UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO
DOCTORADO EN CIENCIAS BIOMÉDICAS
INSTITUTO DE ECOLOGÍA

PAPEL DE LA VARIACIÓN EN LA CONDICIÓN DE MACHOS Y HEMBRAS EN LA ELECCIÓN FEMENINA EN EL ESCARABAJO *TENEBRIO MOLITOR*

TESIS
QUE PARA OPTAR POR EL GRADO DE:
DOCTOR EN CIENCIAS

PRESENTA:
ALICIA REYES RAMÍREZ

DIRECTOR DE TESIS
DR. ALEJANDRO CÓRDOBA AGUILAR
INSTITUTO DE ECOLOGÍA
COMITÉ TUTOR
DR. CARLOS RAFAEL CORDERO MACEDO
INSTITUTO DE ECOLOGÍA
DR. HUMBERTO LANZ MENDOZA
INSTITUTO NACIONAL DE SALUD PÚBLICA, CUERNAVACA

CDMX, OCTUBRE DE 2020

Agradecimientos institucionales

Al Posgrado en Ciencias Biomédicas de la UNAM, así como al CONACYT por la beca otorgada No CVU/BECARIO: 778814/612503. Ambos me brindaron el apoyo académico y económico para llevar a cabo este trabajo de investigación.

Al Instituto de Ecología de la UNAM, por ser mi segunda casa durante estos cuatro años. El lugar donde establecí relaciones enriquecedoras de trabajo y personales.

A PAPIIT por el apoyo financiero otorgado al proyecto: IN 206618.

Al jurado de grado: Dr. Constantino de Jesús Macías García
Dr. Carlos Rafael Cordero Macedo
Dr. Alejandro Emmanuel González Voyer
Dr. Raúl Cueva del Castillo Mendoza
Dra. Wendy Portillo Martínez

Por último, pero no menos importante para la UNAM. La aventura inició hace ya catorce años cuando en Iniciación Universitaria ingresé a la "Máxima Casa de Estudios". Durante todo este tiempo el amor y orgullo por ella no ha hecho más que crecer. Por ser el espacio donde tantos sueños se construyen y se logran.

¡Cachorro, puma feroz!

Agradecimientos a título personal

A mi familia, porque sus nombres no van a estar escritos en ningún artículo, a pesar de todo el apoyo, amor y cuidado que me han brindado. Por estar en los mejores y peores momentos del doctorado. Por aguantar y comprender mi malhumor cuando se acumulaba el estrés. A mi papá, que siempre se las ingenia para crear herramientas, instrumentos y objetos que me faciliten el trabajo. A mi mamá, por pasar horas ayudándome a cuidar a mis bichos, por darme su opinión sobre mis ideas y escritos. A mi hermano, por resistir las manías de la bióloga de la familia y constantemente hacerme sonreír. A mis "JJJ" por esperar mi regreso a casa después de un día cansado en el laboratorio, por madrugar y desvelarse conmigo en muchas ocasiones.

A mis queridos Angee y Ricardo, que saben que esta historia del posgrado a veces parecía estar escrita por Stephen King. Por ser no sólo los mejores amigos, si no además los mejores ayudantes de laboratorio, cuya paga consistió únicamente en risas, charlas y salpicaduras de reactivos. El camino hubiera sido muy difícil sin ustedes.

A Ale, por escuchar muchos términos y chistes de biólogos. Porque a la distancia me sigues animando y motivando.

Un especial agradecimiento a Alex, mi director de tesis, por sus consejos y su preocupación por mi crecimiento profesional y personal. Por creer en mi desde la primera vez que llegué al laboratorio a platicar con él.

A los miembros de mi comité tutor, los doctores Carlos y Humberto, que semestre tras semestre se involucraron en este proyecto.

A mis sinodales, los doctores Constantino, Carlos, Alejandro, Raúl y Wendy, por el tiempo que dedicaron en la revisión de este trabajo. Porque sus consejos y correcciones permitieron una mejor versión de esta tesis

A mis compañeros de laboratorio: Angelita, Schneider D., Aldo, Cata, Edday, Monse, Ulises, Xavi, Memo, Tania, David y Jaime, por todas las anécdotas que hemos compartido. A los bebés del laboratorio Anita e Iván, por siempre estar dispuestos a aprender. A Maya por su constante ayuda con los análisis estadísticos, R hubiera sido una pesadilla sin ti. A Raúl que desde el primer día en el laboratorio me apoyó. Porque sin él encargándose de la logística, muchos experimentos no se realizarían.

A la Licenciada Erika Rodríguez, porque su trabajo es una pieza fundamental para todos los estudiantes del Instituto. Por guiarnos a través de los constantes trámites del posgrado y sobretodo estar al pendiente de nuestro bienestar.

A la Bióloga Irma Acosta, por su paciencia y buena disposición para enseñarme. No sabes lo mucho que me facilitaste el desarrollo de innumerables experimentos.

A mis amigos Fabi, Rosa, Jesús, Mayari, Samuel, Luis, Angélica, Madisson y Bryan, porque a pesar de sus propios proyectos se toman el tiempo de preguntarme cómo estoy y de escuchar los avances de los experimentos.

El camino del doctorado es largo, por lo que es muy probable que me esté olvidando de mencionar a alguien, pero agradezco profundamente a todas las personas que me han acompañado en esta aventura. A todos ustedes muchas gracias.

Dedico esta tesis a

Mis padres, Alicia y José Luis,
y a mi hermano Omar

Alicia estaba ya tan acostumbrada a que todo cuanto le sucediera fuera algo extraordinario, que le pareció de lo más soso y estúpido que la vida siguiera por el camino normal.

Lewis Carroll

La enseñanza es un regalo en sí misma, es el mejor regalo que cualquiera puede dar o recibir.

Stephen King

ÍNDICE

Resumen

Conocemos relativamente poco sobre cómo la variación en la condición femenina impacta sobre sus decisiones. La variación femenina puede cambiar la magnitud y dirección de la selección sexual. Se espera que hembras en buena condición sean más selectivas al elegir pareja, que aquellas en mala condición. Para entender la variación femenina y sus consecuencias, en esta tesis utilicé como modelo de estudio al escarabajo de la harina *Tenebrio molitor*, especie en donde las hembras discriminan entre sus posibles parejas a través de feromonas. Evalué: 1) el efecto de la dieta y un reto inmune con la infección por un hongo, sobre la condición de machos y hembras, a nivel de su ingesta de alimento y la concentración corporal de macronutrientes; 2) los cambios en la supervivencia y las preferencias de las hembras cuando la condición de ambos sexos varía; y 3) los beneficios directos que las hembras reciben del espermatóforo durante la cópula con machos en buena y mala condición. Tanto la dieta como el estado de salud afectaron la cantidad de macronutrientes que los individuos consumen, siendo los machos infectados con el hongo *Metarhizium robertsii* los de menor ingesta. Estas fuentes de variación también afectaron las concentraciones corporales de proteínas, lípidos y carbohidratos totales. Tanto machos como hembras vivieron más en dietas ricas en carbohidratos. Por otro lado, la dieta no afectó las preferencias femeninas, aunque el estado de salud de los machos si tuvo un efecto, sorprendentemente siendo preferidos los infectados con el hongo. Por último, los machos infectados con hongos produjeron los espermatóforos con mayor abundancia de proteínas, lípidos y carbohidratos, lo cual es llamativo dado que estos individuos son los que consumieron menos alimento. Posiblemente los machos realicen una inversión terminal, asignando sus recursos hacia la reproducción mejorando por un lado tanto su cantidad de

feromonas sexuales, como sus espermatóforos.

INTRODUCCIÓN GENERAL

Introducción general

Desde que Darwin propuso el concepto de selección sexual como la fuerza responsable de la diferencia en el éxito reproductivo entre individuos del mismo sexo y especie (Darwin, 1859, 1871), uno de sus mecanismos, la elección de pareja ha sido ampliamente estudiada (Andersson & Simmons, 2006; Bateson, 1983; Kokko et al., 2003; Rosenthal, 2017). El sexo que invierte menos en la reproducción suele ser el que compite más fuertemente por el acceso a las parejas (Bateman, 1948). Dado que casi siempre son los machos los que invierten menos en cada descendencia, es típico que ellos compitan por el acceso a las hembras y presenten rasgos más llamativos (Andersson, 1994). Los ornamentos y despliegues sexuales que los machos exhiben, evolucionan al ser más atractivos para las hembras (Darwin, 1871). Las hembras pueden obtener beneficios al elegir tales rasgos en los machos. Estos beneficios de acuerdo con su efecto sobre la adecuación femenina se dividen en directos e indirectos.

Los beneficios indirectos o genéticos están relacionados con la calidad de la descendencia (Fedorka & Mousseau, 2002; García-González & Simmons, 2007), que se puede reflejar en la resistencia a ciertos patógenos o la producción de hijos más atractivos (Fisher, 1930; Hamilton & Zuk, 1982; Heywood, 1989; Snow et al., 2019). Mientras que los beneficios directos o materiales incluyen entre otros regalos nupciales, el cuidado parental, aumento en la fecundidad y esperanza de vida y la ausencia de enfermedades de transmisión directa (Andersson, 1994; Brooks & Griffith, 2010; Møller & Jennions, 2001). Los beneficios directos son de suma importancia debido a que sus efectos son inmediatos (Møller & Jennions, 2001). Además resulta más sencillo comprender el mantenimiento de su variación en comparación con el mantenimiento de una variación detectable en la calidad genética para los beneficios indirectos (Møller & Jennions, 2001). De tal manera que la variación ambiental por sí sola, puede generar fácilmente diferencias en los beneficios directos que ofrecen los machos. Por ejemplo, si la disponibilidad de alimentos no es uniforme, puede provocar que algunos machos estén en mejor condición que otros para ayudar en la crianza de la progenie (Kokko et al., 2003).

Diversas hipótesis se han propuesto para explicar la evolución y el mantenimiento de las preferencias y los ornamentos sexuales. Entre ellas resaltan la selección desbocada (Fisher, 1930), el sesgo sensorial (West-Eberhard, 1984) y el hándicap (Zahavi, 1975, 1977). La selección desbocada fue propuesta por Fisher como un mecanismo para explicar la correlación genética entre el rasgo sexual secundario expresado en un sexo y la preferencia del rasgo en el otro sexo (Fisher, 1930). Este acoplamiento genético conduciría a bucles de coevolución autorreforzantes que provocan una evolución acelerada de ornamentos exagerados y de preferencias. Fisher planteó que en un inicio el rasgo masculino podría estar confiriendo una ventaja seleccionada de forma natural (Fisher, 1930). Sin embargo, al establecerse el acoplamiento genético, la retroalimentación positiva será tan intensa que desembocará en un rasgo tan exagerado que puede incluso ser perjudicial para la supervivencia. Por lo que la ventaja en la adecuación termina debiéndose únicamente al éxito de apareamiento (Fisher, 1930). El desbocamiento puede evolucionar fuera de la línea de equilibrio (Lande, 1981) o hacia un equilibrio límite donde el rasgo masculino se fija (Kirkpatrick, 1982).

El modelo del sesgo sensorial y la hipótesis de la explotación sensorial de la selección sexual, plantean que las preferencias femeninas en la elección de pareja son subproductos de la fisiología subyacente de sus sistemas sensoriales, que han sido moldeados por la selección natural, y que a su vez, los machos desarrollan rasgos que coinciden con estas características del sistema sensorial (Basolo, 1995; Dawkins & Guilford, 1996; Ryan, 1998; West-Eberhard, 1984). De acuerdo con el sesgo sensorial, los machos poseen rasgos que actúan como señales. Estas señales deben viajar a través del entorno y ser detectadas por los sistemas sensoriales y nerviosos de las hembras, para finalmente determinar las preferencias femeninas (Fuller et al., 2005).

Por otro lado, el biólogo evolutivo Amotz Zahavi propuso en la hipótesis de hándicap, que los ornamentos sexuales deberían estar funcionando como señales honestas de la condición de los machos (Grafen, 1990; Iwasa & Pomiankowski, 1994; Pomiankowski, 1987; Zahavi, 1977). Los machos en buena condición exhibirán su calidad mediante rasgos sexuales más elaborados y/o despliegues más vigorosos a posibles parejas y competidores (Cotton et al., 2004; Gilbert & Uetz, 2016; Zahavi, 1977). Por su parte, los machos en mala condición no serán capaces de asumir los costos de viabilidad ligados a tal extravagancia (Cotton et al., 2004, 2006; Zahavi, 1975). La dependencia de la condición puede evolucionar para señalar la calidad masculina genética, ambiental o ambas (Cotton et al., 2004). Evidencia empírica respalda la correlación entre la calidad de los ornamentos y despliegues sexuales con diversas medidas de condición, así como su efecto sobre la adecuación de las hembras y su progenie.

Por ejemplo, las hembras de la rana cohete dorada *Anomaloglossus beebei*, encuentran más atractivos a machos cuyas llamadas son más largas (Pettitt et al., 2020). A su vez, estos machos atractivos brindan un mayor cuidado parental, asistiendo a los huevos y defendiendo el territorio (Pettitt et al., 2020). Este cuidado parental impacta de manera positiva en la supervivencia de la progenie (Pettitt et al., 2020). Por lo que, a través de la llamada, las hembras reciben información sobre la condición de los machos y los posibles beneficios directos que pueden obtener. Por otro lado, en la araña *Pisaura mirabilis* se encontró que machos con un acceso *ad libitum* de alimento dedicaban más tiempo a la construcción de regalos nupciales y usaban más seda, en comparación con los machos hambrientos (Albo et al., 2011). El regalo se trata de una presa envuelta en seda, que funciona como un indicador honesto de la condición de los machos y por lo tanto un rasgo que las hembras evalúan para hacer su elección de pareja (Albo et al., 2011).

A diferencia de la evidencia teórica y empírica que se tiene para la variación en los ornamentos masculinos, la variación en las preferencias femeninas ha recibido considerablemente menor atención (Jennions & Petrie, 1997; Kelly, 2018; Millan et al., 2020). Conocer y cuantificar el efecto de la variación femenina en sus preferencias es clave, ya que puede aumentar o disminuir la magnitud y dirección de la selección sexual (Poulin & Vickery, 1996; Tomlinson & O'Donald, 1996; Vickery & Poulin, 1998). Por ejemplo, las hembras del grillo de campo negro, *Teleogryllus commodus*, en buena condición (alimentadas en una dieta rica), prefirieron a machos con una alta tasa de canto en comparación con las hembras en condiciones de nutrición pobre (Hunt et al., 2005). La disminución en la capacidad de respuesta y preferencia de las hembras en mala condición se debe a que la elección se vuelve costosa para ellas al no contar con suficientes reservas energéticas para evaluar adecuadamente a su potencial pareja (Bonduriansky, 2001; Hunt et al., 2005).

Los modelos de optimización de la elección de pareja predicen que una disminución en el esfuerzo de muestreo o una selectividad menor por parte de las hembras ocurrirá si (i) no pueden físicamente pagar los costos que implica la búsqueda prolongada de pareja, (ii) tienen menor éxito al competir con su mismo sexo, (iii) tienen menor éxito en atraer al sexo opuesto y/o (iv) tienen más probabilidades de ser abandonadas por su pareja (Fawcett & Johnstone, 2003; Härdling & Kokko, 2005; Johnstone, 1997; McNamara et al., 1999). Se espera que las hembras modulen sus preferencias conforme a la magnitud de los costos y beneficios que reciban (Cotton et al., 2006; Kelly, 2018). Por lo tanto, las preferencias serán débiles cuando representen costos muy altos y más fuertes cuando los beneficios por discriminar entre machos sean mayores (Cotton et al., 2006; Kelly, 2018). Las hembras de mejor condición podrán asumir los costos de la preferencia y obtener mayores beneficios. Esto conlleva a que la magnitud de preferencia evolucione para depender de la condición como ocurre con los ornamentos masculinos (Grafen, 1990; Iwasa et al., 1991; Iwasa & Pomiankowski, 1994, 1999).

Distintos factores pueden afectar la condición de los individuos o el conjunto de recursos disponibles para asignar a los rasgos de historia de vida (Rowe & Houle, 1996) e influir en la elección de pareja. La respuesta inmune, es decir, la capacidad de un organismo para defenderse ante la infección por un agente patógeno (Rauw, 2012; Strand, 2008; Zuk & Stoehr, 2002), forma parte del estado de salud del individuo y, por lo tanto, es un determinante importante de su condición (Tummeleht, 2006). Los parásitos reducen la adecuación de los hospederos al disminuir su reproducción, longevidad o ambas (Moore, 2002). Invertir en la defensa inmune también tiene un costo, ya que los recursos utilizados ya no se encontrarán disponibles para otros rasgos de historia de vida (Sheldon & Verhulst, 1996), por ejemplo, provocando una reducción en la expresión de los ornamentos sexuales (Faivre et al., 2003).

La variación en la condición nutricional a su vez va a afectar la canalización de recursos a la defensa inmune y la producción de señales sexuales, al proveer los recursos disponibles de la alimentación y las reservas corporales (Jacot et al., 2004). A partir del marco geométrico para la nutrición (GF) es posible evaluar los efectos de los nutrientes sobre la condición y la adecuación, mediante dietas con composición definida que permiten una manipulación experimental eficiente (Raubenheimer et al., 2009; Simpson & Raubenheimer, 1999; South et al., 2011). El GF es una metodología analítica basada en la lógica de la geometría del espacio de estados, destinada a caracterizar las variables clave responsables de la regulación de los nutrientes (Raubenheimer et al., 2009). Los diferentes estudios que han empleado el GF han revelado que en insectos la ingesta de proteínas es esencial para producir una mayor respuesta inmune (Povey et al., 2014). Por otra parte, también se ha encontrado que la disponibilidad de carbohidratos es más importante que la de proteínas en la señalización para atraer parejas en insectos (Maklakov et al., 2008; Reifer et al., 2018; South et al., 2011).

En el escarabajo de la harina *Tenebrio molitor*, la dieta en la que se desarrollaron los individuos afectó su susceptibilidad ante una infección por nematodos entomopatógenos (Shapiro-Ilan et al., 2008). Siendo más susceptibles aquellos individuos cuya dieta se basaba en lípidos (Shapiro-Ilan et al., 2008). También se ha encontrado que el estrés nutricional disminuye la actividad de enzimas relacionadas con la respuesta inmune en esta especie (Rantala et al., 2003). Además, los machos que se alimentan de dietas ricas en nutrientes son más grandes, longevos y son más atractivos para las hembras (McConnell & Judge, 2018; Rantala et al., 2003). Por otro lado, en *T. molitor* no hay un consenso en cuanto a cómo afecta un reto inmune el atractivo masculino, ya que, existe evidencia de que los machos sanos son preferidos por las hembras (Worden et al., 2000; Worden & Parker, 2005). No obstante, también existe evidencia que respalda que los machos enfermos son más exitosos en conseguir pareja (Kivleniece et al., 2010; Krams et al., 2014; Nielsen & Holman, 2012; Sadd et al., 2006). En *T. molitor*, la activación de la respuesta inmune y la nutrición provocan un cambio en la expresión del ornamento masculino, que en esta especie se trata de una feromona (Hurd & Parry, 1991; Tanaka et al., 1986). Al parecer la cantidad de feromona sexual es más relevante que la calidad en esta especie como fuente de información (Rantala et al., 2003).

La producción de ornamentos en los machos y la preferencia de las hembras por tales ornamentos están influenciados por la condición de los individuos. Por ello en esta tesis puse a prueba cómo la condición nutricional de ambos sexos afecta la elección femenina de pareja en el escarabajo *T. molitor*, cuando las hembras discriminan entre machos sanos y enfermos desarrollados en dietas con diferentes concentraciones de proteínas respecto a carbohidratos. En el primer capítulo examino el efecto de la dieta y la infección por un hongo entomopatógeno, *Metarhizium robertsii*, sobre la ingesta de alimento, así como la concentración de proteínas, lípidos y carbohidratos presentes en machos y hembras, como proxis de la condición. Este capítulo es novedoso porque examina, usando un patógeno natural y dietas a detalle, el efecto de estas variables en ambos sexos. Este capítulo se sometió para publicación a la revista *Journal of Insect Physiology*. En el segundo capítulo analizo los posibles cambios en las preferencias femeninas cuando la condición de las hembras y los ornamentos masculinos varían. La novedad de esta aportación es que no existía un estudio donde simultáneamente se variara la condición de ambos sexos, aún cuando esta variación es lo más cercano a lo que ocurre en condiciones naturales. Este capítulo cumple con el requisito del doctorado al ser publicado en la revista *Biological Journal of the Linnean Society*. En el tercer capítulo abordo los beneficios directos que las hembras pueden estar obteniendo al aparearse con los machos mediante el espermatóforo, que éstos pasan durante la cópula. La novedad de este estudio es que no se había investigado la composición química (concentración de proteínas, lípidos y carbohidratos totales) de los espermatóforos producidos por machos en buena y mala condición. Este capítulo actualmente está en revisión en la revista *Journal of Insect Behavior*. Presento también un apéndice en donde evalúo la elección de hembras en buena condición por machos sanos y enfermos, así como las consecuencias de su elección en la calidad de su progenie. Este estudio no es parte medular de mi tesis, porque comprende parte de mi tesis de licenciatura (Reyes-Ramírez, 2016) y nuevos datos que obtuve mientras ya realizaba el doctorado. Este apéndice fue publicado en la revista *Ethology*. Finalmente, también incluyo un artículo de divulgación que publiqué en la revista Oikos=, del Instituto de Ecología de la UNAM. Este artículo describe las implicaciones del balance de los principales macronutrientes (proteínas y carbohidratos) en la ecología y evolución de los seres vivos. El material suplementario no se encuentra disponible en esta tesis, estará a disposición mediante la previa solicitud al correo alis_bio@ciencias.unam.mx.

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CAPÍTULO I

Feeding and condition shifts after encountering a pathogen

Feeding and condition shifts after encountering a pathogen

Alicia Reyes-Ramírez[1,2], Maya Rocha-Ortega[1] & Alex Córdoba-Aguilar[1]*

[1]Departamento de Ecología Evolutiva, Instituto de Ecología, Universidad Nacional Autónoma de México, Apdo. P. 70-275, Circuito Exterior, Ciudad Universitaria, 04510, Coyoacán, Ciudad de México, México

[2]Posgrado en Ciencias Biomédicas, Universidad Nacional Autónoma de México, Ciudad de México 04510, México

*acordoba@iecologia.unam.mx

ABSTRACT

Feeding behavior is a dynamic process, especially if an individual is dealing with an infection. Here, we used *Tenebrio molitor* beetles to evaluate the effects of changes in diet macronutrients (protein:carbohydrate) on: a) feeding behavior before and after infection (using the entomopathogenic fungus *Metarhizium robertsii*) in males; and, b) body condition, measured as the amount of proteins, carbohydrates, and lipids in the body, in males and females. Given that females also depend on the nutrients from the spermatophore, we also addressed the impact on female condition of using spermatophores from males whose diets differed in macronutrients whether they were confronting an infection. We found that males with different diets and regardless of their infection status, and females with different diets, all consumed less of the protein-rich diet but more of the carbohydrate-rich diet. In addition, infection in males produced anorexia. The infection resulted in males and the females they mated with, with fewer body proteins and lipids. This suggests that unlike studies in other insects, *T. molitor* does not consume large amounts of protein during the adult stage, even during an infection. Female's condition depended strongly on that of their mates, improving even when paired with infected males. This suggests that females may be using the nutrients that the males transfer during mating for maintenance.

KEYWORDS: condition, carbohydrate, diet, feeding behavior, protein, *Tenebrio molitor*.

INTRODUCTION

Nutrition is key in shaping life's history variables and maximizing fitness (Chown & Nicolson, 2004; Raubenheimer et al., 2009; Simpson & Raubenheimer, 2012; Simpson et al., 2018). However, optimal nutrition is a complex process that involves an interaction between feeding behavior and the processing of food once ingested (Chapman et al., 2013). The geometric framework (GF) of nutrition is a methodological tool that uses a geometric space of nutrients built by two or more axes that represent a nutritional component that may be influencing the organism's responses to its environment (Simpson & Raubenheimer, 1993, 1999, 2012; Simpson et al., 2004). In this sense, the GF has become a tool for evaluating the effects of the main macronutrients on condition and fitness, since it provides control over the diet in terms of a defined composition and efficient experimental manipulation (South et al., 2011). Consequently, the GF has been successfully applied in a wide range of taxa (Simpson et al., 2003, 2004; Dussutour et al., 2010; Rodrigues et al., 2015).

