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CEREBRO Y ELECCIÓN FEMENINA: MECANISMOS PRÓXIMOS Y ÚLTIMOS EN *Tenebrio molitor*

TESIS

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Introducción

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En su teoría de la selección sexual, Darwin (1859; 1871) propuso que las características que favorecen el éxito reproductivo no sólo derivan de la lucha por la supervivencia (selección natural), sino también de la competencia por apareamiento. Su teoría contempló dos mecanismos de selección: la selección intrasexual (competencia entre individuos del mismo sexo para acceder a apareamientos con el sexo opuesto); y la selección intersexual (la preferencia de un sexo por ciertas características del sexo opuesto; Darwin, 1871; Andersson, 1994; Zuk & Simmons, 2018). Darwin argumentó que las hembras, en la mayoría de los casos, realizan la selección intersexual, y lo sustentó con el argumento de que los machos, en la mayoría de las especies, presentan rasgos conspicuos, como los llamativos plumajes de algunas aves (Figura 1).

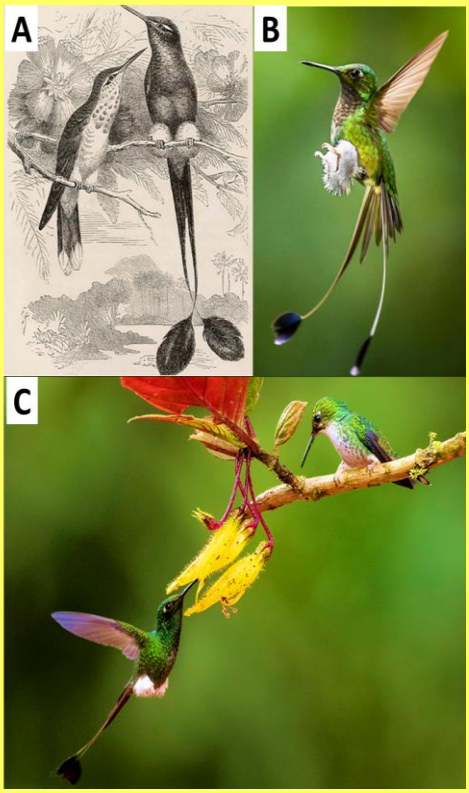

Figura 1. Dimorfismo sexual en el colibrí *Ocreatus underwoodii*.

A) Ilustración de Brehm impresa en el libro "The Descent of Man, and Selection in Relation to Sex" de C. Darwin, en el que habla de lo exagerados que pueden ser algunos rasgos en los machos. *"Casi cada parte del plumaje ha sido aprovechada y modificada; y las modificaciones se han llevado a un extremo maravilloso…en el caso de los colibríes, las aves del paraíso, etc., se debe a la selección sexual, es decir, a la selección por las hembras de los machos más bellos"* (C. Darwin, 1978, p. 78).

Fotografías de B) un macho y de C) una pareja de la especie *O. underwoodii*. (Fotografías por Andres Vasquez Noboa; descargada en Enero del 2024 del sitio: https://ebird.org/species/boorat1)

Estos rasgos, llamados caracteres sexuales secundarios (CSS), no favorecen la supervivencia del macho, pero sí su éxito de apareamiento, ya que las hembras tienden a elegir individuos con estos caracteres. Aunque actualmente la selección sexual se acepta como uno de los principales mecanismos de evolución biológica, en su momento enfrentó resistencia por los contemporáneos de Darwin. La idea de los machos luchando entre si por el acceso a las hembras resultaba comprensible, pero las limitaciones de Darwin para explicar los mecanismos empleados por las hembras para llevar a cabo la elección de los CSS, sumadas a los prejuicios sexistas prevalentes en su época, restringieron la aceptación de la elección femenina como una fuerza evolutiva (Zuk & Simmons, 2018; Rosenthal & Ryan, 2022). Actualmente, este proceso, que también se conoce como elección de pareja, se considera que ocurre cuando los efectos de un sexo conducen a apareamientos no aleatorios con miembros del sexo opuesto (Halliday, 1983; Kokko et al., 2003). Aunque la elección de pareja sucede en ambos sexos, en la mayoría de las especies se ha descrito a las hembras como el sexo que comúnmente elige, y a los machos como el sexo comúnmente elegido (Andersson, 1994). Aunque, la cantidad de publicaciones sobre elección femenina es mayor en comparación con las de elección masculina (Edward & Chapman, 2011; Pollo et al., 2022), la literatura científica evidencia que las hembras están infra estudiadas, y que no se genera suficiente información sobre la diversidad de decisiones reproductivas que toman a través de la elección de pareja (Ah-King, 2022; Rosenthal & Ryan, 2022).

Un aspecto aún menos explorado en la elección de pareja, son sus mecanismos neuronales (Ryan, 2021). Se ha señalado que los artículos sobre selección sexual se centran en los machos, abordando tanto el comportamiento como sus mecanismos neuronales durante la elección de pareja (DeAngelis & Hofmann, 2020; Ryan, 2021; Cordero-Molina et al., 2023).

La importancia de investigar los mecanismos neuronales en la elección femenina radica en que es una decisión que toma lugar en el sistema nervioso de las hembras, es decir que se podrían conocer los mecanismos moleculares y fisiológicos de la elección femenina, cuando típicamente, se han analizado a nivel de individuo. Además, son las hembras quienes perciben, evalúan, rechazan o aceptan a los portadores de los genes que transmitirán a su descendencia. Sin embargo, aún con el avance tecnológico en el estudio del sistema nervioso, la mayoría de los trabajos de mecanismos neuronales en la elección femenina se centran en unos pocos modelos de estudio. Principalmente en vertebrados, mientras que en los invertebrados se ha limitado mayormente a *Drosophila melanogaster* y *Caenorhabditis elegans* (Lenschow & Lima, 2020). Considerando la amplia diversidad de comportamientos durante la elección de pareja y que éstos han sido moldeados por diferentes presiones de selección, es crucial diversificar los modelos de estudio en esta área.

La unión del estudio clásico del comportamiento con sus bases fisiológicas, como la neurobiología, ha sido un objetivo constante en el desarrollo de la etología. En la década de los 60, Ernst Mayr estableció la distinción de dos enfoques en el estudio de la evolución de los rasgos biológicos: los mecanismos próximos que explican cómo ocurren en un sistema biológico, y los mecanismos últimos que explican por qué un rasgo biológico es de determinada forma (Mayr, 1961). Posteriormente, Niko Tinbergen propuso que estos enfoques se aplicaran al estudio del comportamiento a través de preguntas que abordaran tanto las causas próximas (es decir, ¿cómo se genera y se desarrolla un comportamiento en el organismo?), como las causas últimas (es decir, ¿cómo evolucionó y cuál es la función de cierto comportamiento?) (Tinbergen, 1963). En conjunto, los mecanismos próximos del comportamiento explican las respuestas fisiológicas que desencadenan la conducta,

mientras que los mecanismos últimos se centran en las explicaciones evolutivas y adaptativas.

Algunas preguntas de interés en el caso de la elección femenina son: ¿cómo genera el cerebro las respuestas precisas para encontrar a la mejor pareja?; ¿cómo cambia el cerebro en cada etapa reproductiva?; ¿la actividad neuronal se modifica en respuesta a las estrategias reproductivas masculinas?; ¿los procesos neuronales de la elección femenina generan costos en las hembras? Las respuestas a esto ampliarían la comprensión de la importancia de las decisiones de las hembras en varios niveles de estudio, tanto en lo funcional como en relación con su entorno. Además, se podrían realizar estudios simultáneos de los mecanismos próximos y últimos para entender cómo perciben las hembras a los machos, el impacto en los procesos cognitivos en la elección de pareja, y cómo las decisiones femeninas influyen en la adecuación de ambos sexos.

Esta tesis, es producto de mi interés por analizar de forma integrativa la elección femenina. El objetivo fue investigar los mecanismos próximos y últimos de la elección femenina a través del estudio del cerebro y el comportamiento de las hembras, utilizando como modelo de estudio al escarabajo *Tenebrio molitor*. Aunque el comportamiento de elección de pareja y las estrategias masculinas en esta especie han sido estudiadas ampliamente, la investigación sobre las estrategias femeninas en respuesta a los machos, y sobre todo, su respuesta neuronal, ha sido limitada. A continuación, abordo dos temas relevantes de mi tesis, iniciando con una breve exploración sobre el papel del cerebro en la elección de pareja, y posteriormente presento las particularidades del comportamiento de elección femenina en *T. molitor*. Finalmente, describiré cada capítulo de la tesis.

El cerebro durante la elección de pareja

El cerebro es el principal órgano en el sistema nervioso de la mayoría de los animales, y coordina una variedad de procesos fisiológicos, así como el comportamiento. Aunque la estructura del cerebro varía entre especies, funciones como el procesamiento sensorial, regulación endócrina, control homeostático, control del comportamiento y procesos cognitivos como la memoria y el aprendizaje, se conservan a lo largo de distintos linajes (Chittka & Niven, 2009; Burns et al., 2010; Ito et al., 2014) (Figura 2). En selección sexual, investigaciones recientes han dirigido su atención hacia las regiones cerebrales, genes y moléculas implicadas predominantemente en el cortejo masculino, como el canto de aves (Bolhuis & Gahr, 2006), y la danza y canto de *Drosophila* (Clowney et al., 2015; Kallman et al., 2015). Con respecto a los mecanismos neuronales femeninos detrás de la elección femenina, los estudios se han centrado en la recepción de señales durante el cortejo masculino. En *Drosophila*, Immonen y Ritchie (2012) analizaron las respuestas genómicas en el cerebro de hembras expuestas a diferentes canciones de cortejo; encontraron que los genes activados en el lóbulo antenal (responsable de información sensorial química) participaban en la regulación de la receptividad sexual femenina. Además, observaron que las canciones del cortejo incrementan la sensibilidad olfativa de las hembras, activando la

transcripción de genes que codifican para proteínas de unión a las feromonas masculinas (Immonen & Ritchie, 2012).

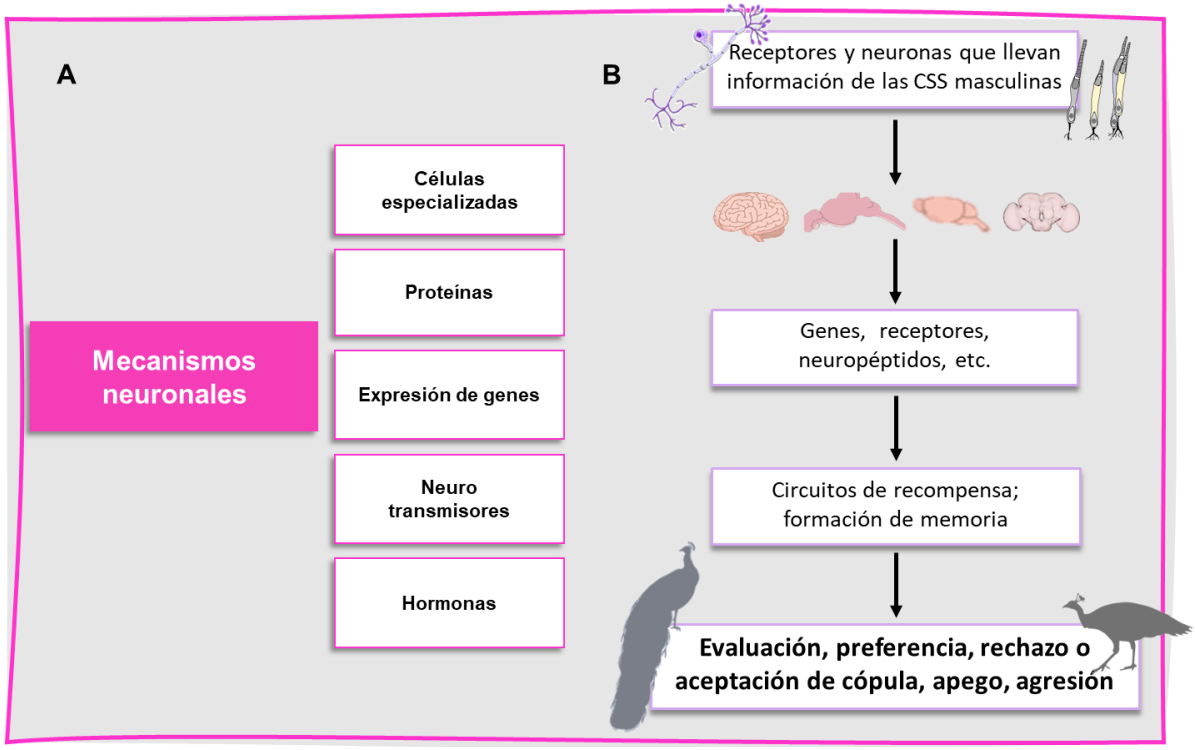

Figura 2. Los mecanismos neuronales en el estudio de la elección femenina.

A) En el estudio de los mecanismos neuronales del comportamiento, se han abordado diversos componentes moleculares y procesos celulares. B) Aunque el cerebro es estructuralmente diferente entre especies, los efectores de su funcionamiento (genes, hormonas, péptidos, células, etc.) y el procesamiento general de información para efectuar comportamientos en la elección de pareja son similares.

Después de la percepción de señales masculinas, las hembras integran en su cerebro la información recabada, llevando a cabo procesos de evaluación que culminan en la toma de decisiones (Figura 2). Aunque esta etapa ha sido menos estudiada, se ha observado que, durante la toma de decisiones, se desencadenan procesos de plasticidad neuronal, liberación de neurohormonas y neurotransmisores, remodelación del citoesqueleto de las neuronas y activación de genes de respuesta inmediata (revisado en: Fisher et al., 2006; DeAngelis & Hoffman, 2020; Ryan, 2020; Cordero-Molina et al., 2024). Algunas de las

evidencias de que la elección femenina es un proceso de cognición complejo, fueron encontradas en la respuesta del cerebro de las hembras de la especie *Xiphophorus nigrensis* al interactuar con machos de diferentes fenotipos. Se encontró que las hembras que mostraban mayor preferencia de pareja por los fenotipos masculinos más atractivos presentan un incremento en la expresión de marcadores de actividad neuronal como el gen *egr-1* y de los genes de la neuroligina, involucrados en generación de nuevas sinapsis (Cummings et al., 2008). Además, se observaron patrones de respuestas genómicas únicos para diferentes tipos de encuentros sociales (frente a hembras, frente a sólo un macho, frente a dos machos de igual o diferente fenotipo) (Cummings et al., 2008; Wong et al., 2012), lo que sugiere que el cerebro de las hembras modula respuestas apropiadas para cada encuentro social a través de la activación de genes de plasticidad sináptica que, a su vez, le permiten discriminar y evaluar el atractivo de los machos.

La experiencia y exposición a machos con variación en sus caracteres sexuales secundarios puede moldear las preferencias en la elección de las hembras por sus parejas futuras. Observaciones en insectos y aves, indican que las hembras ajustan su umbral de aceptación de posibles parejas según las experiencias previas con diferentes tipos de machos (Dukas, 2005; 2006; Bailey & Zuk, 2008; Sockman & Lyons, 2017). En *Melospiza lincolnii*, las hembras expuestas a canciones más atractivas aumentan su umbral de aceptación para canciones de nuevas parejas (Sockman & Lyons, 2017). Este cambio fue atribuido a que los patrones de sonido de los machos preferidos provocan una disminución de la actividad noradrenérgica y serotoninérgica en zonas del cerebro encargadas del procesamiento del sonido (Lyons & Sockman, 2017; Sockman & Lyons, 2017). Cabe destacar que la serotonina y norepinefrina participan en la modulación de la plasticidad

neuronal en procesos auditivos, de atención y la excitación sexual (Sockman & Lyons, 2017).

Dentro de una misma población pueden observarse diversos patrones de elección de pareja con relación al grado de preferencia por algún fenotipo. Sin embargo, el uso exclusivo de la observación a nivel de conducta podría ser insuficiente para explicar apropiadamente la variación entre las hembras. Por otro lado, el estudio del cerebro podría ampliar la comprensión de los patrones de preferencia encontrados en las poblaciones. Por ejemplo, en los peces *Poecilia reticulata,* las hembras con una marcada preferencia por machos grandes y coloridos poseen un cerebro más grande que aquellas sin preferencia (Corral-López et al., 2017). Trabajos posteriores indicaron que la variación en el tamaño del cerebro solo es una característica en el fenotipo de las hembras con preferencia, y no la causa de este comportamiento. Al analizar el transcriptoma del cerebro de ambos tipos de hembras, Bloch et al. (2018) observaron diferencias sólo en áreas de integración y procesamiento de la información (el telencéfalo) y no en las áreas sensoriales (el téctum óptico), indicando que las hembras sin preferencia no tienen dificultades al percibir las señales masculinas, sino que integran la información y toman decisiones de manera distinta a las hembras con cerebro de mayor tamaño. Entre las diferencias en los patrones de transcripción se encontraron genes de plasticidad sináptica y genes de expresión inmediata que permiten tener respuestas más rápidas y cambiantes ante diferentes estímulos. El gen Angiopoyetina-1 (*ang-1*) es el único cuya expresión difiere al exponer a las hembras a los machos, sus funciones incluyen en el desarrollo vascular, angiogénesis y crecimiento neuronal y se expresa más en las hembras con preferencia de pareja (Chen et al., 2015; Bloch et al., 2018). Este trabajo muestra que el estudio del cerebro femenino amplía la

comprensión de la variación en el comportamiento de elección de pareja, evitando asumir explicaciones erróneas.

Por último, estudiar la elección de pareja por medio de la fisiología comparada del cerebro proporcionaría información valiosa sobre la diversidad y evolución de los mecanismos neuronales de las hembras. Por ejemplo, se ha descrito que el sistema neuromodulador oxitocina-vasopresina, es responsable del vínculo de pareja en sistemas monógamos y se conserva en varios grupos de vertebrados (Cummings, 2015; Grinevich et al., 2016). También se ha encontrado que ambos neuropéptidos actúan junto con el sistema de recompensa de la dopamina, y ambos sistemas promueven el comportamiento afiliativo con la pareja, y el mantenimiento del vínculo en la monogamía (Winslow et al., 1993; Liu & Wang, 2003; Young & Wang, 2004; Aragona et al., 2006). Sin embargo, aunque varios linajes de invertebrados poseen genes ortólogos al de la oxitocina (Garrison et al., 2012; Lockard et al., 2017), aún se desconoce si también regulan el apego a la pareja en especies de este grupo.

A pesar de la limitada cantidad de estudios sobre el funcionamiento del cerebro durante la elección de pareja en hembras, las investigaciones existentes demuestran que este proceso es neuronalmente complejo, y va más allá de solamente respuestas instintivas a los estímulos masculinos. La propuesta de Tinbergen de conectar los mecanismos próximos y últimos sigue siendo un método de estudio atractivo y vigente para entender la biología del comportamiento. El análisis del cerebro puede ampliar las explicaciones evolutivas que se han propuesto respecto a la elección femenina, y abrir nuevas perspectivas en este campo. Por ejemplo, aunque la conducta de las hembras parezca sugerir lo contrario, examinar su

actividad cerebral podría revelar si responden defensivamente a los intentos de los machos por anular o manipular la elección femenina. Tal es el caso de las cópulas coercitivas observadas en *Gerris gracilicornis*, donde los machos atraen depredadores si las hembras se niegan a copular con ellos (Han & Jablonski, 2010); o en el caso de los escarabajos *Tenebrio molitor*, donde la elección femenina parece estar manipulada por señales deshonestas de los machos cuando están enfermos (de baja condición), ya que resultan más atractivos para las hembras que aquellos en buena condición (Sadd et al., 2006). La respuesta del cerebro en estos casos permitiría entender mejor el papel de las hembras en la elección de pareja y cómo enfrentan los desafíos cuando surgen conflictos entre los intereses reproductivos de ambos sexos. En resumen, es necesario estudiar al cerebro en la elección de pareja como un componente en interacción con el entorno y el sexo opuesto, y no solamente como un órgano generador de la conducta.

Estrategias reproductivas masculinas y femeninas: el caso de *Tenebrio molitor*

El escarabajo de harina (*Tenebrio molitor*) se considera una plaga en la agricultura ya que su éxito de propagación se atribuye al almacenamiento de harinas y a los desplazamientos de las poblaciones humanas (Cotton, 1927; Howard, 1955). La facilidad en su crianza y las particularidades de su conducta sexual, han propiciado que sea un modelo de estudio de las estrategias reproductivas, especialmente con relación a los compromisos entre la supervivencia y la reproducción que enfrentan los machos. *Tenebrio molitor* es una especie polígama (Drnevich et al., 2000; Worden & Parker, 2001), y ambos sexos se atraen a través de feromonas volátiles (Happ, 1969; August, 1971; Griffith et al., 2020) e hidrocarburos cuticulares de baja volatilidad (Nielsen & Holman, 2012). Existe evidencia de elección de pareja en ambos sexos (Ruiz-Guzman et al., 2021; De León, 2023), pero en la literatura se

describe mayormente la elección femenina. Las hembras prefieren aparearse con los machos que producen más la feromona Z-3- dodecenyl acetato (Bryning et al., 2005). Las feromonas sexuales de estos machos son dependientes de su condición fisiológica (Rantala et al., 2002; 2003; Nielsen & Holman, 2012; Márquez-García et al., 2016; Ruiz-Guzman et al., 2021). Esto significa que funcionan como señales honestas de su condición, ya que la producción de los CSS es costosa para los portadores (Zahavi, 1975). Por lo tanto, aquellos en óptima condición son percibidos como más atractivos (Rantala et al., 2003). Sin embargo, las variaciones ambientales pueden disminuir la condición masculina, alterando la producción de sus feromonas. En tales circunstancias, los machos de *T. molitor* recurren a una estrategia reproductiva alternativa llamada inversión terminal que les permite sobreproducir feromonas, incluso en mayor cantidad que los machos sanos, de condición óptima (Sadd et al., 2006; Kivleniece et al., 2010; Krams et al., 2011; Nielsen & Holman, 2012).

La inversión terminal ocurre cuando los individuos tienen riesgo de morir, y por ende, pierden su reproducción futura (Clutton-Brock, 1984). Por lo tanto, usan sus recursos enérgeticos para maximizar sus oportunidades reproductivas en el presente a costa de la reproducción futura y de su supervivencia (Clutton-Brock, 1984; Duffield et al., 2017). En su trabajo sobre la dinámica de la estrategia de inversión terminal, Duffield et al. (2017) propone que la capacidad de realizar una inversión masiva en la reproducción está condicionada por un umbral de recursos energéticos mínimos, ya que la inversión también demanda gasto energético (Figura 3). Sin embargo, hasta el momento no se ha demostrado experimentalmente la ocurrencia de los umbrales de la inversión reproductiva que inducen la inversión terminal o señalización de condición en una misma especie.

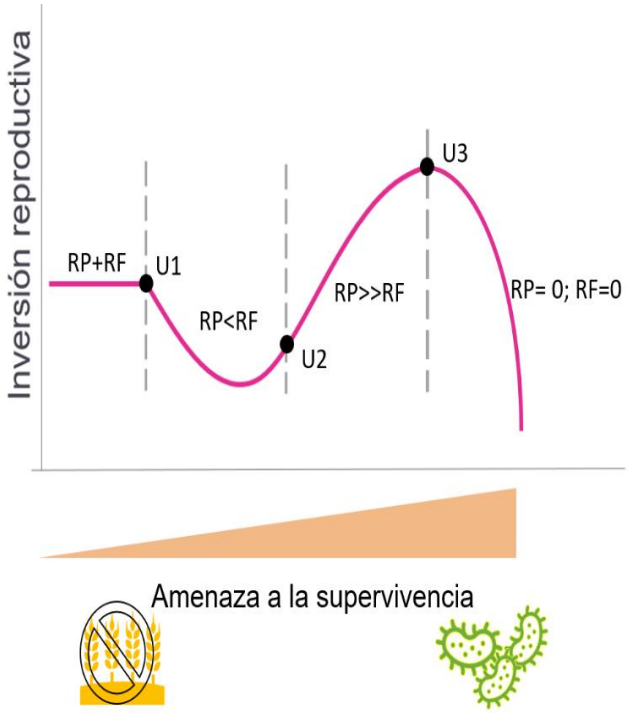

Figura 3. Dinámica de la inversión reproductiva en relación con el nivel de amenaza a la supervivencia durante la inversión terminal.

