entienden como fenómenos sustentados por un gran cuerpo de evidencias cuyas explicaciones parecen estables y, por ello, son adoptadas por la comunidad científica en un momento determinado. Sin embargo, en ocasiones, ocurren cambios en estos paradigmas y pueden ser rechazados por completo, o tal vez, ampliados con nueva evidencia. Un ejemplo actual ocurrió en biología:

La inmunología es el estudio de los mecanismos involucrados en defender a los seres vivos de los enemigos infecciosos (patógenos, parásitos o virus). Esta defensa se logra con células y moléculas muy especializadas, cuya organización evita el daño causado por los enemigos, y eventualmente, controla su crecimiento o los elimina por completo. Estos mecanismos de defensa se han clasificado en dos grupos, la Respuesta Inmunitaria Innata y Respuesta Inmunitaria Adaptativa.

Los libros básicos de inmunología describen un modelo en el que, ante infecciones recurrentes: **(a)** la respuesta innata es rápida en su activación porque es inespecífica en su capacidad de distinguir entre diferentes tipos de patógenos y sin la capacidad de establecer memoria inmunitaria; mientras que **(b)** la respuesta adaptativa requiere un mayor tiempo de activación que la respuesta innata y un alto grado de especificidad (memoria) en su respuesta, siendo las inmunoglobulinas, la recombinación y la presencia de linfocitos B y T, algunos de sus componentes esenciales; finalmente, los libros de



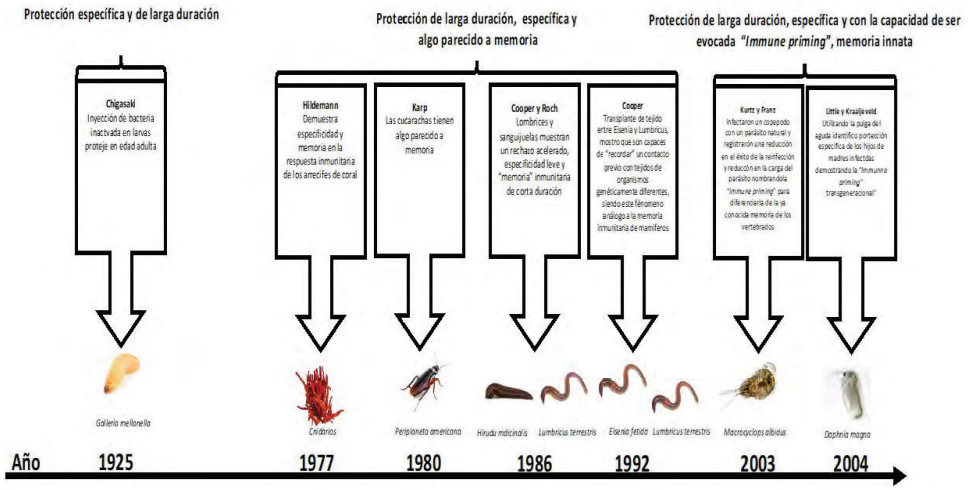
texto en inmunología proponen que **(c)** los invertebrados solo poseen respuesta innata, mientras que los vertebrados poseen respuesta innata y adaptativa. Sin embargo, ahora se tiene una gran evidencia de que estos tres principios han cambiado y, por ello, hay **un nuevo paradigma: tanto vertebrados como invertebrados poseen memoria inmunitaria.** En ambos casos la respuesta es específica, de larga duración y bifásica. No obstante, aún se desconocen los mecanismos de la memoria innata en invertebrados.

¿Cómo se logró cambiar el paradigma?

En 2003, los investigadores alemanes Joachim Kurtz y Karolin Franz, quienes se formaron en el campo de la ecología evolutiva, lograron demostrar de manera contundente la **protección de los invertebrados ante infecciones recurrentes** como memoria inmunitaria, aunque se tiene evidencia de trabajos anteriores que ya intentaban demostrarla.

Kurtz y Franz nombraron al fenómeno *immune priming*, haciendo alusión a que sus mecanismos de memoria son diferentes a los mecanismos de la memoria adaptativa de vertebrados. Actualmente, en inglés se le llama *immune priming* o *innate immune memory*, pero en español le denominaremos **memoria inmunitaria innata**. Esta memoria, al igual que la de vertebrados, se define como un aumento en la protección de los hospederos (en términos de respuesta inmunitaria, eliminación de parásitos y aumento en la supervivencia) después de un encuentro específico con la misma cepa o especie de parásito o patógeno, y se presenta dentro y a través de las generaciones.

En el trabajo de Kurtz y Franz, el copépodo *Macrocyclops albidus* fue infectado experimentalmente con su patógeno natural, el cestodo *Schistocephalus salbidus*. El diseño experimental que planearon para poner a prueba la hipótesis de que los invertebrados poseían memoria inmunitaria innata, fue el siguiente: utilizaron dos grupos de copépodos, pero uno fue infectado con la misma cepa (individuos genéticamente idénticos), y el otro grupo fue infectado con cepas distintas. El tiempo que transcurrió entre un reto y el siguiente, fue de cinco días para que los copépodos generaran la memoria. Encontraron que la infección recurrente con retos similares a nivel de cepa (el grupo con memoria inmunitaria) en comparación con la infección recurrente, pero con cepas distintas (el grupo control) tuvo menor mortalidad, reducción en la tasa de reinfección (menos copépodos se reinfectaron) y me-



Línea del tiempo que muestra las evidencias que permitieron la identificación de la memoria inmunitaria innata. En 2003 se reportó que la memoria inmunitaria innata se presenta dentro de las generaciones y que era altamente específica, mientras que en el trabajo de 2004, presentan la primera evidencia de que esta protección puede ser transmitida de generación en generación.

nos cantidad de patógenos presentes en el interior del copépodo.

Estos resultados se publicaron en la prestigiada revista *Nature*, y fue la **primera evidencia clara** a favor de que la protección específica favorecía la supervivencia (algo similar a la vacuna de vertebrados). Entonces, los investigadores alemanes propusieron que este resultado se debía a la presencia de memoria inmunitaria en invertebrados, y después de un arduo debate, se dio el cambio de paradigma no solamente en inmunología, sino en general en biología.

¿Qué sabemos ahora de la memoria innata?

Los mecanismos de la memoria inmunitaria innata y sus implicaciones evolutivas y ecológicas son un campo nuevo que ha revelado algunos secretos. Se han identificado mecanismos efectores asociados a la activación de la memoria inmunitaria innata, como son **aumento en la cantidad de hemocitos** (células responsables de aniquilar a los parásitos y patógenos en invertebrados), **péptidos antimicrobianos** (moléculas pequeñas que rompen la membrana de los parásitos y patógenos), un **vínculo epigenético** (aumento en modificaciones postraduccionales en términos de metilaciones y acetilaciones en las histonas y ARN o ADN), y una **asociación con el endociclo celular** (replicación de ADN sin división celular). No obstante, aún no sabemos cómo se genera la diversidad de reconocimiento, ni cómo y dónde se guarda la memoria innata. Sin duda, este será un gran reto en esta década.