Immune response expression results from feeding decisions. While the capacity for immune response is shaped by genetics and the environment, the via nutrition is also essential (Chandra, 1996; Lochmiller & Deerenberg, 2000; Lazzaro & Little, 2009; Povey et al., 2014). In this sense, experiments using the GF have demonstrated that the ingestion of protein is key for an optimal immune response in insects (Povey et al., 2014). For example, caterpillars of *Spodoptera littoralis* that had protein rich diets had a better immune response against an infection by nucleopolyhedrovirus (Lee et al., 2006). These experiments have also shown that animals select protein rich diets to improve their immune defense, an adaptive behavior generally referred to as self-medication (Chapman et al., 2013). An illustrative case is that of the African soldier worm *Spodoptera exempta*, whose larvae choose to ingest protein after being infected with a sublethal dose of the bacterium *Bacillus subtilis,* which improves their defense capacity (Povey et al., 2014).

Self-medication is just one of several behavioral mechanisms that animals may use to improve their immune response to a pathogen, which are collectively known as sickness behaviors (Hart, 1988; Dantzer & Kelley, 1989; Aubert, 1999). In this respect, it has been demonstrated that both vertebrates (Dantzer, 2004) and invertebrates (Adamo, 2012), show adaptive behaviors when facing an infection, such as lethargy (i.e. reduced activity), seeking shelter, reducing feeding and reproductive rates, and anorexia (Hart, 1988; Aubert, 1999). The case of anorexia is interesting because it would seem counterintuitive when facing the resource demands of fighting a pathogen the animal would stop eating. The reasons for this, is that anorexia reduces the risk of ingesting more pathogens, it can kill the pathogens through lack of nutrients (Kyriazakis et al., 1998; Adamo et al., 2007) and/or can reduce the costs of over-ingestion or under-ingestion (Simpson et al., 2004; Raubenheimer et al., 2005; Cotter et al., 2011). In relation to over-ingestion or under-ingestion, it may occur when animals with anorexia avoid extreme feeding rates, they evade deficits and excesses of certain macronutrients, like proteins and carbohydrates (e.g. Kyriazakis et al., 1991; Raubenheimer & Simpson, 1997; Berthoud & Seeley, 1999).

Females of a wide variety of insects depend not just on the foods they consume, but also on the nutritional contribution of the spermatophore acquired during copulation (Chapman et al., 2013). Spermatophores are protein-rich structures and in a lesser degree, lipids and carbohydrates that increases fecundity (Stanley-Samuelson & Loher, 1983; Watanabe & Sato, 1993; Karlsson, 1995; Heller et al., 1998). Spermatophores vary in their content of these macronutrients, depending on the male's diet (Cahenzli & Erhardt, 2013; Muller et al., 2015). It is generally known that spermatophores that vary in their caloric contribution, impact the condition of females (e.g. Ferkau & Fischer, 2006). However, little is known about how the quality of a spermatophore affects female condition when the macronutrients in the diet of the male are modified (i.e. when using the GF). In part, this question has remained unanswered because there have not been researches that directly modify the macronutrient composition of male diets to determine its impact on the spermatophore. Perhaps the only investigation that has partially answered this question is a recent study using the beetle *Tenebrio molitor*. In this animal, diets high in proteins and low in carbohydrates yielded spermatophores with higher protein, carbohydrate, and lipid contents, while diets low in proteins and high in carbohydrates led to spermatophores with lower protein, carbohydrate, and lipid contents (Reyes-Ramírez et al., submitted). Interestingly, the same study found that males infected with the fungus *Metharizium robertsii* produced spermatophores with the highest content of the three macronutrients (Reyes-Ramírez et al., submitted), which suggests a diet-infection link as well as a terminal investment (investment of resources in reproduction when survival probability is low (Clutton-Brock, 1984). Given these results, it is worth evaluating how the variation in spermatophore quality affects female condition when males are given different diets after an infection.

In this work, we modified the macronutrients present in the diet to evaluate: a) feeding behavior, before and after an infection in males; b) the effect on body condition in males and females; and, c) the possible dependence of female condition on the spermatophore coming from males whose diets differ in macronutrients and facing an infection. For this purpose, we used the flour beetle, *T. molitor*, and different ratios of proteins to digestible carbohydrates, while for an infection we used the entomopathogenic fungus *Metarhizium robertsii*. *T. molitor* requires a large proportion of carbohydrates in its diet (Fraenkel, 1950). This species has a polygynandric mating system where males and females engage in multiple matings (Drnevich et al., 2001; Carazo et al., 2012). The females require the spermatophores to maximize their reproductive success (Drnevich et al., 2001; Worden & Parker, 2001). With respect to health status and immune response, previous studies in this species have found that both healthy and sick males fed diets rich in carbohydrates and with little or no protein at all, had higher survival rates (Reyes-Ramírez et al., 2019a).

To determine the changes on the feeding behavior before and during infection, the amount of food consumed by males was measured. Here, we expected that males fighting an infection would consume less food. To determine the contribution of the macronutrients to the condition, the total amount of proteins, lipids and carbohydrates present after an infection were measured (in the case of males) and after copulation (in males and females) directly from the animals. For males in good condition, we expected higher protein, carbohydrate and lipid contents than those in poor health condition. For females, we expected would have higher protein, carbohydrate and lipid contents after they mated with healthy males.

MATERIALS AND METHODS

Insect maintenance

Pre-adult *T. molitor* were raised in an environmental chamber at 70% humidity, a temperature of 25 ± 2ºC (mean ± STD) and a photoperiod of 12:12 hours. Larvae were kept in plastic containers (30.5 cm diameter x 10.5 cm height), placing 200-300 individuals in each container to minimize cannibalism (Weaver & McFarlane, 1990). They were fed *ad libitum* with wheat bran (Maxilu® brand) and apple slices. Based on observations of the eighth abdominal segment, the pupae were sexed (Bhattacharya et al., 1970) and placed in plastic containers (22.1 cm length x 15.4 cm width x 5.7 cm height). Recently emerged males and females were separated until the moment of the experiments in individual plastic containers (4.2 cm diameter x 3.8 cm height). Once emerged as adults, both sexes were fed with the synthetic diets described below.

Manipulation of nutritional condition

Once emerged as adults, the nutritional condition of both sexes was manipulated using five artificial diets that contained different proportions of protein (p) with respect to digestible carbohydrates (c) (% dry mass p:c). The proteins consisted of a mixture of casein, peptone and albumin in a proportion of 3:1:1. For carbohydrates, we used sucrose and dextrin in a 1:1 proportion. The diets had the following concentrations: 1) 80% proteins and 0% carbohydrates (p80:c0); 2) 64% proteins and 16 % carbohydrates (p64:c16); 3) 40% proteins and 40% carbohydrates (p40:c40); 4) 16% proteins and 64% carbohydrates (p16:c64); and 5) 0% proteins and 80% carbohydrates (p0:c80). In addition, all diets contained the same proportion (20%) of the following nutrients: Wesson salt mixture (2.4%), cholesterol (0.5%), linoleic acid (0.5%), ascorbic acid (0.3%) and a vitamin mixture (0.2%). Cellulose (16.1%) was added as a non-nutritive agent to complete the diet concentrations. These synthetic diets were prepared following previously described protocols (Dadd, 1960; Simpson & Abisgold, 1985). All reagents were acquired from Sigma-Aldrich.

The diets were provided individually to both sexes from the time they emerged (using the mentioned plastic containers) in "dishes" (the inverted cap of a 1.5 mL Eppendorf tube, 9 mm in diameter, 5 mm deep). Similar dishes were also used to provide water *ad libitum* (20 µl of water per day). The dishes were removed and replaced with a new one every two days. These diets were provided for a total of 15 days. Note that on day 12, some males were infected with the fungus *M. robertsii* (see below) to vary their health status, but all animals continued with their diets for 3 more days. The reasons for such interval are that: a) at 12 days, the animals are sexually mature (thus reaching the differential allocation of resources for different reproductive and somatic tissues (Gerber, 1976)); and b) three days was the amount of time at which all infected males were still alive. In other words, if we had extended the time beyond 3 days post-infection, this would have unbalanced the groups and made the statistical analysis more difficult. Thus, the twelfth day (before) and third day (after the health status manipulation respectively), was the best logistical compromise to test whether there were changes in diet based on the animals' physiology. Once the 15 days passed, both sexes were allowed to copulate, forming pairs of all possible combinations of males (5 diet groups x 4 health status groups = 20 groups) and females (5 diets).

Preparation of the fungus and the LC_{50}

The fungus *M. robertsii* (ARSEF 2134) was obtained from the Agricultural Research Service of the United States Department of Agriculture. Spores were stored in 10% glycerol at -80ºC until their use. Then, spores were plated on Sabouraud Dextrose Agar (SDA) for incubation at 28ºC in the dark for 15 days. The conidiophores were collected at the end of the incubation period by scraping the SDA plates and suspended in Tween 80 at 0.03% (hereafter referred to as Tween). The suspension was formed by vortexing for 5 minutes. To separate the conidia from the mycelium, the mixture was filtered through cotton mesh. A > 95% relative viability of the conidia was determined using the SDA plate count method (Goettel & Inglis, 1997). Using the filtrate, we determined the LC_{50} (median lethal concentration), which has been previously reported for *T. molitor* using the same fungus (Reyes-Ramírez et al., 2019b).

Variation in health status due to pathogens in males

After being fed for 12 days with the diets described above, the males were divided into four groups to manipulate health status (for a schematic description of the experiment see Figure 1). This assignment was made randomly using the random number generator available at http://www.alazar.info/generador-de-numeros-aleatorios-sin-repeticion. Each group was composed by up to 105 individuals: (i) unmanipulated individuals (hereafter, negative control); (ii) Tween control—individuals submerged in Tween for 5 seconds; (iii) non-viable spores—individuals submerged for 5 seconds in Tween with *M. robertsii* (ARSEF 2134) previously exposed to high temperatures; and (iv) fungus—individuals submerged in Tween with $3x10^5$ conidia/mL (CL_{50}) of *M. robertsii* for 5 seconds. This experimental manipulation was only done with males.

Measurement of food consumed

The dishes with food were dried at 40 ºC for 24 hours to eliminate moisture before offering them to the beetles and after removing them from the container. We determined their dry weight (in mg) on an analytical balance (Scientech, SA310). To reduce measurement error, a) any food spilled was returned to the dish (Rho & Lee, 2014); and, b) the person in charge of weighing the food was blind to the individual's treatments. The food consumed was determined as the difference of the dry mass before and after feeding.

Animal condition: Protein, lipid and carbohydrate measurement

After the 15 days of the experiment, 30 males and 30 females of each treatment and diet were randomly chosen using the before mentioned random number generating webpage. They were placed in Eppendorf tubes and frozen at -20ºC until use. Then, 180 µL of aqueous lysis buffer was added to the tubes [100 mm KH2PO4, 1 mm dithiothreitol (DTT) and 1 mm ethylenediaminetetraacetic acid (EDTA), pH 7.4], for later homogenization in a Tissue Lyser-II (Qiagen, Valancia, California) for 30 seconds at 25 Hz. The quantification of proteins, lipids and carbohydrates was done using the unified Foray method (Foray et al., 2012). This method uses multiple solvents to sequentially extract the desired components and quantify them using specific colorimetric techniques. Briefly, after agitation, the proteins in the sample were solubilized in phosphate lysis buffer and quantified using the Bradford procedure (Bradford, 1976). Bovine serum albumin was the standard and the sample was read at an absorbance of 595 nm. Both for lipids and total carbohydrates, we used the Van Handel & Day method (Van Handel, 1985; Van Handel & Day, 1988). For lipids, the vanillin assay was used with trioleate glycerol as a standard and absorbance at 515 nm, while the anthrone colorimetric method was used for carbohydrates with d-glucose as the standard and absorbance at 630 nm (Absorbance Reader ELx800; BioTek Inc., Winooski, Vermont).

Statistical analysis

To determine whether there was a change in the feeding rate (i.e. anorexia) between healthy and infected males from the different diets, two analyses of variance (ANOVA) were constructed. Thus, two ANOVA’s—one for before manipulation and one after manipulation—were carried out. In each of these analyses the amount of food consumed by the individuals was used as the dependent variable while the diet and health status were used as independent variables. Also, in the case of females, two ANOVA's, one for before mating and one for after mating, were used to determine a change in feeding rate. Again, the amount of food consumed by the individuals was used as the dependent variable while the diet and the mate's health status were used as independent variables. Following statistical significances among independent variables, we made comparisons between treatments using the Fisher's Least Significant Difference post hoc test.

To determine the effect of treatments on the individual's condition, ANOVA's were also used to examine the effect of diet, health status and their interaction on the amount of macronutrients (proteins, lipids and total carbohydrates) in males and females. In each model the macronutrient amount corresponded to the dependent variable, while diet and state of health were the independent variables. We used the Fisher's Least Significant Difference post-hoc test for all comparisons. All analyses were carried out in R (Team, 2017).

RESULTS

Amount of food consumed by males before manipulating health status

Males of the 20 treatments varied in their consumption as a function of the p:c ratio ($F_4 = 212.68$, $P < 0.001$; Figure 2A). The differences were as follows: the diet with the highest consumption was p0:c80 while the opposite diet was the least consumed (p80:c0; for all combinations, see supplementary material Table S1). At the moment of the comparison, within each diet group according to the future assignment to health status groups, there was no difference in consumption among males within each diet ($F_3 = 0.0034$, $P > 0.05$). This means that even though the assignments to different health status groups had not yet been made, there were no differences in consumption rate among the future groups (for all combinations, see supplementary material Table S2). But the interaction between diet and health status is also significant ($F_{12} = 2.264$, $P < 0.01$).

Amount of food consumed by males after manipulating health status

Males consumed the artificial diets differentially ($F_4 = 154.436$, $P < 0.001$; Figure 2B). Again, the most consumed diet was p0:c80 while the least consumed was the opposite, p80:c0 (for all combinations, see supplementary material Table S3). Health status also affected the amount of food consumed by the males ($F_3 = 49.006$, $P < 0.001$). Infected males had the lowest consumption, followed by those treated with non-viable spores, and negative control, while the Tween control males consumed the most (see supplementary material Table S4). The interaction between diet and health status was significant ($F_{12} = 6.07$, $P < 0.001$).

Amount of food consumed by females

Females differed in their consumption as a function of p:c ratio, both before mating ($F_4 = 349.63$, $P < 0.001$) and after mating ($F_4 = 265.71$, $P < 0.001$). They consumed the most with the p0:c80 diet and the least with the p80:c0 diet (for all combinations, see supplementary material Table S5 and Table S6). When comparing female condition according to the future assignment to the health status of the males they were mated with, there was no difference among groups within each diet (before $F_3 = 0.001$, $P > 0.05$ and after $F_3 = 0.0002$, $P > 0.05$). That is, females did not differ in their feeding rate before assignment to partners from the different health status groups. The interaction before ($F_{12} = 0.0014$, $P > 0.05$) and after ($F_{12} = 0.001$, $P > 0.05$) yielded non-significant results.

Effects on animal condition: protein

There was an effect of the diet on the proteins present in males ($F_4 = 140.4$, $P < 0.001$; Figure 3A) and females ($F_4 = 133.09$, $P < 0.001$; Figure 3B). Males and females fed with the p16:c64 diet had the lowest amount of proteins. Individuals fed with the p80:c0 diet had the highest amount of proteins (for all combinations, see supplementary material Table S7 and S9 for males and females respectively).

Health status also influenced the amount of protein found in males ($F_3 = 12.62$, $P < 0.001$; Figure 3A). Infected males and negative control males had the lowest amount, followed by Tween control males, while those exposed to non-viable spores had higher amounts of total proteins (for all combinations, see supplementary material Table S8). The interaction between diet and health status was significant ($F_{12} = 26.32$, $P < 0.001$).

The health status of female's mates affected the protein amount ($F_3 = 2.73$, $P < 0.05$; Figure 3B). Females that copulated with infected males, negative control males and Tween control males had lower protein amounts. Females that copulated with non-viable spore males had higher amounts of protein (for all combinations, see supplementary material Table S10). The interaction between diet and health status of the female's mates was significant ($F_{12} = 17.57$, $P < 0.001$).

Effects on animal condition: lipids

The total lipid amount in males ($F_4 = 83.411$, $P < 0.001$; Figure 3C) and females ($F_4 = 57.481$, $P < 0.001$; Figure 3D) varied depending on diet. Individuals of both sexes that were fed the p64:c16 diet presented the lowest amount. The highest amount of total lipids was among animals fed with the p0:c80 diet (for all combinations, see supplementary material Table S11 and S13 for males and females respectively).

Health status influenced lipid amount ($F_3 = 75.794$, $P < 0.001$; Figure 3C). Infected males had the lowest amount, while Tween control males had the highest amount (for all combinations, see supplementary material Table S12). Females followed the same pattern as males ($F_3 = 94.682$, $P < 0.001$) (for all combinations, see supplementary material Table S14). The interaction for males ($F_{12} = 21.48$, $P < 0.001$) and females ($F_{12} = 54.05$, $P < 0.001$) was significant.

Effects on animal condition: carbohydrates

The diet affected the amount of carbohydrates present in males ($F_4 = 172.81$, $P < 0.001$; Figure 3E). The individuals fed with the p80:c0 and p64:c16 diets had the lowest amount. Males fed with the p0:c80 diet had the highest amount of carbohydrates (for all combinations, see supplementary material Table S15). In the case of females, their diet also influenced the amount of present carbohydrates ($F_4 = 156.09$, $P < 0.001$; Figure 3F). The lowest amount was found in females that consumed the p80:c0 and p16:c64 diets, while the highest amount was in females under the p0:c80 diet (for all combinations, see supplementary material Table S17).

Like the other macronutrients, health status affected the amount of carbohydrates in males (F_3 = 38.967, $P < 0.001$; Figure 3E). Tween control and non-viable spore males had the lowest amount of total carbohydrates, while infected males had the highest amount of carbohydrates (for all combinations, see supplementary material Table S16). There was also an effect on the carbohydrate amount of the females they mated with (F_3 = 44.843, $P < 0.001$; Figure 3F). Females that mated with Tween control males had the lowest amount, while females that copulated with negative control and infected males had the highest amounts (for all combinations, see supplementary material Table S18). The interaction between diet and health status was significant for males (F_{12} = 19.24, $P < 0.001$) and females (F_{12} = 15.552, $P < 0.001$).

DISCUSSION

Insects must consume an adequate balance of macronutrients (Jensen et al., 2012; Simpson & Raubenheimer, 2012; Simpson et al., 2018). Imbalances can lead to insects ingesting and processing insufficient or excessive amounts of food (under- or over-ingestion, respectively) without obtaining enough of certain nutrients (Waldbauer & Friedman, 1991; Behmer, 2009; Raubenheimer & Simpson, 2018). Unlike other insects (Lee et al., 2006, 2008; Alaux et al., 2010), adult *T. molitor* consume mostly carbohydrates, even in situations of infection (Reyes-Ramírez et al., 2019a), which we corroborated in our study (note that there were no differences before and after being assigned to experimental groups). In the cockroach *Nauphoeta cinerea*, similar results have been found, males and females selected highly carbohydrate-biased diets (p:c around 1:5), maximizing reproductive traits (e.g. pheromone levels, which increase male attractiveness, female clutch size and gestation time (Bunning et al., 2016)). Females typically require more proteins than males to maximize their reproduction (Maklakov et al., 2008; Harrison et al., 2014), but it is not surprising that both sexes regulate their nutrition similarly when confronted with a nutritionally unbalanced diet (Bunning et al., 2016). With this type of diets, individuals must find a suitable trade-off between over-ingestion of some nutrients and under-ingestion of others, which is sometimes referred to as the "rule of best compromise" (Raubenheimer & Simpson, 1997). In *T. molitor*, there is evidence that both sexes prioritize their ingestion of carbohydrates over proteins (Rho & Lee, 2014), as occurred in this study. Another possible explanation is that there is an intralocus sexual conflict over the selection of nutrients, as has been found in other insects (for example Maklakov et al., 2008; Reddiex et al., 2013; Jensen et al., 2015). Intralocus sexual conflict could occur when the genes for a given trait are linked between sexes (Zajitschek et al., 2007; Bonduriansky & Chenoweth, 2009). It is therefore possible that both sexes are displaced from their nutritional optima, ultimately resulting in a convergence of both sexes on a similar diet that is not necessarily ideal for either (Reddiex et al., 2013). Thus, the result of comparing the diet between sexes does not necessarily reflect the preferences that maximize fitness, but rather the outcome of a conflict that favors one sex (Reddiex et al., 2013). In the case of our subject of study, the fact that the diet of both sexes is biased toward carbohydrates and zero protein, could be interpreted as a conflict if that diet benefits one sex more than the other. This would need to be studied in more detail.

Another result is that animals ate less during an infection, which suggests anorexic behavior. We must wonder why this sick behavior occurs. We know that the infection with *M. robertsii* is produced by spores that adhere to an insect's epicuticle (Lin et al., 2011), which then germinate, and form infection structures called appressoria, which produce enzymes that degrade the cuticle (Leger et al., 1989; St et al., 1996; Wang & Leger, 2007). Lipids are known to be the main nutrient reserve of the spores (Wang & Leger, 2005). However, despite these reserves, *M. robertsii* spores require extracellular nutrients to germinate (Leger et al., 1989), so it is possible that through anorexia and under-ingestion of macronutrients, *T. molitor* restricts the nutrients available to the fungus and therefore decreases the probability of its germination. Given that adult *T. molitor* essentially prefer carbohydrates over proteins, our prediction is that these beetles restrict the carbohydrates available to the fungus to prevent its growth. Another possible explanation is that anorexia may be beneficial by reducing the trade-offs between the immune system and other energetically costly activities, like digestion or somatic growth (Dunn et al., 1994; Freitak et al., 2003; Tye et al., 2020). This temporary behavioral change that led to a reduced food consumption may allow organisms to invest their resources to immunity (Weers & Ryan, 2006; Adamo et al., 2010), increase tolerance towards an infection (Ayres & Schneider, 2009) or reduce the chances of predation while recovering from an infection (Hart, 1988).

The condition of infected males, as well as the females that mated with them, indicated low values of carbohydrates, proteins, and lipids. On one hand, this suggests that there is an impact of infection and diet on the condition of both sexes. On the other, it is likely that the reduction in proteins and lipids indicates that these macronutrients are used to combat the fungus, as we suggest above. These two possibilities are not contradictory with respect to the under-ingestion of proteins because ultimately this macronutrient could be used (though in small amounts) for immune defense. It should be noted that our diet treatments began during the adult stage, so there may have been protein and lipid reserves from the pre-adult stage, which would explain why these insects decrease their ingestion of protein as adults. Either way, infection led to spermatophores to become richer in macronutrients (Reyes-Ramírez et al., submitted). However, this investment does not come free, since it reduces male survival (Reyes-Ramírez et al., 2019b). Interestingly, females should mate with these males as they receive higher quality spermatophores. A spermatophore rich in proteins increases egg production (Engebretson & Mason, 1980; Watanabe & Sato, 1993; Voigt et al., 2006; Gwynne, 2008), although this relationship has yet to be tested in *T. molitor*. As an indirect evidence of this, Worden and Parker recorded a 32% increase in egg laying after four matings with healthy males (Worden & Parker, 2001). If *T. molitor* females mate exclusively with sick males, females could pay the energetic costs of egg production using the nutritional content of spermatophores and not using their own nutrition. This could explain multiple mating and preference for sick over healthy partners in this species (Sadd et al., 2006; Kivleniece et al., 2010; Nielsen & Holman, 2012; Krams et al., 2014; Reyes-Ramírez et al., 2019b). However, these multiple matings can lead to costs to females if these get infected by their male partners (Knell & Webberley, 2004; Zhong et al., 2013).