El grado de daño a la condición puede ser ocasionado por condiciones adversas (ej. escasez de alimento, infección parasitaria). La inversión reproductiva es la estándar en individuos sanos antes de U1. U1: umbral de daño que disminuye la condición y la inversión en RP. Uso de recursos para recuperar la condición e invertir en RF. U2: umbral de daño que amenaza la supervivencia y RF. Los recursos no son suficientes para recuperarse y reproducirse en el futuro, la inversión reproductiva aumenta exponencialmente. U3: umbral en que el daño es severo y la muerte es inminente. La condición es extremadamente baja y no es posible reproducirse. **RP: reproducción presente; RF: reproducción futura**

Se considera que la sobreproducción de feromonas generadas por los machos de *T. molitor* durante la inversión terminal son señales deshonestas porque corresponden a las señales de los machos de buena salud, aunque en realidad, estén a punto de morir (Zahavi, 1978; Sadd et al., 2006; Vainikka et al., 2007; Krams et al., 2011). Se propone que las hembras enfrentan constantemente un ambiente cambiante en la señalización de condición poque los machos usan señales honestas o deshonestas, colocándolas en una posición pasiva y vulnerable a la manipulación masculina. Sin embargo, este tipo de descripciones tradicionales de los roles de los sexos en la selección sexual promueven el desconocimiento del comportamiento femenino (Ah-King & Gowaty, 2015; Ah-King, 2022). Para evitar esto, Ah-King (2022) resalta la necesidad de evitar sesgos, al utilizar adjetivos como "pasivas" y

"manipulables" al referirse a las hembras. En lugar de ello, propone adoptar enfoques más amplios que reconozcan la complejidad y la diversidad de las interacciones entre los sexos.

En este trabajo, mi objetivo fue comprender la perspectiva femenina de *T. molitor* frente a la estrategia de deshonestidad de los machos. Hernández-Villanueva et al. en 2023 reportaron que el cerebro de las hembras que se aparean con machos atractivos producen más proteínas relacionadas con la formación de memoria (i. e. Metiltransferasa LDS-1; Li et al., 2013) (Figura 4), así que investigué si las hembras de *T. molitor* poseen mecanismos neuronales en respuesta a las estrategias masculinas. El aumento en la producción de esta proteína sugiere que las hembras almacenan información de las parejas más atractivas, lo que podría permitir que reconozcan y evalúen constantemente a sus parejas previas. La búsqueda de mecanismos femeninos que permitan enfrentar el engaño masculino se fundamenta en la posibilidad de que las hembras incurran en costos por no poder discernir entre machos de buena o mala condición. Sin embargo, hasta ahora no se ha demostrado que elegir a un macho deshonesto en cuanto a su condición implique consecuencias negativas en la adecuación femenina. De ser así, se esperaría diversidad en las estrategias femeninas y que una parte de la población pueda evitar estos costos, reconociendo a los machos incapaces de mantener una condición óptima a lo largo del tiempo, y evitando a posibles manipuladores de la condición. A continuación, describiré brevemente el contenido de los capítulos que corresponden a los artículos y manuscritos generados a partir de mis resultados.

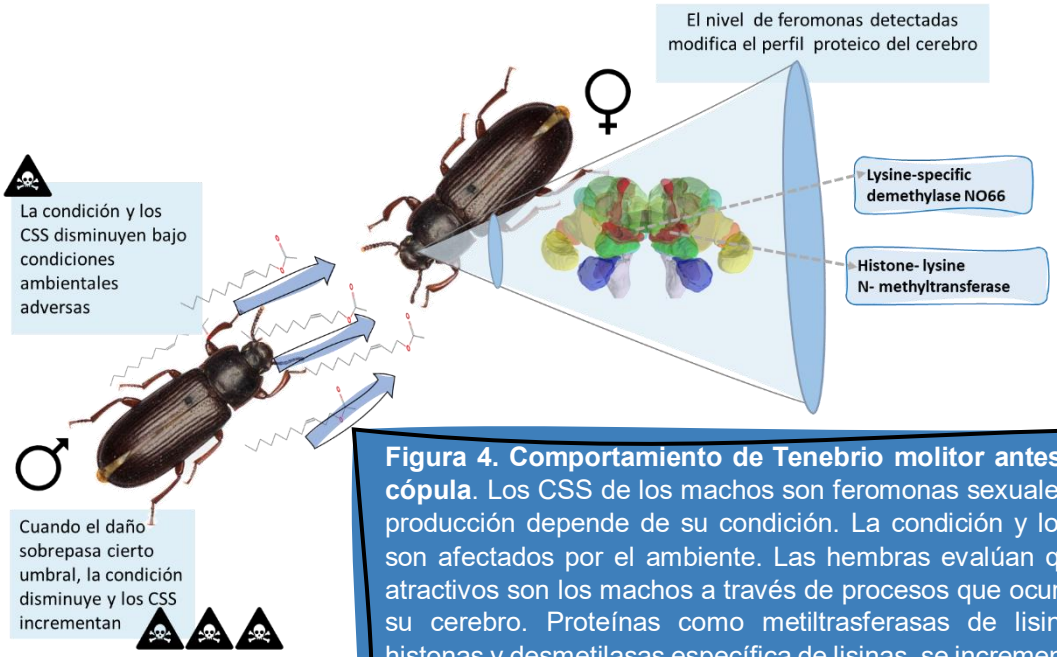

Figura 4. Comportamiento de Tenebrio molitor antes de la cópula. Los CSS de los machos son feromonas sexuales cuya producción depende de su condición. La condición y los CSS son afectados por el ambiente. Las hembras evalúan qué tan atractivos son los machos a través de procesos que ocurren en su cerebro. Proteínas como metiltrasferasas de lisinas de histonas y desmetilasas específica de lisinas, se incrementan en el cerebro de las hembras cuando se aparean con machos altamente atractivos (Hernández-Villanueva et al. 2023), ambas proteínas tienen funciones de formación de memoria. (Modelo de cerebro de *Tribolium castaneum;* fuente: https://www.insectbraindb.org/)

El capítulo II es una revisión de los mecanismos neuronales implicados en la elección femenina en invertebrados. Aquí abordo como el sistema nervioso puede revelar respuestas de las hembras a comportamientos masculinos que no se podrían detectarse únicamente con observaciones de conducta. También propongo posibles rutas neuronales y moléculas que se deben investigar, tomando en cuenta comportamientos poco estudiados como los que revelan conflictos sexuales extremos, y durante la elección postcópula.

En el capítulo III, exploré la participación de la inotocina, un péptido similar a la oxitocina, en la conducta sexual y de elección de pareja en insectos. Utilizando a *T. molitor* como

modelo, investigué la localización de la inotocina en el cerebro de *T. molitor*. Además, administré inotocina en hembras adultas no apareadas y comparé su comportamiento con el de las hembras control. En cuanto a la localización en el cerebro, observé que las neuronas secretoras de inotocina proyectan hacia áreas relacionadas con la formación de memoria en insectos. Encontré que la inotocina redujo la tasa de apareamientos, pero promovió la preferencia por parejas conocidas. Estos resultados sugieren que la inotocina desempeña un papel similar al de la oxitocina en mamíferos, potenciando la selectividad durante la elección de pareja y la formación de memoria social en hembras.

En el capítulo IV, puse a prueba la hipótesis de que las hembras de una especie poliándrica podrían usar la memoria para detectar y evitar a machos de condición subóptima. Además, siguiendo la idea de mis propuestas en el capítulo II, investigué los cambios en el cerebro de las hembras de *T. molitor* en diversas etapas de la elección de pareja. Llevé a cabo observaciones conductuales para evaluar la preferencia de las hembras por machos conocidos o nuevos, y manipulé experimentalmente el nivel de atractividad en machos. Posteriormente, mediante técnicas de proteómica analicé el cerebro de las hembras vírgenes, apareadas, con y sin preferencia por la pareja conocida. Las pruebas conductuales revelaron que las hembras prefieren a los machos conocidos cuando su nivel de feromonas se mantiene alto con el tiempo, pero los rechazan si disminuye. Esta preferencia desapareció al aplicar un amnésico. Este último resultado, junto con los hallazgos del análisis de proteómica en el cerebro sustentan la existencia de un proceso de memoria. Además, los resultados también sugieren que la elección femenina produce un incremento en el metabolismo y estrés oxidante en el cerebro.

En los dos capítulos siguientes, me centré en el estudio de las posibles presiones de selección que han moldeado la memoria de las hembras de *T. molitor* en la elección de pareja.

En el Capítulo V, investigué si un factor ubicuo para los organismos, como el estrés oxidante, podría desencadenar la estrategia de inversión terminal en los machos de *T. molitor*. La siguiente pregunta fue si, además de ser dependiente de la dosis de daño, la inversión terminal respondía al tiempo transcurrido entre el daño y la ocurrencia del apareamiento. Para ello, administré un gradiente de dosis de un prooxidante herbicida y controlé el intervalo de tiempo entre el daño y la inversión reproductiva. Encontré que la ingesta de prooxidantes efectivamente desencadena la estrategia de inversión terminal. Bajo cierta dosis, los machos oxidados resultan más atractivos y mueren antes que aquellos que están sanos. Los resultados también revelaron que esta estrategia no solo depende de la dosis sino también del lapso entre la ingesta y el apareamiento. Los machos oxidados que retrasaron el tiempo de apareamiento lograron recuperarse y no murieron antes que el grupo control. Finalmente, sugerí estudiar los costos asociados a esta estrategia considerando retos de gasto energético, como el apareamiento, para evitar que los costos queden enmascarados. Estos resultados evidencian los costos para los machos de iniciar la estrategia de inversión terminal.

En el capítulo VI, puse a prueba si existen costos para las hembras en la elección de machos deshonestos. Administré un prooxidante a los machos en la dosis requerida para inducir la estrategia de inversión terminal, y analicé la calidad de su eyaculado en términos de proteína, oxidantes y antioxidantes. Encontré que el eyaculado de los machos

deshonestos contiene menor protección antioxidante en comparación con los machos sanos. Este resultado sugiere un potencial costo para la viabilidad de los gametos masculinos, la descendencia e incluso para la condición de la hembra.

En conjunto, mi tesis muestra un análisis integrativo del cerebro de las hembras en diversas etapas de la elección de pareja y examina la diversidad en sus estrategias de elección. Además, proporciona una posible función del comportamiento femenino, analiza los costos asociados a las estrategias de ambos sexos, en las que cada uno busca maximizar su adecuación.

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CAPÍTULO II.

Cordero-Molina, S., Fetter-Pruneda, I., & Contreras-Garduño, J. (2023). Neural mechanisms involved in female mate choice in invertebrates. Frontiers in Endocrinology, 14. doi: 10.3389/fendo.2023.1291635

Abstract

Mate choice is a critical decision with direct implications for fitness. Although it has been recognized for over 150 years, our understanding of its underlying mechanisms is still limited. Most studies on mate choice focus on the evolutionary causes of behavior, with less attention given to the physiological and molecular mechanisms involved. This is especially true for invertebrates, where research on mate choice has largely focused on male behavior. This review summarizes the current state of knowledge on the neural, molecular and neurohormonal mechanisms of female choice in invertebrates, including behaviors before, during, and after copulation. We identify areas of research that have not been extensively explored in invertebrates, suggesting potential directions for future investigation. We hope that this review will stimulate further research in this area.

1 Introduction

Mate choice is one of the most critical decisions organisms make because of its direct impact on their fitness. Mate choice is an evolutionary process that often favors the evolution of conspicuous traits in individuals (1). These traits, called secondary sexual characters (SSC), include structures, colors, odors, and behavior (Box 1). Mate choice occurs when the evolution of SSC in one sex leads to non-random mating with members of the opposite sex based on those characters (2, 3). Although mate choice was recognized over 150 years ago (4), many gaps exist in our understanding of its underlying mechanisms. For example, most studies on mate choice focus on the evolutionary causes of behavior, with less attention given to the physiological and molecular mechanisms involved (5, 6). Furthermore, although female choice is recognized as the more frequent type of mate choice (1, 7, 8), there have

been more studies focusing on male behavior during the process of mate choice compared to female behavior (9). This bias may exist because male behaviors tend to be more conspicuous than female behaviors, resulting in a misinterpretation of females in a passive role in sexual selection. However, it is important to understand that the information conveyed by males through their behavior is interpreted within the female's nervous system (10, 11) (Figure 1). Studying how female brains work during mate choice is crucial for fully understanding these behaviors from mechanism to evolutionary consequences.

While a growing number of studies in vertebrates reveal the neural mechanisms involved in the process of selecting a mate, our comprehension of the neural mechanisms of mate choice in invertebrates remains limited. DeAngelis and Hofmann reviewed the most relevant works in vertebrates on the neural and molecular mechanisms of female choice, focusing mainly on decision-making (5). Although such studies in invertebrates are limited, the existing research provides an opportunity to explore several important research avenues. 1) What are the specific locations and mechanisms within the nervous system that govern the intricate process of mate choice decision-making? and where in the brain does the process of mate choice occur: before, during, or after copulation?; 2) Given the diversity of mating strategies invertebrates possess (12, 13), is there a corresponding diversity in their neural mechanisms, or do a few mechanisms control such diversity? 3) Are the neural mechanisms underlying behaviors that are less explored or absent in invertebrates, such as selective use of stored sperm or sexual cannibalism, controlled by similar mechanisms to better studied behaviors in vertebrates? 4) The mechanistic understanding of mate choice behavior in invertebrates may provide hints to their origin and evolution, and this knowledge may be important for understanding the variety of ways that such behaviors can occur and may additionally allow a better understanding of the evolution and mechanisms of behaviors that we observe in vertebrates.

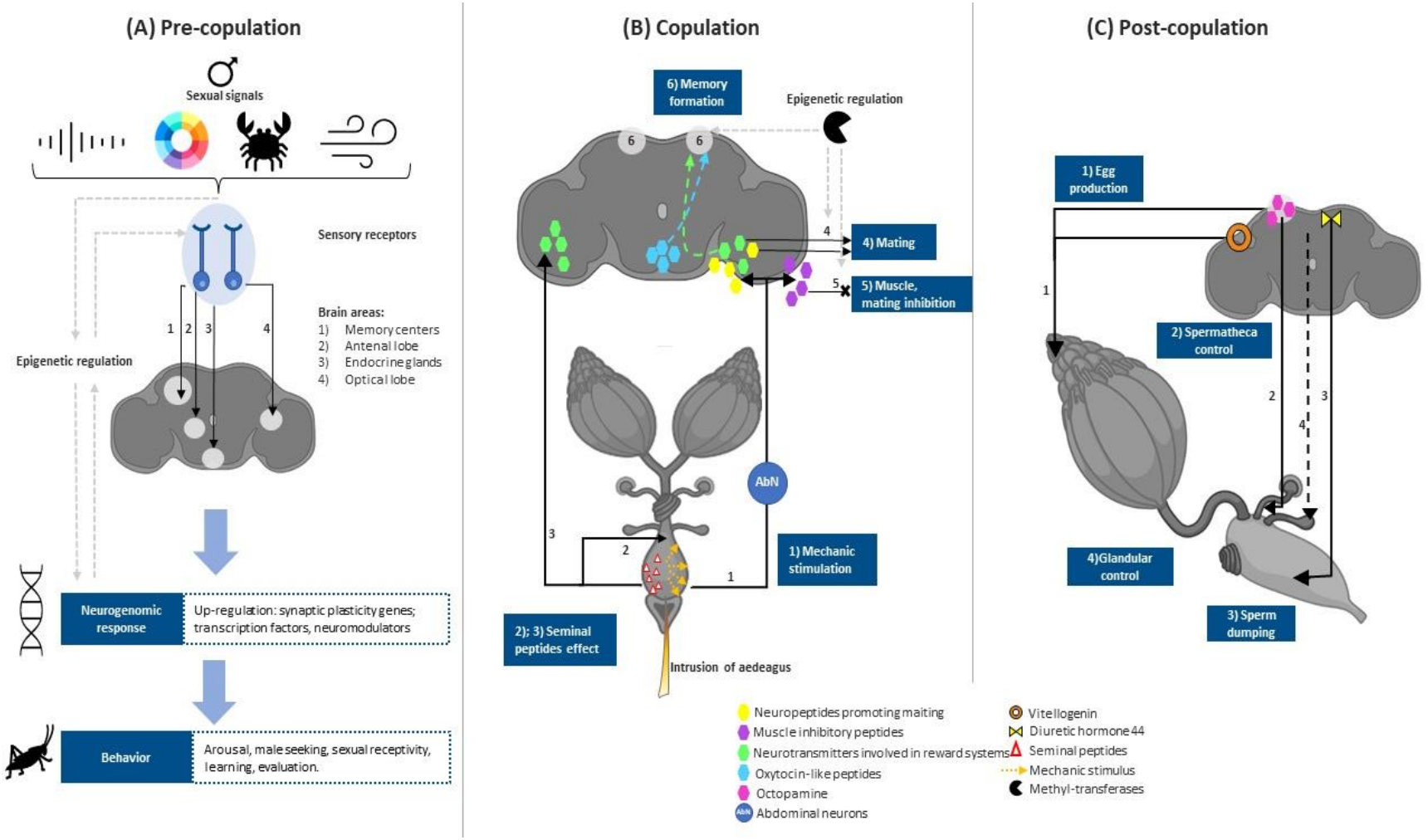

Figure 1. General description of neural pathways during female choice in insects

Figure 1. General description of neural pathways during female choice in insects

(A) Pre-copulation: Female mate choice is influenced by gene expression cascades that respond to stimuli from both the potential mate (e.g., sounds, colors, structures, pheromones) and the environment. The process begins with neural pathways that carry information from the sensory organs to integration points in the corresponding brain areas. There, synaptic plasticity genes activate primarily to produce suitable, plastic, and immediate responses based on the intensity of the signals presented by males (45, 48). If appropriate, such neural activity will prompt arousal, motivation, and reward systems mainly modulated by neurotransmitters (29). Furthermore, all the mentioned steps are subject to epigenetic regulation, while simultaneously being able to influence the same epigenetic regulators. Finally, the neural responses will result in behaviors that favor mating.

(B) Copulation: During copulation, mechanical stimulation from the aedeagus (or pennis-like structure) intrusion stimulates abdominal neurons that carry the signal and triggers neural release of neurotransmitters involved in reward systems (29, 59, 60), such as dopamine (39). Motivation and reward systems facilitate sexual receptivity and copulation. The mechanical stimulus could inhibit re-mating through muscle inhibitor peptides or by blocking rewarding systems. In certain species, neurotransmitters may modulate pair bonding and memory formation mediated by oxytocin-like peptides in the brain (77). Changes in brain areas responsible for memory could trigger partner preference but may also lead to rejection of less attractive males or to former mates in the case of polyandrous females. In addition to mechanic stimulation, male ejaculate contains peptides that have the potential to act in the brain and release other neurotransmitters (34, 101). Other seminal peptides also have effects on the neural control of the female reproductive tract. Some of the neural process occurring during copula may be regulated by epigenetic enzymes, such as methyltransferases (48).

(C) Post-copulation: Following copulation, additional mechanisms enable females to bias the paternity of their offspring or counteract male manipulation prior to or during copulation (108). Because the neural mechanisms of this stage are not well understood, a general description of what occurs in this stage is not yet possible. However, some works provide insights into the neural mechanisms of certain female behaviors. For example, hormones such as Diuretic hormone 44 in *Drosophila*, responsible for controlling neurons in the muscle of the female tract to control sperm retention or dump (126). Octopamine and octopamine receptors located in the decision-making brain areas of insects are required for ovulation, egg laying and muscular contraction of spermathecae (114, 120, 122). Vitellogenin is required for egg production and maturation, but is also located in the brain of social insects during reproduction and mate choice (48, 89). Females bias fertilization through sperm activation and/or deactivation, for example by glandular secretions that preserve the stored sperm (140) or with spermicidal action (139), it is unknown whether females exercise glandular control via neural actions from decision-making areas.

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Here, we review the neural mechanisms of female choice in invertebrates during mate choice. We specifically include works that focus on the role of genes, molecules (such as neurotransmitters, hormones, and proteins), and neurons that participate in female choice. We consider female choice to be a behavior that contributes to non-random mating and/or fertilization, including behaviors before, during, and after copulation. Furthermore, we identify areas of research that have not been extensively explored in invertebrates, suggesting potential directions for future investigation.

2 Precopulatory neural mechanisms

A crucial early phase in mate choice involves the accurate perception of signals from a potential mate, with a primary function being the identification of the sender as a member of the opposite sex. (1). The mechanisms behind this stage will depend mainly on the nature of the signals and the type of organs and sensory cells used to receive the stimulus, e.g. chemoreceptors in the antennae of fruit flies (14, 15), the cuticle of nematodes (16, 17) or the ventral mesosoma of scorpions (18); the mechanoreceptors of drosophila (19), crickets (20) and arachnids (18, 21); UV sensitive and other wavelength photoreceptors in odonates (22) and spiders (23, 24). The type of male signals will determine the neurobiological pathway that will carry this information to the integration centers in the female brain (Box 1) (25, 26). After the individual has been identified as a male of the same species, the female will be able to discriminate between potential mates.

Box 1. Sexual selection

Darwin described sexual selection as "a struggle not for existence concerning other organic beings or external conditions, but a struggle between individuals of the same sex, usually males, for possession of the other sex" (170, p. 59). The result of this fight is not death but the lack of offspring of individuals. This implies that males with the most exaggerated secondary sexual characteristics (SSC) (coloration, plumage, antlers, pheromones, etc.) will be favored by mate choice to leave more progeny. Sexual selection has three components. The first two, mate competition and mate choice were described by Darwin (4). The third, sexual conflict between mating pairs, occurs when males and females compete with their partners to obtain an optimum between the costs and benefits of mating and reproducing, with this optimum differing between the sexes (147, 164, 171).

Box 2. Mating Systems

A mating system refers to the way in which individuals within species establish and maintain sexual pairs, as well as how fertilizations are achieved and which individuals are involved (50, 172). These systems are frequently influenced by a range of factors, encompassing ecological, social, and evolutionary aspects. Studying mating systems is invaluable for comprehending the reproductive strategies of diverse animal species and the dynamics of their populations. In the animal kingdom, various mating systems are observed, and they can be broadly classified into the following main types (50, 172, 173):

Monogamy: In a monogamous mating system, a single male and a single female form a long-term (often lifelong) bond. Monogamy ensures that both parents participate in the care and protection of their offspring. When females exhibit this behavior, it is referred to as monandry.

Polygamy: Polygamy is a mating system where both sexes mates with multiple individuals of the opposite sex. When females display this type of behavior, the term used is polyandry.

Promiscuity: In a promiscuous mating system, individuals have multiple, often brief, sexual encounters with multiple partners, without forming long-term bonds. This is common in many species, including many insects and some mammals.