Ahora sabemos que la memoria innata de invertebrados ocurre en ctenóforos, cnidarios, moluscos y artrópodos (hexápodos y crustáceos). **La clase insecta es el grupo con el que más se ha trabajado,** pero dentro de estos, se ha estudiado la subclase Pterygota, de la cual solamente se han hecho estudios en Blattodea, Diptera, Coleoptera, Hymenoptera y Lepidoptera. Esto revela un gran sesgo de trabajos a unos pocos grupos, por lo que aún se debe analizar con mucho cuidado qué tan extendida es la estrategia de memoria inmunitaria en invertebrados. De los pocos estudios en insectos, se sabe que la virulencia de los parásitos y patógenos es sumamente importante en el alcance de la memoria inmunitaria innata, porque impide

su establecimiento y aumenta el costo metabólico derivado del gasto de energía.

Como se mencionó anteriormente, la memoria innata ocurre dentro de la generación, pero también, a través de las generaciones, lo que significa que los padres pueden transmitir resistencia inmunitaria (la memoria) a su progenie contra patógenos recurrentes en la población. Este fenómeno se denomina *transgeneracional immune priming* y en español lo denominamos **memoria innata transgeneracional**. En escarabajos, se ha observado que las madres y los padres que son retados con un tipo de patógeno pueden transmitir la capacidad de resistencia a sus hijos. Se han identificado mecanismos epigenéticos que involucran cambios tanto en metilaciones, como acetilaciones en ADN, metilaciones en ARN y la transmisión a las crías de pequeñas estructuras de los patógenos que infectaron a sus madres --es como si las madres vacunaran a sus crías con pedacitos de los patógenos que las infectaron--. Todo esto favorece la resistencia contra los patógenos que infectaron a sus padres.

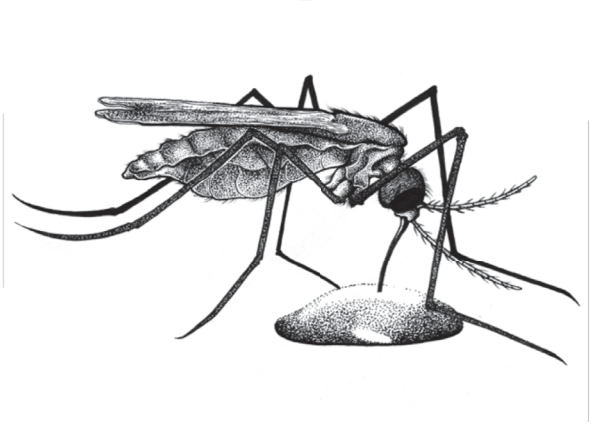
¿Cuáles son las perspectivas en el estudio de la memoria inmunitaria innata?

Los investigadores interesados en inmunología, ecoinmunología, enfermedades infecciosas, biología molecular, bioquímica, control biológico, bioinformática, parasitología evolutiva, epidemiología y ecología evolutiva, deben considerar la ocurrencia de la memoria inmunitaria innata en el abordaje de sus investigaciones y describir sus mecanismos moleculares (cómo se genera la memoria y dónde se almacena), y porque podría o no ocurrir la memoria en condiciones naturales. Además, se deben llevar a cabo **estudios de este tipo en distintas especies de invertebrados** para saber qué tanto se distribuye en este grupo.

¿Cuáles son las aplicaciones de la memoria inmunitaria innata?

En salud pública, producción agrícola, ciencias agroforestales y acuicultura, ya se están analizando los potenciales usos de entender y aplicar los mecanismos efectores relacionados con el establecimiento de la memoria inmunitaria innata. El Dr. Humberto Lanz, del Instituto Nacional de Salud Pública encabeza este esfuerzo en insectos vectores de enfermedades como dengue, malaria, zika y chikungunya.

En el área de ecología evolutiva, se ha estudiado la ocurrencia de memoria inmunitaria de insectos plaga y vectores de enfermedades en humanos (i.e. el escarabajo *Tenebrio molitor*) contra sus enemigos naturales (el hongo entomopatógeno *Metarhizium brunnum*). Este trabajo se lleva a cabo en el laboratorio de ecología evolutiva de la Escuela Nacional de Estudios Superiores (ENES) de la Universidad Nacional Autónoma de México (UNAM), Unidad Morelia. Otro enfoque es la **«vacunación» de los camarones de granja** para eliminar la enfermedad de la mancha blanca, ocasionada por el virus del mismo nombre y que tiene un efecto económico negativo para esta actividad productiva. El Dr. Jorge Olmos del Centro de Investigación Científi-



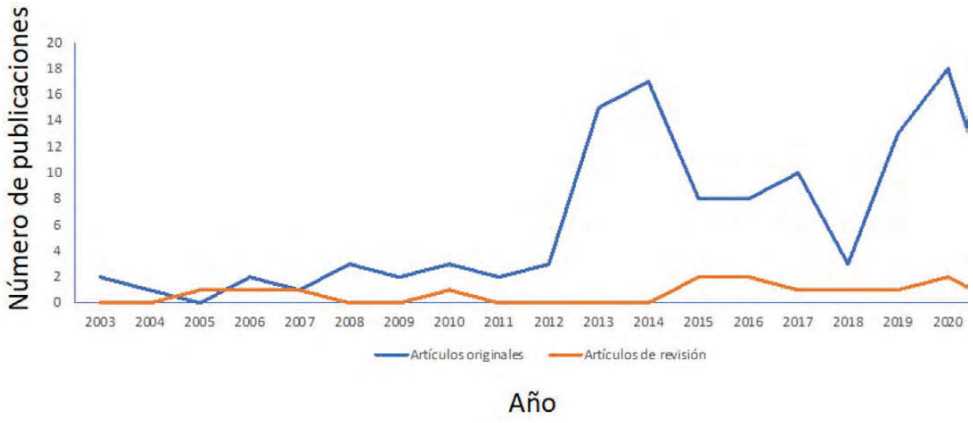
Dibujo del mosquito *Anopheles albimanus* tomando sangre infectada con el parásito de la malaria *Plasmodium vivax*

fica y de Educación Superior de Ensenada (CICESE) en Baja California, utilizó esporas recombinantes derivadas de la bacteria *Bacillus subtilis,* las cuales presentan la proteína CotC::Vp26 en su superficie y las bacterias transformadas fueron adicionadas en paquetes (bolitas) de alimento para camarones e inducir la respuesta de memoria. Este tratamiento, similar a una vacunación, pero en crustáceos, redujo en un cien por ciento la letalidad de la enfermedad en los camarones que enfrentaron los pedazos de virus en el vector. Hasta donde sabemos, hasta ahora, este es el único estudio de su tipo a nivel mundial.