As we mention above, sick male *T. molitor* attract more females due to the pheromones they emit (Sadd et al., 2006; Kivleniece et al., 2010; Nielsen & Holman, 2012; Krams et al., 2014; Reyes-Ramírez et al., 2019b), and produce higher quality spermatophores (Hurd & Ardin, 2003; Reyes-Ramírez et al., submitted). However, this double investment, which compromises male survival, only allows the best of a bad job, since these males do not have higher reproductive success, nor higher success in hatching or quality of their progeny (Reyes-Ramírez et al., 2019b; Reyes-Ramírez et al., submitted). Even though females may take advantage of the opportunity to copulate with males that have nutrient-rich spermatophores, they penalize them (Reyes-Ramírez et al., submitted). Apparently, females have mechanisms to identify these males in poor condition during or after mating, or this bad condition affects the male spermatogenesis. It could be that these identification cues stem from behaviors carried out during copulation. During copulation, males carry out a series of repeated and fast movements with their antennae and legs. Our hypothesis is that the intensity of these movements depends on the nutritional and/or health status of the males, such that only males in good condition can perform them intensely. Related to this, high activity levels and even aggression have been linked to higher carbohydrate content (e.g. sucrose) in ants (Grover et al., 2007). In male moths, however, low carbohydrate intake led to more attractive songs (Cordes et al., 2015). Either way, it is possible that the mechanisms to detect male condition in *T. molitor* occur during copulation and are related to sexual behavior.

Acknowledgements

This study was supported by a PAPIIT grant IN 206618 and Consejo Nacional de Ciencia y Tecnología (CONACyT). Alicia Reyes-Ramírez is a doctoral student in the Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM) and was supported by CONACyT: 778814/612503. The authors declare that they do not have any conflicts of interest.

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Figures

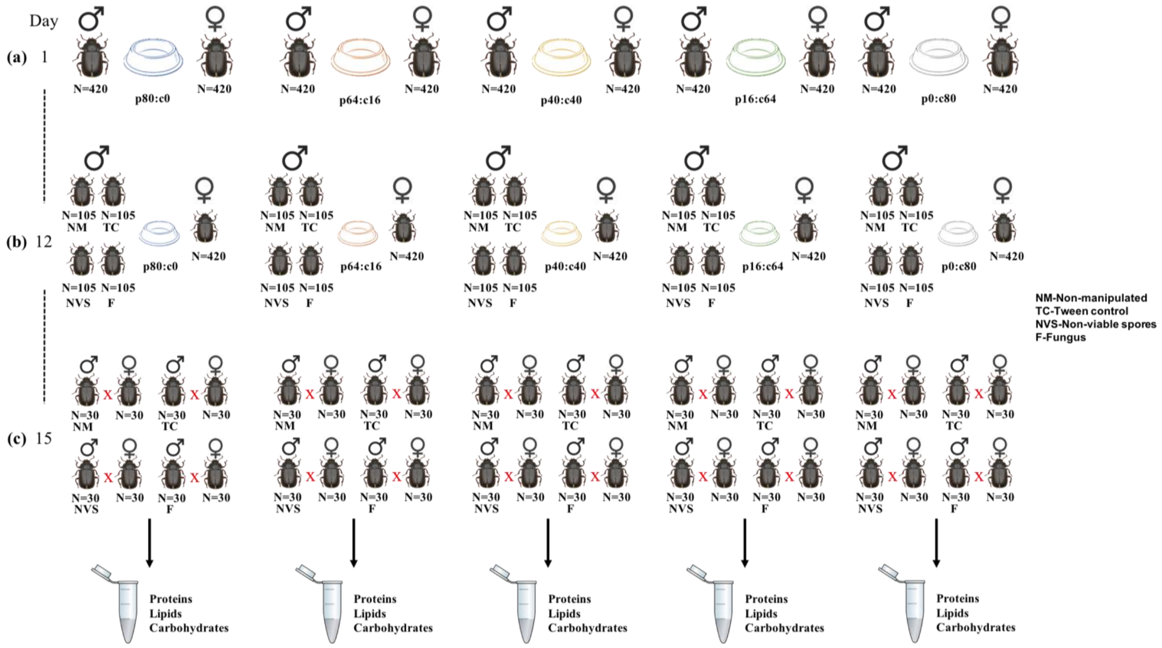

Figure 1. Diagrammatic representation of the experimental design. (a) Once emerged as adults, both sexes were fed with five synthetic diets (protein:carbohydrate ratios, p:c). (b) On day 12 a total of 420 males of *Tenebrio molitor* were randomly divided into four groups to manipulate their state of health. (c) Three days after male manipulation, males and females were allowed to copulate, forming pairs of all possible combinations of males (5 diet groups x 4 health status groups= 20 groups) and females (5 diets).

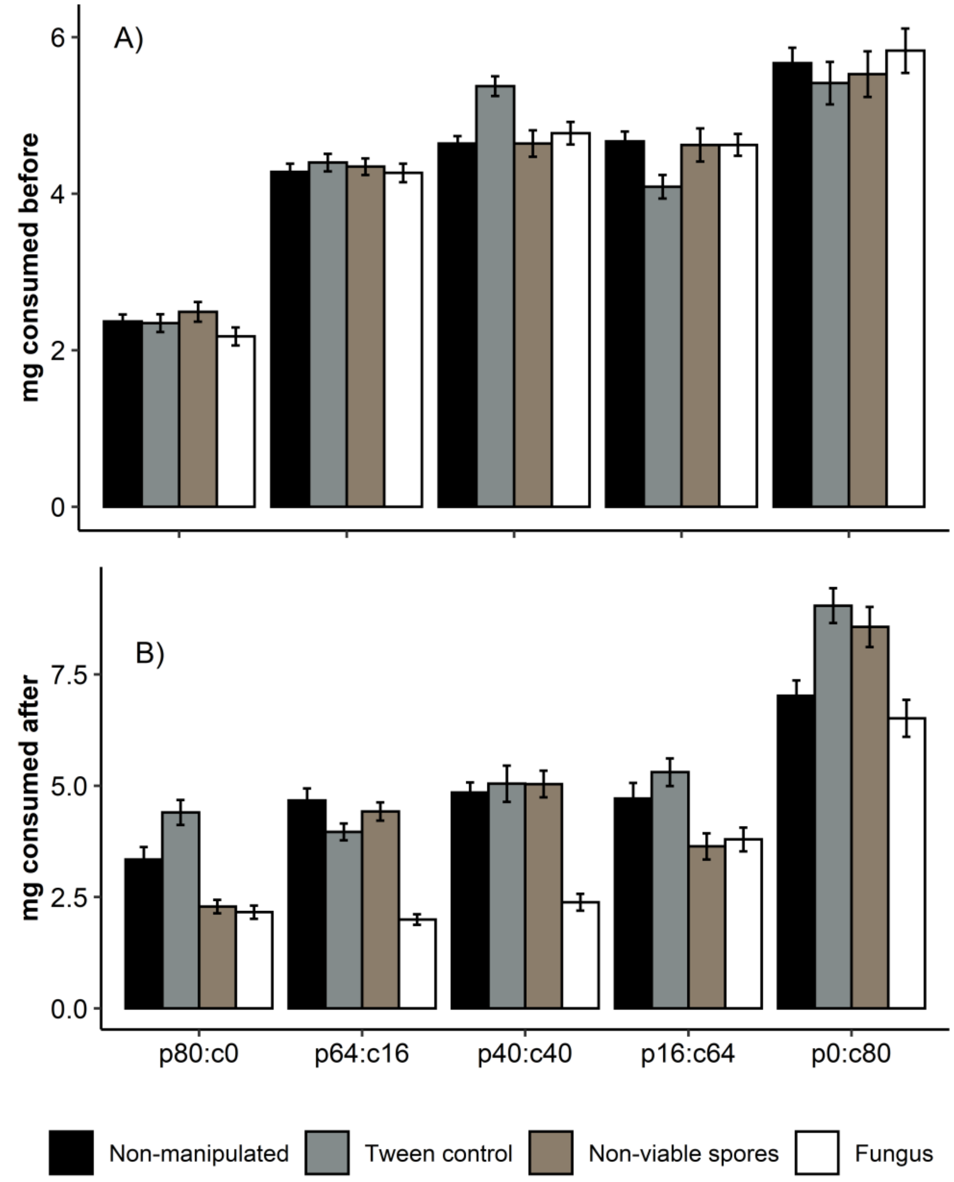

Figure 2. Changes in food consumption (mean ± SE) by males of *Tenebrio molitor* in the different artificial diets. A) Amount of mg consumed before manipulating health status; and, B) Amount of mg consumed after manipulating health status.

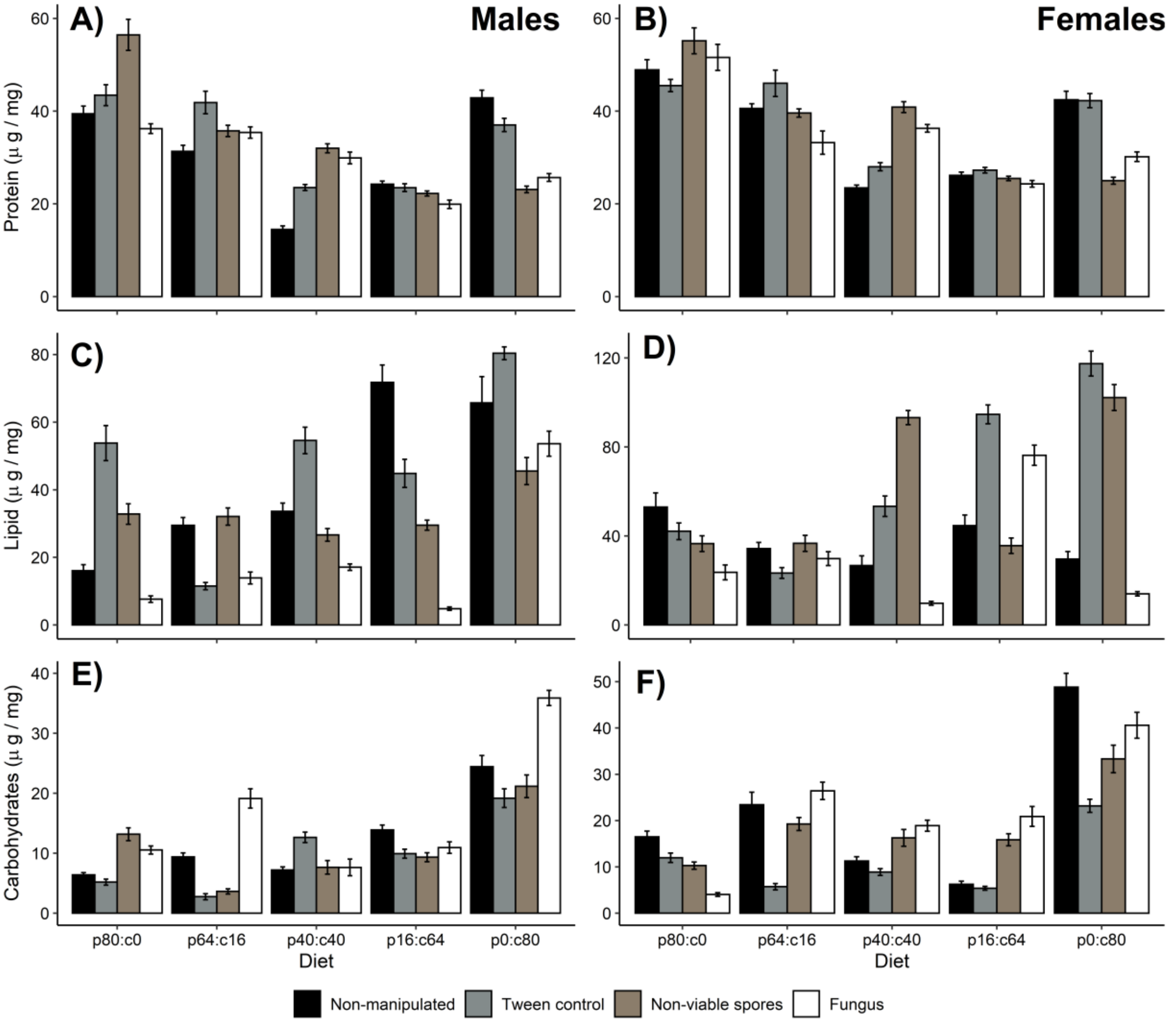

Figure 3. Changes in body nutrient content (mean ± SE) following changing ratios of protein:carbohydrate (p:c) in *Tenebrio molitor* diet. Protein amount in males (A) and females (B); lipid amount in males (C) and females (D); and, carbohydrate amount in males (E) and females (F).

CAPÍTULO II

Female preferences when female condition and male ornament expression vary

Biological Journal of the Linnean Society, 2019, **128**, 828–837. With 3 figures.

Female preferences when female condition and male ornament expression vary

ALICIA REYES-RAMÍREZ, MAYA ROCHA-ORTEGA and ALEX CÓRDOBA-AGUILAR*

Departamento de Ecología Evolutiva, Instituto de Ecología, Universidad Nacional Autónoma de México, Apdo. P. 70–275, Circuito Exterior, Ciudad Universitaria, 04510, Coyoacán, Distrito Federal, México

Received 29 June 2019; revised 13 September 2019; accepted for publication 15 September 2019

Variation in the condition of females can affect their mate preferences. This may explain variation in the expression of male ornaments. We tested these ideas in the mealworm beetle (*Tenebrio molitor*), a species in which females choose males based on their pheromones. We modified female condition using diets that differed in proteins and carbohydrates. We then allowed females to choose among males in which we had previously modified pheromone expression (either by varying diets as in females, or by fungal infection). Females were offered a choice between two males, both of which had been fed the same diet as the female, but which differed in whether they were infected or not. We repeated the same diet and infection treatments to determine whether poor (lower carbohydrate) diets decrease survival in both sexes. There was no effect of female diet on mate choice, but the infection state of the male did have an effect, with infected males being preferred. It is possible that infected males invest their resources in producing pheromones rather than attacking the pathogen. Both sexes, independent of infection, had higher survival when fed carbohydrate-rich diets. The results showed no effect of female condition on their preferences, at least not prior to copulation.

ADDITIONAL KEYWORDS: carbohydrate – condition – diet – female choice – pheromone – protein – *Tenebrio molitor*.

INTRODUCTION

Sexual selection has given rise to both ornaments and preference for those ornaments (Darwin, 1871). Generally, males are the sex that displays these ornaments, while females choose among them (Andersson, 1994). A common question is how to explain variation in the expression of ornaments despite female preferences (Borgia, 1979; Rowe & Houle, 1996). Part of the answer is based on the costs of producing ornaments: only individuals in good condition are able to produce exaggerated ornaments (Zahavi, 1977; Cotton *et al.*, 2004). This relationship has been the basis for understanding and accepting why, despite intense directional selection by females, ornaments vary in their expression. However, this is unlikely to be the only explanation.

Although there is acceptance that preferences are consistent among females, it has been shown that there may be individual variation in their preferences (Jennions & Petrie, 1997; Cotton *et al.*, 2006; Kelly, 2018). This variation in preference is key to understanding the maintenance of variation in ornament expression (Jennions & Petrie, 1997; Qvarnström *et al.*, 2000), especially when preferences are related to female condition. For example, females in better condition on average choose more attractive mates than females in poorer condition (Hunt *et al.*, 2005; Cotton *et al.*, 2006). This is because, compared to females in good condition, females in poor condition cannot afford the costs of searching and/or waiting for a male with the best phenotype, cannot compete with females in better condition, or are less attractive (Fawcett & Johnstone, 2003; Härdling & Kokko, 2005). An example of this occurs in damselflies, where females with higher parasite loads tend to be less selective, mating with males with different degrees of ornament expression, including low-quality males (Córdoba-Aguilar *et al.*, 2003). Several studies have looked at how female condition drives female preferences (e.g. Bailey & Zuk, 2008; Judge *et al.*, 2014; reviewed by Jennions & Petrie, 1997). One such study used finches, in which

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*Corresponding author. E-mail: acordoba@iecologia.unam.mx female quality was manipulated from the chick stage (Holveck & Riebel, 2009). As predicted, there were differences in female preference as a function of quality: high-quality females preferred high-quality males, while low-quality females preferred low-quality males (Holveck & Riebel, 2009).

Two important sources of variation in the expression of ornaments are diet and state of health (Beltran-Bech & Richard, 2014; Rosenthal & Hebets, 2015). While diet has traditionally been manipulated only roughly, recent studies in the geometric framework of nutrition have not only allowed modification of the relative availability of proteins vs. carbohydrates in a detailed way (Raubenheimer *et al.*, 2009; Hosking *et al.*, 2019), but have demonstrated that the balance of these two macronutrients plays an important role in development, survival and reproduction, especially in insects (Chapman, 2013). For example, it has been shown that in different courtship signals in insects, the availability of carbohydrates is more important than that of protein (Maklakov *et al.*, 2008; South *et al.*, 2011; Reifer *et al.*, 2018). An example of this occurs in cockroaches, where males that consumed carbohydrate-rich diets produced more pheromones, making them more attractive to females, than males that consumed protein-rich diets (South *et al.*, 2011). In this way, applying nutritional geometry theory to sexual selection may help manipulate the expression of ornaments with more precision. Regarding health, the cost of defending against or being attacked by a pathogen is known to be high (reviewed by Ayres & Schneider, 2009). This means that individuals may prioritize their energy resources toward defence when their likelihood of reproducing is high (Magnhagen & Vestergaard, 1991). Several examples using pathogens have concluded that infection generally affects ornament expression (e.g. (Gilbert & Uetz, 2016; Suhonen *et al.*, 2018). Moreover, it should be noted that sexual behaviours including courtship vary among species.

Males of the mealworm beetle, *Tenebrio molitor*, produce pheromones to attract females (Tanaka *et al.*, 1986; Hurd & Parry, 1991). These volatile compounds are composed of 3-dodecenyl acetate (Bryning *et al.*, 2005) as well as cuticular hydrocarbons (Nielsen & Holman, 2012). Two sources of variation have been used to manipulate pheromone expression. First, the nutritional condition of males has been manipulated, and females were found to prefer males that had consumed more food (Rantala *et al.*, 2003). However, other studies did not find that pheromone-based attractiveness differed according to diet quality (McConnell & Judge, 2018). In other studies, pathogen attack has been used as a source of variation, but opposite findings were found: while some of these studies showed that females prefer males in good health (Worden *et al.*, 2000; Worden & Parker, 2005), others showed that females prefer sick males (e.g. Sadd *et al.*, 2006; Kivleniece *et al.*, 2010; Nielsen & Holman, 2012; Krams *et al.*, 2014; Reyes-Ramírez *et al.*, 2019). For the latter results, the explanation was that sick males prioritized their energy resources toward pheromone production, analogous to terminal investment (Williams, 1966). According to this idea, when faced with the risk of dying relatively soon, an individual prioritizes resources toward reproduction (Williams, 1966). Indeed, this explanation is consistent with the fact that despite investing in attractiveness, sick males die sooner than healthy males (Reyes-Ramírez *et al.*, 2019).

In this paper, we manipulated condition in both sexes to investigate female preferences using the system of attraction by pheromones in *T. molitor*. First, we experimentally varied the quality of the diet (by varying the protein to carbohydrate ratio) of females to affect their condition and preferences. Second, we varied the expression of male pheromones by manipulating male diets (in the same way as female diets) and their state of health by infecting them with an entomopathogenic fungus (*Metarhizium robertsii*). Using these protocols, we recorded female and male survival depending on their condition. The idea was to test whether our treatments affect survival, and to determine which diet/pathogen attack is a better predictor of individual condition. This experiment suggested that increased carbohydrate content increased survival in both sexes (see Results). We then performed a mate choice experiment offering females to choose between two males in different states of health (an infected vs. a control male) that had been fed the same diet. Using females and males that had all consumed the same diet ensured that state of health (pathogen attack) was the only variable that affected the females' decision, rather than the dietary state of the males. In this context, we predicted that females in better condition (i.e. females that had increased access to carbohydrates) would prefer males in better condition (uninfected males), while females of lower quality (i.e. with decreased access to carbohydrates) would be less choosy (i.e. not showing a preference for infected or non-infected males).

MATERIALS AND METHODS

INSECT MAINTENANCE

Different stadia of *T. molitor* were kept in an environmental chamber set at 70% relative humidity, at 25 ± 2 °C (average ± SD) with a 12:12-h photoperiod. Note that these conditions are the same for all animals indicated below. We kept around 200 beetle larvae in plastic containers (30.5 cm diameter × 10.5 cm height) to reduce cannibalism events as suggested by previous studies (Weaver & McFarlane, 1990). Animals were fed with bran (Maxilu brand) and apple slices, both provided *ad libitum*. The pupae were gathered in plastic containers (22.1 cm length × 15.4 cm width × 5.7 cm height) and sexed by examining the eighth abdominal segment (Bhattacharya *et al.*, 1970). Recently emerged males and females were separated to ensure that all individuals were virgin at the time of the choice experiment. Once adults emerged they were individually placed in plastic containers (4.2 cm diameter × 3.8 cm height) until experiments took place.

SYNTHETIC DIETS

Five different diets were established using different concentrations of proteins (p) and digestible carbohydrates (c) (% dry mass). All diets contained the same concentration (totalling 20%) of the following nutrients: Wesson salt mixture (2.4%), cholesterol (0.5%), linoleic acid (0.5%), ascorbic acid (0.3%) and vitamin mix (0.2%). To complete diet concentration, non-digestible cellulose was added as a non-nutritive agent (16.1%). Thus, the proteins and carbohydrates in each diet had the following proportions: (1) 80% protein and 0% carbohydrates (p80:c0); (2) 64% protein and 16% carbohydrates (p64:c16); (3) 40% protein and 40% carbohydrates (p40:c40); (4) 16% protein and 64% carbohydrates (p16:c64); and (5) 0% protein and 80% carbohydrates (p0:c80). Proteins consisted of a mixture of casein, peptone and albumin in a 3:1:1 ratio. Carbohydrates were composed of sucrose and dextrin in a 1:1 ratio. These synthetic diets were prepared following previously described protocols (Dadd, 1960; Simpson & Abisgold, 1985). All reagents were from Sigma-Aldrich.

Freshly hatched adults were placed in their feeding area consisting of individual plastic containers of dimensions as indicated above. Males and females were randomly assigned to one of five different synthetic diets. During the entire experimental period (day 0 to day 15) each individual had its own food plate [the inverted lid of a 1.5-mL Eppendorf tube (9 mm in diameter and 5 mm deep)] filled with the corresponding dry diet, and *ad libitum* access to water (20 μl of water was added daily). Food plates were removed and replaced by new ones every 2 days.

PREPARATION OF THE FUNGUS

The fungus *M. robertsii* (ARSEF 2134) was obtained from the Agricultural Research Service of the United States Department of Agriculture. Prior to use, spores were stored in 10% glycerol at −80 °C. For inoculum preparation, spores were plated on Sabouraud Dextrose Agar (SDA) and then incubated at 28 °C in darkness. Following 15 days of incubation, conidiophores were harvested by scraping them from the plate and suspending them in 0.03% Tween 80 (hereafter referred to as Tween). The suspension was mixed by vortexing for 5 min and then filtered using a cotton mesh to separate the conidia from the mycelium. Using the SDA plate count technique (Goettel & Inglis, 1997), we found that the relative viability of the conidia was greater than 95%.

DETERMINATION OF 50% LETHAL CONCENTRATION

We inoculated five groups of 15 males with different concentrations of fungal conidia suspended in 10 mL Tween (1×10^4, 1×10^5, 1×10^6, 1×10^7 and 1×10^8 conidia/mL). Insects were inoculated by immersing them in the suspension for 5 s and allowing them to air dry in a 9-cm-diameter Petri dish lined with Whitman No. 1 filter paper. A sixth group was immersed in Tween without conidia and dried in the same way as a control group. Insects were then placed in individual wells in 12-well plates with wheat and incubated for 10 days at 25 °C and 90% humidity, recording mortality every 24 h. Dead insects were incubated at 25 °C in 5-cm-diameter Petri dishes with wet filter paper to promote sporulation and confirm fungal infection. Although the conidium morphology and colour and, in general, fungal growth appears as described for *M. robertsii*, we cannot assume that this was the only fungus species present in the insect (Ment *et al.*, 2012). Mortality was corroborated by ensuring that the individual remained motionless even after tactile manipulation. All initial male ages and weights were between 12 and 15 days and 0.9 and 0.12 g, respectively.