2.1 Sexual receptivity

Before choosing a male, the sexual status of the female determines her receptivity to copulation. A receptive female often displays a series of behaviors facilitating reproduction (27), which may include the release of chemical signals, movements toward males, or ovipositor extrusion. The neural mechanisms for sexual receptivity have been studied in *Drosophila melanogaster*. When immature virgin or recently mated *D. melanogaster* females are not receptive, they do not respond to the male's courtship and instead avoid him by performing specific stereotypical movements: immature virgins curl their abdomen tip downwards, a behavior referred to as "curling;" mated females and immature virgins engage in a behavior called "decamping," which involves running, jumping, or flying away; and mature females and immature virgins kick backward, a behavior termed "kicking." (28) When receptive, however, they pause their movements and interact with male partners to copulate, a behavior referred to as "pausing" (28, 29). Two primary genes regulate *D. melanogaster*'s receptivity, doublesex (*dsx*) and fruitless (*fru*). Both are transcription factors responsible for differences in male and female sexual behaviors, and these differences are also observed in the neuronal circuits (30-33). The description of the neural circuitry of sexual receptivity in Drosophila has allowed the identification of *dsx*- and *fru*-expressing neurons, dendritic projections, and connection sites in the brain and reproductive tract (29, 32, 33). Zhou et al. found that the silencing of *dsx*-expressing neurons reduced female receptivity (33). In addition to *dsx*, Bussell et al. showed that the transcription factor Abdominal-B (*Abd-B*) regulates the female behavior of pausing in front of courting males and interacting with them to copulate (29). Abd-B is present in a group of neurons in the abdominal ganglion with projections to the brain and reproductive tract, which suggests that their function is to integrate the sensory information of courtship and output the observed motor activity, such as "pausing" (29). The nervous system of females can be altered by peptides produced by

themselves, but also by those transferred by males in the spermatic fluid (34). As an example, the sex peptide (SP) is originated in the male accessory glands and transferred during insemination to females. SP elicits alterations in neural activity that reduce receptivity (34). Within the female's reproductive tract, sensory neurons responsive to sex peptide detect its presence and convey the signal to the SP-abdominal ganglia neurons and to myoinhibitory peptide interneurons. Concurrently, SP suppresses the activity of serotonergic projection neurons, leading to a decline in female receptivity (35-38).

2.2 Neural response to male courtship

The neural circuits of sexual behavior in invertebrates have been primarily studied in *Drosophila*. Immonen and Ritchie identified differences in gene expression in *Drosophila simulans* female brains exposed to courtship songs by conspecific and heterospecific males (15). They identified several antennal signaling genes that are part of neuropeptide signaling pathways, including the neuropeptide Corazonin (Crz) (15) which is involved in dopamine regulation and modulates female sexual receptivity in *Drosophila* (39). In addition, they found that exposure to courtship songs influences the expression of genes involved in chemical communication, such as odorant receptor and co-receptor genes and odorant-binding protein-coding genes, most of which are involved in binding pheromones (15). These findings suggest that male courtship sensorial stimulation facilitates receptivity in females by enhancing their sensitivity to male chemical signals.

Female response to courtship may change after mating, as evidenced by olfactometry studies that demonstrate a decreased response to male olfactory cues in mated females of *Anastrepha* fruit flies compared to their unmated counterparts (40). While the neural pathways governing female responses to male courtship have been extensively elucidated

in *Drosophila*, studies on other invertebrate species have identified specific components involved in responding to various types of male signals, including visual and vibrational stimuli (conveyed through leg and pedipalp drumming) in the courtship of wolf spiders, *Schizocosa* (21). Notably, *Schizocosa* females possess vibration receptor organs in their legs comprised of multiple slit sensilla, each innervated by two neurons (21). Research by Knowlton and Gaffin (21) revealed that neurons in all sensilla, both proximal and distal, responded to leg drumming, with proximal sensilla exhibiting a higher response to pedipalpal drumming. It's worth noting that pedipalps are also employed for sperm transfer. Therefore, the diverse responses to vibrational signals from different sources provide clues into the precision and sensitivity involved in detecting signals during courtship. Studying the nervous system during courtship not only enhances our understanding of female responses but also helps determine more precisely which male signals elicit a response in the female. Recent evidence suggests that, during courtship, the leg movements of male *Schizocosa retrorsa* induce air particle movements, which females respond to during mate choice, rather than responding solely to the visual stimulus (41).

2.3 Neural responses during mate evaluation

Experience with different males causes changes in female brain activity and affects how they choose a partner. Experience and exposure to males with variation in their SSC play an essential role in how females choose in future encounters. In flies and crickets, it has been observed that females increase the acceptance threshold of potential partners according to the type of males with whom they have had previous social experiences (42, 43). Although the mechanisms behind the increase in choice threshold remain unknown, one suggestion is that *Drosophila* females become more selective after mating because

copulation increases their Juvenile Hormone (JH) levels, which reduces olfactory sensitivity of Or47b odorant receptor neurons that sense the male sex pheromone (44). Therefore, in a circumstance where a previously mated female encounters more than one male, the chosen male will be the one that produces enough sex pheromones to surpass the threshold, which could lead to strong intra and intersexual competition.

Sensory modalities in mate choice may vary across species. However, other processes involved in information integration and neural response modulation may be evolutionarily conserved. For instance, the process of mate choice in vertebrates includes alterations in gene expression related to neural plasticity (45, 46), along with epigenetic control (47). Hernandez-Villanueva et al. found similar responses in the brain of Tenebrio molitor females, consisting in an increase in proteins related to synaptic plasticity and changes in the levels of methyltransferases and subunits of the methyltransferase complexes when they evaluate and mate with males of different phenotypes (48).

2.4 Attention-directed behaviors of sexual signals from various males

During female choice, females receive male signals from various potential partners and should differentiate one signal from another to choose the best option, that is, the signal that favors their fitness. This could be challenging in systems where several males court females simultaneously. In these cases, the female must be able to differentiate the signals of potential partners and focus her attention only on one male while ignoring the others. In crickets (*Mecopoda elongata*), when females receive acoustic signals from several males that give similar calls at brief time intervals, they prefer the leading male, who generally sets the interval rate that other males follow (49). Omega neuron 1 (ON1) neurons are involved in perceiving acoustic signals and receive excitatory monosynaptic connections from

ipsilateral afferent sensory cells. ON1 neurons also have inhibitory activity on their contralateral connections (20). When females receive identical signals from two males in opposite directions, the ON1 neurons that receive the signal from the leading male are excited and simultaneously inhibit the contralateral ON1 neurons, preventing them from perceiving the other males' acoustic signals (20). This raises the question of how the female brain discerns between males in species in which males court females as a group (lek) and how these mechanisms vary in subsequent encounters.

3 Copulatory neural mechanisms

A species' mating system can be classified as as either monogamous or polygamous (Box 2) (50), which may differ in their copulatory mechanisms. However, there is currently a gap in our knowledge, as molecular studies investigating gene expression and neural changes in the female brain of invertebrates with different mating systems are notably lacking, and potential behavioral disparities between these mating systems may be rooted in variations in the neural mechanisms that drive frequent mating with one or more partners. This information is also essential for determining whether these mechanisms are analogous to those found in vertebrates (e.g. 51 and 52). Furthermore, females may influence the outcome of copulation in several ways, which may vary by mating system. Females may respond positively or negatively to copulatory stimulation, they may control the duration of copulation (53) or may bias sperm utilization by favoring certain males (54, 55).

3.1 Responses to stimulation during copulation

In vertebrates, there is ample evidence that mechanical stimuli received during copulation can exert changes in the neuronal response of the female reproductive tract (56-58). In *Drosophila*, during copulation, intrusion by the male aedeagus has been shown to decrease the activity of neuron clusters in the central brain (possibly pC1 and pCd populations (33)), resulting in reduced sexual receptivity of females independent of the action of seminal molecules that males transfer (59, 60). Female abdominal neurons expressing Piezo mechanosensitive channels are stimulated by insertion of the aedeagus. Then, the signal is transmitted to ascending neurons called LSANs that connect in the central brain (60). Shao et al. (60) proposed that LSAN neurons connect to other neurons that produce a peptide known as muscle-inhibitory peptide (also described as allatostatin-B in Drosophila) resulting in a reduction in female receptivity (60).

3.2 Control of copulation duration

Duration of copulation is an important factor in how much ejaculate is transferred from the male and how likely fertilization is to occur. Abdominal ganglia are heavily involved in regulating muscle contractions in the reproductive tract to influence copulation duration (60). Although the specific neuronal centers involved remain unidentified, the brain also plays a significant role, as supported by observations in three fly species (*Musca domestica* (61), *Anastrepha suspensa* (62), and *Batrocera tryoni* (53)) that copulation with decapitated or decerebrated females lasted longer than with intact females (53, 61, 62).

3.3 Monogamy: Neuromodulatory mechanisms of pair bonding

After sexual attraction and choosing a mating partner, some species may form pair bonds characterized by selective attachments with some degree of durability (52, 63). Much of the knowledge about the neural mechanisms of pair bonding has been generated through the

study of strictly monogamous species, mainly mammals such as the rodent *Microtus ochrogaster* (52, 63, 64). In this species, the peptides oxytocin and vasopressin modulate pair bonding (63, 64), and together with the dopaminergic system in the brain, promote continuous mating with the same partner (52). It has been proposed that the genes encoding oxytocin and vasopressin originated about 600 million years ago (65), with the predecessor molecules to these peptides having a similar role as modulators of social behaviors in different taxa (66-70), including invertebrates (71). However, the role of oxytocin-like peptides during mate choice in invertebrates has yet to be explored, likely because they are absent in flies and bees, precluding their study in honey bees, the most widely studied social insect, and *Drosophila* (72). Most descriptions of the role of oxytocin-like peptides are about behaviors associated with male copulation in nematodes (72), hirudines (73), and gastropods (74, 75). However, its role in female pair bonding and copulation is poorly understood.

Some invertebrate species are described as monogamous, remaining with a single sexual partner for an extended period of time, usually marked by the period of parental care, or because they mate only once in their lives (76). Monogamous behavior and its neural mechanisms have been studied in the biparental beetle *Lethrus apterus*. In this species the expression of genes encoding the insect oxytocin-like peptide, inotocin (*int*), and its receptor (*intr*) in the brain increase during reproductive season and are highest at the beginning of pair formation and during the period of parental care (77). These findings suggest that, as observed with oxytocin in vertebrates, inotocin could modulate temporary mate-attachment behavior in insects. More research is needed on the role of inotocin in species typically described as monogamous or performing biparental care. Additionally, while it is intuitive to study monogamy in species that are commonly considered monogamous or exhibit biparental care, the mating systems in invertebrates are much more complex and flexible

than typical descriptions found in other species. Excluding these models limits our understanding of how different mechanisms have evolved in similar behaviors. For example, in *Gonodactylus bredini* shrimps, it has been observed that males and females reduce their aggression towards their former mates when they meet again (78). Some shrimps of the genus *Alpheus* are considered socially monogamous (pair bond without implying sexual exclusivity between the two partners) since both sexes defend the territory and sometimes provide the nest with food, benefiting more from living in pairs than alone (79). Many molecular pathways can be conserved over long periods of evolutionary time, and understanding what molecules are involved in the modulation of mate attachment of invertebrates would allow a better understanding of the evolution of the molecular systems behind pair bonding that have been widely described in typically monogamous mammals and other socially monogamous vertebrates.

In addition to oxytocin-like peptides, other neuropeptides may be involved in regulating mate attachment. Cunningham et al. reported several neuropeptides that orchestrate biparental care in the beetle *Nicrophorus vespilloides* (80). For example, Natalisin FMRFamide and Sulfokinin have functions that promote mating in various taxa (81-83); Tachykinin is involved in aggression (84); neuropeptide-like precursor 1 (NPLP-1) involved in the division of social labor in honeybee workers (85); and Pheromone-Biosynthesis-Activating Neuropeptide (PBAN) activates pheromone synthesis (86). These neuropeptides might be important for pair bonding because biparental care is one of the most important selective pressures for the evolution of monogamy (50), and in N*. vespilloides* pair bonding and parental care of the larvae are long-lasting.

3.3.1 Other molecules involved in social interactions and pair bonding

Another molecule related to insect social behavior is vitellogenin (Vg), whose primary known function is the production of yolk proteins in oviparous animals (87). Nevertheless, Vg has also been detected in the brains of social insects such as *Apis mellifera* (88) and *N. vespilloides* (89); as well as in the brains of female *Tenebrio molitor*, a polygamous species (48). Vitellogenin is also linked to parental care, with expression of it and its receptor decreasing during active parental care and varying throughout the reproductive cycle in both sexes of the subsocial beetle *N. vespilloides* (89), and to the regulation of genes in the brain that are involved in division of labor in eusocial insects, such as insulin receptor precursor, juvenile hormone epoxide hydrolase, *Impl3* (ecdysone-inducible gene L3); PLRP2 (pancreatic lipase-related protein 2 precursor); *sirt6* (sirt 6 histone deacetylase); TRIP4 (thyroid hormone receptor interactor 4), the transcription factor fruitless in the brain (90, 91). The relationship between Vg expression in the brain and social behavior is not yet clear, but it is also known to function in the brain to regulate energy metabolism of glial cells (88) and to buffer against damage and oxidative stress (92), which may provide some clues. Memory and decision-making demand a large energy expenditure in the brain (93), and cognitive processes such as mate recognition may have a high metabolic demand. A potential area for future investigation could involve examining whether vitellogenin mitigates brain damage in species where females remember previous partners or engage in continuous partner assessment based on previous experiences with males. An example of this possibility is observed in *T. molitor* where vitellogenin levels were higher in the brains of females that evaluated more attractive males compared to those that evaluated less attractive males (48). Furthermore, females exposed to attractive males exhibited elevated levels of catalase in comparison to those interacting with non-attractive males (48). The increase in catalase levels implies the activation of an antioxidant defense mechanism within the brain. Whether

the high metabolic rate in females evaluating different phenotypic males is connected to such a mechanism remains to be answered.

Evidence shows that the peptides and proteins mentioned in the above section are important candidate molecules to study their role as regulators of social behavior during mate choice in invertebrates. However, most approaches focus on analyzing brain gene expression during mate choice, pending behavioral observation when these molecules' functions are altered, for example, through silencing or using agonist and antagonist drugs. Combining different experimental approaches would allow knowing if the selected molecules or genes are the only effectors of the behaviors mentioned or if it is a behavior that responds to more than one effector.

3.4 Neural mechanisms promoting polyandry

Polyandry is the system where a female mates with more than one partner (50). However, many mating systems are flexible, with females able to undergo phases of monandry or polyandry in the face of changing ecological contexts (94), such as the availability of mates in some schistosome species (95) and the damselfly *Ichhnura hastata* (96); or environment resources as seen in the beetle *Ips latidens* (97) and the butterfly *Pieris napi* (98). In addition to the absence of mate recognition and attachment systems described in monogamy, the neural mechanisms of polyandry should address; 1) the motivation to seek and accept copulation with multiple males, and 2) behaviors promoting copulation with new males, such as aggression towards previous partners with the intention of increasing offspring variability (50). Re-mating motivation mechanisms usually involve the same genes and neurons as receptivity mechanisms (63, 99), and differences between mating systems might be due to different activational states of these circuits.

In addition to signals perceived before copulation, seminal proteins and peptides can affect female sexual receptivity circuitry (34, 35, 100), leading the female to change temporarily from polyandry to monandry or vice versa. In the cricket *Teleogryllus oceanicus*, the seminal proteins ToSfp022 and ToSfp0 appear to be responsible for reducing mate-seeking behavior, as females mated with males with a knockdown in the *ToSfp022* and *ToSfp01* genes left their nest significantly more frequently in response to male courtship songs compared to those mated with control males (101). While the mechanism of action of these proteins remains unclear, evidence suggests they interact with the female nervous system in a manner similar to sex peptide in *Drosophila* (34, 35, 38).

Finally, among the benefits of polyandry are increased genetic variability of the offspring and/or receiving more direct benefits from different males (i.e. nuptial gifts, nutrients and high sperm reserves 102, 103). Therefore, in studying the neural mechanisms of polyandry, it is essential to include the behaviors of rejection and aggression toward known males with whom they have previously mated. Such behaviors are described in different taxa, such as the pseudoscorpion *Cordylochernes scorpioides* (104), the spider *Pholcus phalangioides* (105), the moth *Ephestia kuehniella* (106), and the cricket *Gryllodes sigillatus* (107). Although for now this explanation remains hypothetical, these works suggest that females recognize their previous partners through chemical signals that they transfer to males during copulation. These chemical cues might trigger changes in female gene expression that could inactivate neural circuitry for sexual receptivity upon subsequent encounters.

4 Postcopulatory neural mechanisms

Females can skew the paternity of their offspring during and after copulation, a phenomenon called cryptic female choice (108, 109). The mechanisms involved include morphological

traits in the reproductive tract or genitalia, physiology, and behaviors that non-randomly favor paternity in certain males (110, 111). Some behaviors include control of copulation duration, retention, or expulsion of the ejaculate, and cryptic elimination of spermatozoa by substances with spermicidal action (108). Below we describe neural mechanisms studied during cryptic female choice in invertebrates.

4.1 Neural control of spermathecal contraction

Female arthropods have sperm storage organs called spermathecae in which the sperm of one or several males can be stored for use long after copulation has finished. This results in males competing against the sperm of their rivals inside the female's reproductive tract (sperm competition). Based on the neural control of the spermatheca by muscles in insects (112, 113), it has been suggested that females could actively bias fertilization through the nervous control of the spermatheca (111, 114-117). Although it is not yet clear how the spermatheca might bias male paternity, important advances have been made in studying the mechanisms that control spermatheca contractions in some insects. In *Locusta migratoria*, females release sperm stored in the spermatheca through contractions that begin when sensory cells located on the wall of the genital chamber are activated by mechanical stimulation from passage of the egg to be fertilized (118, 119). Joint action of octopamine and tyramine increases the frequency of muscular contractions of the spermatheca in *D. melanogaster* (111) and in *L. migratoria* (120, 121). Furthermore, in *D. melanogaster*, octopamine receptors in the mushroom bodies (OAMB) are required both for ovulation (122) and the release of sperm from spermatheca (111). In *D. melanogaster*, these results imply that the mushroom bodies are involved in controlling reproduction and ovulation. This makes sense with the fact that the mushroom bodies are the primary

information integration centers in the insect brain, where learning processes, memory, odor, and size discrimination occur (123-125). These results support the idea that females may bias the paternity of some males based on cues received from male evaluation before or during copulation.

4.2 Sperm dumping

Another behavior by which females may bias male paternity is the selective expulsion of sperm that the male has transferred to her. The neurobiological pathway behind this behavior has been studied in *D. melanogaster* through the dynamics of Diuretic Hormone 44 (DH44) and its receptor Diuretic Hormone 44 Receptor 1 (DH44R1) in the brain. By silencing transcription of the genes encoding DH44 and its receptor in neurons of the pars intercerebralis region with RNA interference, sperm expulsion occurred much more rapidly than in control females (126). The diuretic hormone 44 (*dh44*) and diuretic hormone 44 receptor 1 (*dh44r1*) genes are orthologous to the corticotropin-releasing factor (*crf*) and corticotropin-releasing factor receptor (*crfr*) genes that have stress response functions in vertebrates (127). Therefore, the authors suggested that sperm expulsion in *Drosophila* could be a stress response caused by the seminal peptides transferred during copulation (126,128).

In addition to the possible effect of seminal peptides, control of sperm expulsion may occur based on the perceived genetic quality of the male before or during copulation as seen in females of the spider *Physocyclus globosus*. In this species, sperm expulsion occurs during or after mating, but females favor paternity for males who display increased courtship behaviors before and during copulation (55). The relationship between courtship intensity and sperm expulsion suggests the possibility that the neural pathways regulating sperm

retention are affected by molecules and circuits implicated during courtship or copulation. Such neural pathways could be connected to reward circuits involving dopamine, which may regulate female decisions during mate choice either by promoting mate search and/or acceptance of mating after evaluation of males (129). Neural circuits promoting mating and courtship in male *Drosophila* have been identified, but female neural responses to courting males are poorly studied. Nonetheless, the transition from rejection to mate acceptance in virgin females may be controlled by the ellipsoid body (EB), a structure of the central complex (130). The interconnected ring neurons (R) in the EB receive input from the PPM3 clustered dopaminergic neurons situated in the superior medial protocerebrum. In the EB, Cholinergic R4d neurons promote rejection behaviors, while the activation of GABAergic and glutamatergic R2/R4m neurons promotes mating acceptance. Additionally, inhibiting R2/R4m neurons leads to an increase in mating latency (130, 131). Although this finding aligns with reports in various vertebrate dopaminergic circuits (129), further research on female neural responses is crucial and may be fruitful considering the possibilities for manipulating the *Drosophila* system (130, 131).

4.3 Control of oviposition

Even after fertilization has occurred, it is possible that females exert some control of oviposition, influencing which eggs are laid (109). Octopamine regulates oviposition in insects (132-134), ticks (135) and nematodes (136, 137) by controlling the contraction rhythms of the oviduct. In *Drosophila*, the Octb2R and OAMB receptors present in octopaminergic and glutamatergic neurons in the abdominal ganglia project to the epithelial and reproductive cells of the oviducts (122, 134, 138). Recent studies indicate that activation of the Octb2R receptor causes relaxation, while activation of the OAMB receptor causes

contraction (138). Additional neuromodulators, such as glutamate, tyramine, and other biogenic amines, may alter the impact of octopamine on the aforementioned neurons. However, their effects remain poorly comprehended and may vary among species (132-138).

4.4 Other behaviors of postcopulatory female choice

There are several exciting behaviors to study in postcopulatory female choice for which there is not yet an approach to study the possible neural mechanisms behind them, for example:

Activation and inactivation of spermatozoa. In several invertebrate species, females may influence fertilization through substances secreted in their reproductive tract that either activate or disable sperm transferred by their partners. For instance, the female reproductive tract of *Drosophila pseudoobscura* provides a spermicidal environment that contributes to sperm competition (139). Another intriguing example comes from spiders, where glandular secretions activate and maintain the sperm stored within spermathecae (140, 141). It has been proposed that the female nervous system regulates the secretion of these glands (142), which may act as a mechanism to bias fertilization towards preferred males, such as those with higher courtship intensity (143).

Selective uptake of sperm in the spermatheca. Paternity may be biased by controlling the reception of sperm in the spermatheca (108). There is some research into the mechanism involved in the release of sperm stored in the spermatheca, but the mechanism controlling the uptake of sperm into the spermatheca remains unknown.

5 Female choice issues that have yet to be explored in invertebrates.

5.1 Sexual cannibalism

One of the most extravagant behaviors in sexual selection is sexual cannibalism, when the female consumes the partner during or after mating and sometimes during courtship without mating. This behavior has only been documented in spiders and mantids. Sexual cannibalism might increase male reproductive success and, in many cases, results from female choice based on the quality of their partners (140, 144, 145). The neural mechanisms of this behavior are yet to be addressed. Based on descriptions of the behavior, it can be hypothesized that the visual sensory system as well as circuits and systems related to foraging and aggression are involved (140), but how these systems relate to mate choice is an open question.

Another example of sexual cannibalism is the mutual “partial” cannibalism of the cockroach *Salganea taiwanensis*. In this monogamous species, both sexes reciprocally consume the wings of their mate. The hypotheses for the evolutionary causes of this behavior propose that consumption of the wings promotes monogamy since, without wings, it is dangerous to leave the nest to look for another mate (146). Because this type of cannibalism promotes pair bonding and does not end in complete consumption of the partner, the neural mechanisms behind this behavior may differ from the aggression mechanisms proposed for sexual cannibalism in spiders and mantids.

5.2 Female neural responses to male manipulation

Sexual conflict arises when some of the following circumstances are in place (reviewed in: 147): 1) males try to overcome filters imposed by the females during mate choice, while

the females respond with counter-adaptations to male strategies (148); 2) males exert sensory exploitation of pre-existing circuits in females (149); 3) females resist exploitation or manipulation by males (149, 150). However, sexual conflict is mostly detected through observation of behavior, and most of the conclusions do not address how the female's nervous system responds to male manipulation. The investigation of the neural responses of females during sexual conflict would advance the comprehension of how sexual selection is taking place across species. For instance, in species where male manipulation occurs, analyzing behavior and reproductive success may not reflect females' resistance to manipulation. However, looking through the nervous system of females could offer a different perspective. By exploring their neural responses, a "physiological attempt" of resistance may be revealed. While these responses are not yet considered counter-adaptive mechanisms, they have an evolutionary potential to develop and empower females to confront manipulation.