Actualmente, y a partir del trabajo pionero de Kurtz y Franz en 2003, existen cerca de 117 artículos científicos y 13 revisiones relacionadas con el tema, con un aumento de los estudios de memoria innata en invertebrados a partir de 2003. Cabe resaltar que este tema también se aborda en el libro *Advances in Comparative Immunology,* editado por el padre de la inmunología comparada y uno de los pioneros en el campo de la memoria innata, el Dr. Edwin L. Cooper. En este libro introduce el tema de la memoria inmunitaria innata y de inmunología en diferentes grupos de animales, y permite el análisis y discusión de diferentes temas por inmunólogos y ecólogos. Asimismo, aporta teoría y definiciones para aquellos grupos de investigación interesados.

Utilizamos los buscadores The Web of Sciences, PubMed y Google Scholar con las palabras clave *Immune priming* e *immune priming review* para realizar un tamizaje de los trabajos publicados por año, que se presentan en este gráfico. De los estudios experimentales, descartamos los trabajos que no se realizaron con invertebrados.

A casi 20 años del descubrimiento de la memoria inmunitaria innata, aún no sabemos sus mecanismos y, sin duda, existen grandes cosas por descubrirse. Quizás el conocimiento de la memoria inmunitaria innata ayude a saber por qué los insectos plaga resisten a sus agentes de control biológico, permita generar conocimiento acerca de la resistencia de insectos vectores ante enfermedades transmitidas al humano, ayude a favorecer la resistencia de invertebrados de interés económico en granjas de pulpos, camarones y polinizadores (i.e. abejas melíferas), pero también, y no menos importante, que dé pistas de las complejas estrategias que han evolucionado en los hospederos del reino animal contra sus parásitos y patógenos.



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sabermás

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APÉNDICE II

Méndez-López T. T., Carrero-Sánchez J. C., Lanz-Mendoza H., Ochoa-Zarzosa A. & Contreras-Garduño J. Metabolism and innate immune memory in invertebrates: are they dissociated? En revisión.

Metabolism and innate immune memory in invertebrates: are they dissociated?

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ABSTRACT

Since the innate immune response in invertebrates was discovered, its response to different microbial and environmental stimuli have been determined. The most recent characteristic of innate immune response is the ability to establish, store and express an immune memory. Different species and/or strains of pathogens and parasites can evoke this innate immune memory. Immune memory has evidenced the close collaboration between the different humoral and cellular components. However, the interaction of the immune system with other host systems and pathways, for example, metabolism, is still unknown. If this immunometabolic interaction exists, it would be interesting to know if there is variation concerning intrinsic attributes, such as development and sex, and extrinsic factors, such as enemy species, food, and temperature. We briefly review the invertebrates' immune response and metabolism. Then, we propose to study the relationship between metabolism and innate immune memory. We propose that cellular endocycle and epigenetic modifications are involved in this relationship. We review the evolutionary costs related to metabolism and innate memory. Finally, we propose future avenues of research.

Keywords: Immunometabolism; immune memory; ecoimmunology; host-parasite relationship; innate immune response.

Introduction

Parasites and pathogens can reduce the lifetime of living beings. One of the interactants (i.e., the parasite or pathogen) invades the host and uses it as a resource to reproduce. The other interactant, the host, kills its enemy or is infected more than once during its life cycle [1]. However, another result of these interactions is tolerance, which is probably more frequent when the pathogen does not harm or does not send danger signals (Polly Matzinger's theory of danger) [2-4]. These recurrent infections represent a challenge for the host, which has shaped the evolution of its immune response [5]. It should be noted that, on the one hand, each species of parasite or pathogen possesses molecules known as virulence factors [5] that allow them to evade, reduce or eliminate the immune response facilitating adhesion, colonization, and the establishment of infection [1,2,5]. On the other hand, the host recognizes parasites and pathogens through diverse and specialized mechanisms that coordinate the immune response defense. This complex network comprises tissues, cells, and molecules that allow the host to reduce or eliminate the infection and repair its damaged tissues [2-6].

Recognition of pathogens by the invertebrate immune system

Pathogens are recognized by the pathogen-associated molecular patterns (PAMPs), recognition of damage-associated molecular patterns (DAMPs) released from damaged tissues, and with molecules such as opsonins [2,3,6]. The recognition of PAMPs is achieved after the enemies are recognized by pattern recognition receptors (PRRs) in the immune cells

[7,8]. In invertebrates, these receptors can be found in the hemolymph or anchored to the membrane of hemocytes [8]. Invertebrate PRRs have high specificity in their recognition and include Toll receptors, PGRP (peptidoglycan recognition proteins), Dscam's (down syndrome cell adhesion molecules), and FREP's (fibrinogen-related proteins) [8]. Upon PAMPs binding, PRRs activate different intracellular signaling pathways [9,10]. The Toll-MyD88-Akt pathway is triggered in response to Gram (+) and Gram (-) bacteria and fungi. Gram (-) bacteria mainly activate the Imd (immune deficient) pathway. Both pathways activate specific transcriptional factors, such as nuclear factor kappa-light chain enhancer of activated B cells (NF-kB), which are translocated into the nucleus of immunocompetent cells, where they regulate the expression of specific genes of the innate immune response [6.8]. In response to infection, immunocompetent cells produce AMPs (antimicrobial peptides), cytokines, reactive oxygen or nitrogen species, growth factors and melanization by the phenoloxidase cascade (PO), all of them are tightly regulated by the nuclear factor NF- kB [9]. All above affects the development, differentiation, and activation of cells that make up the immune system of invertebrates [4,11,12].

Hemocytes are the primary cells of innate immunity in invertebrates. Its principal function is to phagocytose pathogens and participate in tissue repair, metamorphosis, and extracellular matrix production by removing damaged and apoptotic cells and producing growth factors [13]. In addition, hemocytes are involved in the encapsulation and nodulation processes of parasites and foreign elements [11]. Recently, it has been described that hemocytes and epithelial cells respond to infection by activating their endocycle. This endoreplication process is a variant of the cell cycle in which the cell duplicates its genomic DNA without segregating it during mitosis [14-16]. The endocycle is characterized by the

absence of cytokinesis, nuclear envelope breakdown, chromosome condensation, and mitotic cell formation. The activation of immunocompetent cells, including hemocytes in invertebrates, has been associated with epigenetic modifications (EMs) [12,13]. EMs are molecular modifications of DNA and RNA (such as methylation) and histones (such as acetylation) signaling without changing their sequence [12, 16]. The most influential studies regarding the role of EMs in establishing and activating immune memory are those carried out with planaria, in particular with *Schmidtea mediterranea*, by priming them with *Staphylococcus aereus* and testing their activation [13].