VARIATION IN THE STATE OF HEALTH DUE TO PATHOGENS IN MALES

We used males from the five diet modification treatments. Within each diet treatment, four groups were formed to manipulate state of health using the pathogen. Each of the four groups of males was composed of adult males between 12 and 15 days of age (stage at which they reached maturity; Gerber, 1976). These groups consisted of: (i) negative control, that is, unmanipulated individuals; (ii) tween control, individuals submerged in Tween for 5 s; (iii) non-viable spores, individuals submerged for 5 s in Tween with *M. robertsii* (ARSEF 2134) spores that had been previously made inviable by exposure to high temperature; and (iv) fungus, individuals submerged in Tween with 3×10^5 conidia/mL (approximate LC_{50}) of *M. robertsii* for 5 s. Each of these groups had a sample size of 35 animals.

Survival experiments

The same diet treatments were carried out for females and males (p80:c0; p64:c16; p40:c40; p16:c64; p0:c80). In males only, the five diet treatment groups were further divided to generate the four state of health groups (negative control, tween control, fungus and non-viable spores) described in the previous section. At 15 days of age (after males had received their assigned pathogen treatment), the individuals were no longer provided food or water. Thirty individuals from each group were placed in individual containers. Mortality was recorded every 24 h until the last individual died. An individual was considered dead when it did not move any body part (legs, antennae or mouth parts) even after being gently manipulated with dissection forceps for 1 min.

Female choice tests

Once all combinations in females and the diet and pathogen treatments in males had been carried out, each female was allowed to choose between two males. The female and both males were all from the same diet treatment. For example, females from the p80:c0 diet treatment were given the choice between two males that had both been fed the p80:c0 diet. Although the two males had received the same diet, they differed in their pathogen treatment. Females were presented with the choice between two males that had been treated with: (i) the negative control vs. tween control; (ii) negative control vs. non-viable spores; (iii) negative control vs. fungus; (iv) tween control vs. non-viable spores; (v) tween control vs. fungus; and (vi) non-viable spores vs. fungus. Therefore, although females were not allowed to choose males based on male diet treatment, they did choose between males based on their pathogen treatment. For each experiment, 35 virgin females were used from each diet treatment at an approximate age of 12–15 days, when females have reached sexual maturity (Gerber, 1976). We did not an animal more than once, and thus each diet treatment had 210 females and 420 males (to illustrate our experimental approach including N sizes, see Fig. 1). The choice tests were carried out in a darkroom with red light, using a Y-tube olfactometer (central tube: 23.1 cm long × 2.8 cm in diameter; each arm: 18.4 cm long × 2.8 cm in diameter; male and female port dimensions: 10.5 cm long × 2.8 cm diameter; see Supporting Information, Fig. S1), an apparatus which allows the evaluation of volatile compounds in small insects such as *T. molitor* (see also Reyes-Ramírez *et al.*, 2019). At the beginning of each choice trial, a female was placed in a chamber before a release gate to habituate for 2 min. The gate was then opened, and the female's behaviour was recorded. The males were kept separate in each of the two arms of the olfactometer and an air pump placed at the end of each arm was used to circulate the males' pheromones toward the female. At the end of each olfactometer arm, there is a port that can be closed or open to manipulate male access. The side where each male was placed was assigned randomly and each male was used only once. The experiment ended 3 min after the gate was opened. Our criterion that a female had chosen a male

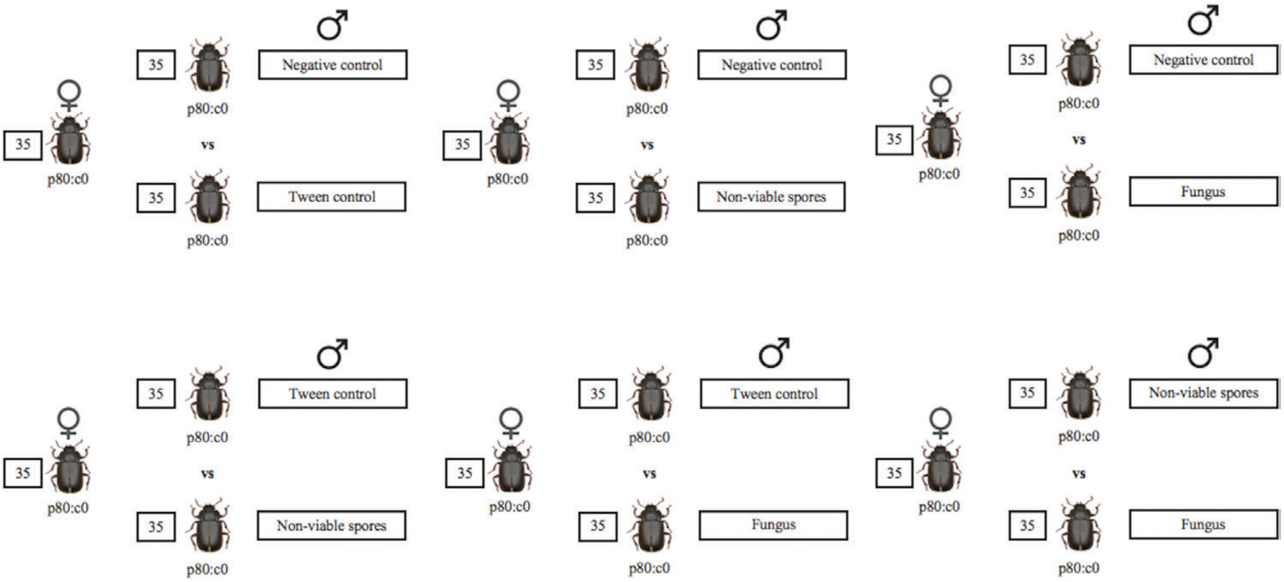

Figure 1. Example of one of six experiments of female choice on a single diet (in this case, p80: c0), where sample sizes are shown. These six tests are repeated for the other five diets.

was that the female reached the end of either arm (right or left) where a male was present on the other side of the distal port. The observer was blind to the treatment of the males used. The olfactometer was cleaned with ethanol after each trial to remove any remaining chemical signals.

Statistical analyses

For the male and female survival experiment, a generalized linear model (GLM) was constructed, using the proportion of individuals that survived from each treatment as the response variable, over time which was entered as an independent variable. Because the interaction between day, state of health and diet was significant for male survival, two independent models were further constructed, the first for diet and the second for state of health. For the former, the individuals that survived each diet were entered as the dependent variable, while diet and time were placed as independent variables. For state of health, the proportion of individuals that survived each treatment was used as the dependent variable while state of health and time were used as independent variables. Both models were adjusted using a quasi-binomial distribution to correct for data overdispersion. Subsequently, a Tukey test was used to compare between treatments. Note that for female survival, the interaction of day, her mate's health and diet were not significant, so the same analysis was held. To examine the effect of female condition on female preferences, we used a generalized linear mixed model (GLMM) using a Poisson distribution, which examined the effect of female diet and state of health due to pathogens in the males on female preferences. In this GLMM, males used were entered as a random factor. A Tukey test was used to compare among treatments. All analyses were performed in R (R Core Team, 2017).

RESULTS

Survival

Male survival was affected by their diet (Table 1) and their state of health due to the pathogen (Table 2). Males in the four treatment groups survived longer when fed with diets with no protein and high carbohydrate (for all combinations see Supporting Information, Table S1) (Fig. 2). Males whose state of health was challenged by the fungus, non-viable spores and Tween died faster than negative control males (for all comparisons see Table S2). No differences emerged between fungus, non-viable spores and Tween treatments (Table S2).

In females, survival was affected by diet treatment (Table 3; Fig. 2) but not state of health of males that they mated with. Females survived longer when provided with a gradually higher proportion of carbohydrates (p16:c64 and p0:c80) (for all combinations see Supporting Information, Table S3).

Female choice tests

The males' state of health due to pathogens affected female choice ($\chi^2 = 10.2031$, $P = 0.01692$), but female diet ($\chi^2 = 0.0030$, $P > 0.05$) and their interaction ($\chi^2 = 5.235$, $P > 0.05$) did not have an effect. Among the different tests and five diets, the only significant difference emerged when females selected between negative control males and infected males (for all combinations see Supporting Information, Table S4). In this case, females preferred fungus-infected males (Fig. 3).

DISCUSSION

While an increased proportion of proteins in the diet has been shown to increase fitness in some insect species (e.g. mass and egg production in crickets; Harrison *et al.*, 2014), in the case of our study species, carbohydrates and state of health were the predictors of condition, as expressed by survival. Note that the effect of carbohydrates differs for the two sexes. While only a high content of carbohydrates (and no protein) increase survival in males, a gradual increase in carbohydrates increases female survival. Similar results have been observed in crickets (Zajitschek *et al.*, 2012) and *Drosophila* (Andersen *et al.*, 2010). One explanation for this is that nutrients that underlie survival differ for both sexes, reflecting different sex-based needs and

Table 1. Results of the GLM evaluating the effects on the survival of males of *T. molitor* by day and diet; significant effects ($P < 0.05$) are in bold

	d.f.	χ^2	P
Day	1	4.236	**< 0.001**
Diet	4	2.397	**< 0.001**
Day × Diet	4	1.553	**< 0.001**

Table 2. Results of the GLM evaluating the effects on the survival of males of *T. molitor* by day and health status; significant effects ($P < 0.05$) are in bold

	d.f.	χ^2	P
Day	1	4.236	**< 0.001**
State of health	3	1.668	**< 0.001**
Day × State of health	3	1.057	**< 0.001**

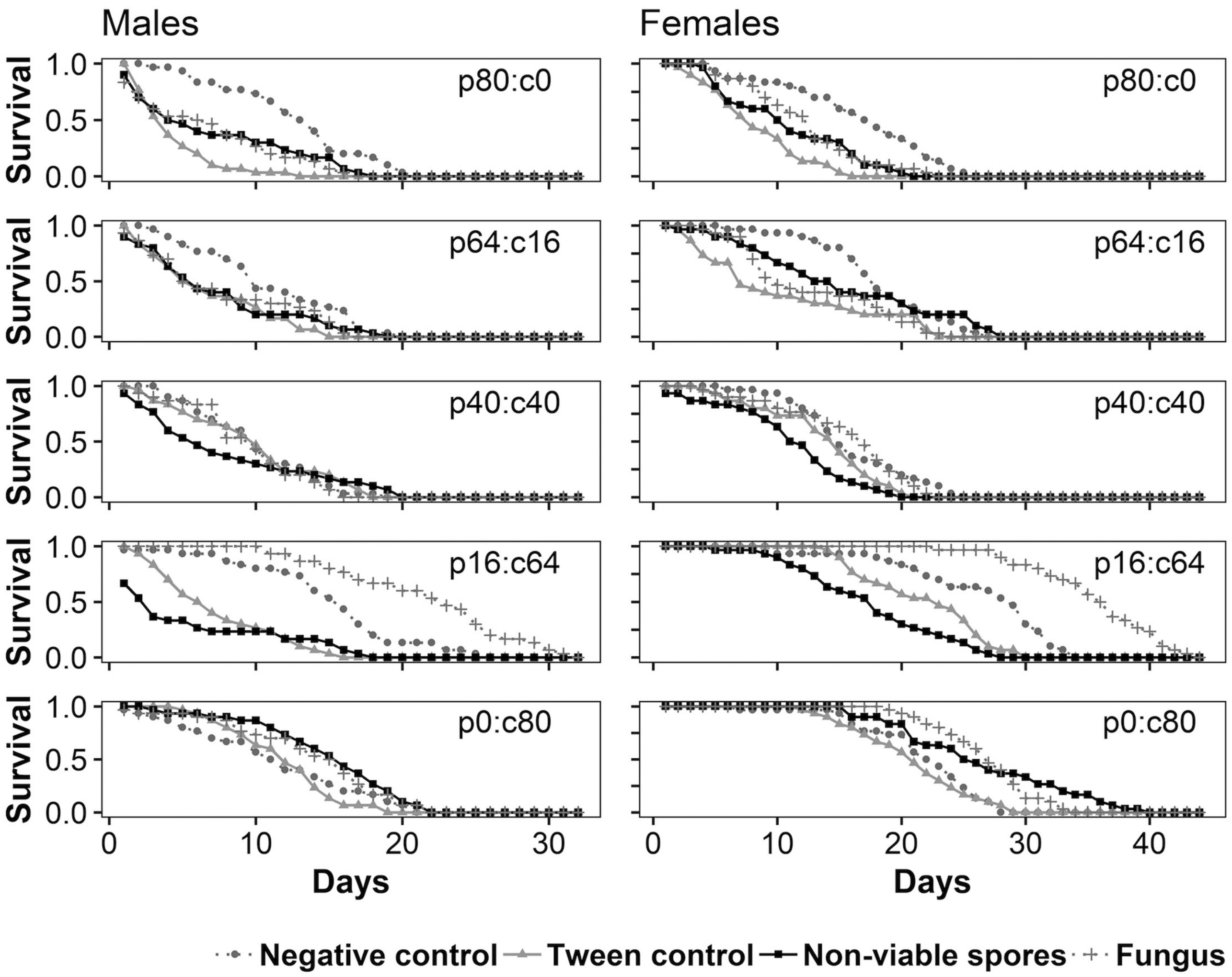

Figure 2. Male and female survival (in days) in *Tenebrio molitor* in relation to state of health due to pathogens (Negative control, Tween control, Non-viable spores and Fungus) and diet [protein (p) and carbohydrate (c), % dry mass: p80:c0, p64:c16, p40:c40, p16:c64 and p0:c80] treatments.

Table 3. Results of the GLM evaluating the effects on the survival of the females of *T. molitor* by day, the health status of their mate and their diet; significant effects ($P < 0.05$) are in bold

	d.f.	χ^2	*P*
Day	1	10.936	**< 0.001**
Mate's state of health	3	1.723	**< 0.001**
Diet	4	10.463	**< 0.001**
Day × Mate's state of health	3	1.475	**< 0.001**
Day × Diet	4	0.936	**< 0.001**
Mate's state of health × Diet	12	3.911	**< 0.001**
Day × Mate's state of health × Diet	12	0.556	0.064

associated trade-offs. For example, tolerance to cold is higher when carbohydrate is supplemented with protein, but this is not the case for males (Andersen *et al.*, 2010). Regardless, the assumption that a higher quality diet (based on increased consumption of carbohydrates) would bias female preferences was not validated. Our impression is that this is the first result of its kind that refutes that idea that

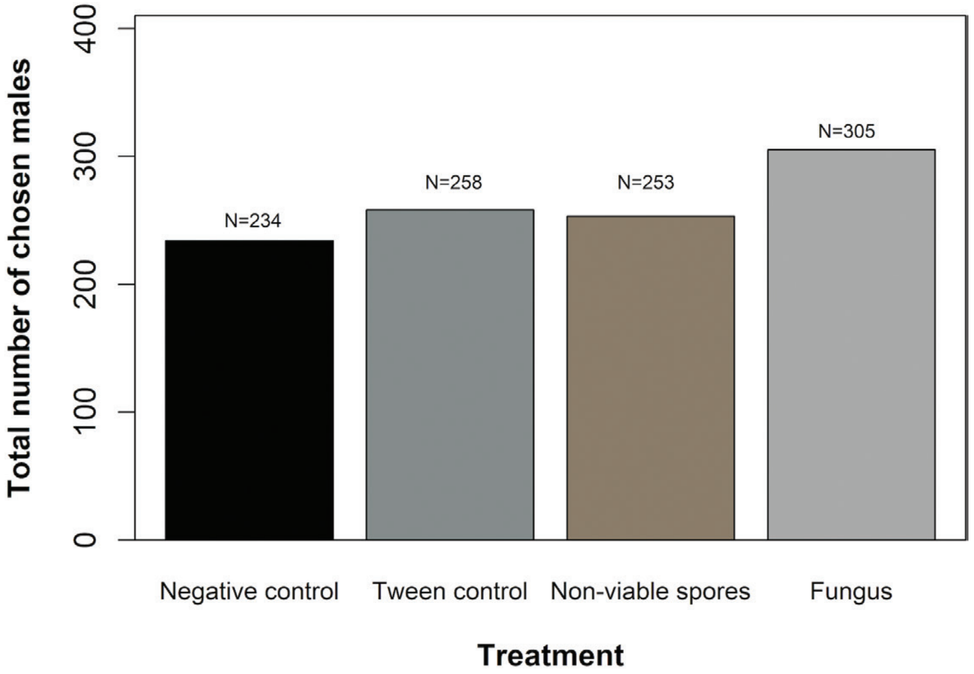

Figure 3. Total number of chosen males by females in *Tenebrio molitor* after olfactometer trials. Only males whose health was manipulated by the fungal pathogen are shown. Number of trials is shown at the top of each bar.

female condition is not relevant in mate choice prior to copulation. As mentioned previously, the reasons why females in poor condition may not have the same preferences as females in good condition are: (1) there are differences in the search or opportunity costs of finding high-quality male phenotypes; (2) when females of different condition compete, females in poor condition will lose; and (c) low-quality females are less attractive than high-quality females. It is important to discuss these reasons to understand our results. Adults of *T. molitor* usually live in colonies where mating is frequent (e.g. Worden & Parker, 2001) and where individuals do not apparently segregate based on their condition (Mellanby, 1932). This assumes that there is no search for mates or that the costs involved in searching are low. However, it is likely that waiting for a good phenotype is crucial, given the number of experiments where females choose males based on their pheromones (e.g. (Worden *et al.*, 2000; Worden & Parker, 2005) or material benefits they receive from males following copulation that increase their fecundity (Worden & Parker, 2001). In this case it may be that the best phenotype is chosen after copulation and that the quality of mates is evaluated during copulatory courtship. Indeed, males perform a number of behaviours that may be interpreted as courtship, such as tapping their antennae on the female's thorax (Carazo *et al.*, 2004). Because we did not examine sequential matings with different males, it is possible that we did not allow females to evaluate males based on their copulatory courtship. With respect to the second possibility, females do not seem to compete with each other given their colonial lifestyle and lack of strong competition for food resources. Finally, females may differ in attractiveness and that this is related to their dietary condition. In insects, size is a good predictor of fecundity (reviewed by Honěk, 1993) and a protein-based diet would make a female more fecund (reviewed by Simpson *et al*, 2018). However, given our experimental design in which females chose, not the other way around, female attractiveness was not tested.

Preference for males with impaired health due to infection by *M. robertsii* is interesting in several respects. On the one hand, it corroborates previous studies where males facing an immune challenge (e.g. Sadd *et al.*, 2006; Kivleniece *et al.*, 2010; Nielsen & Holman, 2012; Krams *et al.*, 2014) including fungi (Reyes-Ramírez *et al.*, 2019) were preferred. These studies concluded that males invested more of their energy resources in pheromone production. The production of pheromones by males of this species is costly. For example, the pheromone of inbred males is less attractive than that of outbred males (Pölkki *et al.*, 2012). Indeed, pheromone production in insects is strongly reduced when they are fed carbohydrate-poor diets (e.g. South *et al.*, 2011). Even though previous studies have not modified the diets of *T. molitor* specifically to decrease pheromone production, our results show that the reduction in carbohydrates still makes fungus-infected males strongly attractive. While the cost of ingesting a lower proportion of carbohydrates and the challenge of fungal infection, Tween and non-viable spores affected mortality in males, investment in pheromones maximizes the opportunity for reproduction. On the other hand, while this was not a study of terminal investment, it does suggest that terminal investment could be operating. This terminal investment would occur even when males vary greatly in their dietary condition. This is important because it could also be expected that males in the best condition (highest-carbohydrate diet, no immune challenge) would not necessarily invest as much in pheromone production. An analogy of this situation is when young individuals, despite an immune challenge, invest less in reproduction than old individuals (e.g. Velando *et al.*, 2006; Creighton *et al.*, 2009). In *T. molitor* the short life expectancy (between 10 and 35 days, depending on condition) may make it worth redirecting resources even when they are young.

Other studies of protein-poor, carbohydrate-enriched diets have also shown an increase in survival in both sexes (Faria *et al.*, 2008; Cotter *et al.*, 2011; Sentinella *et al.*, 2013). Several studies have indicated that effects of diet should be assessed from the juvenile stage (e.g. Sentinella *et al.*, 2013), which was not our case. Even with this possible problem, there are reasons to expect differences in how both sexes assign resources. For example, it has been found that for males, a diet that is balanced in proteins and carbohydrates maximizes survival, while for females, a carbohydrate-rich diet is optimal (Roeder & Behmer, 2014). The fact that females invest in egg production while males have to invest in sexual traits explains the difference in optimal diet (Simpson *et al.*, 2018).

ACKNOWLEDGEMENTS

The project PAPIIT IN206618 and Consejo Nacional de Ciencia y Tecnología (CONACyT) provided financial support. We thank Kevin Judge for his key input to an early version of the paper. A.R.R. is a doctoral student from Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM) and was supported by CONACyT: 778814/612503. The authors declare that they do not to have any conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website.

Figure S1. The olfactometer used for female choice in *Tenebrio molitor*. The two ports where one male was placed and restricted are located in A and A. B is the gate that impeded the access to females previous to the experiment, but that was opened once the experiment started. C is where the female was placed at the onset of the experiment.
Table S1. Comparisons of the different diets and their effect on the survival of *T. molitor* males. All combinations of the five synthetic diets are shown in the first column. These diets vary in their concentration of proteins and carbohydrates (p:c). Significant effects ($P < 0.05$) are in bold.
Table S2. Survival comparisons of the different groups according to their health state, and its effect on the survival of *T. molitor* males. Tukey tests were used. Significant effects ($P < 0.05$) are in bold.
Table S3. Comparisons of the different diets and their effect on the survival of *T. molitor* males. All combinations of the five synthetic diets are shown in the first column. These diets vary in their concentration of proteins and carbohydrates (p:c). Significant effects ($P < 0.05$) are in bold.
Table S4. Survival comparisons of the different groups according to their health state, and its effect on the survival of *T. molitor* males. Tukey tests were used. Significant effects ($P < 0.05$) are in bold.

CAPÍTULO III

Effects of dietary macronutrient balance and fungal infection on spermatophore quality in the flour beetle, *Tenebrio molitor*

Effects of dietary macronutrient balance and fungal infection on spermatophore quality in the flour beetle, *Tenebrio molitor*

Alicia Reyes-Ramírez[1,2], Maya Rocha-Ortega[1] & Alex Córdoba-Aguilar[1]*

[1]Departamento de Ecología Evolutiva, Instituto de Ecología, Universidad Nacional Autónoma de México, Apdo. P. 70-275, Circuito Exterior, Ciudad Universitaria, 04510, Coyoacán, Distrito Federal, México

[2]Posgrado en Ciencias Biomédicas, Universidad Nacional Autónoma de México, Ciudad de México 04510, México

*Corresponding author: acordoba@iecologia.unam.mx

ABSTRACT

1. Males in many insect's species deliver ejaculates with nutritious substances to females in the form of a spermatophore. Different factors can affect spermatophore quality.
2. We manipulated male's diet and health to determine the balance of macronutrients deposited in the spermatophores of *Tenebrio molitor* beetles. For diet, we varied the concentration of proteins and carbohydrates, while for health status we used a fungal infection. Males with different condition copulated with unmanipulated females, and spermatophores were extracted to measure the amount of proteins, lipids and carbohydrates.
3. Diet and infection had an effect on the quality of the spermatophore. Diets with high protein and low carbohydrate contents produced spermatophores with higher protein, carbohydrate, and lipid contents. In contrast, diets with little protein and high in carbohydrates led to poor quality spermatophores. Infected males produced spermatophores with the highest amount of all three macronutrients. In general, spermatophore content was carbohydrates>proteins>=lipids.
4. The fact that sick males produced richer spermatophores can be explained as a terminal investment strategy. On the other hand, the large investment of carbohydrates may be related to the preparation of spermatozoa and eggs.