Some of the male manipulative behaviors are truly extreme, and studies on the neural responses of females when they occur are limited. One species where such a conflict occurs is the true bug *Gerris gracilicornis*. In this species, males attract predators via special leg movements when females they attempt to mount refuse their advances, leading to coercive copulation (151). How females decide to allow or deny copulation in the context of potentially being preyed on, and if the mechanisms of aggression and flight are coordinated and connected to those of sexual receptivity are interesting questions. Another extreme case of sexual conflict during mating is the traumatic insemination observed in the bed bug *Cimex lectularius*. In this species, males pierce the cuticle of females and inseminate them directly into their body cavity without penetrating their genitalia (152, 153). It is unknown whether the circuits connecting the female genitalia to the nervous system are active or inactive in such cases, or if other circuits are involved during insemination or when sperm is released

from their storage organs. Comparison of this behavior with closely related species that do not display extra-genital copulation may provide insight into whether and how the neural response of these females to this type of male behavior has evolved.

In ants of the species *Hypoponera opacior*, some males mate with young nestmate queens even before they eclose from their cocoons (154, 155). It would be interesting to study any behavioral response to this in either the inseminated queens as pupae or post eclosion or in their egg-laying mother.

Mating plugs are another example of male manipulation, and it is not known what happens inside female brains when a mating plug prevents copulation with subsequent males in some species of arachnids (156-158); insects (159); and even nematodes (160). A mating plug could do more than just physically block copulation; it could also mechanically stimulate neurons of the reproductive tract resulting in inhibition of receptivity (161). Mating plugs are also utilized by male vertebrates (162, 163), however, research on the neural responses of females to this behavior is scarce.

5.3 Possible costs of neural processes of female choice

Sexual differences in reproductive strategies are usually explained as arising from different reproductive costs for each sex (164, 165), but could this same argument be applied to neural mechanisms? In numerous species, females select mates using a cognitive process that may incur ecological costs (48, 93, 166, 167); however, males may not face equivalent consequences because of the absence of neural elements that enable females to be more selective in their mates. Nevertheless, if there is male choice, they too may experience comparable costs. One such cost is intoxicating male sperm in *Drosophila* females (168, 169). In this case, the role of the female detoxification systems is unknown. It is certainly

possible to address these questions in vertebrates, but simpler nervous systems, shorter generation times and faster metabolic rates may make invertebrates better candidates.

6 Conclusions

Study of the neural mechanisms of mate choice is essential for a comprehensive understanding of the unusual behaviors we observe in nature. There are an increasing number of studies on the neural mechanisms of mate choice. However, those on female choice are less common and usually have a broad descriptive approach and not under the theoretical framework of sexual selection. Females of various species display a broad diversity of mate choice mechanisms, and it is possible that this variety in mechanisms corresponds to variation in mating system, life history and evolutionary history. For this reason, it is essential that models used to study female mate choice are equally diverse.

As with other social behaviors, the mechanisms behind female choice are complex and difficult to study because several often occur simultaneously. As techniques, including single-cell sequencing, genome and epigenome sequencing, proteomics, and access to invertebrate genomes advance, it will be possible to disentangle mate choice behaviors with careful experimental design. Working together to understand the immediate causes behind the behaviors will complete the picture of the evolution of mate choice.

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CAPÍTULO III.

Cordero-Molina, S., Fetter-Pruneda, I., & Contreras-Garduño, J. Inotocin may favor mate choice and pair bonding in insects. (**submitted**).

Abstract

Mate choice and pair bonding play pivotal roles in reproductive success and shaping mating systems. Within vertebrates, oxytocin and vasopressin are crucial for facilitating this process: higher levels of oxytocin and vasopressin production are associated with increased likelihood of monogamy and pair bonding. However, it remains largely unknown which molecules might promote pair bonding in invertebrates. Notably, oxytocin/arginine-vasopressin are conserved across animal phylogeny. Therefore, this study aims to investigate the hypothesis that inotocin, an analogue of the oxytocin peptide, influences female mate choice behavior and pair bonding. To assess this hypothesis in the insect *Tenebrio molitor*, we conducted histological and immunofluorescence localization studies, pharmacological tests, and behavioral observations. Our findings revealed that inotocin originated in the neurosecretory cells within the suboesophageal ganglia projects to brain, reaching the mushroom bodies areas. Females treated with inotocin displayed greater selectivity compared to untreated females, exhibiting a reduced likelihood of mating and shorter mating durations. Furthermore, inotocin promoted pair bonding, as females showed a preference for mating with their previous partners over novel potential partners. These results offer new insights into the neuroendocrine mechanisms underlying mate choice in invertebrates, highlighting the role of inotocin as a modulator of pair bonding and female reproductive behavior.

Introduction

Mate choice by females plays a crucial role in shaping mating systems (Fromonteil et al., 2023). The extent to which individuals prefer multiple partners versus a single mate leads to a polygamous or monogamous behavior (Emlen & Oring, 1977). These behaviors involve complex physiological and neural processes that facilitate the processing of information, such as the sexual traits influencing preferences for specific partners (DeAngelis & Hofmann, 2020; Ryan, 2021; Cordero-Molina et al. 2024), or the recognition of previous mates, potentially leading to selective affiliation and copulation with a particular partner (Newman & Halpin, 1988; Walum & Young, 2018). One of the most studied mechanisms behind selective mate preference is the oxytocin-vasopressin system in mammals and its equivalents in other vertebrates (Insel, 1992; Insel et al., 1998; Bielsky & Young, 2004). Oxytocin and oxytocin-related peptides in vertebrates regulate the females reproductive physiology (Caldeyro-Barcia & Poseiro, 1959; Young III et al. 1996; Nishimori, et al. 1996), maternal care behavior (Olazabal & Young, 2006; Da Costa et al., 1996; Pedersen, et al. 1982), sexual intercourse, parturition, mother-infant bond selectivity, lactation (Donaldson & Young, 2008), and pair bonding (mammals: Young & Wang, 2004; Smith et al., 2010 ; birds: Klatt & Goodson, 2013; fishes: Oldfield & Hofmann, 2011; Okuyama, et al. 2014). Pair bonding is defined as a selective preference for a particular mate (Donaldson & Young, 2008) and this led to the monogamous behavior (Wang et al., 1997; Young & Wang, 2004; Barret et al., 2013; Blumenthal & Young, 2023). For example, oxytocin facilitates remating with the same partner, as females spend more time with the familiar “partner” than with the unfamiliar “stranger” (Winslow et al., 1993; Young & Wang, 2004; Donaldson & Young, 2008; Bosch & Young, 2018). However, an unresolved question is which molecule might promote pair bonding and mate choice in invertebrates.

Oxytocin-related peptides evolved more than 600 millon years ago, are present in vertebrates and invertebrates because derived from a common ancestor of metazoans (Garrison et al., 2012; Lockard et al., 2016). Despite this evolutionary conservation, the behavioral roles of these peptides in invertebrates have not received adequate attention. The invertebrate oxytocin-related peptides have been implicated in sexual behavior, such as male copulatory behavior in nematodes (Garrison et al., 2012), leeches (Wagenaar et al., 2010), snails (Van Kesteren et al., 1995; De Boer et al., 1997); and oviposition in earthworms (Oumi et al., 1996). However, research on their involvement in social behavior is even scarcer, with exploration into division of labor in eusocial ants (Fetter-Pruneda et al., 2021) and parental care in beetles (Nagy et al., 2021). Furthermore, the role of invertebrate oxytocin-related peptides in modulating mate preference and pair bonding has not even been tested.

Insects constitute a remarkably diverse group, exhibiting a wide array of sexual behaviors and reproductive strategies. In addition, some groups like coleopterans poses inotocin, the insect homolog of oxytocin (Lockard et al., 2016). Hence, in this study, we aimed to investigate whether inotocin is involved in female reproductive behavior, mate choice and pair bonding. We used the mealworm beetle *Tenebrio molitor*, which is a polygamous and gregarious species with no parental care. Female mate choice has been reported in *T. molitor*, with females exhibiting a preference for males emitting higher levels of pheromones and cuticular hydrocarbons (Rantala et al., 2003; Nielsen & Holman, 2012). Furthermore, it has been observed that the female brain produces memory-related proteins during mating, suggesting that females store information about their partners (Hernández-Villanueva et al.,

2023). These characteristics makes *T. molitor* a good model to investigate whether Inotocin modulates sexual behavior, mate choice and pair bonding as it has been reported in vertebrates. First, we aimed to identify the localization of the inotocin signal in the brain of this species. Second, we aimed to test whether inotocin administration increases mating rate in females and whether affects mate choice. Third, we tested if inotocin favors pair bonding.

Methods

Beetles

Pupae were obtained from the insectary at the ENES, UNAM, in Morelia, México. Pupae were sexed and individually housed in well boxes to register the day of emergence as adults. After emergence, they remained individually housed and they were provided with *ad-libitum* food (sterilized mixture of corn flour and wheat bran) and a piece of apple every other day. All experiments started when males and females reached 10 days of adult stage.

Immunostaining

Whole brain from 5 females were dissected in cold PBS. Then, samples were fixated over night at 4°C in 4% paraformaldehyde in 1X PBS. Using a shaker at room temperature, samples were washed in PBS-Triton X (PBS containing 5%Titron-X) three times for 20

minutes each. Samples were blocked in 1X PBS and 1% BSA (bovine serum albumin) for 30 minutes. Then washed in 1X PBS and 0.01% Tween 20 for 5 minutes. Primary antibody stain: samples were incubated over night at 4˚C with a rabbit anti-arginin-vasopressin antibody (AVP ab) (1:10,000) diluted in 1XPBS 0.5% Triton X and 1% BSA. Next day, samples were washed three times for 10 minutes each, with PBS Tween (0.01%). Second antibody incubation: samples were incubated with a secondary antibody (Alexa Fluor 488 1:500 + DAPI 1:1000 + Phalloidin 1:400) diluted in 1XPBS 0.5% Triton X and 1% BSA solution for 2 hours at room temperature, using a shaker. After incubation, samples were washed 5 times in PBS for 10 minutes each. Brains were equilibrated in mounting medium VECTASHIELD® PLUS overnight, and then mounted between 2 cover slips separated by a stack of 2 labels on a slide, and sealed using clear nail polish. Brains were imaged in a confocal microscope Nikon A1R+ STORM, at Instituto de Investigaciones Biomedicas, UNAM. Images were acquired keeping configuration settings fixed within experiments. We used the ImageJ open source program FIJI to process images and adquires 3D projections for display.

Treatments

Inotocin: We administered females with a solution of Inotocin (Phoenix Pharmaceuticals, Inc.) 0.1 mM diluted in sterile distilled water. Females were injected with 1 µl of inotocin, or 1 µl of sterile distilled water for the control group. Injections were administered using 10 µl Hamilton syringes. The injection was applied in the pleural cavity between the abdomen and one of the second pair of limbs.

Female sexual behavior

Each group, control or inotocin, consisted of thirty unmated females. Female sexual behavior was observed one hour after treatment. To observe the behavior, an unmated male was placed in a 100mm diameter glass Petri dish and the dish was closed for a five-minute habituation period. After that, the female was introduced into the dish. The number and duration of copulations were recorded for the next ten minutes.

Mate choice

After a mating event, as described in Experiment 1, the mated individuals were housed individually for five days. Following this period, females that had mated on the first encounter were subjected to a mate choice test, in which they were given the option of choosing between the same male they had mated with five days earlier (partner) and an unfamiliar male (new). Both males were of the same age, size, treatment, and sexual experience, having mated at 10 days of age.

The behavior of the females was observed. Both male were placed in a Petri dish for a 5-minute habituation period. Following this, a female was introduced into the dish, and her copulations were recorded for the next 10 minutes. No treatment was given to the female during this second encounter. If the female copulated with both males, her last preference was considered as the male she chose, as this species follows a male sexual precedence system, P2 (Siva-Jothy et al., 1996; Drnevich, 2003).

Statistic analysis

To analyze mating frequencies, we performed a Chi-square test. We used a Mann Whitney U test to analyze mating times because they do not follow a normal distribution. For mate preference trials, we compared both conditions using chi-square tests and looked for differences within each group. All analyses were performed using the program SPSS Statistics, 22.0.0.0.

Results y Discussion

Neurohormones are involved in mediating mate choice behavior across diverse animal taxa, modulating sensory perception, neural processing, and decision-making processes. Understanding the neurobiological mechanisms underlying mate choice in a wide range of animal models provides deeper insights into the evolutionary dynamics of reproductive behavior. Here, we present evidence suggesting the involvement of inotocin in female sexual behavior and its influence on female preference for previous mates in a polygamous insect model.

Regarding the exploration of inotocin in the brain of *T. molitor*, we have confirmed the signal we detected corresponds to inotocin by identifying the two neurosecretory cells of inotocin in the suboesophageal ganglia (SOG), as reported in previous studies on beetles (Rémy & Girardie, 1980; Aikins et al, 2008). The inotocin signal follows a route that connects inotocin

neurosecretory cells in the (SOG) to areas surrounding the mushroom bodies (MB) (Figure 1, supplementary video). The MB are key regions where learning and memorization take place in insects (Hourcade et al., 2010; Krashes et al., 2007; Menzel, 2014). Although our images do not definitively show Inotocin entering the MBs, its presence is well described in ants' brains (Koto et al., 2019). In our images, inotocin appears to reach these areas through other neurons rather than solely being transported in the hemolymph. This finding could facilitate the study of a potential social memory circuit in insects modulated by inotocin. These results suggest the need for further investigation into the mechanisms of mate choice in insects, which may not differ significantly from those already understood in vertebrates. Future research could investigate the endogenous levels of inotocin peptide in females under different social contexts, as well as the gene expression of the inotocin gene in the SOG. Another interesting approach would be to localize the inotocin receptors in the brain of insects with different mating systems. It has been established that differences in the localization of oxytocin and vasopressin receptors in the brain determine the differences between monogamous and polygamous behavior in rodents of the genus *Microtus* (Insel & Shapiro, 1992; Insel Wang & Ferris, 1994).

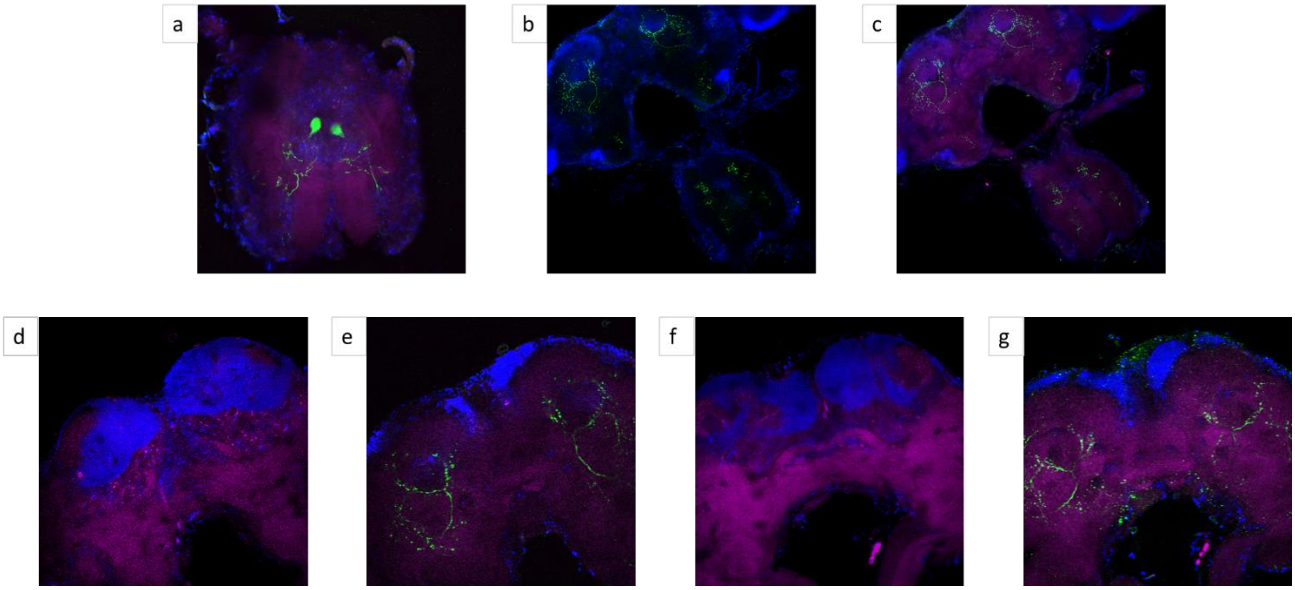

Figure 1 Inotocin immunofluorescence localization. Green: anti AVP; Blue: DAPI; Magenta: Phalloidin. a) SOG, the two inotocin neurosecretory cells are shown in green. b), c) same sample in 10X. Inotocin signal in brain (near to MB) and SOG, b) inotocin and DAPI signal, c) inotocin and phalloidin signal. d), e) same sample in 20X. Inotocin signal in brain (near to MB), d) inotocin and DAPI signal, e) inotocin and phalloidin signal. f), g) same sample in 20X. Inotocin signal in brain (near to MB), f) inotocin and DAPI signal, g) inotocin and phalloidin signal.

While oxytocin has been demonstrated to promote sexual behavior in females across several species (Benelli et al. 1994; Arletti & Bertolini, 1985), our findings indicate that females exhibited reduced mating rates when administered Inotocin compared to controls (26.67% vs. 60%, X^2= 5.45, df= 1, $p < 0.05$; see Figure 2a). Correspondingly, mating duration was also decreased in inotocin-treated females (U=273, n=30, p<0.005; figure 2b). This finding support similar results in the *Gambusia affinis* fish, where females treated with isotocin exhibited avoidance behavior and reduced interaction time with males compared to control females (Ramsey, Fry & Cummings, 2019). Notably, *G. affinis* is known to have a coercive mating system, where males frequently harass females and force them to mate (Ramsey, Fry & Cummings, 2019). A possible reason for females to avoid males is to reduce

the costs associated to mating like male aggressiveness or manipulation (Watson et al. 1998; Arnqvist & Nilsson, 2000; Parker, 2006), as seen in *Nauphoeta cinerea* cockroaches. *N. cinerea* females avoid attractive males due to their pheromone blend alters female's time reproduction and resulting in delayed parturation and reduced lifespan (Moore, Gowaty & Moore, 2003). Although in our model, there is female choice without coercion, it is possible that inotocin enables females to modulate social interactions during courtship, possibly enhancing sensitivity and selectivity towards male chemical cues, intensifying olfactory processing as seen in mammals. Studies have demonstrated that oxytocin can alter conspecific odor processing in rats by increasing olfactory exploration through temporary modifications in the circuits that connect the olfactory bulb and olfactory cortex (Oettl et al., 2016). This effect has been observed not only in social contexts but also in general odor perception. For instance, in humans, oxytocin enhances the detection of certain artificial floral scents (Woolley et al., 2015). It is of great interest to test whether and how inotocin alters the olfactory circuits used in social communication among insects.

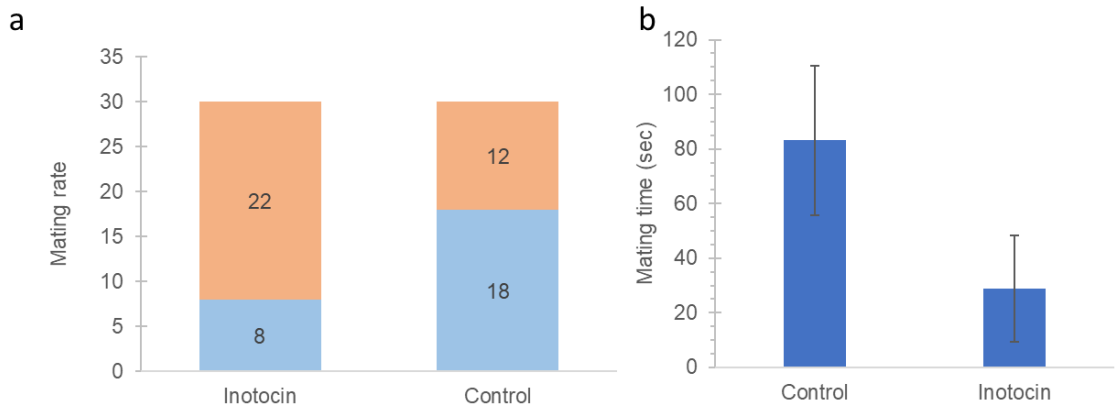

Figure 2. Inotocin reduces female sexual behavior. a) Mating rate between inotocin and control

treatments; blue: copulation frequencies, orange: non copulation frequencies (X^2= 5.45, df= 1, p < 0.05). b) Mating time between inotocin and control treatments (U= 273, n= 30, p<0.005). Error bars represent standard error.

Our initial findings suggest that inotocin may cause females to become more selective in choosing a mate. If this is true, it is possible that inotocin promotes a preference for the previously chosen male in a subsequent encounter, similar to the way oxytocin acts on mammals. Although female *T. molitor* are polyandrous (Worden & Parker, 2001; Drnevich, 2001; Drnevich et al. 2001), the results of our mate choice experiment suggests that inotocin promotes recognition of previous mates and preference for mating with known partners (70%, X^2= 4.8, df= 1, n= 30; p=0.02; figure 2). Control females showed no preference for either previous partner (43.4%) or a new male (54.67%; X^2= 1.2, df= 1, n= 30; p=0.27; figure 2). This finding suggests that inotocin enhances memory formation during the first encounter with a mate, and that females are able to recall the information from the first encounter. Previous studies in humans (Rimmele et al., 2009) and rodents (Ferguson et al., 2000; 2001) have shown that administering oxytocin during initial social exposure leads to improved social memory, enabling individuals to recognize others more quickly in subsequent encounters. Furthermore, oxytocin administration in humans has been found to promote a preference for heterosexual romantic relationship partners while creating social distance between potential new partners (Scheele et al. 2012; Freeman et al. 2021). Additionally, it may promote mate preference and recognition in species with flexible mating systems, such as the medaka fish (Yokoi et al. 2020). Therefore, the conservation of the oxytocin gene and its homologues may contribute to the flexibility of mating systems, whether they are strictly monogamous or polygamous.

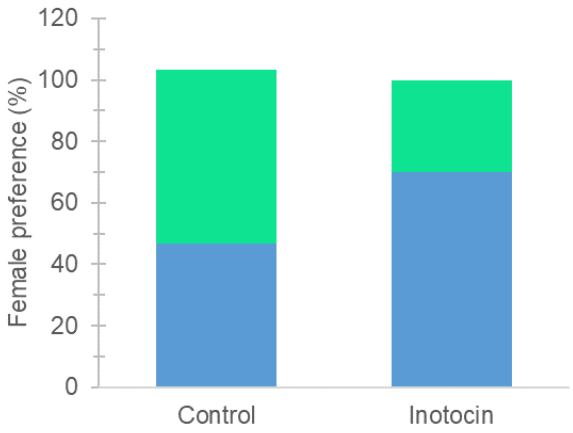

Figure 3. Inotocin affects female mate choice. Females treated with inotocin preferred significantly more the previous male (partner, blue) over a new male (new, green).

In summary, our findings provide evidence of the evolutionarily conserved function of oxytocin-related neuropeptides, as well as the possible shared origins of mate preference behavior through affiliative or selective tendencies towards previous partners. The adaptative significance of this behavior remains to be investigated in each species where it is found. While the preference for previous mates in our polyandrous model may not be a natural behavior, it underscores the existence of neural substrates shared with mammals, potentially facilitating the emergence of similar behaviors. However, it has not been addressed whether females could find advantageous to mate with known partners in a non-monogamous species. Future research should aim to unravel the complexities of neurohormonal regulation in mate choice to broaden our understanding of the interplay between genes, brains, and behavior in the context of evolutionary biology.

Ethics: All procedures in this study were approved by UNAM. Author contributions: S.C.M., C.M.G, G.R.R. and J.C.G. designed the experiments. S.C.M. conducted experiments and analyzed data. All authors wrote the manuscript. Competing interests: The authors declare no competing interests. **Funding**: J.C.G received a grant from Dirección General del Asuntos del Personal Académico/ Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica/Universidad Nacional Autónoma de México (Grant No. IN225120). S.C.M. is a doctoral student from Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM) and received fellowship 632723 from CONAHCyT, México.

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CAPÍTULO IV.

Cordero-Molina, S., Macías García, C., Ríos Castro, E., Roldán Roldán, G. & Contreras-Garduño, J. Have we mated before? The females' behavioral and brain changes due to multiple mating. (submitted).