The new paradigm in innate immunity is its ability to develop immune memory [15,16]. This phenomenon is termed innate immune memory (or immune priming) to distinguish it from the vertebrate's adaptive memory because, although both improve protection from reinfection in terms of the immune response, parasite elimination, and survival, the mechanisms of the innate immune memory are not understood [17,18]. Since its identification in invertebrates [19], some attributes of immune priming have been described: it is specific at the pathogen strain or species level [20,21], it protects the host in the long term, and the response is biphasic, that is, it increases after a first encounter, returns to basal levels, and is evoked with greater intensity in subsequent encounters [22,23]. At present, how immune priming is stored and maintained, how specific challenges are remembered, and the role of other mechanisms, such as EMs in reprogramming, is unknown [24-27]. In addition, it is essential to know its possible interaction with other physiological pathways that regulate metabolism and allow the adaptation of invertebrates.

A novel field that integrates various physiological responses of invertebrates with the immune response is the immunometabolism, which refers to the close interactions between the immune systems and the energy metabolism of immunocompetent cells [28] and their feedback during an infectious process [29,30]. Some aspects of immunometabolism are: 1) the use of energy by the immune response; 2) the available metabolic energy [28]; and 3) the distribution of metabolic energy among immunocompetent cells or tissues. This distribution occurs under different environmental conditions, such as temperature [30], since it defines life history features such as growth, reproduction, and maintenance of cellular functions [28,31,32], attributes of which interact with immune memory [17,31-33]. Here, the evidence of interaction between the immunometabolism and innate immune memory in invertebrates is analyzed. We will show the relationship between energetic costs, metabolism, endocycle activation and epigenetic modifications during the establishment and activation of the innate immune memory in invertebrates.

Immunometabolism

The metabolic system is critically involved in a range of physiological activities of organisms by generation and distributing energy, including the activation of an immune response. These two biological systems, usually independently studied, have recently been found to be tightly interrelated in animal physiology [32]. This Immune-metabolic interaction is an evolutionarily conserved phenomenon observed in all metazoans. A key molecule in the metabolic response is insulin, which regulates animal glucose homeostasis [34]. In invertebrates, insulin-like peptides (ILP) are released in the hemolymph after feeding. When they are recognized by their receptor (InR), a series of intracellular reactions are triggered

[35], which begin with the phosphorylation of Kinase Akt and subsequent inactivation of the transcription factor, such as forkhead box- containing protein subfamily (FOXO) [36]. The insulin/TOR pathway is a nutrient-sensitive signaling cascade of cellular metabolism [37,38]. The InR triggers an intracellular signaling pathway with metabolic and immune implications. InR activates glucose uptake, lipogenesis, and glycogen synthesis and affects the size and number of mitochondria, cell division, and differentiation [38-40]. In *Drosophila melanogaster*, a mutation of the chico gene (insulin receptor homolog), which regulates the signaling pathway dependent on the presence of ILP, increases the survival of flies infected with the Gram-negative *Entomobacter faecalis* and with the Gram-positive *Staphylococcus aureus*.

A relevant aspect in invertebrates is that their energy metabolism is affected during an infectious process in response to the type of pathogen and its virulence factors [41] as some metabolic signals are necessary for activating and regulating the innate immune response [28,42,43]. Such is the case of the insulin pathway, which can be activated upon infection [44,45], and the diverse metabolic phenotypes acquired by hemocytes during their differentiation [36,42]. *D. melanogaster* allows the evaluation of the impact of pathogens on the immune response and energy metabolism [27]. *Mycobacterium marinum* invades the hemocoel of *D. melanogaster*, reducing anabolism and decreasing triglycerides (TGA) and glycogen biosynthesis. This increases cellular catabolism, which is reflected in an increase in glycolysis and lipolysis [12,28,34]. The inactivation of the insulin signaling pathway is characterized by an increase in the synthesis of antimicrobial peptides (AMP´s) [3,9,39]. The activation of the Toll pathway via Akt in the fat body of *D. melanogaster*, reduces its growth and energy reserves [36]. Interestingly, a reduction in gene expression of the biosynthesis of

lipids, glucose, and vitamins has been identified during the activation of the insulin-like pathway [45]. These mechanisms are related to inhibiting the expression of hexamerin, a molecule of energy reserves in insects. On the other hand, in *T. molitor*, the immune response gene expression is favored by the metabolic response [44]. Therefore, gene expression patterns describe the process triggered by the presence of a pathogen, from the expression of receptors, activation of transcription factors, and the inducible immune response such as AMPs. Depending on the entomopathogen, the hemocytes activate specific signaling pathways that maintain a cross-talk with metabolism [33].

The constitutive activation of Toll in insects is sufficient to inactivate Akt and the loss of triacylglyceride. However, the molecular relationship between Toll signaling and ILP during the immune response is still unknown. Studies following a stimulus from a PAMP or ILP to sites in the DNA involved in synthesizing metabolic and immune response genes have highlighted the role of transcription factors such as FOXO, NF k-B, and Hypoxia-inducible transcription factor (HIF) in both responses (Figure 1). Thus, for example, the FOXO transcription factor regulates the number of cells and tissue growth in insects by promoting anabolic metabolism [46]. In contrast, in invertebrates with mutated insulin pathways, increased AMP expression has been shown due to the constitutive activation of this transcription factor [47]. In contrast, as mentioned before, a molecule involved in immunometabolic regulation is Akt, a negative regulator of FOXO activity [35,40]. These events depend on the availability of energy necessary for coordinating the immune response, restoring homeostasis, and driving the expression of other metabolic response genes HIF are involved in the activity of metabolic and immune response genes [48]. The activation of the insulin-PI3K/TOR pathway, in addition, to inducing the transduction activity of target genes

related to a metabolic response [48], also induces the HIF-1 transcriptional response, promoting its nuclear localization [49]. Regarding NF- kB, the infection with *E. coli* of a *D. melanogaster* lacking the inhibitor of the homologous gene of IKK (called ird5), which is involved in the inhibition of the activity of the NF- kB, and showed a decrease in the synthesis of the antimicrobial peptide like drosomycin gene and also in the expression of HIF-1a and HIF-1b homologues genes, called sima and tango respectively in *D. melanogaster*, demonstrating the importance of the crosstalk between the metabolic in the repression of the immune response by controlling the expression of NF- kB (dorsal and dif in Drosophila) [49,50]. These changes also occur in the mutant flies of the sima gene leading to a decreased functioning of NF- kB [48,50].

Therefore, the implications of O_2 concentrations, the availability of metabolic energy, ILPs, and infection in regulating the expression of target genes involved in the metabolic response, the immune response, and the regulation established between them are evident [50].