KEYWORDS: condition, diet, carbohydrate, protein, spermatophore, *Tenebrio molitor*, terminal investment

Running title: Spermatophore, diet and disease

INTRODUCTION

Traditionally it was thought that reproductive success of males was limited only by female access (Andersson 1994) and therefore their investment in gametes should be low (Bateman 1948; Trivers and Campbell 1972). However, we now know that this investment is not trivial (Vahed 1998; Gwynne 2008; Parker and Pizzari 2010; Lehmann 2012). In fact, by selecting high quality males, females increase their fitness or that of their progeny through indirect and direct benefits they receive from males (South et al. 2011; Pischedda and Chippindale 2017; Bachmann et al. 2019). While indirect benefits occur via genes that increase the viability or attractiveness of the progeny (Zahavi 1977; Lande 1981), direct benefits are material resources that increase female's fitness, as is the case of, for example, nuptial gifts (e.g. spermatophores) (South et al. 2011; Lewis et al. 2014; Levin et al. 2016).

In many species of insects, the ejaculate is transferred to the female within a spermatophore – a capsule of albuminous material (Chapman et al. 2013). Females maximize their direct fitness benefits when the spermatophore functions as a nuptial gift (that is, when it provides additional energetic resources to females) (Gwynne 2008; Lewis and South 2012; Boggs 2018). The direct benefits gained will vary with the nutritional value/composition of the spermatophore. In particular, spermatophores rich in proteins promote increased fecundity (Karlsson 1995; Karlsson 1996; Lehmann 2012), which explains why these compounds are the most abundant (e.g. Marshall 1982). However, spermatophores also contain lipids and carbohydrates, though in smaller amounts (Stanley-Samuelson and Loher 1983; Marshall and McNeil 1989; Watanabe and Sato 1993). In the case of lipids, these are also used to produce eggs and synthesize hormones (e.g. Friend 1958; Levinson 1962), while carbohydrates are an additional source of energy, though these are less abundant than proteins and lipids (e.g. Marshall 1982; Mann 2012).

One of the most important factors determining spermatophore quality is male diet (Cahenzli and Erhardt 2013; Muller et al. 2015). For example, restricting diet induced males to produce smaller spermatophores in butterflies (Ferkau and Fischer 2006), katydids (Gwynne 1993) and bush-crickets (Hare and Simmons 2020). While studies of this kind are conclusive in explaining the role of calories in the diet, we know little about the contribution of specific dietary components (i.e. macronutrients). One way for determining this is the geometric framework (GF) of nutrition, which allows us to examine the effect of specific nutrients on the expression of traits and measures of fitness (Simpson and Raubenheimer 1993; South et al. 2011; Simpson et al. 2018). The GF is an analytical methodology that is based on the logic of state-space geometry aimed to characterize the key variables responsible for the regulation of nutrients (Raubenheimer et al. 2009). The geometric space may include one or more nutrients, the current and optimal nutritional states of the organism, the efficiency with which such nutrients are used as well as the rates of excretion and/or any other performance measure (Raubenheimer et al. 2009). Studies that have determined the effect of diet on spermatophores, have used the GF to understand ejaculate quality like sperm count and viability in cockroaches (Bunning et al. 2015), ants (Dávila and Aron 2017) and crickets (Ng et al. 2018). However, the results of these studies are contradictory. While in cockroaches a diet restricted in proteins maximized sperm production, in ants restricting the same macronutrient led to a decrease in sperm number. On the other hand, a low protein diet increased sperm viability in crickets but had no effect in cockroaches or ants. Thus, these three studies do not show the same directions in sperm traits (of course, sperm traits [as documented by these three studies] are different to spermatophore quality traits). Thus, it is difficult to make predictions about what to expect in terms of the effect of the balance of proteins, lipids, and carbohydrates in the diet on the general quality of the spermatophore. Given that spermatophore's macronutrient composition involves a high quantity of proteins followed by lipids and carbohydrates (Marshall 1982), it implies that this should be the natural balance of male's spermatophore investment.

Diet restrictions are not the only pressures that organisms face in nature. Another type of pressure is infection, where pathogens weaken the state of health and therefore, condition. The combined effect of dietary macronutrient and infection has been investigated in several organisms (Lee et al. 2008; Nestel et al. 2016). In this respect, diets with higher protein content led to a more effective response to disease (Lee et al. 2008; Alaux et al. 2010; Povey et al. 2014; Cotter et al. 2019). However, while a diet low in protein will negatively affect an immune response against infections in insects, it would allow to maintain the balance of the proteins>lipids=carbohydrates in the spermatophore, although its investment will be less so than with a protein rich diet.

The flour beetle *Tenebrio molitor* has been used as a study subject to investigate the effects of diet and pathogens on reproduction. Males of this animal transfer a spermatophore to the female during copulation, which increases the number of eggs laid (Drnevich et al. 2001; Worden and Parker 2001). A recent study did not detect differences in spermatophore quality when varying the proportion of digestible food in the male's diets (McConnell and Judge 2018). However, McConnell & Judge's study did not use synthetic diets, which does not allow assessment of the effect of a particular macronutrient on the spermatophore. Related to this, the protein:carbohydrate ratio (p:c) has been studied in this animal in terms of life expectancy and fecundity. In an initial study, Rho and Lee (Rho and Lee 2016) found that while reproductive success should be maximized at a p:c ratio of 1:1, an excess of protein reduces life expectancy in both sexes. In a second study on the same animal, lifespan increased when proteins were absent, but carbohydrates elevated in healthy males and males infected with the entomopathogenic fungus *Metarhizium robertsii* (Reyes-Ramírez et al. 2019a). Thus, these two studies coincide in their findings that increased protein consumption negatively affects longevity in males, independent of health status.

In this study we investigated the effects of varying nutritional and health status using *T. molitor* male adults on spermatophore quality. First, we varied the nutritional status using different p:c proportions following the GF for nutrition. Second, we varied health status by infecting males with the fungus *Metarhizium robertsii* versus uninfected groups. As a response variable, we measured the quality of the spermatophore in terms of proteins, carbohydrates, and lipids. Although a protein-based diet is harmful for animal's survival (Rho and Lee 2016; Reyes-Ramírez et al. 2019a), we predict that protein must be consumed to satisfy the demand of high investment in the spermatophore. This is, independently of how much protein has been consumed and the state of health, a large amount of this macronutrient must be allocated to the spermatophore. With respect to carbohydrate and lipids, these macronutrients must always be found in the spermatophore but to a less degree than proteins.

MATERIALS AND METHODS

Insect maintenance

Individuals were kept in a controlled environment chamber at 70% humidity and 25 ± 2ºC (average ± standard deviation) with a 12:12 photoperiod. These conditions were maintained during all life stages of *T. molitor*. We placed 200-300 larvae in plastic containers (30.5 cm diameter x 10.5 cm height) to decrease cannibalism (Weaver and McFarlane 1990). Larvae were fed with wheat germ (Maxilu® brand) and apple slices. Pupae were sexed by observing the eighth abdominal segment (Bhattacharya et al. 1970) and were placed in plastic containers (22.1 cm length x 15.4 cm width x 5.7 cm height). Males and females were separated as soon as they emerged to ensure they were virgins at the time of the experiment. Both sexes were placed individually in plastic containers (4.2 cm diameter x 3.8 cm tall). Females were fed the same diet as when they were larvae and males were fed synthetic diets (see below).

Synthetic diets

To vary the nutritional condition of males only, five synthetic diets were established (Dadd 1960; Simpson and Abisgold 1985) that differed in their proportion of proteins to digestible carbohydrates (% dry mass p:c). Proteins consisted of a mix of casein, peptone, and albumin in a 3:1:1 proportion, while carbohydrates were sucrose and dextrin in a 1:1 ratio. The diets were composed of the following proportions: 1) 80% proteins and 0% carbohydrates (p80:c0); 2) 64% proteins and 16 % carbohydrates (p64:c16); 3) 40% proteins and 40% carbohydrates (p40:c40); 4) 16% proteins and 64% carbohydrates (p16:c64); and, 5) 0% proteins and 80% carbohydrates (p0:c80). All diets contained the same proportion (20%) of the following nutrients: Wesson salt mixture (2.4%), cholesterol (0.5%), linoleic acid (0.5%), ascorbic acid (0.3%), and a vitamin mixture (0.2%). To complete the diet, cellulose (16.1%) was added as a non-nutritious agent. The reagents used were all purchased from Sigma-Aldrich.

Recently emerged adult males were randomized into one of the five synthetic diets (for graphical illustration of experimental design, see Figure 1). These diets were provided *ad libitum* in feeders (the previously mentioned plastic containers) in a “food dish” (inverted top of a 1.5 mL Eppendorf tube [9 mm diameter, 5 mm depth]) during the experimental period (day 0 to day 15). The males also had access to water *ad libitum* (20 µl of water per day). The “dishes” were removed and replaced with new ones every other day.

Preparation of the fungus and determination of LD_{50}

The fungus *M. robertsii* (ARSEF 2134) was obtained from the Agricultural Research Service of the United States Department of Agriculture. Spores were stored in 10% glycerol at -80ºC until use. The inoculum was prepared by seeding spores on Sabouraud Dextrose Agar (SDA) plates and then incubating in the dark at 28ºC for 15 days. After this period, the conidiophores were harvested by scraping the plates and suspending them in 0.03% Tween 80 solution (hereafter referred to as Tween). The suspension was mixed for 5 minutes in a vortex, then filtered using cotton mesh to separate the conidia from the mycelium. Using the SDA plate technique (Goettel and Inglis 1997), it was determined that the relative viability of the conidia was above 95%. The LD_{50} (median lethal dose) was obtained from this filtrate. This LD_{50} ($3x10^5$ conidia/mL) has previously been reported by Reyes-Ramírez et al. (2019b).

Variation in the state of health due to pathogens in males

To vary health status in males, we used the same males whose nutritional condition was modified (see Figure 1). Within each diet group, four health status groups were formed. Each group contained 30 mature individuals of 12-15 days of age (Gerber 1976) named as follows: (i) negative control (unmanipulated individuals); (ii) Tween control, individuals submerged in Tween for 5 seconds; (iii) spore-treated (non-viable spores) individuals submerged for 5 seconds in Tween with *M. robertsii* (ARSEF 2134) spores that had been previously exposed to high temperatures to make them inviable; and (iv) fungus-treated, individuals submerged in Tween with $3x10^5$ conidia/mL (LD_{50}) of *M. robertsii* for 5 seconds.

Obtaining and dissecting spermatophores

Three days after the male's health status was modified, pairs were randomly formed using females in good condition and the different groups of males (variation in nutritional and health status). Each pair was placed in plastic containers (4.2 cm diameter x 3.8 cm height) to allow them to copulate. The males touch the females with their legs and antennae until they manage to insert the copulatory organ (Font and Desfilis 2003). After the intrusion, the pair remains together for a variable period of time, usually 1 to 2 minutes; copulation ends once the male removes the copulatory organ (Font and Desfilis 2003). Immediately after completing one copulation, females were removed, frozen dry at −20ºC and refrigerated to later extract the spermatophores. The spermatophores were gently removed from the bursa copulatrix with forceps and dissecting pins. All dissections were carried out in *Tenebrio* saline buffer (Butz 1957), which was also used to store them individually in 2 mL Eppendorf tubes. The spermatophores were kept at -20ºC until macronutrient measurement.

Protein, lipid and carbohydrate measurement

The spermatophores were transferred to new Eppendorf tubes with 180 µL of aqueous lysis buffer [100 mm KH2PO4, 1 mm dithiothreitol (DTT) and 1 mm ethylenediaminetetraacetic acid (EDTA), pH 7.4], to later be disrupted in a Tissue Lyser-II (Qiagen, Valancia, California) for 30 s at 25 Hz. To quantify the amount of nutrients present in the spermatophores, we used the unified Foray method (Foray et al. 2012). In this method, different solvents are used to sequentially extract the desired components and quantify them using specific colorimetric techniques. Briefly, after agitating, the proteins in the samples were solubilized in a phosphate lysis buffer and quantified with the Bradford method (Bradford 1976). Bovine serum albumin was used as a standard and absorbance was determined at 595 nm. In the case of total lipids and carbohydrates we used the Van Handel & Day method (Van Handel 1985; Van Handel and Day 1988). For lipids, we did a vanillin assay with trioleate glycerol as a standard and measured absorbance at 515 nm. For carbohydrates, the anthrone colorimetric method was used with d-glucose as a standard and absorbance determined at 630 nm. All absorbance measurements were done on an absorbance reader (Absorbance Reader ELx800; BioTek Inc., Winooski, Vermont). The concentration of the three macronutrients was calculated taking into account the size (mm^2) of the spermatophores. The size was obtained from photos analyzed in a publicly available image program (ImageJ).

Statistical analysis

To determine how diet, state of health and the interaction between diet and state of health affected the amount of macronutrients present in spermatophores, an analysis of variance (ANOVA) per nutrient was constructed. In the first model, the amount of protein corresponded to the dependent variable, while diet and state of health were the independent variables. In the second model, the amount of lipids corresponded to the dependent variable, while diet and state of health were the independent variables. In the third model, the amount of carbohydrates corresponded to the dependent variable, while diet and state of health were the independent variables. Following statistical significances among independent variables, we made comparisons between treatments using the post-hoc Fisher's Least Significant Difference test (LSD). All analyses were carried out in R (Team 2017).

RESULTS

Protein

The results indicated that diet, health status, and the interaction between diet and health status, had an effect on the amount of total protein in the spermatophore (Table 1). Individuals that were fed p80:c0, p64:c16 and p0:c80 diets had spermatophores with higher protein, while individuals fed with the p16:c64 diet had lower protein amount (for all combinations, see supplementary material Table S1).

The health status of the males also affected the amount of proteins in their spermatophores. The spermatophores produced by males treated with the fungus had the highest protein content, followed by Tween control and spore-treated males, and finally the negative control (for all combinations see supplementary material Table S2).

Lipids

The total lipid amount in the spermatophores varied depending on diet, health status, and the interaction between diet and health status (Table 1). The diet with which males invested the most lipids in spermatophores was p64:c16, while the p80:c0, produced the lowest lipid content (for all combinations see supplementary material Table S3).

Likewise, the health status of the males influenced on the lipids deposited in the spermatophore. Tween control males and those treated with fungus produced spermatophores with the highest total lipid amount, followed by the negative control, and finally the spore-treated males (for all combinations see supplementary material Table S4).

Carbohydrates

Finally, diet, health status, and the interaction between diet and health status affected the total carbohydrate content present in the spermatophores (Table 1). Individuals fed with the p64:c16 diet invested more carbohydrates in their spermatophores, while those fed with the p16:c64 showed the lowest investment (for all combinations see supplementary material Table S5).

Health status also had an effect. Fungus-treated males again produced the spermatophores with the highest carbohydrate content, followed by Tween controls, spore-treated males, then negative controls (for all combinations see supplementary material Table S6).

Balance of proteins/lipids/carbohydrates in spermatophores

In general, visual inspection of the proportion of the three components (see supplementary material Figure 1) suggested the following. Most treatments yielded an order of c>p>l in spermatophores. Some exceptions to this were the negative control males and spore-treated males under the 80:0 diet, in which the balance was protein=carbohydrate>lipid, and the Tween control and fungus-treated males on the 64:16 diet and the negative control on the 40:40 diet, in which the balance was carbohydrate>protein=lipid (see also Figure 2).

Given the trends described above, the proportion assigned to each macronutrient also showed notable differences (see Supplementary material Figure 1). For example, carbohydrates were considerably higher than proteins and lipids in most treatments, especially in fungus-treated males and, to a lesser degree, Tween controls. These differences, however, were not as dramatic in the negative control or spore-treated males.

DISCUSSION

As has been corroborated in other studies where animal's condition is manipulated to observe the effects on the spermatophore (e.g. Ferkau and Fischer 2006; Kelly and Gwynne 2016; Duplouy et al. 2018), in *T. molitor* the macronutrient composition of the spermatophore was sensitive to changes in male's diet and health status. This condition-dependence is expected for energetically demanding characteristics (Kotiaho 2001; Pitnick et al. 2009; Macartney et al. 2018). The case of the spermatophore may be very costly both in quality (Wiklund and Kaitala 1995; Wagner JR 2005; Duplouy et al. 2018) and quantity (del Castillo and Gwynne 2007; Lehmann and Lehmann 2009; Kerr et al. 2010). In the case of diet, we found that in general the p64:c16 ratio was the diet that led males to invest more in the three macronutrients, while the inverse diet, p16:c64, reduced levels. In this way, it can be said that the ideal diet to produce a robust spermatophore is p64:c16, though this would need to be corroborated with a study of reproductive success. In fact, it is common not to find a consensus on a single diet that maximizes the ejaculate traits of different insects (e.g. Bunning et al. 2015; Dávila and Aron 2017; Ng et al. 2018). The most similar study is one in *Drosophila melanogaster*. In this animal egg production is depressed in flies that are fed with a diet without proteins and they laid the most eggs when fed with a high protein diet, in a proportion of 4:1 (Lee 2015), similar to our p64:c16 diet. This suggests that protein is essential for offspring survival. However, it should be noted that that such ideal diet is not optimal for the survival of the male, since protein ingestion reduces lifespan (Rho and Lee 2016; Reyes-Ramírez et al. 2019a). Perhaps the consumption of protein by adults serves to resolve a trade-off between the demands of the spermatophore and what the animal needs to survive. Findings were similar in the cricket *Teleogryllus oceanicus*, where protein consumption similarly decreased survival but increased the song used for courtship and the production of cuticular hydrocarbons (Ng et al. 2018). Like in *T. molitor*, this would assume a trade-off between survival and reproductive traits. In the case of health status, male *T. molitor* infected with the fungus invariably deposited more of all three macronutrients than the other groups. This investment was again clearest under the p64:c16 diet (and considerably less in the p16:c64 diet), which would be expected if it is ideal. In *T. molitor*, protein ingestion does not increase the probability of surviving an attack by *M. robertsii* (Reyes-Ramírez et al.

2019a) but protein is necessary for the spermatophore. It is striking that individuals fed on the diet that did not have any protein intake (p0:c80) did not differ in the content they invested of this nutrient in their spermatophores. On the one hand, we must remember that nutritional variation in this study occurred in the adult stage. So, it is possible that the reserves that accumulated during the larval stage influence. On the other hand, in another study it was found that in the p0:c80 diet, both healthy and sick males increased their life expectancy (Reyes-Ramírez et al. 2019a). In this case, by decreasing the risk of dying, males could be allocating their protein reserves towards the production of spermatophores.

Why do sick males invest more in the spermatophore compared to unmanipulated males? One explanation is what is known as terminal investment, where individuals whose survival is at risk invest more in reproduction to maximize their reproductive opportunities (Williams 1966; Clutton-Brock 1984; Duffield et al. 2017). In the case of *T. molitor*, the traits that will yield the most direct benefits are the spermatophores. There are several sources of evidence supporting terminal investment in this animal. The first has to do with male pre-copulatory attractiveness: males challenged with inert materials like nylon implants or lipopolysaccharide (Sadd et al. 2006; Kivleniece et al. 2010; Nielsen and Holman 2012; Krams et al. 2014) as well as males infected with the same fungus used in this experiment, *M. robertsii* (Reyes-Ramírez et al. 2019b), were more successful in attracting females via pheromones. The second source has to do with post-copulatory success: males infected with pathogens provided more proteins in their spermatophores (Hurd and Ardin 2003), which is similar to what we found here. Paradoxically, increased investment in the spermatophore did not lead to higher reproductive success in *T. molitor* as males infected with *M. robertsii* gave rise to fewer eggs, with lower hatching success and lower lipid content (Reyes-Ramírez et al. 2019b). Thus, it may be that females are attracted to males that are sick and have a higher macronutrient content in their spermatophores but then penalize them with a lower egg number and quality. This supposes that females recognize males that are carrying out terminal investment. Interestingly, females of this species benefit substantially by using the spermatophores from several males to increase their reproductive success (Drnevich et al. 2001; Worden and Parker 2001). This ability to "collect" spermatophores as an additional nutrient source but discard the sperm of terminally investing males would imply a large degree of reproductive control by females of this species.

Contrary to our expectation, the nutrient balance of the spermatophore was almost always carbohydrates > proteins > lipids. The protein contribution is expected to be considerably higher to promote increase egg production (Marshall 1982; Murphy and Krupke 2011; Mann 2012). However, an alternative explanation for our results is that carbohydrates play a role in sperm success. In this respect, it has been shown that the seminal fluid of several species of insects generally contains a mixture of short chain carbohydrates or sugars such as fructose, glucose and trehalose (Yaginuma and Happ 1988). Indeed, in *T. molitor* the presence of glucose and trehalose has been reported in both the bean-shaped accessory glands and in the spermatophore, with trehalose being the more abundant (Gadzama and Happ 1974). There are two possible functions of trehalose: 1) it plays a key role in the successful evacuation of the spermatophore, and/or 2) it participates in the nutrition and activation of the sperm (Yaginuma and Happ 1988). Trehalose has also been found in the eggs of this beetle, where it is converted to glucose for use by the embryo during development (Ludwig et al. 1968). In fact, in insects, trehalose is the main metabolic energy source (Thompson

2003; Tang et al. 2014), synthesized mainly in the fat body and rapidly released into the hemolymph (Tang et al. 2010).

Finally, our study shows that spermatophore characteristics are dynamic, influenced by the male's condition. While the adaptive function of the changes to macronutrient composition we observed is not clear, they are expected to have differential effects on reproduction. One key topic would be to measure food intake to see how animals respond to infection. For example, given a pathogen attack, animals may either show a reduction (to reduce the ingested food that would be available for the pathogen) (Kyriazakis et al. 1998; Adamo et al. 2007) or increase (to provide energetic resources to their immune system) (Slansky Jr 1986; Ponton et al. 2013) in food intake. Our study should be replicated in other systems where the spermatophore plays a key role in the evolution of mating systems. Examples include the cases of butterflies (Oberhauser 1988; Arnqvist and Nilsson 2000; Cardoso and Silva 2015) and crickets (Burpee and Sakaluk 1993; Kerr et al. 2010; Sturm 2014) where experiments have not included analysis of macronutrients but whose effects can be key to reproductive success. In the case of *T. molitor*, the differential effects of diet probably combine with female's parental investment decisions.

Acknowledgements

This study was supported by a PAPIIT grant IN 206618 and Consejo Nacional de Ciencia y Tecnología (CONACyT). Alicia is a doctoral student from Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM) and was supported by CONACyT: 778814/612503. The authors declare that they do not have any conflicts of interest.