ABSTRACT

1. The influence of females on the outcome of the mating system and the underlying molecular mechanisms remains largely unexplored in invertebrates. For example, females may remember the attractiveness of their mating partners and choose to re-mate with attractive males or seek novel mates if their previous partners lose attractiveness. However, the mechanisms underlying this behavior in their brains are poorly understood.
2. To test this, we conducted a study on *Tenebrio molitor*, whose females produce differential brain proteins associated with memory following mating with attractive partners. The more attractive males produce more pheromones than less attractive males.
3. Through behavioral observations, a pharmacological experiment, and proteomic analysis, we support the hypothesis because: a) females prefered their previous mating partners over new males, but reject them in subsequent encounters if loss attractiveness, b) amnesic treatment hindered females' preference for re-mating with attractive partners, and c) more memory-related proteins were found in females mating with previous partners compared to those mating with novel males. Importantly, d) only mating behavior induced differential brain protein expression in females.
4. We propose that rejecting partners due to their loss of attractiveness lead females to avoid mating with deceitful males, and that male attractiveness can influence a female's decision to engage in multiple mating or remain with the same male. These novel findings in invertebrates highlight further research to understand how females shape the mating system.

1. INTRODUCTION

Females choose mating partners based on the elaboration of males' Secondary Sexual Characters (SSC), encompassing traits like colors, songs, plumage, pheromones, and courtship displays (Andersson, 1994; Darwin, 1859, 1871). While research has predominantly focused on how males produce SSC and their success in attracting mates, the perception of SSC and male attractiveness in the female brain has received far less attention (DeAngelis & Hofmann, 2020; Ryan, 2021; Rosenthal & Ryan, 2022). Consequently, most studies on sexual selection have concentrated on the signals transmitted by males during mate choice, while the mechanisms by which females process this information in their brains (DeAngelis & Hofmann, 2020; Ryan, 2021; Rosenthal & Ryan, 2022; Cordero-Molina et al. 2023), and the resulting effects on the species' mating system (Fromonteil et al., 2023) remain poorly understood. Particularly, limited attention has been given to the role of cognitive memory and its impact on the production of specific brain proteins during mate choice in polygamous species, as well as its influence on decisions regarding repeated mating with the same partner or selecting different mates. Memory enables females to retain information about multiple males encountered, aiding in their subsequent mate choice. For example, female bowerbirds *Ptilonorrynchus violaceus* evaluate the bowers and courtship displays of various territorial males before ultimately selecting their preferred mate, necessitating the retention of information from each encounter (Uy et al., 2001). Mate memory also plays a role in avoiding previous partners and favoring polygyny. In the pseudoscorpion *Cordylochernes scorpioides*, females use memory to discriminate against prior partners (Zeh et al., 1998). Furthermore, female mating decisions can be flexibly modified in response to changes in population demographics, such as an increased availability of males (Gowaty, 2013). However, not only does the availability of males vary, but their attractiveness can also fluctuate, presenting females with the

dilemma of choosing between previous partners who are still attractive and novel males if the attractiveness of their previous partner diminishes. Despite female memory can contribute to shaping the brain differential responses according to male attractiveness and the mating system, this hypothesis remains largely unexplored. Hence, the function of mate memory in sexual selection, particularly in polygamous species, has received limited examination. Investigating these questions and understanding the accompanying changes within the female brain may illuminate the proximate and ultimate mechanisms of mate choice and mating systems, offering valuable insights into the female perspective on mate choice (Ryan, 2021; Rosenthal & Ryan, 2022).

In this study, we investigated whether females of the polygamous species *Tenebrio molitor* (Siva-Jothy et al., 1996; Drnevich et al., 2000; Worden & Parker, 2001; Drnevich, 2003) use their memory for choosing a previous or novel males according to their attractiveness and how their brain proteins change accordingly. The mate choice in this species relies solely on chemical communication (August, 1971; Happ, 1969; Rantala et al., 2002, 2003a,b; Carazo et al., 2004; Krams et al., 2011; Nielsen & Holman, 2012; McCallum et al., 2013; Vanderwel et al., 2017; Griffith et al., 2020). The more chemical signals a male produce, the more likely it is to be preferred by the opposite sex for mating (Rantala et al., 2002; Rantala et al., 2003; Nielsen & Holman, 2012; Márquez-García et al., 2016; Contreras-Garduño et al., 2019). This increased attractiveness of males can be attributed to their higher resistance to infection, better immune response (Hurd & Parry, 1991; Nielsen & Holman, 2012; Rantala, Kortet et al., 2003; Rantala, Vainikka et al., 2003; Worden & Parker, 2005; Worden et al., 2000), enhanced resistance to oxidative stress (Ruiz-Guzmán et al., 2021) and improved nutritional status (Rantala, Kortet et al., 2003). Notably, the juvenile hormone (JH) is the molecule that maintains an honest relationship between attractiveness

and male condition, as higher doses of JH promote pheromone production but reduces survival, genital size and immune response and increase oxidative stress (Rantala et al. 2002, 2003a, 2003b; Márquez-García et al., 2016; Ruiz-Guzmán et al., 2021). This could explain why males treated with JH are less likely to be rejected by females and result in increased larval production by females, compared to non-treated males (Hernández-Villanueva et al., 2023).

Recently, a study by Hernández-Villanueva et al. (2023) demonstrated differential production of brain proteins in females based on male attractiveness. Experimentally attractive males treated with the juvenile hormone analogue, methoprene (JHa), exhibited higher production of proteins associated with memory and synaptic plasticity (AF4/FMR2 family member 4, Lysine-specific demethylase NO66, Histone-lysine N-methyltransferase pr-set7, Protein virilizer), while showing reduced production of proteins involved in olfactory learning (phenoloxidase subunit A3) and neuronal architecture (Profilin). It has been proposed that females continuously evaluate potential mates (Møller, 1997) and remember the attractiveness of males, potentially choosing to re-mate only with those that maintain their attractiveness (Hernández-Villanueva et al., 2023). Consequently, we tested the aforementioned hypothesis, and we made the following predictions: 1) females, when faced with a choice between a previous mating partner and a new one, would prefer the former male and their odor if the partners maintain their attractiveness; 2) a pharmacological inhibition of the memory before the second encounter may cause females to lose their initial preference to re-mate with the male partner; 3) if the partners lose their initial attractiveness and novel males are more attractive, females will prefer the novel males over re-mating with the first partner; and 4) females that re-mate with their partners will exhibit differential brain proteins related to memory, compared to females mated with novel males.

To address these predictions, we enhanced male attractiveness experimentally by applying methoprene (JHa) or kept males without artificial enhancement (Hernández-Villanueva et al., 2023). The results confirmed the predictions and supported the hypothesis that females remember male attractiveness and re-mate with them only if males maintain their attractiveness. Additional to investigate the proteins related to memory formation for prediction 4, we searched for brain proteins involved in other stages of mate choice. We compared brain proteins from unmated females with the brain of females that mated once, and females with preference for previous partners against females without this preference. These comparations provide insights into the brain mechanisms and plasticity of female behavior in response to mating decisions through the previous stages of mate choice and with two different strategies of mate choice. The results from protein comparation confirmed the presence of differential brain proteins in the females' brains when mating with a novel male *versus* re-mating with their previous partner. These proteins were associated with memory, synaptic communication, metabolism, locomotion and antioxidant response. Notably, we also discovered differential brain proteins involved in detoxification, general stress, dopamine (pleasure, motivation, arousal, and memory), and glutamate signaling (olfactory memory). Furthermore, we observed differential brain proteins in the females' brains that mated, compared to the brains of virgin females, related to detoxification, memory, brain plasticity, antioxidant activity, sleep, longevity, and fecundity. Although our study is correlative, provides a broad sight of brain dynamics through different stages of female choice in a species different to the classical models used to study the brain during mate choice. The identification of these proteins warrants further research to understand their specific roles in mate choice and how females may shape the mating system, as females remated with their previous partner or mated with two different males.

1. MATERIAL AND METHODS

Insects

Tenebrio molitor pupae were obtained from larvae breeding colonies that were reared under insectarium conditions (27 ± 1 °C in the dark). They were fed with bran and corn meal ad libitum and supplemented with apple pieces. Pupae were separated by sex and when they reached the adult stage, they were individually housed in six-well culture plates (Corning™) and fed the same insectarium diet ad libitum. Treatments initiated in male individuals upon reaching the age of 10 ± 1, as this age corresponds to the peak of reproduction for both sexes (Cole et al. 2003).

Male treatments

JH promotes pheromone production (Rantala et al. 2003a), and this can be used to manipulate the male attractiveness experimentally (Hernández-Villanueva et al., 2023). Males were assigned to each of the following experimental groups: 1) Control; which were applied 1 µL of Acetone (the vehicle used for the JH treatment); 2) 1 µL of Methoprene (JH III analog) 200 µg/mL. Both treatments were applied between the head and the prothorax with a micropipette. The use of either group depended on the prediction that we tested (see below).

Prediction 1

Partner memory

We performed a two-encounter female preference test to assess whether having mated with one male influence the probability that a female chooses it as a mating partner in a second encounter (Figure 1A). First encounter: two and a half hours after being applied JH analogue (JHa), a virgin 10 ± 1-day old male (the partner male) was placed in a glass petri dish (Pyrex 100 X 15 mm) for a habituation period of 5 minutes, then a virgin 10 ± 1-day old female was placed in the dish with the male. Focal observations were carried out for 10 minutes to determine if the pair copulated (Hernández-Villanueva et al., 2023). If copulation occurred, male and female were transferred to individual wells in 6-well microplates (Corning™) and fed as above. Beetles were marked with acrylic paint (Baco™) of different colors to distinguish between the sexes and between males in the second encounter (see below). Second encounter: five days after the copulation in the first encounter, the female was placed in a Petri dish with the partner male and another, new male from the same cohort. To ensure that females would not select between males that differed in attractiveness, both males were treated with JHa 2.5 h in advance. Males were also matched for mating experience (with a different female) and manipulation (both had been treated previously with JHa, Figure 1A). We followed the same protocol as in the first encounter: males (together) were given a 5 min habituation period, then the female was introduced and a continuous behavioral record followed, during which we recorded the occurrence and latency (s) of copulations and the identity of the beetles involved, for the next 10 minutes. We deemed that females remembered the partner male if in the second encounter they a) copulated only

with the partner male; or b) copulated with both males but the last copulation was with the partner male and lasted for ≥ 30 seconds (as there is last male sperm precedence –P2- in *T. molitor*; Siva-Jothy et al. 1996). All behavioral observations in second encounter were blinded.

Figure 1.

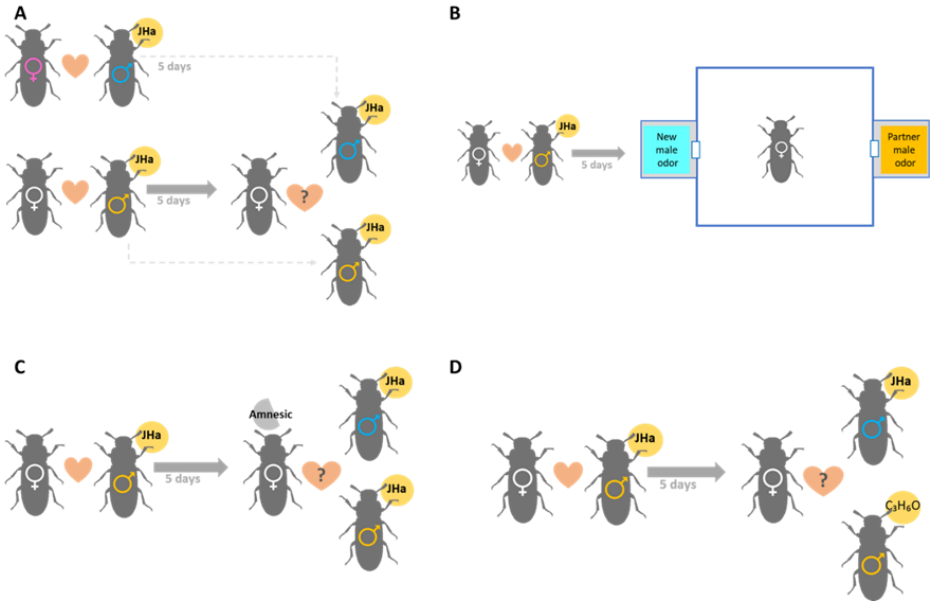

Figure 1. Experimental design. A) Experiment 1. Evaluates whether when confronted with males of equal size, mating history and attractiveness (JHa), females prefer their initial partner (orange male symbol) over the novel one (blue male symbol). If females remember the males' attractiveness, we predicted females' preference for initial partner. (In all next experiments, the new male was selected as in A). **B) Experiment 2**. After the first encounter (as in A), odor preference is tested in a behavioral arena. In the main chamber (big square), a female was free to walk and enter one of two chambers (small squares) that contained the partners odor in one side or new male odor on the opposite side. **C) Experiment 3**. Evaluates the role of memory disruption with zebularine (amnesic) before the females were exposed to males. We predicted that females would mate randomly with initial or new males. **D) Experiment 4**. Evaluated whether the preference for initial partner disappears if it becomes less attractive (treatment with acetone: C_3H_6O) in the second encounter that new males. We predicted that females would prefer the new male.

Olfactory memory

Given that in *T. molitor* male volatile pheromones are used by females to choose amongst males, we evaluated whether the recognition of mates in subsequent encounters is also mediated by pheromones (Figure 1B). We applied JHa to 10- day adult males and allowed the male to copulate with one female as before (first encounter). Then two males, the partner and the new one, we treated with JHa, as in the previous tests, and kept in individual pretri dishes lined with sterile filter paper for the next 2.5 hours. Subsequently, instead of choosing between the males, females choose between the paper impregnated with the odor of their partner and the paper impregnated with odor of the new male (Rantala et al. 2003a; Kivleniece et al. 2010). For this, each female was placed in an acrylic choice arena with a main chamber and two smaller chambers on each side (Figure 1B). Each of the small chambers contained the filter paper impregnated with the odor of one of the males, the positions being alternated between trials. Each female was allowed to habituate for 5 min after gently placing her within a movable restriction container at the center of the main chamber. After the habituation period, the restriction container was removed, and the female was allowed to move freely around the arena and to enter any or both of the entering the two male chambers. We recorded the behavior of the female for 10 minutes registered each time she entered a chamber (Hernández-Villanueva et al., 2023).

Prediction 2

Memory inhibition

We used a common protocol to inhibit the evocation of memory (e. g. Neelamegam et al. 2012; Wang et al. 2013). We conducted a female preference test like that described above, but after the first encounter, we applied 1 µL of Zebularine (1 mM; Merck) between the head and prothorax of female 90 minutes before the second encounter (mate choice test; Figure 1C). Zebularine is an amnesic agent that inhibits methyltransferases (Biergans et al. 2012), known to be involved in memory formation (Neelamegam et al. 2012). The dose we used is known to interrupt memory during foraging behaviors in insects (Biergans et al. 2012).

Prediction 3

Female memory when male attractiveness decreases

This experiment was similar to the partner memory test (prediction 1), but in this case, in the second encounter, the partner male was treated with acetone and the new male with JHa (Figure 1D). Thus, in the second encounter, the partner male had lower pheromone production than during the first encounter, and the new male was more attractive than the male partner. Both males were placed in a Petri dish for 5 minutes, after which the female was introduced. We recorded the sexual behaviors of the triad for 10 min as described above.

Prediction 4.

Proteomics

We formed 4 groups of females to dissect and extrat their brains for posterior proteomic analyses. First group was compraised by aldult unmated females of age 10 day, second group was females mated once as described in the protocol of first encounter. For third and fourth group, we repeated the experiment of mate choice memory (prediction 1), and we selected females that preferred to mate with the previous male for third group, and females with preference for the new male, to form the fourth group. After preference test, we anesthezied females on ice and they were sacrifized to extract their brain. We placed brain in vials with cold PBS pH 7.4 buffer (Hernández-Villanueva et al 2023). We pooled ten brains per treatment per vial for subsequent analysis. We repeated this procedure thrice, resulting in 30 brains per treatment divided into three pools of 10 brains.

Proteomic analyses were conducted in the Unidad de Genómica, Proteómica y Metabolómica, at LaNSE-CINVESTAV. They were not aware of our hypotheses, and the samples were only labeled with numerical identifiers (1, 2, 3 or 4) instead of using the name of each group: unmated females, mated females, females mated with two different males or females re-mated with the same male. Extended methodology for proteomics is found on the Supplementary material.

Statistical Analyses

The number of copulations was analyzed using Chi-square tests (X^2). The total time spent visiting the chambers where the odor of the males was compared with Student's- t test taking into account the homogeneity of variances and normality. All analyzes were performed with the SPSS 20.0 program (IBM Corporation).

RESULTS

Prediction 1. Females remembered their previous partner and males' odors are sufficient to evoke the females' memory.

In an encounter taking place five days after mating, most females mated again (67.5%) with it when a novel male was also present (32.5%; $X_1^2 = 4.9$; $p = 0.02$, n = 40 pairs; Figure 2A).

Given that in *T. molitor* chemical communication is known to mediate mate choice (Rantala et al. 2002; Rantala et al. 2003 a, b; Ruiz-Guzman et al. 2020), we tested whether females prefer their previous partner because they remember its odor. We recorded the time each female spent in front of the filter paper impregnated with the odor of each male. Females spent more time on the source of their partner's odor (177 ± 28.82 s) than on the paper containing the odor of the new male (89.71 ± 15.72s; $t = 2.16$; d.f. = 27, $p = 0.03$; Figure 2B).

Figure 2.

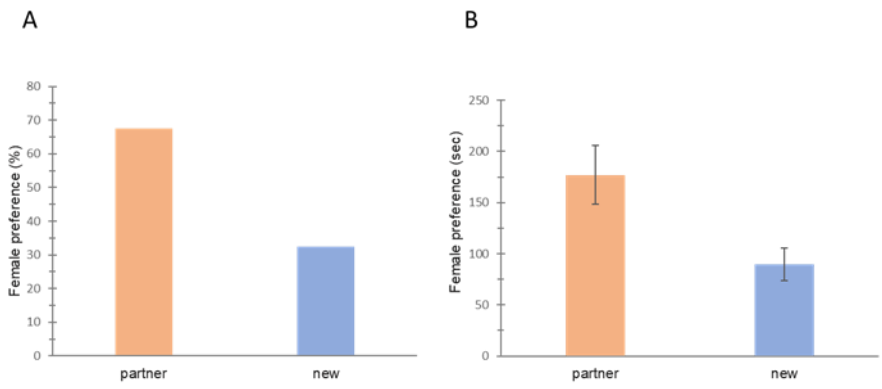

Figure 2. A) Significantly more females mated with their previous partner than with a new male. B) Females spent more time in the vicinity of the partner odor than close to the paper containing the odor of the new male. Error bars represent standard error of the mean.

Prediction 2. Females experimentally become amnesic did not show a sked preference to mate with their previous partners.

To confirm that memory was involved, we then applied amnesic zebularine to females 90 min before the trial in which they would choose between their previous partner and a new male. As predicted, zebularine-treated females did not show a mating preference for their previous mates (51.28%) over the new males (48.8%; $X_1^2 = 0.02$; p = 0.87, n = 39 pairs).

Prediction 3. If the partners attractiveness decreases, females lose the previous partner preference.

When the novel male, but not the previous partner, is treated with JHa, so that the latter is less attractive in the second than the first encounter, females no longer prefer him (55%) over a new male (45%; $X_1^2 = 0.4$, p = 0.52, n = 40).

Prediction 4. Brain proteins confirmed that females memorize their male's identity.

This study confirms the differential brain proteins in the females' brains associated with memory, but we also found proteins related to oxidative stress, metabolism, and locomotion (Table 1, 2). We also found differential brain proteins involved in detoxification, general stress, and JH production, as well as differential presence of glutamate signaling and dopamine (Table 1). We also found differential brain proteins in the females' brains that mated, compared to the brains of virgin females. These proteins were related to detoxification, memory, brain plasticity, antioxidant activity, sleep, JH, longevity, and fecundity (Table 3, 4).

Table 1.

REMATED VS MULTIPLE MATING UPREGULATED		
Protein	**Quantity**	**Function**
Cytochrome P450 6a14 · Tenebrio molitor · Gene: GEV33_000005 · 475 amino acids · Inferred from homology	8.43439989	Metabolism of insect hormones and in the decomposition of synthetic insecticides
Chitinase · Tenebrio molitor · Gene: GEV33_002890 · Inferred from homology	6.90501491	Digestion, inflammatory response
Fibronectin type-III domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_002585 PE=4 SV=1	5.52145642	Cell adhesion, cell morphology, cell migration
Ionotropic glutamate receptor C-terminal domain-containing protein· Inferred from homology OS=Tenebrio molitor OX=7067 GN=GEV33_011884 PE=3 SV=1	2.73421325	Synaptic communication; detection of odors and tastants; transmembrane transport
Prominin-like protein · Tenebrio molitor · Gene: GEV33_006021 · 402 amino acids · Inferred from homology	2.07433729	Regulates locomotion; dopamine synthesis
Synapsin_C domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_002561 PE=3 SV=1	1.75127227	Regulate synaptic transmission and plasticity; release of neurotransmitters from synaptic vesicles
Carboxylesterase type B domain-containing protein · Tenebrio molitor · Gene: GEV33_001427 · 1035 amino acids · Predicted ·	1.64637363	Detoxification; resistance to insecticides
CRAL-TRIO domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_004285 PE=4 SV=1	1.54396816	Actin remodeling, cell migration and growth

Table 2.

REMATED VS MULTIPLE MATING DOWNREGULATED		
Protein	**Quantity**	**Function**
Armadillo segment polarity protein OS=Tenebrio molitor OX=7067 GN=GEV33_013186 PE=4 SV=1	-7.37456574	Associated to cell-contact and cytoskeleton-associated proteins; gene expression.
Tropomyosin OS=Tenebrio molitor OX=7067 GN=GEV33_007527 PE=2 SV=1	-6.39374991	Regulates of actin-myosin interaction in muscle and nonmuscle cells.
Heat shock protein 60 · T. molitor · Gene: GEV33_008105 ·Inferred from homology	-.362850221	Protein protection during stress; refolding and degradation after stress.

Peroxiredoxin-5 OS=Tenebrio molitor OX=7067 GN=GEV33_002862 PE=3 SV=1	-2.1403614	Cell protection against oxidative stress; sensor of hydrogen peroxide-mediated signaling events.
Spermatogenesis-associated protein 20 · Tenebrio molitor · Gene: GEV33_002575 (t. madens)	-1.87730254	Fertility regulation; cell differentiation; and spermatogenesis; oxidative stress response.
FAS1 domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_003150 PE=4 SV=1	-1.68925547	Axonal guidance; cell adhesion.
Cytochrome c oxidase polypeptide Va · T molitor · Gene: GEV33_004642 · Predicted (Asbolus verrucosus)	-1.54239535	Mitochondrial respiratory chain.
Predicted: BAR domain-containing protein [Oryctes borbonicus] (A0A0T6BF68) (91.3%, 1.9e-118	-1.67649631	Formation of local membrane curvatures; endocytic mechanisms.
Inferred from homology: Ras-related protein Rab-3 [Tribolium castaneum] (D6X1H3) (100%, 8.9e-74)	-1.66424478	Neurotransmitter release; protein transport and vesicular traffic.

Table 3.

MATED VS UNMATED UPREGULATED		
Protein	**Quantity**	**Function**
Ankyrin repeat protein · Tenebrio molitor · Gene: GEV33_013627 · Predicted ·	23.8674998	Cell growth; cell adhesion; neurodevelopment.
Laminin G domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_008636 PE=4 SV=1	3.82184028	Cell adhesion, migration, assembly and differentiation.
Ion_trans domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_007934 PE=3 SV=1	3.51431849	Transmembrane transport.
Choline transporter-like protein OS=Tenebrio molitor OX=7067 GN=GEV33_003302 PE=3 SV=1	2.44297181	Cell membrane transporter; carries choline into acetylcholine-synthesizing neurons.