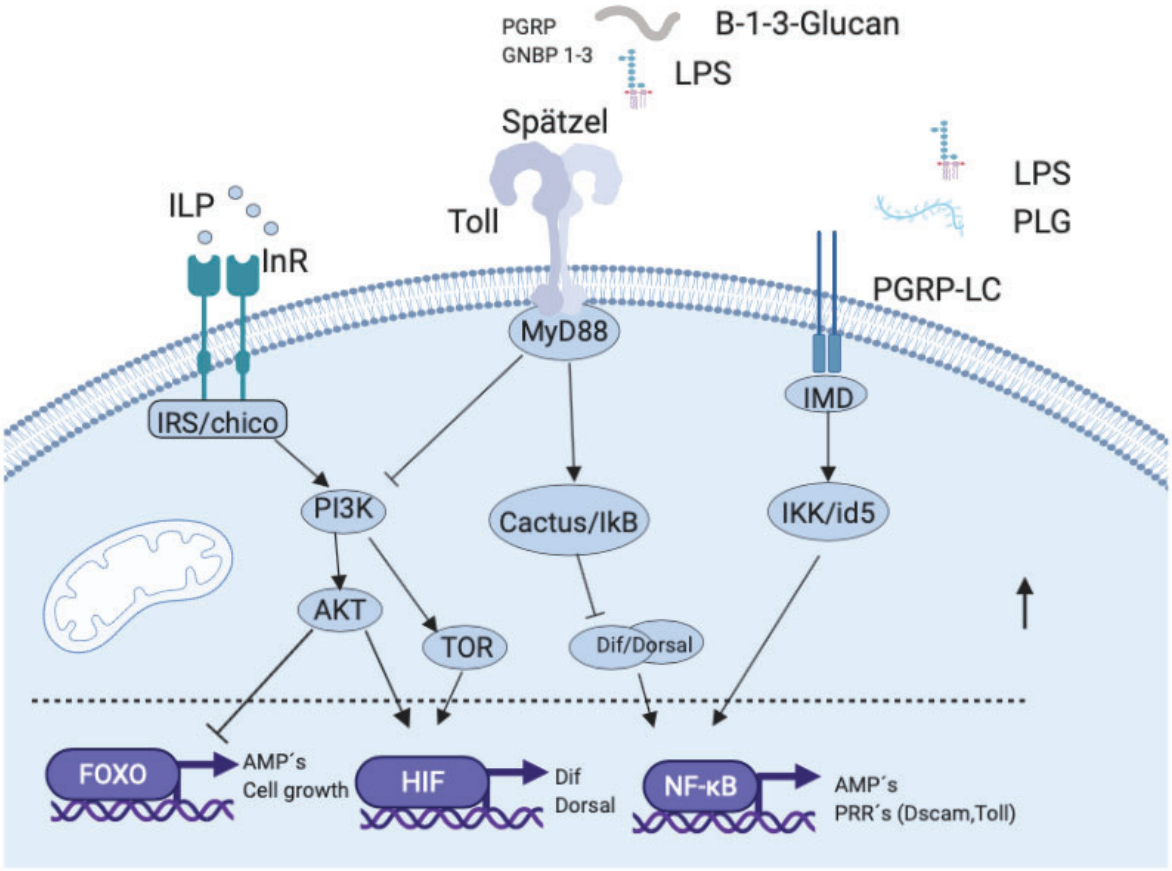


Figure 1. Immunometabolic relationship in an immunocompetent invertebrate cell, between the activation of insulin-like pathways (IIS), by the ligand of ILP; insulin-like peptide; InR: to the insulin receptor; IRS/chico, and the signaling pathway via TOR to the synthesis of dimer to form NF-kB and via AKT the inhibition of cell growth and the synthesis of AMP´s by the transcriptor factor FOXO and HIF 1 α/β. Following the activation of the receptor Toll, by the bind of Spätzel of the PAMP´s (Gram (-) and Gram (+) LPS and B1-3 glucan), activated the signaling pathway MyD 88 (myeloid differentiation factor 88), Cactus/ IkB (Inhibitor of kB in *Drosophila*), Dif (Dorsal related immunity factor), Dorsal (transcription factor) to the activation of innate immune response and Imd (immune deficiency pathway), IKK (IkB kinase) ligand to the PAMP´s LPS and Peptidoglycan via PGRP-LC receptor.

Innate immune memory

Since innate immune memory was identified in invertebrates [17], its efficiency has been tested in different host species against parasites and pathogens [51-53]. Some effector mechanisms related to innate memory activation in invertebrates have been identified (Figure 2). Factors affecting memory establishment include the microenvironment of tissues and organs, host health, sex, age, and metabolic condition [13,54,55]. An important aspect of innate memory is how it modulates the pathogens' virulence [19,56,57]. Studies on *T. molitor* larvae challenged with two strains of *M. anisopliae*, which varied in virulence, showed induction of innate memory against the less virulent strain but not the virulent strain [56]. In the same species, memory was found against the less virulent species but not against the more virulent species [19]. Finally, in *T. molitor* larvae challenged with two strains of the entomopathogenic nematode *Rhabditis regina* did not show any evidence of innate and also, no differences were found in the strain's virulence [58,59].

An essential feature of innate memory is the ability to be inherited by subsequent generations, a process called transgenerational innate immune memory (TGIM) [60,61]. The TGIM is affected by pathogens and the parental sex but is also proposed to play a key role in epigenetic mechanisms [62-67].