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Table

Table 1. Results of three analyses of variance whereby each evaluates the effects of diet and state of health treatments on each of three nutrients (proteins, lipids and carbohydrates) present in the spermatophore of *Tenebrio molitor* beetles. Significant differences appear in bold.

		d.f.	**F**	***P***
Proteins				
	Diet	4	4.4	**<0.01**
	State of health	3	14.36	**<0.001**
	Diet × State of health	12	3.26	**<0.001**
Lipids				
	Diet	4	49.82	**<0.001**
	State of health	3	29.04	**<0.001**
	Diet × State if health	12	9.46	**<0.001**
Carbohydrates				
	Diet	4	21.92	**<0.001**
	State of health	3	37.2	**<0.001**
	Diet × State of health	12	3.49	**<0.001**

Figures

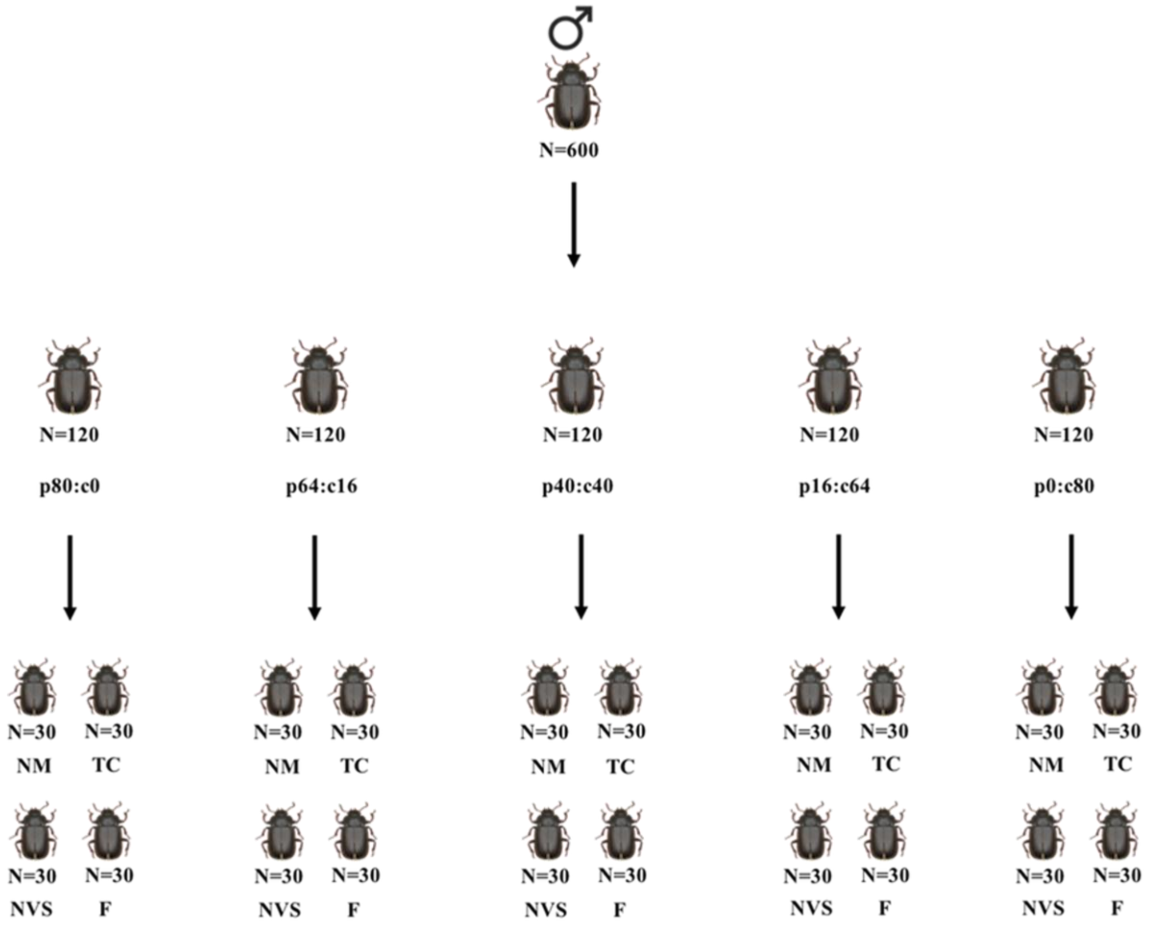


Figure 1. Diagrammatic representation of the experimental design showing the distribution in the five artificial diets (p:c) as well as treatments according to male state of health (NM: non-manipulated, TC: Tween control, NVS: non-viable spores and F: fungus-treated) in *Tenebrio molitor*.

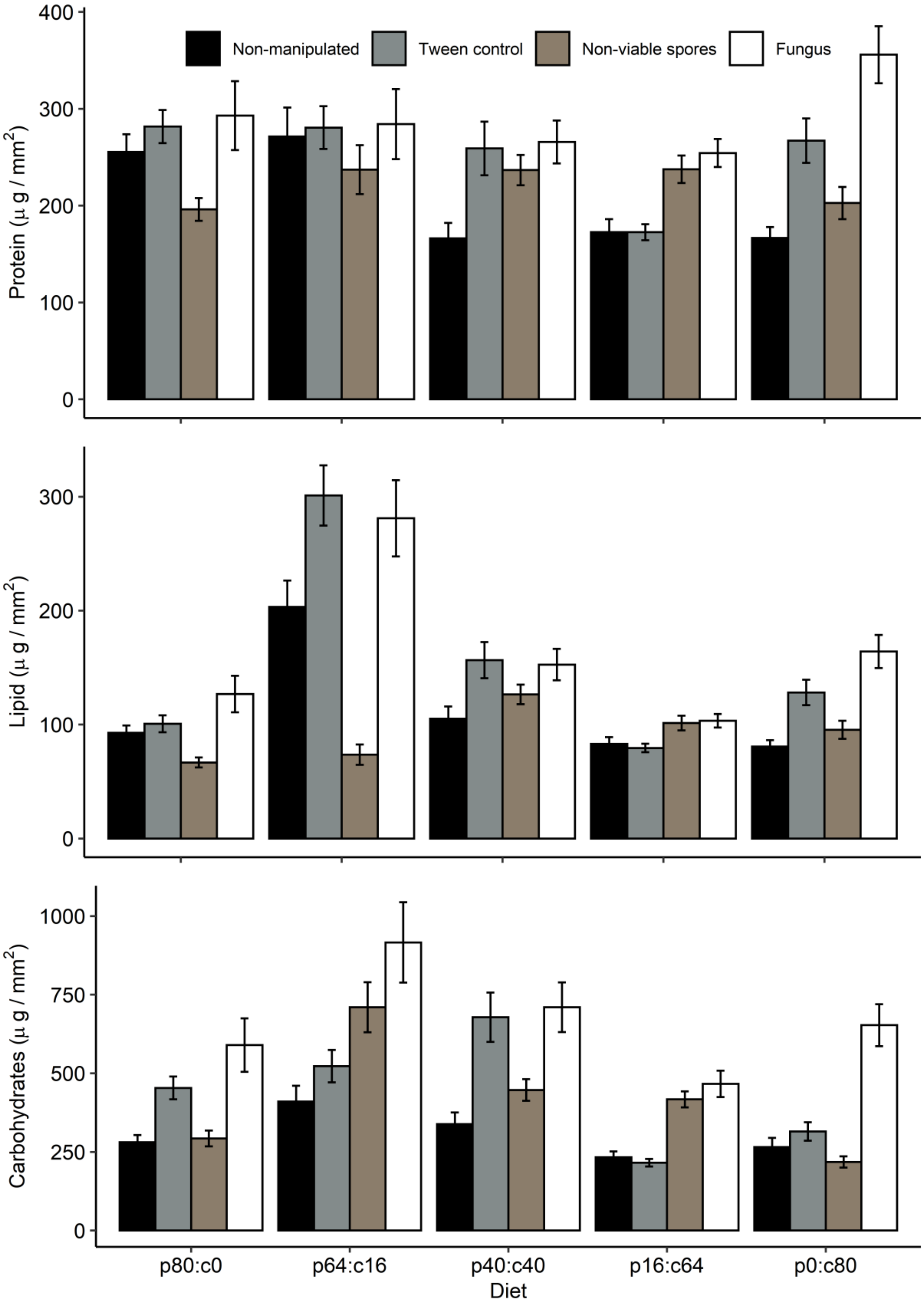
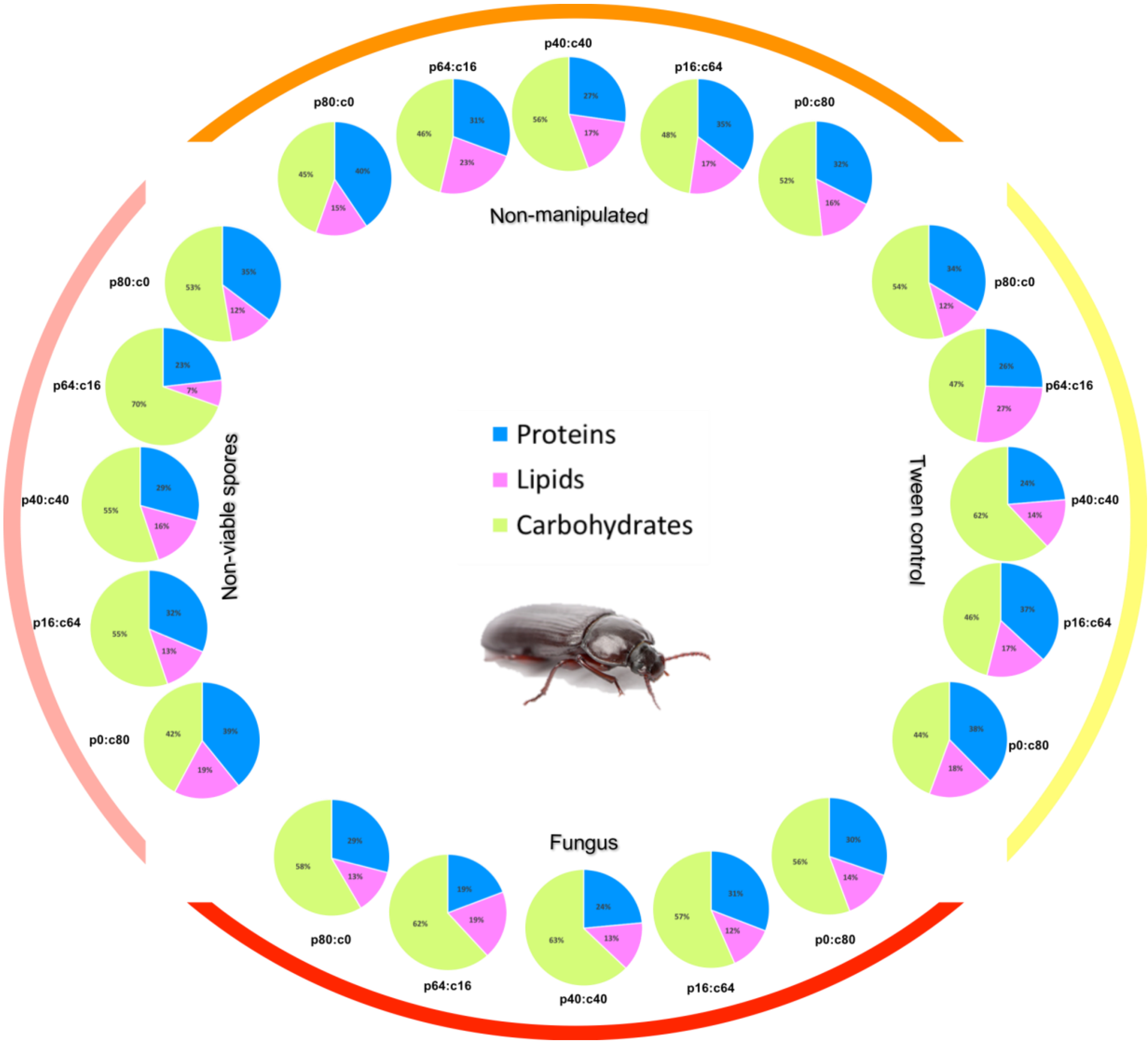

Non-manipulated
Tween control
Non-viable spores
Fungus
Protein (μ g / mm^2)
400
300
200
100
0
Lipid (μ g / mm^2)
300
200
100
0
Carbohydrates (μ g / mm^2)
1000
750
500
250
0
p80:c0
p64:c16
p40:c40
p16:c64
p0:c80
Diet

Figure 2. Changes in spermatophore nutrient content (carbohydrate, lipid and protein; mean ±SE) following changing ratios of protein:carbohydrate (p:c) and experimental manipulation of status of health in the *Tenebrio molitor* males.



Supplementary material Figure 1. Visual comparisons of relative carbohydrate, lipid and protein allocation to spermatophore following changing ratios of protein:carbohydrate (p:c) in the male diet and manipulation of health status (fungus: fungal challenged males; and controls: Tween manipulated, non-viable spores, and non-manipulated) in *Tenebrio molitor* males.

DISCUSIÓN Y CONCLUSIONES GENERALES

Discusión general

Para los insectos como para otros animales, es fundamental consumir de manera equilibrada los distintos nutrientes que requieren (Jensen et al., 2012; Simpson & Raubenheimer, 2012; Simpson et al., 2018). En el caso de *T. molitor* encontré que los adultos consumen mayores cantidades de las dietas ricas en carbohidratos en comparación con las dietas ricas en proteínas, inclusive después de haber pasado por un reto inmune. En un principio esta conducta es contraria a lo reportado para otros insectos, en donde se ha demostrado el papel positivo que tienen las proteínas para aumentar la respuesta inmune (Lee et al., 2006; Povey et al., 2014). Sin embargo, en otras especies en donde al igual que en *T. molitor*, se prefieren dietas sesgadas a carbohidratos, se encontró que los individuos maximizaron la expresión de rasgos reproductivos (por ejemplo, mayor producción de feromona, machos más atractivos, mayor producción de descendencia, etc.) (Bunning et al., 2016; Maklakov et al., 2008; South et al., 2011). Es sabido que la expresión de estos rasgos determinantes de la adecuación, dependen de la cantidad y combinación de nutrientes ingeridos. Aún así, los óptimos dietéticos pueden ser específicos para cada rasgo y, por lo tanto, la mejor dieta para una mayor adecuación general estará influenciada por la edad y condición del individuo, así como por el ambiente (Lihoreau et al., 2015; Senior et al., 2015). Por otro lado, una sobreingesta o subingesta puede representar grandes costos en la adecuación (South et al., 2011). Por ejemplo, en las cucarachas se ha encontrado que una ingesta excesiva de proteínas disminuye su longevidad (Haydak, 1953; Mullins & DE, 1975). Es probable que la preferencia observada hacia los carbohidratos esté relacionada con una variedad de indicadores de adecuación que afectan la fecundidad y la supervivencia.

Los machos infectados con el hongo fueron los que consumieron la menor cantidad de alimento en las distintas dietas. La respuesta inmune se ha demostrado que demanda una gran cantidad de energía (Ardia et al., 2012; Bonneaud et al., 2003; Schmid-Hempel, 2005), por lo cual, en un inicio, no parece tener sentido que los machos enfermos disminuyan su ingesta de alimento. Sin embargo, como señalo en el primer capítulo, la anorexia en insectos podría ser una conducta adaptativa que limita el acceso de nutrientes para los patógenos (Adamo et al., 2007; Kyriazakis et al., 1998). En el caso particular del hongo con el que infecté a los escarabajos, *M. robertsii*, sabemos que a pesar de que las esporas cuentan con reservas de lípidos (Wang & Leger, 2005), éstas no son suficientes para su germinación (Leger et al., 1989). De tal manera que los machos al comer menos estarían disminuyendo las posibilidades de que el hongo se desarrolle. Es necesario realizar nuevos experimentos para poner a prueba esta hipótesis.

La anorexia tuvo un impacto en el contenido de macronutrientes que presentaron los adultos de ambos sexos, ya que los machos enfermos tuvieron menos reservas de proteínas y lípidos. A su vez, las hembras con las que se aparearon también se vieron afectadas, pues presentaron un patrón similar en su contenido de nutrientes. Aunque en el caso particular de los carbohidratos los machos enfermos y las parejas con las que se aparearon presentaron la mayor cantidad. Los carbohidratos en esta especie, además, parecen estar incrementando la expectativa de vida tanto de los machos como de las hembras. Los machos sanos vivieron más en comparación con los otros tratamientos (control Tween, retados con esporas no viables y retados con el hongo), excepto en la dieta rica en carbohidratos en donde no se encontraron diferencias entre ellos. Este patrón sugiere que la dieta sesgada a carbohidratos está aportando los nutrientes necesarios para que los grupos manipulados no entren en una disyuntiva en la asignación de sus recursos y de esta manera puedan tener un desempeño similar al de los individuos sanos. En el caso de las hembras, no afectó su supervivencia el estado de salud del macho con el que se aparearon, pero también vivieron más en las dietas ricas en carbohidratos. En diferentes insectos, como moscas, grillos, hormigas y abejas se han encontrado resultados similares, en donde dietas bajas en concentraciones de proteínas y altas en carbohidratos están asociadas con una mayor esperanza de vida (Dussutour & Simpson, 2012; Fanson & Taylor, 2012; Harrison et al., 2014; Jensen et al., 2015; Lee et al., 2008). Cabe resaltar que estos estudios también han utilizado el marco geométrico para la nutrición. La evidencia de los mecanismos que vinculan este tipo de dietas con un aumento en la esperanza de vida es limitada (Solon-Biet et al., 2015). Se cree que el efecto se da a nivel del metabolismo y la función mitocondrial, lo cual influye en el envejecimiento de los organismos, aunque las rutas se han estudiado en ratones (Le Couteur et al., 2016; Solon-Biet et al., 2015).

En cuanto a la elección femenina de pareja, la dieta no modificó las preferencias de las hembras, pero el estado de salud de los machos sí influyó en su elección. Cuando las hembras tuvieron la opción de elegir entre machos sanos y machos infectados con el hongo, prefirieron en las cinco dietas a los machos enfermos. Con otro tipo de retos inmunes (por ejemplo, implantes de nylon y la tenia *H. diminuta*) se han reportado resultados similares, los machos enfermos de *T. molitor* resultan ser más atractivos para las hembras (Kivleniece et al., 2010; Krams et al., 2014; Nielsen & Holman, 2012; Sadd et al., 2006). Los machos enfermos son exitosos en conseguir pareja, pero su supervivencia es menor, lo que sugiere una posible inversión terminal. La hipótesis de la inversión terminal propone que los individuos al censar que sus posibilidades de sobrevivir son muy bajas, invierten sus recursos hacia la reproducción en lugar de a la respuesta inmune para atacar al patógeno (Williams, 1966). La producción de feromonas es costosa, además en *T. molitor* su función no se limita a la atracción de pareja, mediante ellas se transmite información importante sobre la calidad individual de una posible pareja (August, 1971; Hurd & Parry, 1991; Vainikka et al., 2006). El aumento en el atractivo puede entonces deberse a una mayor asignación de recursos hacia la señalización sexual, en este caso a la producción de feromonas (Tanaka et al., 1986), como una última oportunidad de aumentar su adecuación mediante la reproducción (Clutton-Brock, 1984).

Elegir a los machos enfermos como pareja tiene repercusiones en la adecuación de las hembras al producir progenie de menor calidad (menor cantidad de lípidos, volumen y probabilidades de eclosionar) cuando ellas se encuentran en buena condición de salud (Reyes-Ramírez et al., 2019). En este proyecto investigué la calidad de los regalos nupciales que las hembras reciben durante la cópula como una posible fuente de recursos para la producción de huevos. Contrario a lo que esperaba, los machos enfermos produjeron los espermatóforos con la mayor concentración de proteínas, lípidos y carbohidratos. Este resultado refuerza la hipótesis de que los machos de *T. molitor* están realizando una inversión terminal, no sólo reasignando sus recursos hacia la producción de feromonas, sino también a la producción de espermatóforos. El hecho de que una mayor inversión en el espermatóforo no se traduzca ni en una mayor calidad de los huevos ni en una alta tasa de eclosión, puede deberse a que la espermatogénesis de los machos se está viendo afectada (Biwot et al., 2020; Radhakrishnan & Fedorka, 2012), o a que esté ocurriendo una elección críptica de pareja, en donde las hembras estén sesgando los recursos que invierten en la progenie (Eberhard, 1991; Eberhard & Cordero, 1995). Si bien previo a la cópula las hembras prefieren a los machos infectados, durante y después de la cópula es posible que detecten que tales machos no se encuentran en buena condición, ya sea por un mal desempeño en el cortejo u otro tipo de señales. Estas hipótesis fueron evaluadas, determinamos si la conducta de cortejo copulatorio (movimientos de patas y antenas) que el macho ejecuta cuando se encuentra sobre la hembra se relaciona con su éxito reproductivo, en términos del número de huevos. Los machos utilizados fueron tanto sanos como infectados con el hongo *M. robertsii*. No encontramos relación entre la intensidad de cortejo y la condición experimental del macho. Esto indica que las hembras no obtienen información de la condición del macho mediante los rasgos de cortejo evaluados. Una alternativa es que otros posibles rasgos masculinos que no medimos estén involucrados como la conducta de resguardo que se presenta al finalizar la cópula (Font & Desfilis, 2003) o bien características más fisiológicas como la viabilidad y el número de espermatozoides.

En esta investigación se planteó un escenario más realista de la elección femenina de pareja, en donde la condición de ambos sexos varía. Usando dos fuentes relevantes para la variación, como lo son la nutrición y la inmunidad de los individuos. Los resultados demuestran que existe una interacción entre la dieta y la respuesta inmune, así como sus repercusiones en otros rasgos de historia de vida. Utilizar al marco geométrico para la nutrición permitió una comprensión más detallada sobre la relevancia de un nutriente sobre el otro, en este caso los carbohidratos parecen tener un mayor impacto positivo en la condición de los individuos en comparación con las proteínas. La variación del estado nutricional en este estudio ocurrió en la etapa adulta del escarabajo, por lo que en posteriores trabajos se puede aplicar esta manipulación desde el estadio larval. En la mosca *Telostylinus angusticollis*, la esperanza de vida de los adultos de ambos sexos era más corta, cuando en el estadio larval fueron alimentados con una dieta pobre en proteínas, mientras que los carbohidratos no tuvieron un efecto (Runagall-McNaull et al., 2015). Estos resultados respaldan la hipótesis de que las fuentes de proteína están sirviendo en el mantenimiento de un cuerpo duradero que permita aumentar las expectativas de vida de los adultos, pero que un consumo excesivo de proteínas por parte de larvas o adultos revierte este efecto e incluso puede llegar a ser letal. De ahí la importancia de seguir estudiando el efecto de los nutrientes no sólo en diferentes rasgos, si no también en diferentes etapas del ciclo de vida de los organismos.

Conclusiones generales

En esta tesis se demostró que la dieta y el estado de salud son fuentes de variación relevantes en la condición del escarabajo *T. molitor*. Tanto machos como hembras consumieron más de la dieta rica en carbohidratos y menos de la dieta rica en proteínas. Por su parte los machos infectados con el hongo redujeron su ingesta, posiblemente como una estrategia para combatir al patógeno, al limitar los nutrientes disponibles. Los machos tratados con Tween y retados con esporas no viables presentaron la mayor concentración de proteínas. A su vez, los machos de Tween también presentaron la mayor concentración de lípidos. Mientras que los retados con el hongo tuvieron la mayor concentración de carbohidratos totales. El estado de salud de la pareja con la que se aparearon las hembras influyó en la concentración de nutrientes que presentaron. Las hembras que copularon con machos sanos, tratados con Tween y retados con esporas no viables, presentaron mayor concentración de proteínas. Las hembras cuya pareja pertenecía al control de Tween tuvieron más lípidos. Siendo las hembras que copularon con machos sanos e infectados con el hongo las de mayor concentración de carbohidratos totales.

La supervivencia de los individuos también se vio afectada por la dieta y el estado de salud. Como se esperaba, los machos sanos fueron lo que vivieron más. Sin embargo, no hubo diferencias entre los tres tratamientos restantes (control de Tween, retados con esporas no viables y retados con el hongo). Únicamente los cuatro tratamientos aumentaron sus expectativas de vida en la dieta con la mayor concentración de carbohidratos. En el caso de las hembras, el estado de salud de la pareja con la que se aparearon no modificó su supervivencia. Al igual que los machos su expectativa de vida se incrementó al consumir la dieta rica en carbohidratos.

Contrario a lo que se esperaba, la variación en el estado nutricional de las hembras no cambió sus preferencias de pareja. Las preferencias por los distintos machos experimentales se mantuvieron similares a lo largo de las cinco dietas sintéticas que se utilizaron. Sin embargo, el estado de salud de los machos sí influyó en la elección de pareja, ya que las hembras encontraron más atractivos a los machos retados con el hongo, cuando podían elegir entre éstos y machos sanos. Es posible que los machos infectados con el hongo estén destinando sus recursos hacia la reproducción, en este caso en aumentar la producción de feromonas, en lugar de combatir al patógeno. Es decir, que los machos estarían realizando una inversión terminal al censar que sus probabilidades de sobrevivir son muy bajas.