Protein Wnt OS=Tenebrio molitor OX=7067 GN=GEV33_001200 PE=3 SV=1	2.3355636	Cell proliferation, gene transcription and cytoskeleton reorganization, dendritic development, and synaptic assembly.
Protein quiver OS=Tenebrio molitor OX=7067 GN=GEV33_007652 PE=3 SV=1	1.87992577	Homoeostatic regulation of sleep; regulation of circadian sleep/wake cycle.
Cytochrome P450 monooxygenase OS=Tenebrio molitor OX=7067 GN=GEV33_000493 PE=3 SV=1	1.75164485	Detoxification of xenobiotics; cellular metabolism; homeostasis.
Protein i m not dead yet · Tenebrio molitor · Gene: GEV33_009865 · Inferred from homology ·	1.747393	Plasma membrane transporter of Krebs cycle intermediates.
Vitellogenin domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_012739 PE=4 SV=1	1.7289204	Lipid transport protein; an egg-yolk precursor; protection against oxidative stress.
J domain-containing protein · Tenebrio molitor · Gene: GEV33_010764m· Inferred from homology ·	1.66550743	Family of Hsp70 co-chaperones; protein folding; protein trafficking, remodeling, disaggregation and degradation.
Inferred from homology: DnaJ homolog subfamily A member 1-like Protein [Tribolium castaneum] (D6W9F5) (96.2%, 0)	1.61149199	Act as heat shock protein 70 cochaperones; cellular stress response
F-box domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_013555 PE=3 SV=1	1.64647378	Signal transduction and regulation of the cell cycle.
Homeobox domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_008906 PE=4 SV=1	1.62449452	Anatomical development
14_3_3 domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_009685 PE=3 SV=1	1.57540126	Signal transduction; synaptic vesicle dynamics; memory and learning.
Heat shock 70 kDa protein cognate 4· Tenebrio molitor · Gene: GEV33_012327 · Inferred from homology ·	1.55782183	Molecular chaperone; stress response.
PDZ domain-containing protein Tenebrio molitor · Gene: GEV33_013944 · Predicted ·	1.53330931	Cytoskeletal and cellular membrane organization; trafficking of synaptic proteins.

Heat shock protein 83 OS=Tenebrio molitor OX=7067 GN=GEV33_000826 PE=3 SV=1	1.53202664	Pleiotropic roles in embryogenesis, longevity, and fecundity.
Microsomal epoxide hydrolase OS=Tenebrio molitor OX=7067 GN=GEV33_000480 PE=3 SV=1	1.52924571	Detoxifying metabolism of xenobiotics.
Integrin_alpha2 domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_000613 PE=3 SV=1	3.83689191	Cell adhesion molecules; actively regulate cellular growth, differentiation, and apoptosis.
Ras-related protein Rab-3 [Diabrotica virgifera virgifera] (94.4%, 3.5e-87)	1.599003332	Exocytosis and secretion. It is involved in calcium-triggered exocytosis in neuron

Table 4.

MATED VS UNMATED DOWNREGULATED		
Protein	**Ratio**	**Function**
Dynein regulatory complex subunit 2 OS=Tenebrio molitor OX=7067 GN=GEV33_011590 PE=4 SV=1	-13.5802933	Cilliar/flagelar motility.
Thioredoxin domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_009817 PE=4 SV=1	-12.1912721	Cellular redox homeostasis; antioxidant element.
UDP-glycosyltransferases domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_012933 PE=3 SV=1	-3.69427859	Metabolism of nucleotide UDP-sugar and ectysteroids; detoxification of xenobiotics in insects,
Peptidase M14 carboxypeptidase A domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_007344 PE=3 SV=1	-2.74670431	Digestion; degradation of proteins, neuropeptides and peptide hormones.
Activator of Hsp90 ATPase AHSA1-like N-terminal domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_013636 PE=3 SV=1	-1.84342462	Regulator of Hsp90 function, stress response.
Cilia- and flagella-associated protein 99 · Tenebrio molitor · Gene: GEV33_005118 · 592 amino acids · Predicted ·	-1.58940716	Cilium- and flagellum-specific protein that plays a role in axonemal structure organization and motility. CFAP69 is a highly conserved protein enriched in

		olfactory sensory neuron (OSN) cilia.

DISCUSSION

We conducted a study on female mealworm beetles, demonstrating their ability to recognize previous mating partners and their associated odors. We observed that when we interfered with their memory or made the experimental partners less attractive, the preference to mate with familiar partners was eliminated, but females did not favored novel males instead. Interestingly, females who had mated with familiar partners exhibited a higher presence of proteins related to memory in their brains compared to those who mated with novel males. Previous research has described *Tenebrio molitor*, as polygamous (Drnevich et al., 2000; Siva-Jothy et al., 1996; Worden & Parker, 2001). However, a significant portion of females in our experiment displayed monogamous re-mating behavior when presented with both familiar and novel males. This sequential monogamy relied on the females' ability to recall previous encounters and the continued attractiveness of the males. Our findings support the notion that females can identify their mating partners and selectively choose to mate with the same male in subsequent interactions (Zeh & Zeh, 1996; Endler & Basolo, 1998). Recent proposals have highlighted the significant role females, not just males, play in shaping the mating systems of animals (Cummings, 2015; Fromonteil et al., 2023). These perspectives show a relatively untested point of view of the females' brain mechanisms on their mating behavior according to the males' attractiveness (Cummings, 2015; Ryan, 2021; Rosenthal & Ryan, 2022; Hernández-Villanueva et al., 2023). Memory in mate choice enables repeated mating when engaging in polygamy comes with costs and, when there are, benefits associated with remating (Zeh & Zeh, 1996). Prior to remating, females rely on pre-copulatory male traits to identify potential mates (Jennions & Petrie, 2000), and it has been

observed that females who remate with attractive males produce sons that possess attractive traits as well (Kiyose et al., 2022). This observation sheds light on why the polygamous females of *T. molitor* remember their attractive male partners and actively choose them for subsequent mating instead of pursuing polygamous behavior. Conversely, when female partners lose their attractiveness, females opt for polygyny and mate with either the familiar male or a new one. This flexibility displayed by *T. molitor* females, wherein they can shift between monogamy and polygyny based on the attractiveness of their partners, can be attributed to a key male strategy known as terminal investment.

According to Zahavi (1975), SSC are honest signals of the bearer's condition because such traits are costly to produce and hamper survival. Hence, only males in good condition can deal with the disadvantage imposed by exaggerated SSC (Zahavi, 1975). However, some males display SSC of a magnitude beyond their regular capacity, albeit for short periods, when its survival is at risk (Duffield et al. 2017). For instance, male fiddler crabs (*Uca annulipes*; Backwell et al., 2000), crickets (*Allonemobius socius*; Copeland & Fedorka, 2012) and yellow mealworm beetles (*Tenebrio molitor*; Kivleniece et al., 2010, 2011, 2015; Sadd et al., 2006; Cordero-Molina et al., 2024) increase their investment in sexual displays when ill, and hence when they are prompt to die. Given that the male terminal investment can impose a sub-optimal mate choice on females (Duffield et al., 2017; Sadd et al., 2006), it is expected that females in such species have evolved means to tease apart the males that signal honestly from those that produce deceivingly attractive SSC. Most research on terminal investment in SSC evaluates the disparity between the condition of the males and of their sexual displays, and infer that females are doomed to mate suboptimal with those males until they either die or cannot longer sustain such high investment in SSC (Kivleniece et al., 2010; Sadd et al., 2006; Vainikka et al., 2007). Yet it has been shown that females can

escape such evolutionary traps (Macías Garcia & Ramirez, 2005; Buchinger et al., 2020; Martínez Villar et al., 2021). One prerequisite to avoid re-mating with males that initially resorted to terminal investment in courtship signals is that females remember both the identity and the attractiveness of the males they mate with. Whether female *T. molitor* use mate memory to escape such trap would require a formal test. However, we propose the hypothesis that the ability of females to remember their previous partner's attractiveness allows them to avoid potential male cheaters who might employ a terminal investment strategy to initially secure a mating, but subsequently fail to maintain their attractiveness honestly. Therefore, by utilizing their memory, 1) females may be deceived by a male once, but not twice, as they can recognize and avoid repeated encounters with dishonest males; and 2) females can mitigate the risk of mating with a potentially dishonest, albeit attractive, unfamiliar male by selecting a familiar partner known to sustain his attractiveness.

The present study offers insights into how female animals utilize memory to choose among potential mates and how the attractiveness of males influences such memory. While the mechanisms underlying these behaviors have primarily been studied in fish, birds, and mammals (Gräff & Tsai, 2013; Wang et al., 2013; Cummings, 2015; Johnson & Young, 2015; Lieberwirth & Wang, 2016), it is important to examine these mechanisms in other animal groups to gain a comprehensive understanding of the evolution of social cognition (Weitekamp & Hofmann, 2014), including mate choice cognition and the underlying mechanisms (Ryan, 2021; Rosenthal & Ryan, 2022; Cordero-Molina et al. 2023). We investigated an insect and found evidence supporting the occurrence of brain protein changes during mate choice according to re-mating or multiple mating. We found 59 dysregulated proteins (Table 1, 2, S1 and S2), these protein changes are associated with memory, metabolism, cell transport, odor recognition as well as stress response, such as antioxidant defense. Regarding memory-related proteins, the increase in Synapsin protein

levels in the brains of females engaging in remating is noteworthy. Synapsin regulates the dynamics of synaptic vesicles and neurotransmitter release at synaptic terminals (Cesca et al., 2010). Furthermore, its presence has been reported to be crucial for neuronal plasticity in social insects (Fahrbach & Van Nest, 2016), and in *Drosophila*, it is required for enhancing memory and learning (Niewalda et al., 2015; Kleber et al., 2016). Another interesting protein that exhibited higher production in females that re-mated compared to those that mated multiple times was a Ionotropic glutamate receptor domain containing protein (iGluR), which are known to be involved in synaptic communication (Mayer & Armstrong, 2004; Madden, 2002). The release of glutamate in the mushroom bodies of insects is associated with olfactory memory and long-term memory (Locatelli et al., 2005; Hourcade et al., 2010). Additionally, it has been reported that iGluRs in insects play a role in chemical signal detection (Rytz et al., 2013) and are also involved in regulating juvenile hormone (JH) synthesis (Chiang et al., 2002). JH regulates various reproductive behaviors such as vitellogenesis (Li et al., 2019) and processing of sexual pheromones (Anton & Gadenne, 1999). The upregulation of this receptor could signify that females preferring a familiar mate have a lower threshold of response to male pheromones, possibly due to a consolidated memory. Moreover, the prominin-like protein is involved in locomotion (Ryu et al., 2022), the visual system (Gurudev et al., 2013), and dopamine signaling, which is implicated in arousal and insect memory (Verlinden, 2018). These findings align with our behavioral and pharmacological experiments, suggesting that females may retain memory of their previous mating partners. While in the brains of multiply mated females, there is an upregulation of other proteins related to cytoskeletal architecture and vesicular traffic, there isn't a specific association with memory function.

We also observed lower levels of and Peroxiredoxin-5, a protein involved in the response to stress and oxidative stress (Radyuk et al., 2009), in females that re-mated with attractive

males compared to those that mated twice with different males. This finding aligns with a similar result reported by Hernández-Villanueva et al. (2023), where mating with attractive males led to an induction of proteins associated with oxidative stress. In addition, we also found downregulated in this group of females an atypical protein, the spermatogenesis-associated protein 20 (SPATA20). SPATA family it has been described as proteins that favors spermatogenesis and male fertility (Shi et al., 2004; Ge et al., 2016; Sujit et al., 2020). Nonetheless, SPATA 20 possess a thioredoxin like domain (Shi et al., 2004) which it could confer an antioxidant function as well even in other tissues different from male gonads such as brain. Simultaneously, cytochrome P450, a protein involved in detoxification processes (Kuban & Daniel, 2021) was upregulated in remated females with preference. In *Tribolium castaneum*, this protein plays a role in protecting the insect brain from the toxicity of the insecticide deltamethrin (Zhu et al., 2010), and its gene is more expressed in resistant insects against insecticides compared to susceptible ones (Liu et al., 2015). A similar protective function has been associated with the Carboxylesterase type B protein (Wheelock et al., 2005), which is also upregulated in this group of females. However, cytochrome P450 has also been implicated in a transductory cascade, starting with brain stimulus sensing, and resulting in ecdysteroid release (Gilbert, 2015; Dermauw et al., 2020), as well as the suppression of juvenile hormone during fertilization (Sutherland et al., 1998). Furthermore, cytochrome P450 has been associated with pheromone production and chemical communication (Dermauw et al., 2020). Consequently, further investigation into the role of cytochrome P450 during mate choice is warranted. It's noteworthy the unusual presence of chitinase and luciferin monooxygenase (S2) in the insect brain, for which we lack a comprehensive understanding of their general functions in this organ. Their presence highlights the importance of deeper exploration into insect brain physiology. Although information is limited, chitinases have been linked to inflammatory processes and brain

injury in mammals (Eurich, et al. 2009; Wiley et al., 2015;), while the activity of luciferin monooxygenase suggests its potential involvement as a long-chain fatty acyl-CoA synthetase (Oba et al., 2003). Nevertheless, further research is essential to elucidate their roles conclusively. The imbalance of antioxidant and detoxification proteins could suggest not only that mating is a demanding task that requires brain protection (Hernández-Villanueva et al., 2023) but also choosing a partner possibly through a memory dependent process. It is possible that the process of memory formation results in cellular damage and reduced lifespan, as observed in studies with *D. melanogaster* (Haddadi et al., 2014; Mery & Kawecki, 2005). To further explore this notion, future studies should investigate whether memory during mate choice imposes costs in terms of oxidative stress and reduced lifespan.

Along with the proteomic analysis from females remated or multiply mated, we sought to understand the changes that occur in the brains of females after mating. Results indicate that mating causes broad and severe changes in protein expression in the brain of females, we observed the dysregulation of 92 proteins due to mating (Supplementary material). Notably, mated females exhibited an increased level of proteins associated with detoxification, protein formation and translation, folding and protection of protein, transcription, dna binding and replication, cellular transport, cytoskeleton architecture, chromatin remodeling and cell proliferation. Some of these proteins are consistent as previously observed in females who mated with males of different phenotypes, such as vitellogenin (Hernández-Villanueva et al., 2023). We found vitellogenin increased in mated females. Vitellogenin presence in the brain has been related to oxidative protection in social insects (Münch et al., 2015; Roy-Zokan et al. 2015; Kohlmeier, Feldmeyer & Foitzik, 2018). Moreover, we discovered other proteins induced by mating, such as the cytochrome P450 monooxygenase that plays a role in inhibiting juvenile hormone during fertilization

(Sutherland et al., 1998) and is involved in pheromone production and chemical communication (Dermauw et al., 2020). Furthermore, the heat shock protein 83, known for its associations with longevity, embryogenesis, and fecundity, has been reported to be induced in female insects (Will et al., 2017). Additionally, another heat shock protein, Hsc70-4, associated with synaptic turnover and the promotion of neurotransmitter release, was found to be upregulated (Uytterhoeven et al., 2015), and the Hsp90 which also responds to stress such as diapause, dehydratation and cold temperatures (King & MacRae, 2015) was downregulated . Two other upregulated proteins with similar functions are protein quiver, also known as SLEEPLESS protein in *Drosophila,* and the I am not dead yet protein (Indy). The first one has been reported to enable sleep and suppress brain excitability (Koh et al., 2008). The second one is a transporter of Krebs cycle intermediates (Rogina, 2017). Its presence has been shown to affect metabolic rate and, when suppressed in *Drosophila,* extend lifespan (Neretti et al., 2009; Wang et al., 2009). These differential proteins, found in higher levels in mated females compared to virgin females, may play a role in preparing females for fertilization and egg production. They may also be influenced by males to suppress female receptivity and inhibit egg production (Amaro et al., 2021). These hypotheses warrant further investigation in future research.

Besides metabolism, it is notable the upregulation of cytoskeletal architecture proteins. Changes in the expression levels of key proteins such as integrin, laminin, PZD domain containing protein in females following mating, suggests alterations in the organization of brain cells, potentially impacting synaptic plasticity (Takeichi, 2007; Colognato & Yurchenco, 2000; Nakamura et al., 2017; Kim & Sheng, 2004; Lee & Zheng, 2010). Notably, proteins required for establishing synaptic connections and facilitating memory were found upregulated in mated females. An example of such proteins is ankyrin; reduced expression

of this protein in *Drosophila* results in decreased locomotion, impaired memory, and shortened lifespan (Koch et al., 2008; Higham et al., 2019). In addition to ankyrin, various proteins with diverse biological functions, including those involved in neurodevelopment and cell proliferation such as the 14_3_3 domain protein (Broadie et al., 1997; Skoulakis & Davis, 1996), homeobox domain (Hobert, 2021; Hildebrandt et al., 2022), F-box domain (Atkin et al., 2015; Quadros et al., 2022) and Wnt protein (Murat & McGregor, 2010; Yang & Luo, 2011), as well as memory-related proteins like the 14_3_3 domain protein (Broadie et al., 1997; Skoulakis & Davis, 1996), were found to be upregulated following mating. Notably, there was a significant dysregulation observed in transcription factors and chromatin remodeling-related proteins (refer to Tables S3 and S4). Additionally, a reduction in proteolysis-related proteins was evident when compared to unmated female brains (refer to Tables 4 and S4). These findings underscore the complexity of molecular changes occurring in the brain post-mating, potentially influencing various aspects of neuronal function and behavior.

Typically, research on mating systems has primarily focused on understanding the influence of males and the benefits that females gain by avoiding harassment by males or gaining genetic diversity (Fromonteil et al., 2023). However, it is important to recognize that females also play a significant role in shaping mating systems, as they exhibit their own behavioral plasticity and can gain benefits (Fromonteil et al., 2023). In our study, we employed a comprehensive experimental approach that considered behavior, pharmacology, and proteomics to show that females of a polygamous species can choose to mate repeatedly with the same males or engage in polyandry based on the attractiveness of their partners. While our findings highlight the female's ability to shape the mating system, it is crucial for future studies to explore in deep whether this strategy is influenced by male reproductive

strategies. Additionally, it would be worthwhile to examine whether the female's decision-making process is adaptive. Specifically, researchers could investigate whether the female's plasticity in remating with the same male or engaging in multiple mating, is a response aimed at avoiding the costs associated with mating with males employing a terminal investment strategy. Further research in these areas would deepen our understanding of the complex interactions and strategies employed by both males and females in the context of mating systems.

Ethics: All procedures in this study were approved by UNAM. **Author contributions:** S.C.M., C.M.G, G.R.R. and J.C.G. designed the experiments. S.C.M. conducted experiments and analyzed data. All authors wrote the manuscript. **Competing interests:** The authors declare no competing interests. **Funding:** J.C.G received a grant from Dirección General del Asuntos del Personal Académico/ Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica/Universidad Nacional Autónoma de México (Grant No. IN225120). S.C.M. is a doctoral student from Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM) and received fellowship 632723 from CONAHCyT, México.

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Supplementary tables

Table S1

REMATED VS MULTIPLE MATING UPREGULATED (GENERAL CELLULAR PROCESSES)			
	Protein	**Quantity**	**Function**
Replication and transcription	RNA-directed DNA polymerase OS=Tenebrio molitor OX=7067 GN=GEV33_008369 PE=4 SV=1	1.74467315	Synthesizes of DNA on an RNA template.
	Reverse transcriptase domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_005537 PE=4 SV=1	1.67696862	
	Reverse transcriptase domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_012716 PE=4 SV=1	1.58028416	
	Reverse transcriptase domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_014262 PE=4 SV=1	2.34437363	
	RT_RNaseH domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_008020 PE=4 SV=1	2.26930987	Replication, transcription, and DNA repair
	CCHC-type domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_006199 PE=4 SV=1	1.54932702	DNA recognition, RNA packaging, Transcription activation, Apoptosis regulation, Lipid binding.
	PBC domain-containing protein · Tenebrio molitor · Gene: GEV33_001042 · Inferred from homology	2.89476083	DNA-binding; transcriptional activity
	PRE_C2HC domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_006785 PE=4 SV=1	1.79908148	RNA binding or single strand DNA binding.
	TATA-binding protein interacting TIP120 domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_004310 PE=3 SV=1	2.20034771	Transcription.

Transposable elements and DNA modification	-	-	-
Chromatin remodeling	HMG box domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_012140 PE=4 SV=1	1.60627822	Remodeling the assembly of chromatin.
Signal transduction	CHK domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_002854 PE=4 SV=1	1.60020599	Protein kinase
	Diacylglycerol kinase OS=Tenebrio molitor OX=7067 GN=GEV33_009744 PE=3 SV=1	1.51420278	Catalysis diacylglycerol to phosphatidic acid; regulation of PKC
	INPP1(Inositol Polyphosphate-1-Phosphatase) · Tenebrio molitor · Gene: GEV33_013779 · Inferred from homology ·	2.83062928	Inositol phosphate dephosphorylation
Proteolytic activity	Proteasome activator Blm10 mid region domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_013613 PE=4 SV=1	23.5876524	Degradation of unstructured proteins
	Peptidase C1A papain C-terminal domain- protein OS=Tenebrio molitor OX=7067 GN=GEV33_005805 PE=3 SV=1	12.6810098	Degradation of protein; cystein proteases
	Peptidase M14 carboxypeptidase A domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_007344 PE=3 SV=1	1.79121727	Proteolysis
	Peptidase A2 domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_004684 PE=4 SV=1	1.62231433	Aspartic-type endopeptidase activity; proteolysis
Protein formation	Saccharopine dehydrogenase OS=Tenebrio molitor OX=7067 GN=GEV33_015386 PE=4 SV=1	8.53466791	Involved in metabolism of lysine

Cell proliferation	Pleiotropic regulator 1 · Tenebrio molitor · Gene: GEV33_013589 · 701 amino acids · Inferred from homology ·	1.62290872	Regulator of cell proliferation and apoptosis
Metabolism (lipids, carbohydrates, nucleotides, Krebs cycle enzymes and intermediaries)	Fatty acyl-CoA reductase OS=Tenebrio molitor OX=7067 GN=GEV33_012789 PE=3 SV=1	1.5897709	Metabolism of lipids/plasmogens
	Trehalase OS=Tenebrio molitor OX=7067 GN=GEV33_012772 PE=3 SV=1	2.14708741	Hydrolyses the disaccharide trehalose (main sugar in insect haemolymph); recovery after abiotic stress.
	Glycogen synthase OS=Tenebrio molitor OX=7067 GN=GEV33_010932 PE=3 SV=1	2.13432549	Glycogen synthesis
Cell transport	Small G protein signaling modulator 2 · Tenebrio molitor · Gene: GEV33_012909 · Inferred from homology	2.18724266	GTPase activator; membrane trafficking.
	Predicted: ABC transporter domain-containing protein [Tenebrio molitor] (A0A8J6HGK2) (91.5%, 0)	1.79519217	Membrane transportation ATP dependant
	Predicted: ABC transporter domain-containing protein [Tenebrio molitor] (A0A8J6LBM4) (92.4%, 0)	2.10318231	

Table S2

REMATED VS MULTIPLE MATING DOWNREGULATED (GENERAL CELLULAR PROCESSES)			
	Protein	**Quantity**	**Function**
Replication and transcription	RNA-directed DNA polymerase OS=Tenebrio molitor OX=7067	-2.21201671	Synthesizes of DNA on an RNA template.