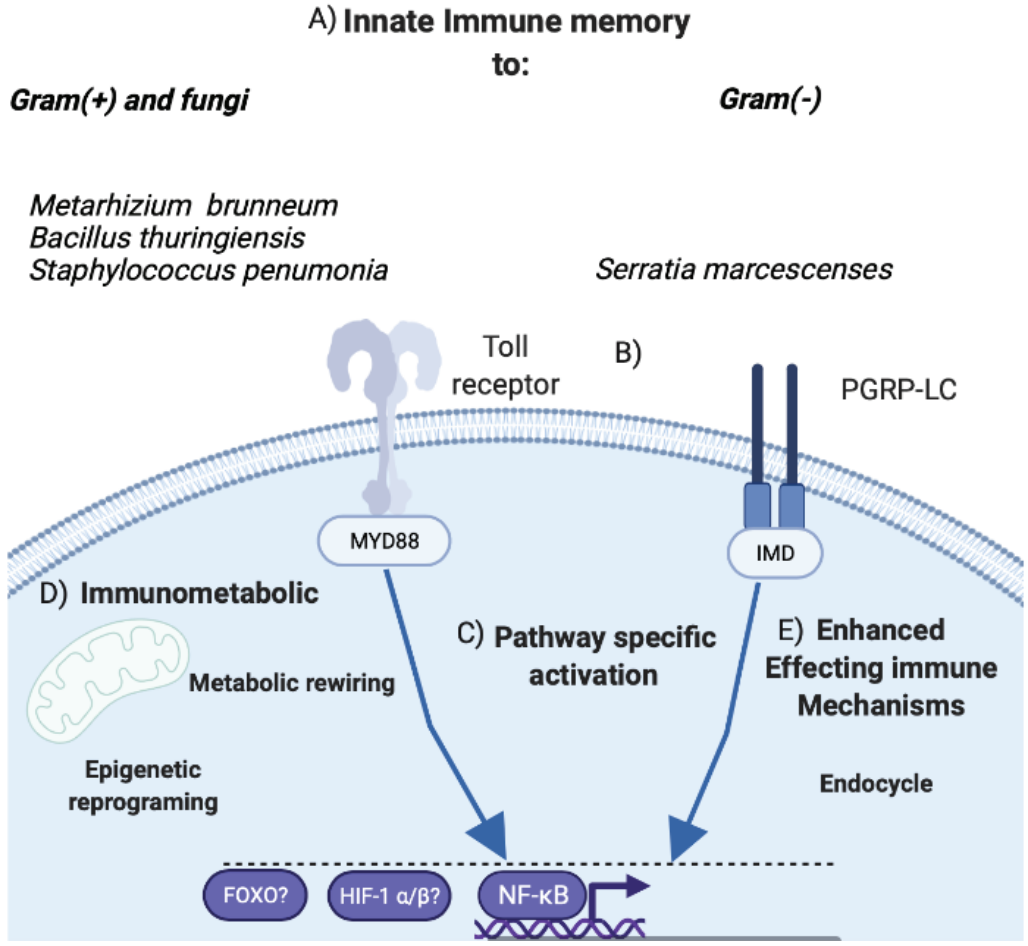
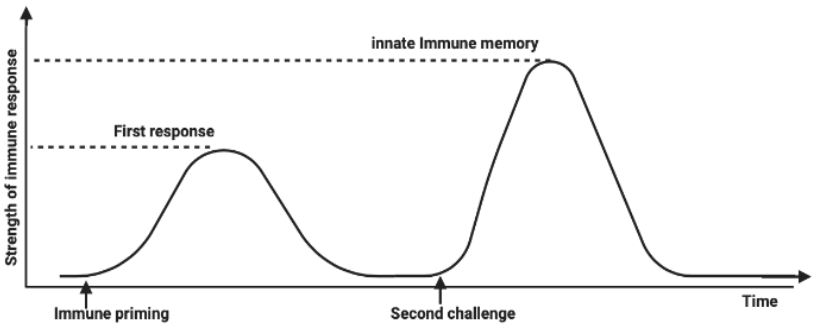


FIGURE 2. General scheme of innate immune memory activation, starting with the establishment of innate immune memory by the effect of different types of pathogens (A), activating different PRRs like Toll and PGRP-LC receptors (B), whose stimulation is following by the activation of signaling pathways (C) related to a high degree of specificity (MyD88 and IMD) that result in the translocation to the nucleus of expression-controlling transcriptional factors. One of the identified responses is the metabolic reconfigurations by way of the metabolic rewiring (D) and the epigenetic reprogramming which result in the activation of innate memory and enhanced effecting mechanisms, process to which the activation of the endocycle has been related.

It is crucial to know that some immune and metabolic mechanisms related to the development and activation of an innate immune memory are shown in Figure 3. In addition,

hemocytes are essential in establishing the innate memory in invertebrates by phagocytosis; the increasing number of circulating hemocytes, the differentiation of prohemocytes into hemocytes [13, 68,69,71-76]. A study in *D. melanogaster* showed an increase in Toll and PGRP-LB PRRs during innate memory activation [68]. Regarding the humoral response, AMPs are induced with biphasic kinetics in *Anopheles albimanus*, similar to the known kinetic for antibodies in mammals [22]. Furthermore, a complex transcriptional reprogramming of histones [32] and a relationship between immune and metabolic systems interaction during innate immune memory has been reported [77,78]. In the snail *Biomphalaria glabrata*, a change in the effector immune response from cellular to humoral mechanisms were observed due to the activation of innate immune memory and the differential expression of several families of genes [79]. Recently, different components of immunometabolism have been described in invertebrates after the first and second immune challenges (Figure 3). However, how they interact during immune memory (recognition, storage, and memory recall) remains to be tested.



Metabolic activation	**Metabolic innate immune memory activated**	**References**
FOXO and HIF-1α trascription factors	Metabolic high energetic demand,	
ILP	anabolic activation and insulin like activation	(77,82,83)
Metabolic rewiring	CO2 increse metabolic costs	(30,59)
Epigenetic	**Epigenetic reprograming**	
Metilasestranferasa and	Metabolic actived histone and H3 metilation	(84-86)
acetilatetranferasa of histones	RNA metilation	(24)
Chromatin remodeling proteins	**Endocycle**	
	hnt synthesis and genome dupliction	(22,87,88)
Immune effectors:	**Enhanced immune effectors**	
AMP´s synthesis	AMP´s biphasic activation	(22,75,85,89-100)
Hemocites activation and diferentiation	Hemocites incresse and differentiation,	
Phagocytic activation	specific phagocytocis activation	(68,101-106)
NF-kB	Melanization incresse and PO activitie	(102,105,106)
Toll and IMD activation pathway	Toll and IMD expression and especific pathways	(85,107-110)
	NF-kB specific regulaion of transpcription	(111)
	Dscam receptor expression	(97,112,113)
	FREP receptor expression	(114,115)
	Antiviral response	(85,97,116)

FIGURE 3. Comparison in the expression of immune and metabolic parameters that characterize the immune response from invertebrates, first a) in an initial challenge (priming) with metabolic activation, epigenetic and the expression of immune effectors mechanisms, and followed by b) activation of innate immune memory after a second challenge, during which metabolic response, activation of innate immune memory, epigenetic reprogramming, activation of the endocycle, and finally expression of immune effectors, are all enhanced .

Hemocytes are important in establishing innate memory and are related to the endocycle [14]. The endocycle is a response strategy to high metabolic demands in which the cell synthesizes a large amount of DNA without entering mitosis (Figure 4) [14,117-119]. Endocycle activation occurs in arthropods including crustaceans and insects [14,22,72,122], but also in vertebrates [121]. It has been involved in cellular functions such as cell growth, embryonic development, and innate memory activation [72,120-121]. Therefore, the endocycle is activated in hemocytes, in response to energetic demands [31,123,124], such as

a viral infection [124,125]. Still, it has also been studied in different tissues such as salivary glands, fat body, intestine, trachea, and renal tubules in larvae, neurons, glia, intestine, ovaries, and follicles in adults [121]. The activation of the endocycle is regulated by the zinc finger protein Hindsight or *hnt*, whose function is the negative regulation of cyclins responsible for the activation of the cell cycle [125,126].