Por último, se encontró que las hembras están recibiendo regalos nupciales, en forma de espermatóforos, de distintas calidades de acuerdo con el estado nutricional y de salud de los machos. De manera general, los machos infectados con el hongo produjeron los espermatóforos con la mayor concentración de proteínas totales. Tanto el tratamiento de Tween como de machos infectados con el hongo, tuvieron la mayor concentración de lípidos presentes en los espermatóforos. Nuevamente los machos infectados con el hongo fueron los de mayor concentración, en este caso de carbohidratos totales en sus espermatóforos. El hecho de que los machos enfermos estén destinando sus recursos hacia la reproducción, en forma de espermatóforo, es otra evidencia que apoyaría que en esta especie los machos están realizando la inversión terminal.

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APÉNDICES

Received: 16 November 2018 | Revised: 8 January 2019 | Accepted: 25 January 2019

DOI: 10.1111/eth.12854

RESEARCH PAPER

WILEY ethology

Female choice for sick males over healthy males: Consequences for offspring

Alicia Reyes-Ramírez[1] | **Jhony N. Enríquez-Vara**[2] | **Maya Rocha-Ortega**[1] | **Aldo Téllez-García**[1] | **Alex Córdoba-Aguilar**[1]

[1]Departamento de Ecología Evolutiva, Instituto de Ecología, Ciudad Universitaria, Universidad Nacional Autónoma de México, Distrito Federal, México

[2]CONACYT-Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco AC, Zapopan, Mexico

Correspondence
Alex Córdoba-Aguilar, Departamento de Ecología Evolutiva, Instituto de Ecología, Ciudad Universitaria, Universidad Nacional Autónoma de México, Distrito Federal, México.
Email: acordoba@iecologia.unam.mx

Funding information
Dirección General de Asuntos del Personal Académico, Universidad Nacional Autónoma de México, Grant/Award Number: IN206618

Editor: Susan Bertram

Abstract

Sexual selection theory indicates that ornament expression in males is in close relation to their condition. This "honesty" relationship serves as the basis for female choice: Females would mate with healthy males over sick males after assessing male ornament signal expression and derive benefits for their progeny. Here, we investigated female mate choice for infected and non-infected males, male survival after infection (to corroborate the negative effect of infection), and fitness consequences of female preferences using *Tenebrio molitor* beetles. Male infection was produced having two types of challenges as follows: males infected with entomopathogenic fungi and males infected with nylon implants. Similar to previous studies, we corroborated that females preferred fungus-infected males over positive control, negative control, and nylon-challenged males. Survival was the lowest for fungus-treated males followed by nylon-treated and control males. Females mated with fungus-treated males laid fewer and smaller eggs, and the laid eggs had less lipid content with a reduced eclosion success compared to females mated with non-challenged males. Our interpretation is that fungus-treated males invested their energetic resources to increase their attractiveness at the risk of survival, in a terminal investment fashion. Females, however, would have corrected their choice by investing less in their offspring.

KEYWORDS

female choice, male signal, offspring viability, *Tenebrio molitor*

1 | INTRODUCTION

The handicap principle (Zahavi, 1975) proposes that male sexual ornaments are honest signals that females use to assess quality of potential mates (Andersson & Simmons, 2006; Bradbury & Vehrencamp, 2011; Palau-Daval, Gardette, & Joly, 2018). One way this "honesty" relationship can be interpreted is that signals reliably indicate the ability of organisms to resist parasites (Balenger & Zuk, 2014; Hamilton & Zuk, 1982). According to this idea, honesty via male ability to deal with parasites relies on two premises. On one hand, parasites affect hosts' survival if hosts are not capable to resist them (e.g., Ebert, Lipsitch, & Mangin, 2000; Davies, Fairbrother, & Webster, 2002). On the other hand, the maintenance and expression of the immune machinery to deal with parasites are energetically costly (e.g., Fellowes & Godfray, 2000; Sol, Jovani, & Torres, 2003; Ardia, Gantz, Schneider, & Strebel, 2012). In this fashion, signals are considered honest indicators of animal condition (Bradbury & Vehrencamp, 2011) and this is the reason why females use their expression when choosing a mate (Andersson & Simmons, 2006; Hamilton & Zuk, 1982). Such choice will grant females to secure parasite-resistant genes that can be passed on to their offspring.

According to life-history theory, organisms adjust their investment in current reproduction considering the gains and future events of reproduction (Ratikainen & Kokko, 2009; Stearns, 1992). An important factor that affects this investment is the quality of mates. For the case of females, it is expected that these can adjust their reproductive investment depending on male attractiveness (Burley, 1986; Harris & Uller, 2009; Sheldon, 2000). Such adjustment can be seen as maternal effects that can alter the strength of sexual selection and male fitness (reviewed by Harris & Uller, 2009). In particular, the differential allocation hypothesis indicates a positive relation between male quality and maternal investment: That is, a reduced resource allocation by females when mated with males in poor condition (Sheldon, 2000). Ways by which females can adjust their resources can be expressed in different ways, many of these directly related to egg traits such as an increase in egg size (e.g., Kolm, 2001), number (e.g., Locatello & Neat, 2005), antibody concentration (e.g., Saino, Romano, Ferrari, Martinelli, & Moller, 2003), and lipid and protein content (e.g., Braga et al., 2010).

One study system where female choice based on male ability to resist pathogens and female resource allocation to offspring can be investigated is that of *Tenebrio molitor* beetles. In this species, males signal their quality to females by producing volatile pheromones (mainly composed of 3-dodecenyl acetate, Bryning, Chambers, & Wakefield, 2005) and a set of sex-specific cuticular hydrocarbons (Nielsen & Holman, 2012). However, studies where males have been immune-challenged to see female choice patterns for male pheromones have found opposite findings: While some studies indicated that males with low levels of infection or no infection were more attractive (Worden & Parker, 2005; Worden, Parker, & Pappas, 2000), other studies determined that immune-challenged males were more attractive (Kivleniece, Krams, Daukšte, Krama, & Rantala, 2010; Krams et al., 2011; Nielsen & Holman, 2012; Sadd et al., 2006). Following these controversial results, in this paper we have set the question of whether females choose healthy males over sick males. Note, however, that our study differs from previous examinations of female choice in the same study system for several reasons. Apart from nylon challenges, we also used real pathogens in the same experimental design. The reason for this is that compared with an artificial challenge, a real pathogen can multiply within its host thereby intensifying its debilitating effects (Moreno-García, Córdoba-Aguilar, Condé, & Lanz-Mendoza, 2013). As a matter of fact, a study in this animal showed that a fungal challenge induced a more rapid mortality than a nylon implant (Krams, Daukste, Kivleniece, Krama, & Rantala, 2013). For these reasons, we used *Metarhizium robertsii*—an entomopathogenic fungus—and nylon implants to infect males. *M. robertsii* (formerly known as *M. anisopliae* var. Anisopliae; Bischoff, Rehner, & Humber, 2009) is widely distributed in soil and has been used as a biological control agent (Faria & Wraight, 2007; Maniania et al., 2003; Wang & Feng, 2014). Second, we monitored survival after a challenge, via an experimental design that includes all animals that died in all treatments. This unlike previous studies where mortality was assessed one month following manipulation where a fraction of surviving animals was left aside (Kivleniece et al., 2010; Krams et al., 2011). By removing a fraction of animals, survival estimates may be obscured. Third, we assessed offspring condition and viability of chosen males to examine whether the differential allocation hypothesis applies. For this, we measured (i) the volume and concentration of proteins and lipids of the eggs. Insect egg volume is in general, a good predictor of egg survival (i.e., Sota & Mogi, 1992), development and future size in both the immature and mature stage (i.e., Fox, 1994). On the other hand, while proteins participate in egg immune protection (Gillepie, Kanost, & Trenczek, 1997), lipids can provide energy for growth during extended periods of non-feeding (Arrese & Soulages, 2010); and (ii) number of eggs laid over one week as well as emerging larvae. Our predictions were that (i) females should prefer healthy males over non-healthy males with fungus-treated males being less selected than nylon-treated males; (ii) negative effects of challenge imply that fungus-treated males will be the first to die followed by nylon-treated and control males; (iii) challenged males will sire fewer eggs, with poorer condition (smaller, with less protein and lipid concentration) and/or eclosion success compared to control males.

2 | MATERIAL AND METHODS

2.1 | Insect breeding

Tenebrio molitor larvae were obtained from four different commercial suppliers in Mexico City and the State of Mexico. Larvae were kept at 25 ± 2°C (mean ±standard deviation) in a 12:12-hr photoperiod cycle and fed with wheat ad libitum and apple slices. Pupae were collected, sexed by examination of the eighth abdominal segment (Bhattacharya, Ameel, & Waldbauer, 1970), and individually separated to ensure that all adults were virgin at the moment of experimentation.

2.2 | Fungus cultivation and inoculum preparation

Metarhizium robertsii (ARSEF 2134) was obtained from the entomopathogenic fungi collection of the Agricultural Research Service of the United States Department of Agriculture. Spores were stored in 10% glycerol at −80°C until their use. For preparation of the inoculum, spores were plated on sabouraud dextrose agar (SDA) and incubated at 28°C in darkness. After 15 days of incubation, conidiophores were harvested by scraping them out gently from the plate with a scalpel and suspended in 0.03% Tween 80 (hereafter referred as Tween). The suspension was stirred for 5 min on a vortex and filtered through a cotton mesh to separate out the conidia from the mycelium. Conidia were counted using a Neubauer chamber. The percent viability of the conidia was greater than 95%, and it was estimated using the SDA plate count technique (Goettel & Inglis, 1997).

2.3 | Determination of the LC_{50}

Five groups of 15 males of *T. molitor* each were inoculated with five different concentrations of *M. robertsii* conidia suspended in 10 ml of Tween (1×10^4, 1×10^5, 1×10^6, 1×10^7, and 1×10^8 conidia/ml). Inoculation was carried out by immersing insects into the conidial suspensions for five seconds and air-dried in a 9-cm diameter Petri dish with a Whitman No. 1 filter paper at the bottom. A sixth control group was immersed in Tween without conidia and dried in the same way. Insects were placed individually in a cavity of a 12-well plate with wheat and incubated for 10 days at 25°C and 90% humidity after inoculation. Mortality was recorded every 24 hr for ten days and dead insects were incubated at 25°C in a 5-cm diameter Petri dishes with wet filter papers to promote sporulation and confirm fungal infection. That an animal was dead was corroborated by making sure that it remained motionless even after tactile manipulation. All male ages and weights ranged between 12 to 15 days and 0.9 to 0.12 g, respectively.

2.4 | Male challenge treatments

Four treatment groups of sexually mature males (12 to 15 days old; Gerber, 1976) were established in the following way: (i) negative control, non-manipulated animals; (ii) fungus, animals immersed in Tween with 3×10^5 conidia/mL (the approximate LC_{50}) for five seconds; (iii) positive control, animals submerged in Tween for five seconds; and (iv) nylon, animals with three nylon filaments (2-mm long by 0.5-mm diameter) fully inserted between the second and third abdominal sternites. Nylon filaments were previously disinfected by storing them in 96% ethanol for 24 hr. before insertion. All animals were kept for three days before female choice trials.

2.5 | Female choice trials

Five different experiments of female choice were carried out arranging the treatment groups as follows: (i) negative control versus fungus; (ii) negative control versus implant; (iii) negative control versus positive control; (iv) positive control versus fungus; and (v) implant versus fungus. Each experiment consisted in 30 tests (for a total of 150 trials) where a sexually mature, virgin female (20 days old; Gerber, 1976) was allowed to choose between two males in a two-armed olfactometer (Supporting Information video S1). At the beginning of each test, the female was placed for 2 min in the release port for acclimatization. Then, the gate of the release port was opened and the behavior of the female recorded. Males remained restrained at the end of the arms of the olfactometer during the whole test, and an air pump connected to the arms was used to push males' scents to the female. Males were randomly assigned to each arm of the olfactometer. The test was concluded 3 min after opening the gate. Two behavioral responses were recorded as follows: female choice (decision to take right or left olfactometer arm) and the time it took females to choose one of the arms (from port opening until reaching the end of the arm). The observer did not know the experimental origin of all males. The olfactometer was cleaned with ethanol after every replicate to remove the remnants of the chemical signals (Ríos-Delgado et al., 2008). Since this species usually mates in the dark, tests were carried out in a dark room with a red light which cannot be detected by the animal but allowed us to record its behavior (for a rationale see Briscoe & Chittka, 2001).

2.6 | Male survival after challenge

We had the same male experimental groups (negative control, fungus, positive control, and nylon) as indicated above, using 30 virgin males of 12–15 days of age. After manipulation, each animal was individually separated, and mortality was recorded every 24 hr. until all animals died. Similar to LC_{50} determination described above, we corroborated that an animal was dead if it remained motionless after tactile manipulation.

2.7 | Offspring condition and viability

The same five experimental combinations of female choice described above were carried out again but this time the number of tests was increased to reach 20 pairs for each male–female combination (for a total of 200 trials). After tests were performed, each pair (female and chosen male) was transferred to a plastic container (4.6-cm diameter by 4.6-cm height) with 9 grams of commercial wheat flour and maintained at room temperature for 1 week. Our previous experience indicated that such food provision is needed by females to lay eggs (all authors' unpub. data). After this time, 20 mating pairs per treatment (thus having a total of 100 pairs) were randomly selected and 5 eggs per female were randomly separated to measure their volume, protein, and lipid concentration. The remaining 20 mating pairs from each treatment were used to record the number of eggs females laid for one week and eclosion success of these eggs measured as the number of hatching larvae.

2.8 | Egg volume, protein, and lipid concentrations

Soon after being laid, eggs were photographed under a stereoscopic microscope and their volume was depicted using ImageJ (Schneider, Rasband, & Eliceiri, 2012). Volume was calculated using the formula reported by Berrigan (1991): $V = 1/6\ \pi\ w^2\ L$, where w and L are egg width and length, respectively.

Total protein and lipid concentrations were determined using Foray et al.'s (2012) methodology using the same 5 eggs whose volume was measured. Eggs were ground with 200 µl of PBS pH 7.4 using a micropestle. The resultant suspension was centrifuged at 4°C for 10 min at 10,000 RPM. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Rockford, IL, USA; Smith et al., 1985). According to this, 10 µl of the sample supernatant were mixed with 40 µl of PBS and 150 µl of the kit reagents and then incubated in a 96-microwell plate for 30 min at 37°C. Bovine serum albumin was used to perform the standard reference curve, and the absorbance was recorded at 562 nanometers in a microplate reader. Lipid concentration was determined by the Van Handel (1985) method. In brief, 180 µl of the sample supernatant were mixed with 1 ml of chloroform-methanol (1:1) and vortexed for 2 min. 100 µl of this sample were taken and heated at 90°C to evaporate the solvent.

Then, 10 µl of sulfuric acid (98%) was added to the sample and incubated at 90°C for 2 min. After cooling, each sample was placed in a 96-microwell plate with 190 µl of phospho-vanillin (1.2 g/L). Glyceryl trioleate was used to perform the standard reference curve, and the absorbance was recorded at 562 nanometers.

2.9 | Statistical analysis

LC_{50} was determined with a Probit analysis using the mortality data at day 5. LC_{50} was estimated at 3.9×10^5 conidia/ml with the 95% confidence interval between 8.11×10^4 and -1.43×10^6 conidia/ml. For the female choice experiment, a *G* test of goodness-of-fit was performed to determine whether there were differences in female preference of the two males tested in the olfactometer. The time it took females to choose a male from each treatment group in these experiments was compared with a Mann–Whitney *U* test since data did not meet the parametric assumptions even after transformation. For the male survival experiment, we used a generalized linear model (GLM) using the proportion of individuals that survived according to time from challenge and experimental treatment. In this GLM, we fitted a quasibinomial error distribution to correct for data overdispersion. We used Tukey tests to compare among treatments. As for egg traits, we first tested if male treatment affected the volume of eggs, so that we used a nested ANOVA having treatment as a fixed factor and female identity as a random factor nested within treatments. A Tukey–Kramer test was used as a post hoc test to observe significant differences between treatments. A Welch's ANOVA was used to compare protein and lipid concentrations according to male origin female mated with, since data did not meet the assumption of homogeneity of variances. A Games-Howell test was used as a post hoc test to detect significant differences. Normality and homoscedasticity were tested with the Shapiro–Wilk and Levene's tests, respectively. To assess whether egg number and eclosion were related to treatment, we used a GLM where we set a Poisson and a binomial distribution for egg number and eclosion success, respectively, to correct for overdispersion. Tukey tests were used to compare differences among treatments. All analyses were carried out in R (R Core Team, 2017), except the Probit analysis, which was carried out in the Polo Plus™ software (LeOra Software, 2002). R packages used were car (Fox & Weisberg, 2011), DescTools (Signorell, 2017), ggplot2 (Wickham, 2009), multcomp (Hothorn, Bretz, & Westfall, 2008), nlme (Pinheiro, Bates, DebRoy, & Sarkar, 2017), Rmisc (Hope, 2013), and userfriendlyscience (Peters, 2017).

3 | RESULTS

3.1 | Female choice

Preferences for males were not equally distributed in all comparisons (Table 1), and so females preferred (i) fungus males over negative control males (*G* = 5.795, *p* = 0.008) and (ii) fungus males over positive control males (*G* = 8.398, *p* = 0.001). No difference emerged between negative control males versus positive control males (G = 5.15, *p* = 0.237), nylon males versus negative control males (G = 0.057, *p* = 0.406) and fungus males versus nylon males (G = 5.15, *p* = 0.237).

TABLE 1 Number of males chosen by females as mates in *Tenebrio molitor* according to five different male combinations

Treatment	Chosen males	G test	*p* value
Negative control versus fungus	25 versus 45	5.795	0.008*
Negative control versus nylon	34 versus 36	0.057	0.4060
Negative control versus positive control	38 versus 32	5.15	0.237
Positive control versus fungus	23 versus 47	8.398	0.001*
Nylon versus fungus	32 versus 38	5.15	0.237

Note. The two numbers in the "Chosen males" column refer to the number of males females chose from each respective treatment. For example, 25 negative control males and 45 fungus-challenged males were chosen from the first treatment.

The time it took females to select a male did not differ among groups in all experiments (*p* values for all four Mann–Whitney *U* test-based experiments>0.05).

3.2 | Male survival effects after challenge

Male survival differed across groups ($\chi^2 = 2.05$, $p < 0.001$; Figure 1). Also, there was an interaction between treatment and time from exposure ($\chi^2 = 2.77$, $p < 0.001$). Comparison between groups showed that fungus males died sooner than the other three groups (against nylon implant, *z* = 2.01, *p* = 0.04; against negative control, *z* = 7.16, *p* < 0.001; and against positive control, *z* = 2.70, *p* = 0.007). Conversely, negative control animals took longer to die than the other three groups (against fungus, *z* = 7.25, *p* < 0.001; against positive control, *z* = 5.71, *p* < 0.001; and against nylon implant, *z* = −6.18, *p* < 0.001). Nylon implant animals died sooner than negative control (*z* = −6.18, *p* < 0.001) but had no difference with positive control animals (*z* = −0.631, *p* = 0.921).

3.3 | Offspring condition and viability

There were differences in egg volume across treatments (nested ANOVA: $F_{3,36} = 8.04$, $p < 0.001$; Figure 2). Tukey–Kramer post hoc tests revealed that the eggs sired from fungus males were significantly smaller than those sired by positive control and implant male groups. However, eggs from fungus males were not different than those of the negative control group (Figure 2).

There were no significant effects of male treatment on total protein concentration (Welch's ANOVA, $F_{3,36} = 1.41$, $p = 0.256$). However, total lipid content was significantly different among treatments (Welch's ANOVA: $F_{3,36} = 12.38$, $p < 0.001$; Figure 3). A Games-Howell test showed that eggs sired by males of the positive control (*z* = 5.81, *p* < 0.001), implant (*z* = 4.38, *p* < 0.001), and fungus treatment (*z* = 3.91, *p* < 0.001) groups had less lipid content

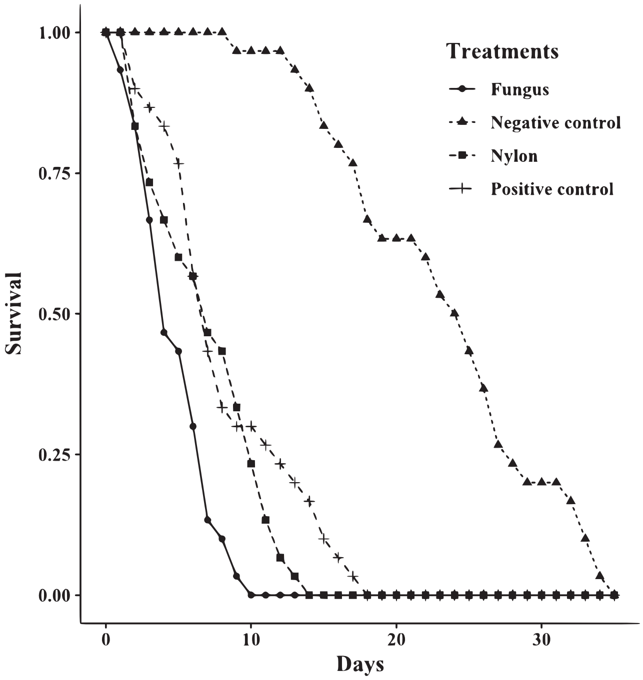

FIGURE 1 Male survival (in days) following experimental treatments in *Tenebrio molitor* adults: a) fungus-challenged males; b) negative control males; c) nylon-challenged males; and d) positive control males

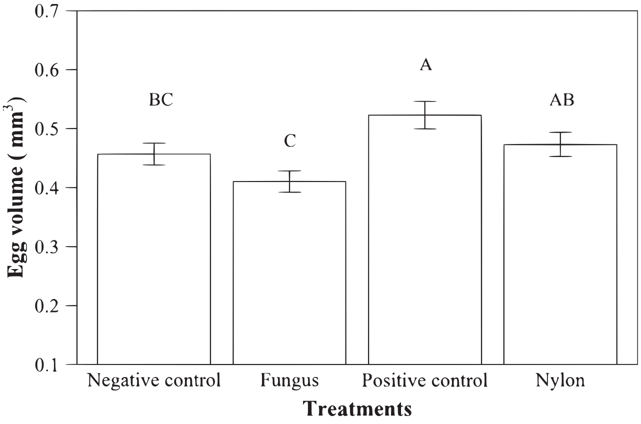

FIGURE 2 Egg volume (mean ±*SD*) from siring males after female preferences for four different male treatments in *Tenebrio molitor*. Means with different letters indicate significant differences

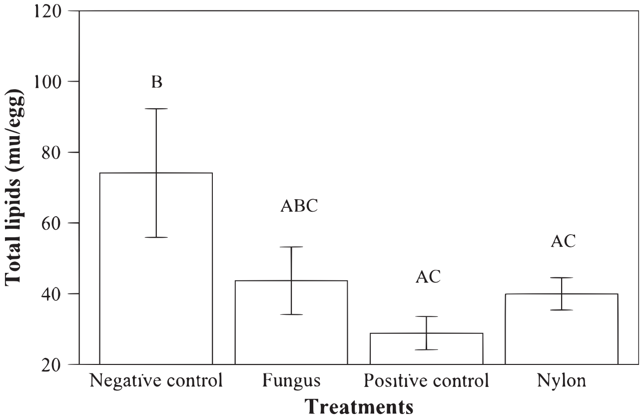

FIGURE 3 Total concentration of lipids (mean ±*SD*) from siring males after female preferences for four different male treatments in *Tenebrio molitor*. Means with different letters indicate significant differences

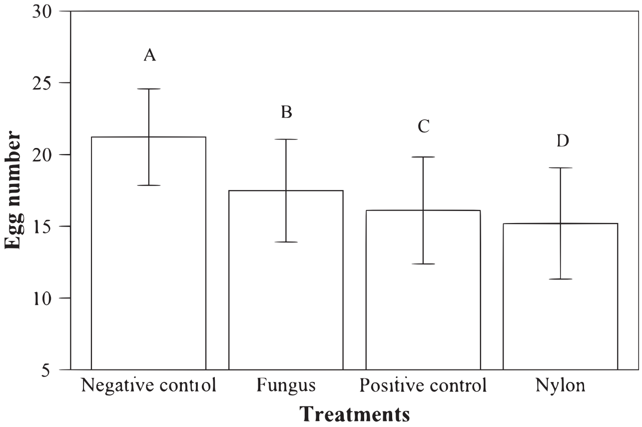

FIGURE 4 Number of eggs (mean ±*SD*) laid from siring males after female preferences for four different male treatments in *Tenebrio molitor*. Means with different letters indicate significant differences

compared to eggs sired by males of the negative control group. Eggs from fungus group did not differ in lipid content from those of positive control ($z = 1.90$, $p = 0.22$) and implant ($z = 0.47$, $p = 0.96$).