	GN=GEV33_008362 PE=4 SV=1		
	RNA-directed DNA polymerase OS=Tenebrio molitor OX=7067 GN=GEV33_009124 PE=4 SV=1	-1.81556803	
	RNA-directed DNA polymerase OS=Tenebrio molitor OX=7067 GN=GEV33_007077 PE=4 SV=1	-1.62989775	
	Reverse transcriptase domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_004294 PE=4 SV=1	-4.18722749	
	Endonuclease · Tenebrio molitor · Gene: GEV33_002047 · Predicted	-1.76253	DNA repair; incision of DNA.
	RNA helicase OS=Tenebrio molitor OX=7067 GN=GEV33_011842 PE=4 SV=1	-1.57590967	RNA metabolism; gene expression
	CCHC-type domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_001905 PE=4 SV=1	-2.35216518	DNA recognition, RNA packaging, Transcription activation, Apoptosis regulation, Lipid binding.
	Mothers against decapentaplegic homolog OS=Tenebrio molitor OX=7067 GN=GEV33_003281 PE=3 SV=1	-2.03143223	Transcription factors, regulation of cell proliferation, apoptosis, and cell differentiation.
	DDE_3 domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_010733 PE=4 SV=1	-1.55053209	Nucleic acid binding
Transposable elements and DNA modification	-	-	-
Chromatin remodeling	RuvB-like helicase OS=Tenebrio molitor OX=7067 GN=GEV33_007601 PE=3 SV=1	-2.00695922	Chromatin remodeling.
Signal transduction	-	-	-
Proteolytic activity	-	-	-
Protein formation	Transglutaminase C domain-containing protein · Tenebrio molitor · Gene: GEV33_000537, GEV33_000816 · Predicted ·	-.327569718	Establish of covalent links between proteins; hydrolysis of peptide bonds.
Metabolism (lipids,	Nucleoside phosphorylase domain-containing protein OS=Tenebrio molitor OX=7067	-1.53277549	Nucleoside catabolism

carbohydrates, nucleotides, Krebs cycle enzymes and intermediaries)	GN=GEV33_001180 PE=3 SV=1		
	Alcohol-forming fatty acyl-CoA reductase · T molitor · Gene: GEV33_009973 · 1043 amino acids · Inferred from homology	-1.55582606	Reduction of fatty acyl-CoA to fatty alcohols.
	ATP synthase subunit d_ mitochondrial OS=Tenebrio molitor OX=7067 GN=GEV33_005226 PE=3 SV=1	-1.90592708	Production of ATP from ADP; involved in electron transport complexes of the respiratory chain.
Cell transport	GRIP domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_014800 PE=4 SV=1	-1.51556142	Vesicular traffic within the secretory pathway.
unknown	Band 7 domain-containing protein · T molitor- Gene: GEV33_009798 · Inferred from homology · KAH0821975.1	-.252248212	unknown
	Inferred from homology: Luciferin 4-monooxygenase [Tribolium castaneum] (A0A139WH95) (90.9%, 1.4e180)	-1.86523387	Unknown in brain.

Table S3

MATED VS UNMATED UPREGULATED (GENERAL CELLULAR PROCESSES)			
	Protein	**Quantity**	**Function**
Replication and transcription	Reverse transcriptase domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_009643 PE=4 SV=1	1.51059617	Synthesizes of DNA on an RNA template.
	Reverse transcriptase domain-containing protein · Tenebrio molitor · Gene: GEV33_000014 · Predicted	1.51915265	
	Reverse transcriptase domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_011267 PE=4 SV=1	32.8897467	
	Reverse transcriptase domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_001760 PE=4 SV=1	1.83368662	
	Reverse transcriptase domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_004517 PE=4 SV=1	2.36260847	
	Reverse transcriptase domain-containing protein OS=Tenebrio	2.18975923	

	molitor OX=7067 GN=GEV33_008006 PE=4 SV=1		
	RNA-directed DNA polymerase OS=Tenebrio molitor OX=7067 GN=GEV33_007077 PE=4 SV=1	1.74083409	
	RNA helicase · Tenebrio molitor· Gene: GEV33_012480 · Predicted	3.07000778	RNA metabolism, including: RNA synthesis, RNA folding, RNA-RNA interactions, RNA localization, RNA degradation
	HTH CENPB-type domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_014104 PE=3 SV=1	4.54763958	Nuclear acid binding; transcription; DNA repair and replication, RNA metabolism, and protein–protein.
	CCHC-type domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_010892 PE=4 SV=1	2.04054527	DNA recognition, RNA packaging, Transcription activation, Apoptosis regulation, Lipid binding.
	C2H2-type domain-containing protein	1.537635003	Transcription factor.
Transposable elements and DNA modification	Tc1-like transposase DDE domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_006738 PE=3 SV=1	2.01524796	DNA cleavage, DNA modifications
	Tc1-like transposase DDE domain-containing protein [Tenebrio molitor (94.5%,1.1 e-62)(A0A8J6LEL9)	3.066596464	
	Retrotransposon gag domain-containing protein (Tenebrio molitor) (97.7%,1.5 e-43)(A0A8J6HL35)	2.077156416	
	Integrase catalytic domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_012577 PE=4 SV=1	2.00012542	
Chromatin remodeling	PHD-type domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_008331 PE=4 SV=1	2.43641055	Histone post-translational modifications (methylation and acetylation).
	BAH domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_010783 PE=4 SV=1	2.10545316	Chromatin protein-protein interactions; recognition of methylated histones; nucleosome binding.

Signal transduction	DH domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_005499 PE=4 SV=1	1.76723671	Signal transduction; activation of GTPase activity
	DH domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_003144 PE=4 SV=1	2.53642505	
Proteolytic activity	DZIP3-like HEPN domain-containing protein · Tenebrio molitor · Gene: GEV33_001470 · Predicted	18.8945405	Ubiquitin-protein transferase activity
	Peptidase S1 domain-containing protein· Tenebrio molitor · Gene: GEV33_007722 · Predicted	1.77173224	Proteolysis.
Protein formation and transport	BRO1 domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_005918 PE=4 SV=1	1.663516	Protein targeting to the vacuole or lysosome.
	Pre-rRNA-processing protein TSR1 homolog OS=Tenebrio molitor OX=7067 GN=GEV33_002939 PE=4 SV=1	2.89999839	Maturation of the ribosomal subunits in the nucleolus.
	WD repeat-containing protein on Y chromosome OS=Tenebrio molitor OX=7067 GN=GEV33_004928 PE=3 SV=1	1.59469903	Fertility factor; pre-mRNA processing, transcription factor.
	GTP-binding protein SAR1 · Tenebrio molitor · Gene: GEV33_007784 · Inferred from homology	1.99815791	Protein transport from the endoplasmic reticulum to the Golgi apparatus.
	Large ribosomal subunit protein mL53 · Tenebrio molitor · Gene: GEV33_004899 · Inferred from homology	1.98247965	Formation of peptide bonds; protein synthesis in mithocondria.
	Transglut_N domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_002814 PE=3 SV=1	1.62369597	Catalisis of calcium-dependent formation of isopeptide bonds between protein-bound glutamine and lysine substrates
	60S ribosomal protein L13 OS=Tenebrio molitor OX=7067 GN=GEV33_006890 PE=3 SV=1	1.54485006	Component of the ribosome.
	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1 OS=Tenebrio molitor OX=7067 GN=GEV33_002623 PE=3 SV=1	1.54462905	Tansfer of high-mannose oligosaccharides to asparagine residues on nascent polypeptides in rough endoplasmic reticulum.
	Nucleolar GTP-binding protein 2 OS=Tenebrio molitor OX=7067 GN=GEV33_010964 PE=3 SV=1	1.52161875	Nuclear export and maturation of ribosomal subunits.

Metabolism (lipids, carbohydrates, nucleotides, Krebs cycle enzymes and intermediaries)	Fatty acyl-CoA reductase OS=Tenebrio molitor OX=7067 GN=GEV33_009174 PE=3 SV=1	1.81713607	Synthesis of ether lipids/plasmalogens.
	Aldehyde dehydrogenase domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_010339 PE=3 SV=1	1.6995529	Metabolism; oxidation of aldehydes to carboxylic acids.
	Mitochondrial 12S rRNA dimethylase 1 OS=Tenebrio molitor OX=7067 GN=GEV33_002641 PE=3 SV=1	1.5388399	Transcription of mitochondrial DNA.
	Complex I-49kD OS=Tenebrio molitor OX=7067 GN=GEV33_005271 PE=3 SV=1	1.51513049	Metabolism; enzyme in the respiratory chain.
	Peroxisomal multifunctional enzyme type 2· Tenebrio molitor · Gene: GEV33_008911 · Predicted ·	1.52980037	Lipid metabolism; fatty acid beta-oxidation.
	Hexosyltransferase OS=Tenebrio molitor OX=7067 GN=GEV33_007686 PE=3 SV=1	8.64887646	Glycosyltransferase; metabolism.
Cell transport	t-SNARE coiled-coil homology domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_010165 PE=3 SV=1	1.70569875	Vesicular membrane traffic.
	V-type proton ATPase subunit a OS=Tenebrio molitor OX=7067 GN=GEV33_015432 PE=3 SV=1	2.63906077	Proton transport across intracellular and plasma membranes.
	Secretory carrier-associated membrane protein OS=Tenebrio molitor OX=7067 GN=GEV33_006541 PE=3 SV=1	1.58924412	Membrane trafficking; recycling carriers to the cell surface.
unknow	MOSC domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_000989 PE=4 SV=1	2.29918617	Unknown.

Table S4

MATED VS UNMATED DOWNREGULATED (GENERAL CELLULAR PROCESSES)			
	Protein	**Quantity**	**Function**
Replication and transcription	DNA-directed DNA polymerase OS=Tenebrio molitor OX=7067 GN=GEV33_001795 PE=4 SV=1	-1.72024212	DNA synthesis.
	RNA-directed DNA polymerase from transposon X-element · Tenebrio molitor · Gene: GEV33_006084 · Predicted	-1.7182471	Synthesizes DNA on an RNA template.
	RNA-directed DNA polymerase OS=Tenebrio	-1.80674254	

	molitor OX=7067 GN=GEV33_006882 PE=4 SV=1		
	Reverse transcriptase · Tenebrio molitor · Gene: GEV33_012241 · Predicted	-29.4072214	
	Reverse transcriptase domain-containing protein · Tenebrio molitor · Gene: GEV33_004265 · Predicted	-9.13580108	
	Reverse transcriptase domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_004588 PE=4 SV=1	-6.38005537	
	Reverse transcriptase domain-containing protein [Tenebrio molitor] (99.2%, 3.4e-173)	-5.85486221	
	Predicted: Reverse transcriptase domain-containing protein [Tenebrio molitor] (A0A8J6HHG7) (99.2%, 3.4e-173)	-2.332237265	
	Predicted: Hrp65 protein [Asbolus verrucosus] (A0A482VYK3) (90.7%, 0)	-1.8439883	RNA binding, and its biological process is mRNA export from the nucleus; actin binding protein.
Transposable elements and DNA modification	Retrotrans_gag domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_006739 PE=4 SV=1	-2.01060334	Transposable elements promote various chromosomal rearrangements more efficiently.
Chromatin remodeling			
Signal transduction	Protein kinase domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_005374 PE=4 SV=1	-2.41051305	Cellular signaling pathways; phosphorylation.
	EF-hand domain-containing protein · Tenebrio molitor · Gene: GEV33_008442 · Inferred from homology	-4.36783161	Signal transduction of calcium as a secondary messenger.
Proteolytic activity	Proteasome subunit alpha type-2 · Tenebrio molitor · Gene: GEV33_010382 · Inferred from homology	-2.64762812	Protein degradation, protein homesostasis.
	COP9 signalosome complex subunit 4 OS=Tenebrio	-1.67938468	Modification to proteins; Ubiquitylation.

	molitor OX=7067 GN=GEV33_012900 PE=3 SV=1		
	Serpin C1 · Tenebrio molitor · Gene: GEV33_008233 · Inferred from homology ·	-2.21508832	Protease inhibitor; anticoagulation and anti-inflammation.
Protein formation and transport	Asparagine--tRNA ligase OS=Tenebrio molitor OX=7067 GN=GEV33_011708 PE=4 SV=1	-1.64051811	Catalyzes the attachment of asparagine to tRNA(Asn)
	Signal recognition particle subunit SRP68 · Tenebrio molitor · Gene: GEV33_010278 · Predicted	-2.05417667	Ribonucleoprotein complex that mediates the cotranslational targeting of secretory and membrane proteins to the endoplasmic reticulum for processing.
	S1 motif domain-containing protein OS=Tenebrio molitor OX=7067 GN=GEV33_013813 PE=4 SV=1	-4.60849479	Interacts with the ribosome and messenger RNA
Metabolism (lipids, carbohydrates, nucleotides, Krebs cycle enzymes and intermediaries)	3-oxoacyl-[acyl-carrier-protein] synthase OS=Tenebrio molitor OX=7067 GN=GEV33_009844 PE=3 SV=1	-2.02648832	Fatty acid synthesis.
	Aldehyde dehydrogenase domain-containing protein protein · Tenebrio molitor · Gene: GEV33_006390 · Predicted ·	-5.46948906	Metabolism; cellular homeostasis; oxidation of aldehydes to carboxylic acids.
	NADP-dependent oxidoreductase domain-containing protein · Tenebrio molitor· Gene: GEV33_006236 · Predicted	-2.34405759	Metabolism; oxidation or reduction of a nicotinamide adenine dinucleotide cofactor NAD(P)H or NAD(P)+.
	Hydroxymethylglutaryl-CoA synthase · Tenebrio molitor · Gene: GEV33_008014 · Inferred from homology	-1.80840845	Synthesis and degradation of ketone bodies.
	Group XV phospholipase A2 · Tenebrio molitor · Gene: GEV33_006809 · 392 amino acids · Predicted	-1.67300743	Phospholipid digestion and metabolism; signal transduction.
	D-3-phosphoglycerate dehydrogenase · Tenebrio molitor · Gene: GEV33_004922 · 246 amino acids · Inferred from homology ·	-1.77212054	Metabolism; glycolysis.

Cell transport	Vacuolar protein sorting-associated protein 53 homolog OS=Tenebrio molitor OX=7067 GN=GEV33_003213 PE=3 SV=1	-5.85090199	Transport in Golgi apparatus.
	Proton-coupled zinc antiporter SLC30A9, mitochondrial OS=Tenebrio molitor OX=7067 GN=GEV33_010799 PE=3 SV=1	-2.29736038	Zinc homeostasis, zinc mobilization.

Supplementary methodology

Protein extraction and sample preparation for mass spectrometry

40-50 mg of protein extraction beads (Diagenode, Liège, Belgium) were added to a microcentrifuge tube containing brain tissue, subsequently, 100 µL of RIPA Buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% (v:v) NP-40, 0.25% (w/v) sodium deoxycholate and 0.1% SDS) and mix of protease inhibitors (1X) were added to the tubes and samples were placed in a holder and sonicated at 4°C for 25 cycles of 30 seconds per cycle using a BioRuptor Pico® (Diagenode, Liège, Belgium). Then, the supernatants were transferred to the new microcentrifuge tubes and, the remnant protein extraction beads were washed with 25 µL of RIPA Buffer shaking eventually in a vortex; this washing was transferred to the original supernatants and, the samples were centrifuged at 4°C using 14000 rpm during 15 min. Again, resulting supernatants were transferred onto a new microcentrifuge tube to eliminate debris.

Protein was quantified using a 2D Quant KIT (Cytiva, Marlborough, MA, USA) and 12 µg precipitated with were precipitated using MeOH/Chloroform in 4:1 ratio. The resulting pellets were resuspended with urea 8M and loaded in a 12% 1D SDS-PAGE gel, separated ≈1 cm

inside the resolving gel to concentrate the proteins in a small gel zone, and dyed with silver staining using a Silver Quest^{TM} Staining Kit (Invitrogen, Waltham, MA, USA).

After the electrophoretic run, gels were digested “in-gel”, briefly, gel pieces were excised from the gel and cut into ≈1 mm pieces; cut pieces were transferred into centrifuge microtubes to be destained according to the protocol established by the manufacturer of the Silver Quest^{TM} Staining Kit with some modifications; briefly, gels were destained with 100 µL from a mix of 50:50 (v:v) of destainer A and destainer B during 15 min, then gels were washed with ultrapure water and immediately dehydrated with Methanol (MeOH) then, gels were hydrated with 30% MeOH and, again, be dehydrated with MeOH, the remaining solvent was eliminated in a Savant DNA120 SpeedVac Concentrator (Thermo Fisher Scientific,Waltham, MA, USA) for 10 min. Then, proteins contained in dried bands were reduced with 10 mM DTT (Sigma-Aldrich, St. Louis, MO, USA) in 100 mM ammonium bicarbonate (ABC) (Sigma-Aldrich) and alkylated with 50 mM iodoacetamide (IAA) (Sigma-Aldrich) in 100 mM ABC. Afterward, cut bands were washed with 100 mM ABC and dehydrated with ACN; subsequently, cut bands were hydrated and washed anew with 100 mM ABC and dehydrated with Acetonitrile (ACN); then, the excess solvent was removed using the SpeedVac for 10 min. Proteins in gel were enzymatically digested overnight using 20 ng/µL trypsin (Sigma-Aldrich) in 50 mM ABC at 37 °C in a Precision water bath (Thermo Fisher Scientific, Waltham, MA, USA). Once the time had passed, the reaction was stopped with 40 µL of 5% Formic Acid (FA) for 10 min at room temperature; subsequently, peptides were eluted from the gel for two cycles using 40 µL of a solution of 5% FA and 50% ACN. Peptides were concentrated in the SpeedVac and desalted using PierceTM C18 spin columns (Thermo Fisher Scientific,Waltham, MA, USA); finally, the resulting peptides were again concentrated and stored at -20 °C until LC–MS analysis.

Mass spectrometry and data analysis

Peptides were injected into the mass spectrometer Synapt G2–S*i* (Waters, Milford,MA, USA) in MS^E mode to calculate the area under the curve (AUC) of the total ion chromatogram (TIC), to normalize the injection prior to LC–MS analysis and, therefore, inject a comparable sample quantity for each condition (inserter reference https://pubs.acs.org/doi/10.1021/jasms.0c00134). Afterwards, peptides were loaded and separated on HSS T3 C18 Column (Waters, Milford, MA, USA); 75 µm × 150 mm, 100 Å pore size, 1.8 µm particle size, using an UPLC ACQUITY M-Class using mobile phase A 0.1% formic acid (FA) in H_2O and mobile phase B, 0.1% FA in acetonitrile (ACN), under the following gradient: 0 min 7% B, 121.49 min 40% B, 123.15 to 126.46 min 85% B, 129 to 130 min 7% B, at a flow of 400 nL/min at 45°C. The spectra data were acquired in a Synapt G2-S*i* mass spectrometer (Waters, Milford, MA, USA) using nanoelectrospray ionization (nanoESI) and ion mobility separation (IMS) with a data-independent acquisition (DIA) approach through UDMSE mode. Values for the ionization source were as follows: 2.75 kV in the capillary emitter, 30 V in the sampling cone, 30 V in the source offset, 70°C for the source temperature, 0.5 Bar for the nanoflow gas and 150 L/hour for the purge gas flow. Low and high energy chromatograms were acquired in positive mode using m/z 50–2000 range with a scan time of 500 ms. No collision energy was applied to obtain the low energy chromatogram, while for the high energy chromatograms, the precursor ions were fragmented in the transfer using a collision energy ramp from 19 to 55 eV. (https://pubs.acs.org/doi/10.1021/jasms.0c00134).

The MS and MS/MS measurements contained in the generated *.raw files were analyzed and relatively quantified using Progenesis QI for Proteomics software *v*4.2 (https://doi.org/10.1002/pmic.200800564) (Nonlinear Dynamics, Milford, MA) using a target decoy strategy against the *Tenebrio mollitor* *.fasta database (obtained from Uniprot,

UP000719412, 15190 protein sequences), which was concatenated with the same *.fasta file in the reverse sense (inserter https://doi.org/10.1021/pr700600n y https://link.springer.com/protocol/10.1007/978-1-60761-444-9_5). The identification of proteins includes cysteine carbamidomethylation as fixed modification, methionine oxidation and serine, threonine and tyrosine phosphorylation as variable modifications, trypsin as cut enzyme and one missed cleavage allowed; default peptide and fragment tolerance (maximum normal distribution of 10 ppm and 20 ppm, respectively) and false discovery rate ≤ 4%. Synapt G2–S*i* was calibrated with [Glu1]–fibrinopeptide fragments through the precursor ion $[M+ 2H]^{2+}$ = 785.84261 fragmentation of 32 eV with a result less than 1.5 ppm across all MS/MS measurements.

The average intensity of the three most abundant peptides per protein (Top3) was used for label-free quantitation according to a previously described method (*inserter doi:10.1074/mcp.M500230-MCP200*). All proteins considered differentially expressed (DEPs) in this work displayed at least a fold change (FC) of ±1 (expressed as log2), calculated on the Top3 signal of each characterized protein “mated” over “unmated”; and “remated” over “multiply mated”. All DEPs have a p-value ≤ 0.05, at least two total peptides, including at least one unique peptide.

CAPÍTULO V.

Cordero-Molina, S., Mendoza, L., Alvarado, Y., Krams, I., & Contreras-Garduño, J. (2024). Exploring the males' terminal investment strategy: Impact of the dose of stress and the time lapse between stress and mating. Ecological Entomology, 49, 21-30. doi.org/10.1111/een.13275

Abstract

1. In the terminal investment strategy, organisms allocate resources to maximize current reproduction when future reproductive opportunities are uncertain due to a high risk of mortality. However, whether the time-lapse between stress and sexual advertisement might trigger the terminal investment strategy remains unknown.
2. We examined this hypothesis in the beetle *Tenebrio molitor* using oxidative stress as the stressor. Given that this is the first time that oxidative stress is used to trigger terminal investment, we first analyzed the male mortality against 5, 20, 40, and 80 mM of the pro-oxidant, paraquat (PQ). Subsequently, we conducted mate choice trials where males exposed to each of these engaged in courtship behavior towards the females.
3. Males exposed to a five mM treatment exhibited higher survival rates compared to the control group, while males exposed to 80 mM experienced a shorter lifespan. No significant differences in mortality were observed between the control group and those treated with 20 or 40 mM. Notably, only males exposed to 40 mM were preferred over the control group in the mate choice trials.
4. Finally, we conducted an experiment to measure the survival costs when male organisms courted and mated with the females at varying time intervals after oxidative stress. When males courted and mated three hours after experiencing oxidative stress (40 mM), their mortality rates significantly increased, compared to males that confronted oxidative stress, courted, and mated 24 hours later.
5. The results suggest that 40 mM PQ induces terminal investment in males. Upon exposure to this dose and engaging in courtship and mating 3 hours after facing oxidative stress, their survival decreases, but not after 24 hours.

INTRODUCTION

According to the theory of sexual selection, secondary sexual characteristics (SSCs) serve as honest signals for individuals to attract mates by honestly communicating their condition to others of the same species. These SSCs are considered reliable indicators of good condition because they require significant resources to develop and maintain (Zahavi, 1975; Andersson, 1994). Unfavorable environmental conditions can be detrimental to the organisms' condition (Hill, 2011). As a result, only individuals in the best condition can bear such damage and simultaneously invest resources in the development of exaggerated SSCs to ensure their immediate and future reproductive success (Zahavi, 1975; Andersson, 1982).

Elaborated SSCs provide valuable information about the organisms' condition after an immune challenge (Siva-Jothy, 2000; Faivre et al., 2003; Contreras-Garduño et al., 2007), food scarcity (Hill & Montgomerie, 1994; Krams et al., 2015), or oxidative stress (Martínez-Lendech et al., 2015; Ruiz-Guzman et al., 2020). However, some organisms strategically hide their poor condition by investing in SSCs, giving the appearance of being in good condition. This strategy, known as terminal investment, prioritizes immediate reproductive success over recovery (Williams, 1966; Clutton-Brook, 1984; Nielsen & Holman, 2012; Duffield et al., 2015; An & Waldman, 2016). Terminal investment is triggered by intrinsic factors, such as senescence (Velando, Drummond & Torres, 2006), as well as extrinsic factors like immune challenges (Sadd et al., 2006; An & Waldman, 2016) or food scarcity (Krams et al., 2015; Miyashita et al., 2019). However, terminal investment is a dynamic strategy influenced by both the intrinsic condition of the organism and its environmental context, which determine the terminal investment threshold (Duffield et al.,

2017). Studies have addressed terminal investment by considering mainly intrinsic factors in a dynamic context (Sanz et al., 2001; Krams et al., 2015; Duffield et al., 2018; Miyashita et al., 2019; Farchmin et al., 2020; Hudson et al., 2020), but additional factors that induce terminal investment should provide new insights into reproductive strategies. For this purpose, terminal investment must be tested considering for example, the doses of stress and reproductive opportunities (Duffield et al., 2017; Foo et al., 2023).