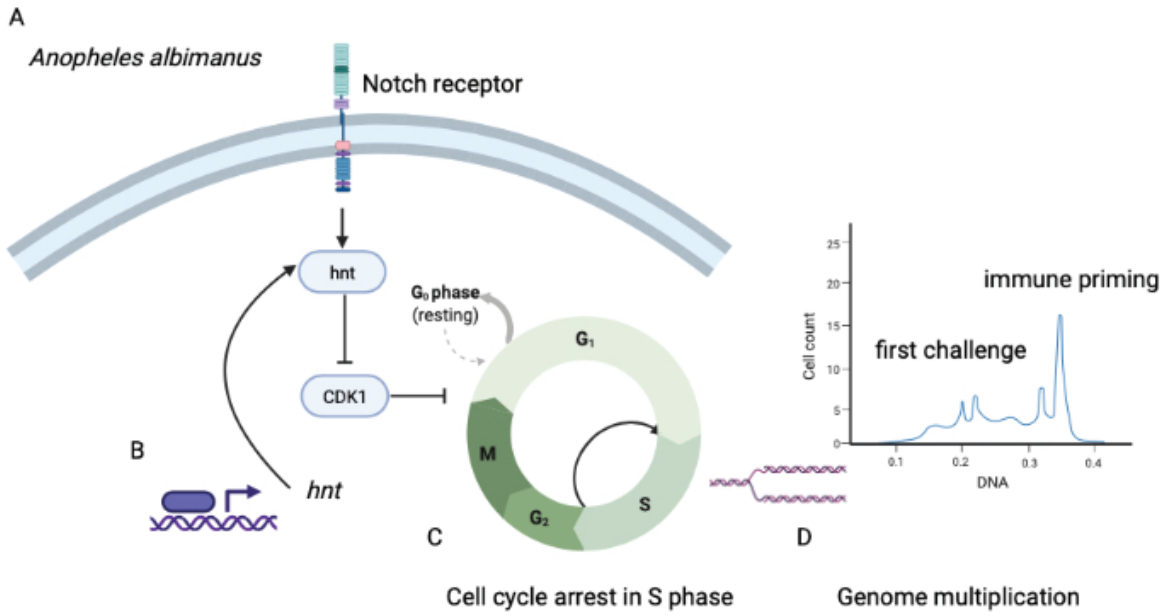


FIGURE 4. The cell endocycle is an effector mechanism related to the activation of innate immune memory. An example of innate immune memory activation by Anopheles albimanus is shown. A) activation of innate immune memory by Notch receptor recognition B) increase in the number of *hnt* gene copies and inhibition of the cell cycle regulator CDK1, C) activation and arrest of the cell cycle in the S stage and D) increase in the amount of DNA due to the duplication of the genome.

The metabolic reconfiguration might favor the invertebrate host to get the energy to combat recurrent infections, including the energy consumption related to the activation of

the endocycle (22,87,88) and the posttranscriptional modifications in specific regions at the epigenetic regulation of chromatin or specific regions of histones (84-86). The communication between epigenetics and energy consumption involves the activation and maintenance of inducible immune responses. This waste of energy can be measured by the changes in the basal metabolic rate (BMR), which is defined as the individual's energy expenditure at rest, represented by the amount of CO_2 expired in the activation of innate immune memory [127,128]. These parameters have been used to know how expensive innate immune memory is.

Costs of innate memory in terms of metabolic rate

The basal metabolic rate (BMR) is characterized by variation between invertebrates and vertebrates [129] and is affected by body mass, temperature, and stress [130,131]. Interestingly, there is variation in BMR during an immune challenge or tissue damage [132,133]. Therefore, the BMR reveals energy depletion and distribution among different functions, such as the trade-off between immune response and reproduction [133,134]. Since parasitism induces a high energy demand for the host, it increases BMR [134]. In invertebrates and vertebrates, immune system activation increases BMR [132]. So that energy reserves are used to mount an immune response [134-136] and, thus, the energy requirements in homeostatic recovery [55,137]. Therefore, the relevance of approaching the study of innate immune memory by integrating immune and metabolic responses in invertebrates is evident [138]. Consequently, the study of the evolutionary costs of the innate memory in invertebrates and vertebrates [31,59,77,139] has shown that the activation of

immune defenses [137-139], such as AMP, PO, and hemocytes proliferation, induce CO_2, which is released as a by-product of the energetic demand by the immunocompetent cells [131,137,139]. Therefore, the measurement of CO_2, in ppm, can be used as an indicator of immune activation [137,140]. Hence in the host, the activation of innate memory demands energy in terms of waste of glycogen, lipids, and proteins [129]. The energy demands can determine the costs of the activation of innate memory, the evolutionary costs, and their relationship with the biphasic and sustained response [17,31,59,141,142]: the higher the energetic demand, the higher the cost. Finally, another important aspect to consider is diet since dietary restriction affects memory [24,133,137,138]. It is relevant to indicate that, in *T. molitor*, inserting a nylon monofilament it results in decreased survival and increased CO_2 production [133]. In addition, the metabolic costs of innate memory activation, changes in basal metabolic rate, and expired CO_2 production by *T. molitor* larvae against two strains of the fungus *M. brunneum* (Ma10 and CAT) have been evaluated. The first dose was sublethal (5 conidia killed 20% of the insects), and the second was lethal (100 conidia killed 50% of the insects). The results showed an increase in CO_2 expiration depending on the fungus strain used to establish innate immune memory (Ma10) and on both priming and lethal doses, which implies an adaptive cost that affects larvae in its development [31,56]. Therefore, in the cost of activating the metabolism due to immune memory is higher than activating a general response, and more CO_2 is produced in the former than in the latter [22,31,59]. The cost might be due to synthesizing specific immune effector mechanisms [77,112,115,143,144]. It is important to consider that these scenarios where BMR is increased or maintained are ideal for describing how metabolism is modulated according to innate immune memory in the future.

Immunometabolism in innate memory

The forefront in the study of the relationship between innate immune memory and the metabolism of the cellular response, comes from the study of immune training, which has been the way to name the phenomenon of immune priming in the vertebrate's model. Immune training is better protection after an unspecific immune challenge [145]. Related research has been done with mouse models and some cell lines, which have contributed to significant advances in the immunometabolism of the activation of immune training [20,145-148]. Such is the case of the innate immune response of mice that received a stimulus with β-glucans derived from *Candida albicans*, in which protection derived from immune training was observed [87]. In invertebrates, some molecules have been identified, and are the mTOR signaling pathway, who is responsible for modulating the activation of HIF-1α/β, an evolutionarily conserved mechanism responsible for regulating glucose metabolism in vertebrates [143,146-149]. Likewise, the vertebrate models used for testing immune memory have shown changes in glucose metabolism, from oxidative phosphorylation to aerobic glycolysis, as well as an increase in glutamine metabolism, cholesterol synthesis, and in non-steroidal RNA long coding (IncRNAs) [31,143] (Figure 5). In vertebrates, epigenetic modifications have been identified in metabolic activation via enzyme post-transductional regulation [145-147]. Invertebrates have an information gap to explain the connection between the metabolic response and the post-transductional regulation; some of the information we have is limited. Briefly, it describes part of the known finds. In the T. molitor larvae and adults, RNA methylation is implicated, and epigenetic modifications have been characterized [13,17,143,144]. As well histone epigenetic modifications are also involved in

TGIP [24,84,86,113]. Eventually, the innate memory might reconfigure the immunometabolic network through the differential expression of molecules in the hemocytes from insects [77,82,83,143,144,148,149]. This reprogramming in cellular metabolism is crucial since it determines the regulation of certain epigenetic changes in the trained memory mechanisms [32,143]. However, the relationship between epigenetic mechanisms with the innate immune response and metabolism remains unanswered in invertebrates.