In terms of egg number, there were significant differences among all treatments ($X^2 = 60.035$, $p < 0.001$; Figure 4). Negative control males sired more eggs than fungus males ($z = 35.95$, $p < 0.001$), positive control males ($z = 44.21$, $p < 0.001$), and nylon males ($z = 52.40$, $p < 0.001$).

Male treatment had an effect on eclosion success ($X^2 = 8.737$, $p < 0.05$). Eclosion was higher for eggs sired by negative control males compared to fungus ($z = 14.39$, $p < 0.001$), nylon ($z = 9.28$, $p < 0.001$), and positive control males ($z = -22.12$, $p < 0.001$). Eggs from fungus males had a higher eclosion success than those from positive control males ($z = -9.28$, $p < 0.001$).

4 | DISCUSSION

Similar to some previous studies (Kivleniece et al., 2010; Krams et al., 2011; Nielsen & Holman, 2012; Sadd et al., 2006), *T. molitor* females showed a preference for sick males over healthy ones. Notice that this applies to situations when males were infected with fungus but not to males treated with nylons. In this extent, we used the same number of nylon implants that elicited an increase in pheromone production at the expense of immune ability by previous studies (Kivleniece et al., 2010; Krams et al., 2011). However, when comparing fungus-treated males versus nylon-challenged males, no difference emerged meaning that females were similarly attracted to both so that either male challenge elicited similar pheromone production. Contrary to our expectation, preferences expressed in all trials indicated that females chose challenged males no faster than non-challenged males. This can be interpreted as females being able to detect and process scents at a similar rate for all treatments.

To complement our experimental manipulation of male condition, our protocol included the survival cost of challenges for all males. In this regard, we provided evidence for such negative effect: Fungus-treated males showed an impaired survival compared to nylon, positive, and negative control males. Interestingly, nylon implants did not lead to reduced survival compared to control groups. This lack of difference, as well as those anomalous results of female preferences expressed above, suggests that experiments using nylon implants must be interpreted with care (Moreno-García et al., 2013). One fundamental difference between a fungus pathogen and nylon implants is that the former can lead to a generalized weakening of the cuticle structure (e.g., Lacey, Lacey, & Roberts, 1988; Rangel, Alston, & Roberts, 2008; Wang & Leger, 2006) which is unlikely to be the case for the latter. These fungal effects may induce a resource allocation in the host to increase survival at the cost of activity and fecundity (Scholte, Knols, & Takken, 2006) and, in general, explain more costly effects compared to nylon challenges. Note that positive control males died at a similar rate than challenged males. A few studies using Tween 80 have also found negative effects on insect survival (e.g., Yazgan, 1981; Luz, Silva, Magalhães, Cordeiro, & Tigano, 1999; Enríquez-Vara, Córdoba-Aguilar, Guzmán-Franco, Alatorre-Rosas, & Contreras-Garduño, 2012). One explanation is that the Tween immersion that animals experienced may have obstructed some of the insect body openings such as the mouth or spiracles. This may have rendered Tween-treated animals to impair their feeding or respiration activities. This explanation means that Tween may be still a safe control but that the way this should be provided is not via immersion.

Why do females prefer mating with immune-challenged males? According to theory and in the face of an infection, the limited amount of resources that animals can secure would induce a trade-off between survival and other life-history traits, such as current reproductive effort (McKean & Lazzaro, 2011; Stearns, 1989). If the survival expectancy is perceived as low, animals should invest more in current reproduction as a last-ditch effort to maximize their fitness even if they die soon (Clutton-Brock, 1984; Kivleniece et al., 2010). This last strategy is known as terminal investment (Williams, 1966) and it can be elicited not only by pathogens, but also by any factor that reduces the residual reproductive value of an individual (e.g., Creighton, Heflin, & Belk, 2009; Heinze & Schrempf, 2012; Billman & Belk, 2014). So, possibly our challenged beetles may have re-allocated their resources to make them more attractive at the risk of surviving less in a terminal investment fashion. Evidence for the terminal investment comes not only from other animals (e.g., Copeland & Fedorka, 2012; González-Tokman, González-Santoyo, & Córdoba-Aguilar, 2013) but also our study species (Kivleniece et al., 2010; Krams et al., 2011). For example, experimentally infected male damselflies tend to defend their mating territories for longer compared to non-infected males (González-Tokman et al., 2013). Thus, infected males increased their chances to mate although they lived less than non-infected males (González-Tokman et al., 2013).

Assuming a terminal investment basis for our study species, there was a possible penalization for terminally investing males in terms of reduced fitness for offspring as fungus-treated males gave rise to smaller and fewer eggs. Furthermore, although there was difference in protein concentration across treatments, eggs sired by challenged males had less lipid content than those sired control males. Perhaps this lipid difference may explain why eggs from fungus-treated males had a reduced eclosion success. This lipid-based explanation may be related to spermatophore quality. Related to this, evidence from different insects indicate that spermatophores can serve as a lipid source for egg production, especially those lipids that cannot be synthesized such as cholesterol (reviewed by Marshall, 1982; Lewis & South, 2012). Thus, we suggest that terminally investing male beetles were penalized despite their large investment in attracting females. These ideas need to be formally tested.

Another explanation for why fungus-treated males were more attractive is that, rather than the insect, it is the fungus what makes the animal attractive. In fact, fungal manipulation of invertebrate hosts includes modification of the host's behavior (reviewed by Roy, Steinkraus, Eilenberg, Hajek, & Pell, 2006). For example, recent evidence has suggested that *Beauveria bassiana* entomopathogenic fungus is attractive to mosquitoes (George, Jenkins, Blanford, Thomas, & Baker, 2013). Also, fungus-killed female house flies are more attractive to males than non-infected females (Møller, 1993). Thus, fungal manipulation may induce fungus free animals to become attracted to fungus-infected animals (Roy et al., 2006). From the fungus perspective, this is an effective strategy to disperse and colonize new hosts (Watson & Petersen, 1993). One mechanism for this is that the fungus increases the insect's volatile production. If this is the case, then infected males may die sooner due to the a) cost of the infection and/or b) increased production of volatiles in a short period (although it may also be that it is the fungus the one that produces semiochemicals of sexual nature). Whatever the cost, it is clearly advantageous for the infected male as it becomes more effective at attracting females.

How are sick males attractive but then end up with reduced fitness? Sick males may indeed enhance their volatile production to attract females more intensively so that, according to theory, females take such production as an honest signal of male quality. Females may have used pheromone as a first trait to evaluate males to then correct their decision after copulation as implied by the differential allocation hypothesis. As a matter of fact, females of several species use a number of traits to have a “balanced” assessment of male quality (e.g., Hill, Enstrom, Ketterson, Nolan, & Ziegenfus, 1999; Hankison & Morris, 2003; Hasegawa, 2018) which is in agreement with theory (Candolin, 2003; Møller & Pomiankowski, 1993). Perhaps extended filters occur after copulation, whereby females assess other traits that may be coupled with male condition (e.g., Evans, Zane, Francescato, & Pilastro, 2003). One related example is that of *Tribolium castaneum* beetles whose females assess male condition during copulation (Fedina, 2007). However, cryptic female choice can be ruled out if males are able to modulate egg production by providing material benefits to females. As indicated before, this possibility can apply to *T. molitor* as males provide a spermatophore whose quality may affect offspring traits (Drnevich, Papke, Rauser, & Rutowski, 2001; Worden & Parker, 2001). Thus, we hypothesize that variation in spermatophore quality may affect egg production and condition. How females gather such benefits from the spermatophore is unclear. One way is if females use the lipoprotein materials that conform the spermatophore wall (Gadzama & Happ, 1974), for egg production. According to this material benefits possibility, perhaps fungus-treated males re-allocated more resources to pheromone but ended up producing spermatophores of reduced quality that affected offspring' fitness. One way to look at this relation is testing whether pheromone production is directly related to spermatophore quality. There is evidence that pheromone production is costly (Harari, Zahavi, & Thiéry, 2011; Rantala, Kortet, Kotiaho, Vainikka, & Suhonen, 2003) but whether there is trade-off between this trait and spermatophore production is unknown.

In conclusion, our study indicates that *T. molitor* females were more attracted to sick males, but this led females to lay fewer and smaller eggs, and with less lipid content and eclosion success This negative fitness outcome may be due to either females corrected their choice by investing less in their eggs or that sick males provided fewer energetic resources to females. This second explanation is in agreement with the differential allocation hypothesis which predicts a positive correlation between male quality and maternal investment (Sheldon, 2000). The fact that sick males invested considerably more than healthy males, to attract females is also coherent with the terminal investment hypothesis (Williams, 1966).

ACKNOWLEDGEMENTS

To a PAPIIT grant IN206618 to AC-A, CONACyT grants to MR-O and JE-V. This paper constitutes a partial fulfillment of the Doctorado en Ciencias Biomédicas of the Universidad Nacional Autónoma de México (UNAM) for Alicia Reyes Ramírez. Thanks to Raúl I. Martínez Becerril for logistic support. Authors state not to have any conflict of interests.

ORCID

Alex Córdoba-Aguilar https://orcid.org/0000-0002-5978-1660

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How to cite this article: Reyes-Ramírez A, Enríquez-Vara JN, Rocha-Ortega M, Téllez-García A, Córdoba-Aguilar A. Female choice for sick males over healthy males: Consequences for offspring. *Ethology*. 2019;00:1–9. https://doi.org/10.1111/eth.12854

Artículo

Un vistazo a la ecología nutricional

Alicia Reyes Ramírez y Alejandro Córdoba Aguilar

El motor de la vida

Dicen que "el comer y el rascar, todo es empezar", pero este dicho popular no considera los beneficios de una alimentación sana y equilibrada. Para los seres vivos, obtener una dieta equilibrada no es una tarea sencilla. Los animales requieren alimentos para desarrollarse, conquistar territorios y parejas y defenderse de los patógenos, entre otras cosas, y sus necesidades específicas de nutrición cambian con el tiempo. La búsqueda y el consumo del alimento implican por sí mismos un gasto energético considerable. Además, un animal que está buscando comida puede convertirse en el alimento de otro, así que también se trata de una actividad peligrosa. Por lo tanto, aun si dicho animal busca equilibrar su dieta acorde con sus diversas necesidades, las presiones que tiene que enfrentar hacen que no siempre lo logre. En este sentido, la ecología nutricional trata de entender cómo la alimentación afecta a los individuos, directa o indirectamente, en diferentes aspectos de su vida.

La necesidad de una dieta equilibrada nos ha llevado a estudiar la ecología de animales que se pensaba que no eran selectivos al momento de alimentarse. Por ejemplo, se creía que los carnívoros simplemente obtenían más energía al comer un mayor número de presas. Sin embargo, esto no es así. Ahora se sabe que son capaces de forrajear selectivamente para obtener ciertas grasas y proteínas según las requieren. Además, los depredadores no están exentos de llevar a cabo funciones clave como la reproducción. A este respecto, en estudios de nuestro laboratorio, hemos observado que cuando los machos adultos del caballito del diablo *Hetaerina americana* (que son carnívoros) están infectados con cierta bacteria (ver En la salud y en la enfermedad de A. Córdoba), además de usar su tiempo para encontrar hembras, aumentan el tiempo de forrajeo en comparación con machos que no están enfermos, para obtener nutrientes que les ayuden a recuperar su salud.

Forrajear son todas aquellas conductas asociadas con la obtención y consumo del alimento. Por ejemplo: buscar, manipular y digerir la comida.

Para el apetito descontrolado

La alimentación es un proceso intermitente. Es decir, los períodos de alimentación se intercalan con períodos en los que se realizan otras actividades. Estos ciclos son muy variables entre los animales. En el caso de las ratas hooded (*Rattus norvegicus*) criadas en laboratorio, por ejemplo, la cantidad de comida que consumen cuando los intervalos entre comidas son relativamente largos (24 a 72 horas) varía según el tamaño de los alimentos; mientras que en intervalos relativamente cortos (3 a 12 horas), la cantidad de alimento consumido ya no depende del tamaño. En las ratas también puede cambiar la ingesta diaria si cambia la temperatura ambiental. La codorniz japonesa (*Coturnix japonica*) es un caso que ilustra muy bien el fenómeno de la variación en los intervalos de alimentación dependiendo del tipo de comida que

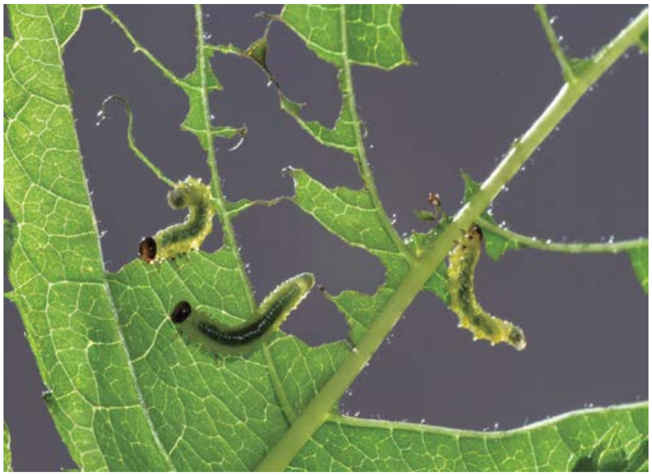
El herrerillo común *Cyanistes caeruleus*, es una especie en la cual el tamaño de larvas con las que alimentan a sus polluelos varía dependiendo de la duración de los intervalos entre cada comida. Fotografía: Pixabay.com se le administre ya que, experimentalmente, se ha encontrado que comen con más frecuencia y en intervalos más cortos cuando se alimentan con puré diluido, que contiene 40% de celulosa, que cuando consumen puré sin diluir o pellets.

Si no puedes comer sólo una: regula tu ingesta diaria

A pesar de las presiones, los animales escogen su alimento y regulan la ingesta de nutrientes con base en dos parámetros. Uno es la composición y la calidad nutricional del alimento, lo cual implica que detectan los nutrientes clave por medio de ciertos receptores; es decir, que de alguna manera perciben los azúcares, algunos aminoácidos, sales y agua. El otro es el estado nutricional del animal. Por ejemplo, los insectos herbívoros que han estado sometidos a dietas desequilibradas regulan su ingesta para consumir una mayor cantidad de los nutrientes que les faltan y una menor cantidad de los nutrientes que tienen en exceso. Para lograr este balance llegan incluso a ingerir sustancias con efectos adversos, como compuestos *aleloquímicos* de los tejidos de las plantas, ya que les ayudan en el proceso de digestión.

La energía del triunfo

El estudio de la fisiología de un animal y el papel de los microbios asociados a su organismo y la diversidad de su dieta, es una de las áreas más activas de la ecología nutricional. Se ha encontrado que no todas las relaciones microbio-hospedero (en este caso el hospedero es el animal) son benéficas para los animales, y hay evidencias de "carreras armamentistas" en las que ambas partes buscan tener el control de la conducta alimentaria. Por ejemplo, los hospederos pueden automedicarse por medio de la alimentación. Esto incluye el consumo de compuestos orgánicos, como los metabolitos secundarios que producen las plantas para atraer o repeler a otros organismos y que robustecen la inmunidad del hospedero. Las larvas de la mosca de la fruta, por ejemplo, obtienen etanol al consumir frutos en descomposición, lo cual le dificulta a la avispa que las parasita poner huevecillos en su cavidad o hemocele. Incluso si una larva de la mosca ya tiene parásitos, el etanol provoca que una cantidad considerable de las larvas de la avispa mueran.



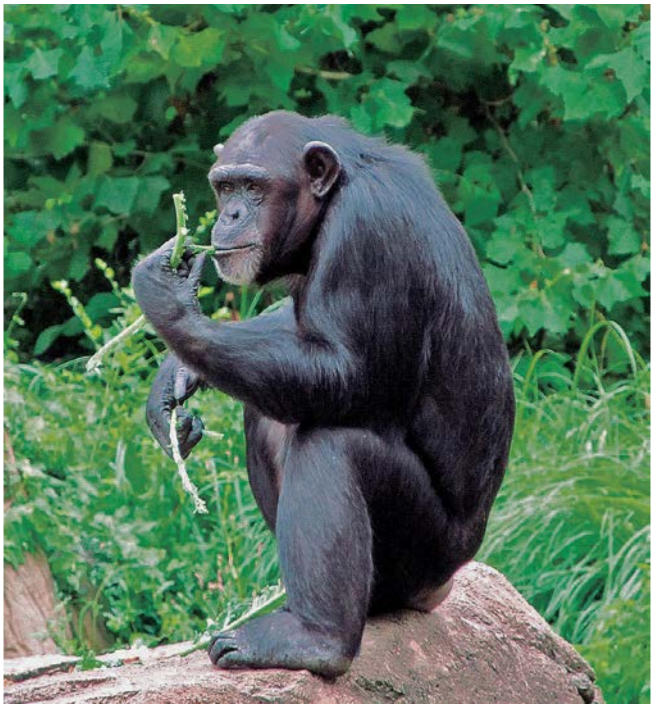
Algunas larvas de insectos, como las orugas, consumen compuestos aleloquímicos que les ayudan a regular la ingesta de nutrientes. Fotografía: Pixabay.com

Los *aleloquímicos* son compuestos químicos liberados por una planta, que tienen efectos conductuales o fisiológicos, ya sea negativos o benéficos, sobre otros organismos (plantas, hongos, animales o bacterias).

La automedicación no es la única estrategia para enfrentar una enfermedad; un caso curioso es la falta de apetito. Esta conducta, en principio, no parece la estrategia más conveniente, dadas las altas demandas energéticas que conlleva la respuesta inmune necesaria para enfrentar a un patógeno. En los insectos se ha encontrado que la *apolipoforina III* es la misma proteína que participa tanto en la respuesta inmune, como en la digestión (en el transporte de lípidos). Así, los episodios de falta de apetito o anorexia en un organismo enfermo, en realidad evitan conflictos fisiológicos entre la digestión y la inmunidad, provocando que la *apolipoforina III* trabaje exclusivamente en combatir al patógeno. Aparentemente, mecanismos semejantes podrían operar en los vertebrados, de modo que no hay que asustarse si, ante la enfermedad, uno no siente hambre.

Todo comienza con un buen marco

La ecología nutricional estudia una amplia gama de organismos y abarca una gran cantidad de métodos, conceptos y objetivos que se centran en su morfología, fisiología, desarrollo, conducta, ecología (por ejemplo, los efectos de las restricciones en el alimento sobre ciertas funciones para la preservación de la vida, como el crecimiento, la reproducción y la supervivencia), y abarca también a su evolución fenotípica, tanto desde el punto de vista de la función como del mecanismo. Es por eso que se ha propuesto la integración de marcos conceptuales como el del forrajeo óptimo (que analiza el proceso de alimentación visto como una cuestión de costos y ganancias), la ecología nutricional (que analiza la interacción entre el estado nutricional del individuo y el medio ambiente), la geometría de la nutrición (que mide el efecto de uno o más alimentos en el estado nutricional, composición corporal y eficiencia en la utilización de los nutrientes de los organismos) y la estequiometría ecológica (que analiza el flujo de materia y energía en el ecosistema, es decir, compara la composición de elementos como nitrógeno, fósforo y carbono presentes en los consumidores y sus recursos). En conjunto, estos cuatro enfoques conforman y le dan cohesión teórica y metodológica a lo que se conoce como el marco geométrico, que describe las relaciones entre el consumo de nutrientes y distintas variables importantes en la vida de los organismos, como la esperanza de vida, la reproducción, la inmunidad y la microbiota.



Chimpancé consumiendo hojas. En este género de primates se ha documentado la automedicación con plantas para combatir los parásitos intestinales. Fotografía: Pixabay.com

Actualmente, la ciencia busca entender el impacto de la conducta alimenticia en la ecología y evolución fenotípica de los organismos y en sus diferentes niveles de organización. Estos son temas que ayudarán también a entender aspectos sobre la salud alimenticia de los seres humanos, como el incremento en la incidencia de la diabetes y de los ataques al corazón.

Sabiduría y obesidad

La Organización Mundial de la Salud ha señalado que la tasa de obesidad en los humanos (y sus mascotas, perros y gatos) va en aumento. De hecho, este organismo estima que más de mil millones de personas en todo el mundo tienen sobrepeso u obesidad. Pero el riesgo que conlleva el consumo de grasas no es exclusivo de nuestra especie. La relación entre el contenido de grasa corporal y el riesgo de muerte prematura también se ha estudiado en insectos como las langostas y grillos, orugas o moscas de la fruta. Sin embargo, a diferencia de nosotros, estos animales parecen tener una "sabiduría nutricional", ya que mantienen un consumo objetivo de macronutrientes, lo que les permite minimizar el riesgo de morir en etapas tempranas por enfermedades asociadas a las grasas.

Si otras especies han logrado llevar un equilibrio entre lo que consumen y su fisiología, entonces ¿por qué a nosotros, los seres humanos, nos va tan mal? Para entenderlo, tenemos que remontarnos a una transición importante en la nutrición humana, que ocurrió cuando el estilo de vida dejó de ser el del cazador-recolector para convertirse principalmente en el de agricultor. A pesar de que esto sucedió en momentos distintos en diferentes lugares del mundo, los resultados han sido similares. Uno de los más relevantes es el aumento en los carbohidratos disponibles. A lo largo de nuestra historia, la incorporación de carbohidratos a la dieta ha aumentado progresivamente, sobre todo a partir de la revolución industrial, que trajo consigo la tecnología para refinar el azúcar y una gran facilidad para transportarla del sitio de producción al consumidor final. Consecuentemente, hoy en día tenemos un acceso sin precedentes a todo tipo de alimentos, y el ritmo al que ha cambiado nuestro ambiente nutricional rebasa la velocidad de nuestro metabolismo para adaptarse, en términos evolutivos.

Al utilizar el marco geométrico, se ha logrado saber más sobre el papel que juega el porcentaje de proteínas ingeridas en la dieta humana. Por ejemplo, los científicos han identificado que cuando tenemos un déficit de proteínas en nuestra dieta, muchas veces lo tratamos de compensar consumiendo en exceso bebidas azucaradas (jugos de fruta y refrescos) y bocadillos con

Mediante el marco geométrico para la nutrición, podemos estudiar la forma en que el estado nutricional de un insecto como la mantis religiosa o de un mamífero como un zorro afecta, su forrajeo, entre otras cosas. Fotografía: Pixabay.com

La obesidad es una de las principales enfermedades en el mundo, debido a los hábitos alimentarios del ser humano. Fotografía: Pixabay.com altos contenidos de grasas y carbohidratos. Sin embargo, al final estos alimentos no logran satisfacer el hambre de proteínas, pero sí se acumulan en nuestro tejido adiposo. Es por ello que, en cuestiones de salud y gestión de la dieta tenemos, sí o sí, que tomar en cuenta la biología; de otra manera, nuestro esfuerzo por combatir el hambre de proteínas estará destinado al fracaso.

Actualmente, en el laboratorio trabajamos con el escarabajo de la harina (*Tenebrio molitor*) para comprender cómo diferentes proporciones de carbohidratos y proteínas en dietas sintéticas afectan diferentes aspectos de la vida de este animal. La idea es usar a este insecto como un sujeto de estudio ejemplar para encontrar el "balance perfecto" en la dieta, para obtener el mayor éxito en cuanto a adecuación.

Alicia Reyes Ramírez. Estudia el doctorado en Ciencias Biomédicas e investiga cómo la condición de los individuos afecta la elección de pareja y el efecto que esto tiene, a su vez, en la progenie.

Alejandro Cordoba Aguilar. Es investigador del Instituto de Ecología con intereses en temas como selección sexual, inmunidad, control hormonal y biología de la conservación de insectos.

Para saber más

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