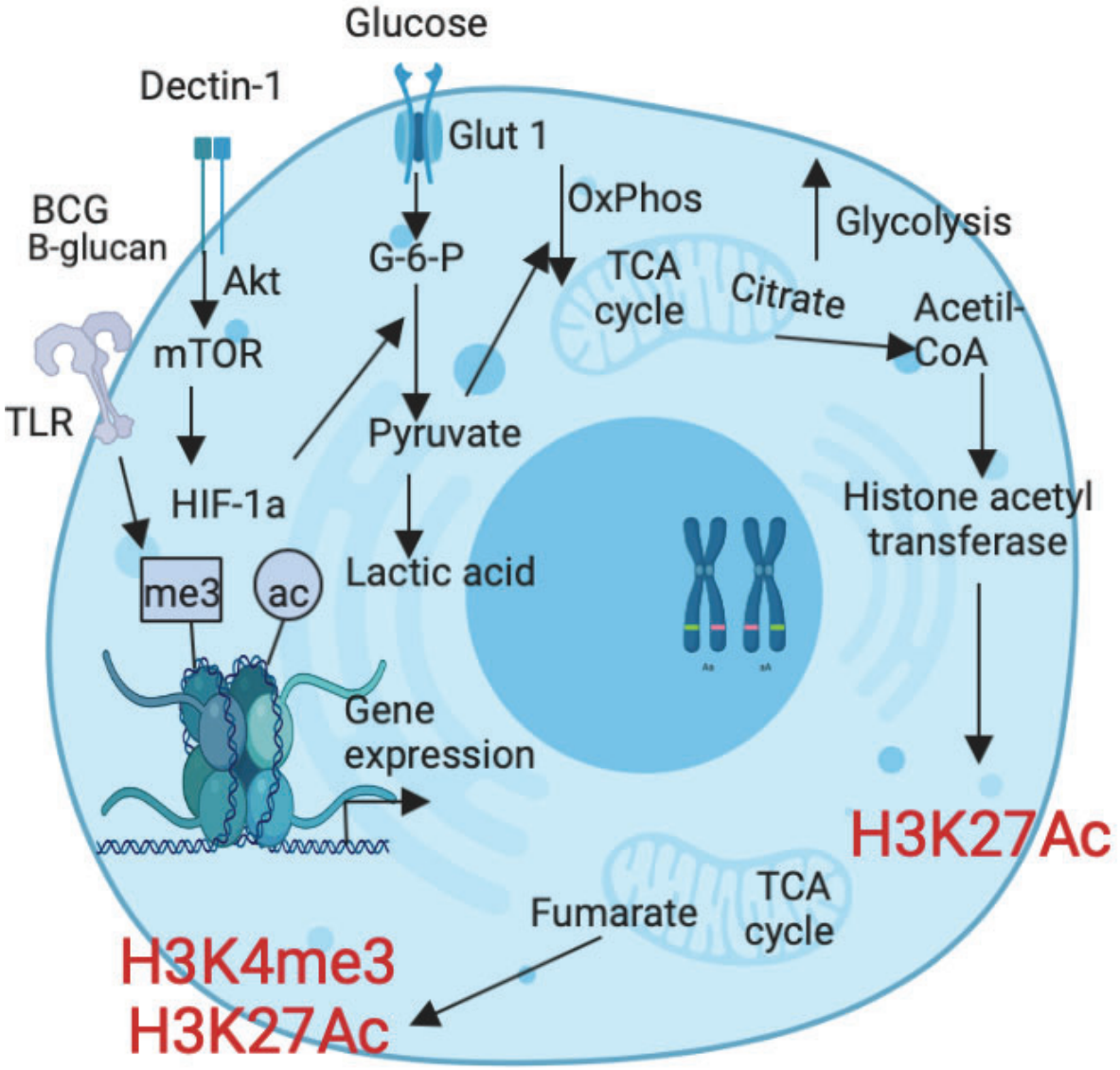


Figure 5. Metabolic pathways involved in epigenetic reconfiguration of macrophages stimulated with β-glucan or BCG vaccine to generate trained memory. First the posttranslational epigenetic modification of histones in a triple methylation and acetylation; The Dectin 1 receptor activates Akt-mTOR-HIF-1α pathway, which….??? Glucose transporter 1 Glut 1), C-type NK cell lectin receptor this affect the change from G-6-P to pyruvate affecting the oxidative phosphorylation (OxPhos) this change affect the tricarboxylic acid cycle (TCA) and at the same time increase of glycolysis and decrease of the change of citrate to Acetil-CoA; it affects the enzyme Histone acetyl transferase with influence in the epigenetic modification in Histone 33 in particular at lysine 27 with an acetylation; and in other way affect the fumarate that have effects in Histone 3 at lysin 4 or 27 with a triple methylation and acetylation.

Future directions to study immunometabolism in immune priming

It is important to highlight one of the most relevant aspects in invertebrates that has not yet been fully resolved: the role of histones, particularly in histone 3, to understand the immunometabolism of hemocytes, as well as the epigenetic regulation of metabolic pathways such as the transcription factor HIF-1, FOXO, and its relevance in the immunometabolic connection of innate memory. The study of the endocycle continues to be an essential topic in the study of innate memory, so it is important to know its role in the post-transcriptional regulation of histones as well as its role in the plasticity of the innate immune response during the activation of innate memory. It is important to highlight that the intimate interaction that metabolic mechanisms establish with the differential expression of effector immune mechanisms during the activation of innate immune memory in invertebrates is a field of research that can help understand innate immune memory.

The implications of understanding the evolutionary ecology of animals and their applications in fields such as pest control, aquaculture, and biomedicine are some of the impacts of the study of short-term innate immune memory. It would be interesting to study immunometabolism during the TGIM, to know the effect on inheritance.

In vertebrates, there is little evidence of the relationship between immunometabolic and epigenetic pathways with trained immunity (unspecific response after secondary infections). Still, these relationships remain to be tested in innate immune memory (in both vertebrates and invertebrates). In invertebrates, post-translational epigenetic modifications might reconfigure immune signaling pathways, and the derived epigenetic landscapes may favor specific responses during immune memory. This topic will help to understand better the host-parasite relationship, its implications in evolutionary processes, and interconnections between physiological feedback mechanisms, in which the redistribution of metabolic energy and its costs have an important role from a study approach in ecoimmunology. Using parasites with different virulence in different host species, PAMPs and DAMPs will help identify the defense mechanisms against recurrent infections and might provide insights into the evolution of immune memory.

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Disclosures

The authors declare that we have no conflict of interest

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