In this study, we examined the influence of oxidative stress and male reproductive investment, on terminal investment. Male reproductive investment refers to the energy allocated to reproduction during a reproductive episode, such as courting a mating partner (Trivers, 2002). In this study, we focused on the impact of oxidative stress and male reproductive investment (courtship or courtship plus mating) on the terminal investment strategy. On the one hand, oxidative stress poses a significant selective pressure on all living organisms due to its association with metabolic processes (Finkel & Holbrook, 2000). It can also arise from external anthropogenic agents like pollution (Isaksson, 2010) or pesticides (Rutherford & Krieger-Liszkay, 2001), which generate free radicals capable of harming the organism's health. When there is an excessive production of free radicals with insufficient antioxidant production, an imbalance known as oxidative stress occurs (Finkel & Holbrook, 2000; Garratt & Brooks, 2012). Considering that oxidative stress can compromise the integrity of SSCs (von Schantz et al., 1999), the extent of pro-oxidant exposure is expected to impact reproductive strategies, such as terminal investment, which aim to offset this damage. We predicted that: 1) as the level of oxidative stress increases, males would be likely to become less attractive to females, and 2) the greater investment in SSCs (courtship and hence, mate choice) by organisms under oxidative stress should result in shorter survival. On the other hand, the male reproductive investment (courtship or courtship

plus mating) modulates reproductive strategies (Hubbell & Johnson, 1987; Gowaty & Hubbell, 2009) such as terminal investment strategy. Considering the substantial costs associated with reproduction (Lawniczak et al., 2007; Speakman & Garratt, 2014; Blount et al., 2016), we formulated the hypothesis that terminal investment is influenced by the time interval between an oxidative challenge and subsequent male reproductive investment (courtship plus mating). We predicted that individuals engaging in reproductive investment immediately after experiencing an oxidative stress challenge (3 hours) would exhibit terminal investment, leading to a decrease in their survival time. Conversely, individuals exposed to reproductive investment after a relatively longer period following the challenge (24 hours) might have adequate time for recovery, resulting in the absence of terminal investment and no reduction in survival.

The hypotheses of the effect induced by oxidative stress and reproductive investment on terminal investment was tested by using the mealworm beetle *Tenebrio molitor*. Male produces sex pheromones to advertise about their sexual status and condition, and these traits are evaluated by females during mate choice (Tschinkel et al.,1967; Rantala et al., 2003; Bryning, et al., 2005). Terminal investment has been found in this species when a poor diet impairs the condition of males (Krams et al., 2015), and when they face immunological challenges (Rantala et al., 2002; Sadd et al., 2006; Nielsen & Hoffmann, 2012). Under experimental stressful conditions, males exhibited an elevation in their sexual courtship behavior, through increased pheromone production (SSCs), surpassing the attractiveness of males in good condition (Rantala et al., 2003; Nielsen & Hoffman, 2012; Krams et al., 2015). Consequently, female individuals of *T. molitor* evaluate the pheromone production of males to gauge their oxidative stress status after exposure to paraquat (PQ), a pro-oxidant herbicide (Ruiz-Guzman et al., 2020). It is essential to emphasize that the

theory of terminal investment is based on the fundamental assumption that redirecting resources from survival-related processes (such as somatic maintenance or health recovery) towards reproduction (such as the production of SSCs) carries a significant cost on future reproductive endeavors, ultimately resulting in a shortened lifespan (Williams, 1966; Clutton-Brook, 1984; Krams et al., 2014). Surprisingly, only a limited number of experimental studies have investigated the cost associated with simultaneous evaluations of survival and reproduction (see Table 1; reviewed in Duffield et al., 2017). Therefore, as this is the initial examination of oxidative stress in relation to terminal investment, we conducted an analysis of male mortality across varying concentrations of PQ (5, 20, 40, and 80 mM). Furthermore, we assessed the influence of these stress levels on male courtship, which was evaluated through female choice (Rantala et al., 2002, 2003; Kivleniece et al., 2010; Krams et al., 2011, 2015; Márquez-García et al., 2016; Ruiz-Guzman et al., 2020). In a subsequent experiment, we exposed *T. molitor* males to reproductive investment, consisting of male courtship and mating, after receiving 40 mM of PQ for either 3 or 24 hours. This allowed us to determine the impact of courtship alone and courtship in conjunction with mating on the males' reproductive strategy for attracting females, including both honest and dishonest signals.

Table 1. Survival recordings in studies investigating intrinsic and extrinsic factors that induce terminal investment. Only studies explicitly supporting the terminal investment hypothesis were included. The table include papers reviewed by Duffield et al. (2017) and recent studies obtained by searching the term "terminal investment" in Scopus from January 2017 to May 2022. The table highlights that although terminal investment is strongly indicated, survival is typically not reported (only 32.3% of studies include survival data). Furthermore, the table reveals a lack of consideration for the dynamic context in which terminal investment can be triggered. Among the studies involving intrinsic factors (12.9%) or extrinsic factors (9.7%), survival or survival after mating is seldom recorded (except for Tarwater & Arcese, 2017). Additionally, only 21.9% of studies measured survival following a reproductive event, supporting the notion that terminal investment is often not viewed as a dynamic strategy influenced by behavioral factors, as suggested by Duffield et al. (2017). STI = Survival measured after performing terminal investment; STIM = Survival measured after performing terminal investment and after mating; EF = Extrinsic factors; IF = Intrinsic factors; Differences = Significant differences in survival between treatment and control groups.

Species	Survival STI	Survival STIM	EF	IF	Dif	Reference
Taeniopygia guttata						Sköld-Chiriac et al. 2019
Drosophila melanogaster	X	X			X	Hudson et al. 2019
Gryllodes sigillatus	X					Duffield et al. 2018
Gryllus texensis	X				X	Miyashita et al. 2018
Ephippiger diurnus	X	X			X	Rebar & Greenfield 2017
Melospiza melodia	X	X		X	X	Tarwater & Arcese 2017
Litoria r heocola						Roznik et al. 2015
Tenebrio molitor						Krams et al. 2015
Gryllodes sigillatus						Duffield et al. 2015
Gambusia affinis	X	X			X	Billman & Belk 2014
Acyrthosiphon pisum	X				X	Leventhal et al. 2014
Cyanistes caeruleus			X			Podmoka et al. 2014

Nicrophorus vespilloides	X	X			X	Benowitz et al. 2013
Daphnia magna						Vale & Little 2012
Allonemobius socius						Copeland & Fedorka 2012
Acyrthosiphon pisum						Barribeau et al. 2010
Nicrophorus vespilloides				X		Cotter et al. 2010
Nicrophorus orbicollis	X	X			X	Creighton et al. 2009
Syngnathus typhle				X		Billing et al. 2007
Somateria mollissima						Hanssen 2006
Sula nebouxii						Velando et al. 2006
Daphnia magna						Chadwick & Little 2005
Peromyscus leucopus						Derting & Virk 2005
Passer domesticus						Bonneaud et al. 2004
Gryllus campestris						Jacot et al. 2004
Sinapis arvensis			X			Poveda et al. 2003
Ficedula hypoleuca						Sanz et al. 2001
Belostoma flumineum			X	X		Kight et al. 2000
Plasmodium falciparum						Bluckling et al. 1999
Acheta domesticus						Adamo 1999
Drosophila nigrospiracua	X	X				Polak & Starmer 1998

MATERIALS AND METHODS

1.1 Tenebrio molitor breeding

Pupae of *T. molitor* were acquired from the insectary at the Escuela Nacional de Estudios Superiores (ENES) of UNAM, Morelia campus. The insects were maintained at a controlled temperature of 27 ºC under complete darkness (Márquez-García et al., 2016). They were provided *ad libitum* with sterilized wheat bran and corn flour as their food source, supplemented with apple every other day (Punzo & Mutchmor, 1980). The food was sterilized (125 ± 2 °C for 15 minutes) to avoid any infection (Márquez-García et al. 2016; Castro-Vargas et al. 2017). The pupae were individually sexed and placed in plastic containers (12-well Corning plates) with food. We used males and males aged 12 ± 1 days to ensure consistent age across treatments (Márquez-García et al., 2016). This age corresponds to the reproductive peak for both females and males (Cole et al., 2003).

1.2 Oxidative stress and mate choice

To investigate the hypothesis that exposure to pro-oxidants promotes terminal investment, we employed a dose-dependent gradient of pro-oxidant levels. This approach allowed us to determine the specific threshold required for male courtship in terms of pheromone production and the corresponding female choice (Figure 1A).

1.3 Treatments

Males were subjected to oxidative stress by administering the paraquat herbicide (PQ; Sigma) diluted in sterilized distilled water, while the control group received only sterilized distilled water (Martínez-Lendech et al., 2018, 2019; Ruiz-Guzmán et al., 2021). Each

treatment was administered orally using a micropipette, with a volume of 1 µL for water or PQ. To determine the threshold at which terminal investment could be triggered, PQ was administered at various concentrations (5, 20, 40, or 80 millimolar (mM)).

1.3 Mate choice regarding the dose of PQ

Mate choice trials were conducted three hours after the administration of PQ at each of the four doses and their respective control groups (Figure 1A). Virgin females were used as the focal subjects for the behavioral trials, with a new female used for each trial. The behavioral observations took place in the insectary under red light, maintaining a consistent temperature to avoid disrupting the animals' behavior (Márquez-García et al., 2016). Transparent acrylic arenas consisting of a main chamber connected to secondary chambers were utilized, following the design described by Márquez-García et al. (2016). The secondary chambers included a compartment for each male, ensuring isolation from the female and the other male while allowing chemical signals to be released into the main chamber through small perforations (Márquez-García et al., 2016; Ruiz-Guzmán et al., 2021). Once the males were positioned in their respective chambers, the female was placed in a small compartment with perforations within the main chamber, allowing a five-minute habituation period (Márquez-García et al., 2016). Following the habituation period, the female was released and allowed to freely move between the chambers (Kivleniece et al., 2010; Krams et al., 2011; Márquez-García et al., 2016; Ruiz-Guzmán et al., 2021). To determine the female's mate choice, the amount of time she spent in each chamber was recorded for a total of ten minutes per trial (Márquez-García et al., 2016; Ruiz-Guzmán et al., 2021). Mate choice is an accurate estimator of the amount of courtship in terms of the male chemical signals (Rantala et al., 2002, 2003; Carazo et al., 2004; Kivleniece et al.,

2010; Krams et al., 2011, 2015; Nielsen & Hoffman, 2012; Márquez-García et al., 2016; Ruiz-Guzmán et al., 2021). The criterion for female choice was met when the female completely entered the male chamber (Márquez-García et al., 2016; Ruiz-Guzmán et al., 2021). After the 10-minute trial, the procedure was repeated with new individuals. Instead of relying solely on male scents, we chose to use the entire male because it provides additional information about the female and the male competitor and allows for pheromone production (Kivleniece et al., 2010; Márquez-García et al. 2016; Contreras-Garduño et al., 2019; Ruiz-Guzmán et al., 2021). To prevent saturation of the chamber and potential interference with female choice, we limited each trial to a maximum of two males presented to each female (Kivleniece et al., 2010). All observations were conducted in a blinded manner, ensuring that the researchers were unaware of the group to which each male belonged during the behavioral experiments. Furthermore, between each observation, all chambers were cleaned with 70% ethanol to maintain hygiene standards.

1.4 Terminal investment costs in face of a dynamic environment

To investigate whether terminal investment is modulated by reproductive investment, the survival of male individuals was recorded under three different scenarios: 1) No reproductive event (Figure 1A), 2) reproductive investment shortly after exposure to PQ (Figure 1C), and 3) reproductive investment 24 hours after exposure to PQ (Figure 1C). Importantly, all survival experiments were conducted in a blinded manner, with researchers unaware of the experimental conditions from the first day of survival recording until the final day when all individuals had perished.

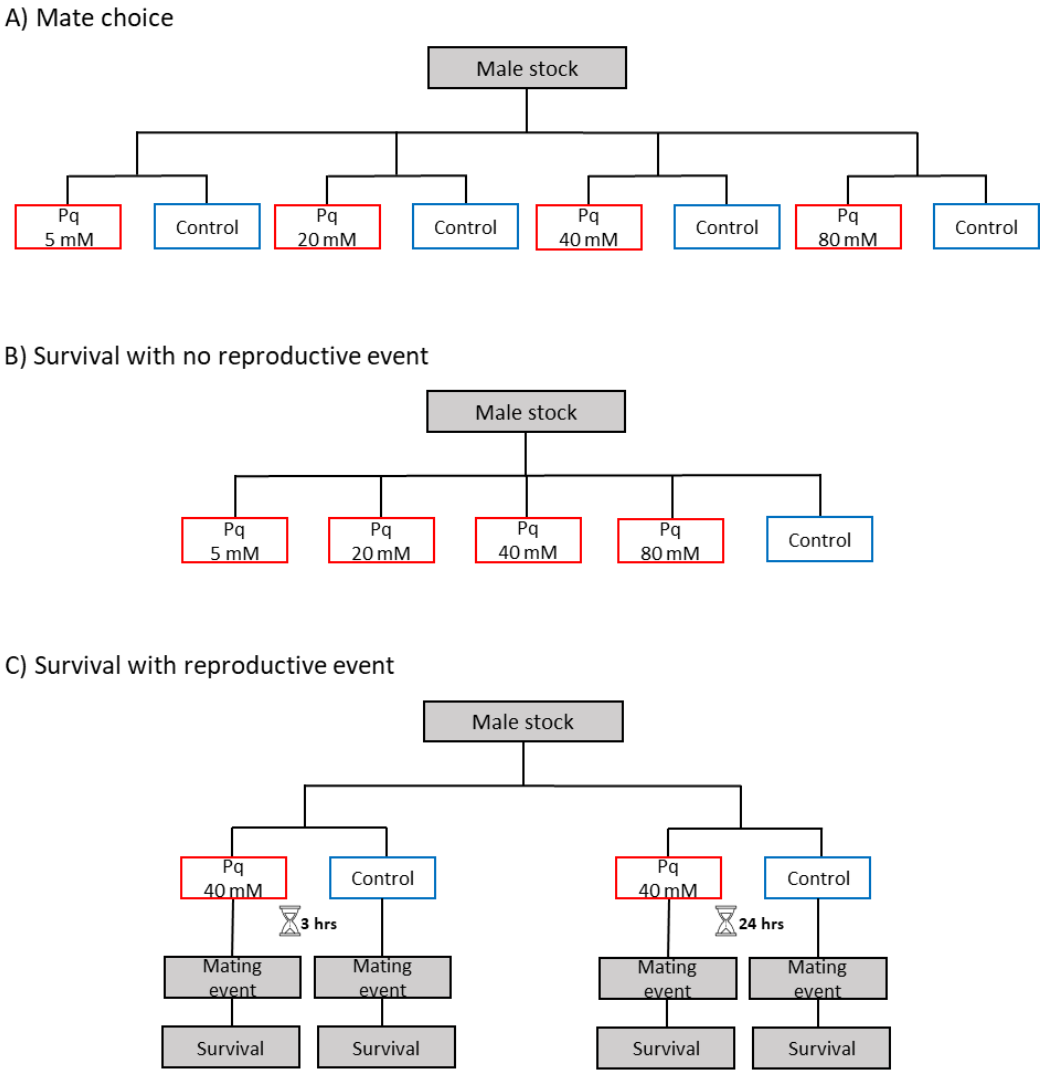

Figure 1. Experimental design. A) Mate choice and B) survival regarding PQ dosage without mating. C) Survival in the face of oxidative stress with mating 3 or 24 hours after PQ treatment.

1.5 Survival in the face of oxidative stress with no reproductive events

Following the administration of treatments (PQ at concentrations of 5, 20, 40, or 80 mM, and control), each male was placed back into its respective container (Corning), and their survival was recorded every other day (Figure 1B). The death of an individual was documented when it ceased to exhibit movement or response to touch with tweezers.

Throughout the experiment, the beetles were provided with a diet of wheat bran and corn flour, while the inclusion of apple was deliberately avoided for two reasons. Firstly, to prevent potential confounding effects stemming from the antioxidants present in apples, as they could potentially impact positively on the condition outcomes. For example, the addition of antioxidants mascaraed the effect of oxidative stress (Medina-Gómez et al., 2018). Secondly, this decision was made based on the recognition that organisms can compensate for their nutritional requirements by increasing their intake of available resources (Valtonen et al., 2010).

1.6 Survival in the face of oxidative stress with reproductive investment

Only the dosage that clearly induced a reproductive investment was used for this experiment. Each group of males with treatment was separated into two sub-groups (as shown in figure 1). These groups were: 1) Control 3h, 2) PQ 3h, 3) Control 24h, 4) PQ 24h. The 4 groups had reproductive investment (courtship plus mating) either 3 or 24 hours after the treatment was administered as shown below (Figure 1C). After finalizing the reproductive event, each male was returned to its original container for registering their survival (Figure 1C). For reproductive investment**,** each male was placed for 5 minutes in a glass Petri disc with a circular filter paper as substrate. The female was then placed, and behavior was recorded for the following 10 minutes. Latency time to first mating (intromission of the aedeagus) was recorded. Males that succeeded in a mating event longer than 30 seconds were returned to their container for survival registration. Thirty second (or longer) mating events are considered successful as it is the time required for a male to transfer its spermatophore (Gadzama & Happ, 1974).

1.7 Statistical analyses

Mate choice was analyzed using a General Linear Model, comparing the time each female spent with each male, either control or experimental. Bonferroni post-hoc test was used to detect significant differences between groups. Survival was analyzed using Log-Rank (Mantel-Cox) or Breslow (Generalized Wilcoxon) with multiple pairwise comparations. All tests were performed with the program SPSS Statistics, 22.0.0.0.

RESULTS

1.1 Mate choice according to the PQ dosage

Significant differences were found in the time that females spend with either, control or experimental males ($F = 3630.36$, d.f. = 7, $p < 0.0001$; Figure 2). Control males (209.45 ± 30.55) were preferred over those with the 5mM pro-oxidant challenge (102.61 ± 21.32; Bonferroni $P < 0.001$); the same effect was observed in the 20mM (Control 207 ± 28.44; PQ 92.69 ± 16.52; Bonferroni $P < 0.001$) and 80 mM (Control 221.72 ± 26.94; PQ 129.59 ± 23.25; Bonferroni $P < 0.001$) doses; except for the 40mM dosage where the males challenged had higher visit time (235.92 ± 28.88) than control (121.08 ± 24.73; Bonferroni $P < 0.001$).

1.2 Survival after oxidative stress and courtship

Results are shown in Figure 3. The lowest dosage (5 mM) showed higher survival than control (Log Rank: $X^2_1 = 5.42$; p = 0.02). Dose of 20 mM (Log Rank: $X^2_1 = 0.56$; p = 0.45) and 40 mM (Log Rank: $X^2_1 = 0.49$; p = 0.48) did not have a negative impact on survival, even though the dose of 40 mM provoked a greater investment in production of the SSC (Figure 2). The dose of 80 mM lowered survival even without mating (Breslow: $X^2_1 = 5.11$; p = 0.02).

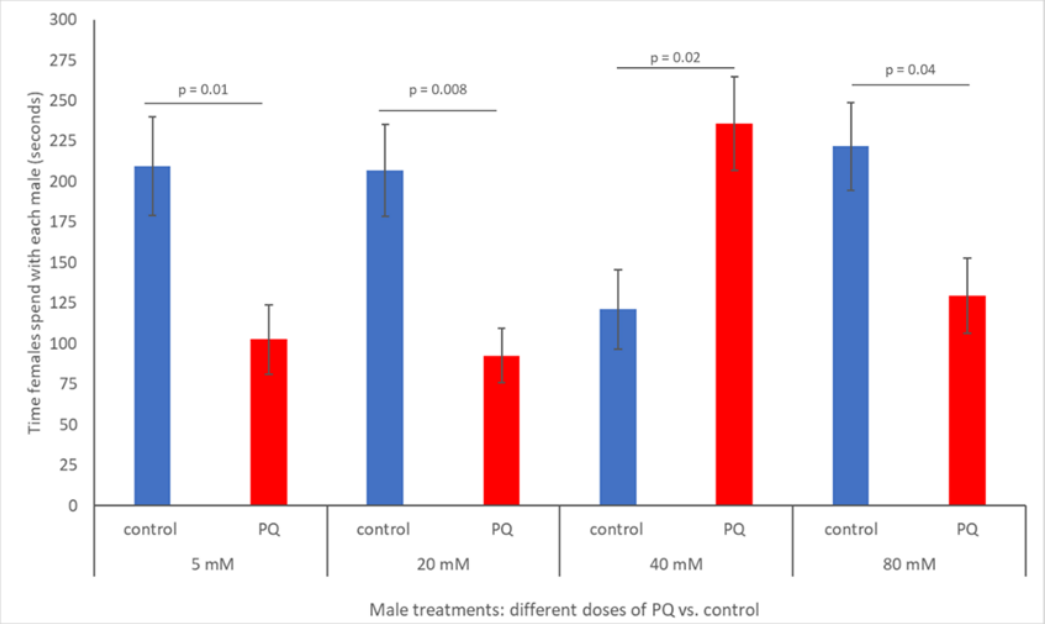

Figure 2. Female preference for odors of males after oxidative stress treatment (PQ) under different dosage or control treatment males. Female preference was recorded for 10 minutes. All comparisons showed statistical differences, p value is showed above each pair of bars. Number of replicates for each pair: 5 mM vs. control, n=30; 20 mM vs. control, n=28; 40 mM vs. control, n=30; 80 mM vs. control, n=39. (mean ± s.e.m.).

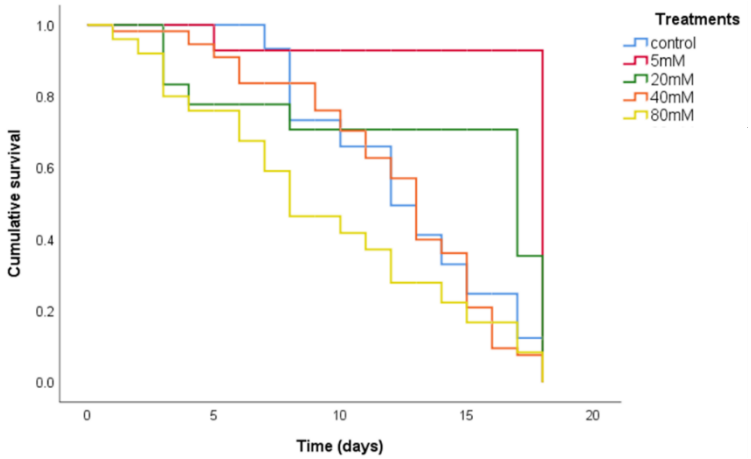

Figure 3: Male survival after oxidative stress (PQ) in different doses and without a reproductive event (only courthiship). Log Rank: Control vs. PQ 5 mM (X^2_1= 5.42; p = 0.02); Control vs. PQ 20 mM (X^2_1= 0.56; p = 0.45); Control vs. PQ 40 mM (X^2_1= 0.49; p = 0.48); Control vs. PQ 80 mM (X^2_1= 3.03; p = 0.08); PQ 5 mM vs. PQ 20 mM (X^2_1= 3.41; p = 0.06); PQ 5 mM vs. PQ 40 mM (X^2_1= 11.22; p = 0.001); PQ 5 mM vs. PQ 80 mM (X^2_1= 13.83; p < 0.0001); PQ 20 mM vs. PQ 40 mM (X^2_1= 3.1; p = 0.07); PQ 20 mM vs. PQ 80 mM (X^2_1= 5.56; p = 0.01); PQ 40 mM vs. PQ 80 mM (X^2_1= 2.49; p = 0.11).

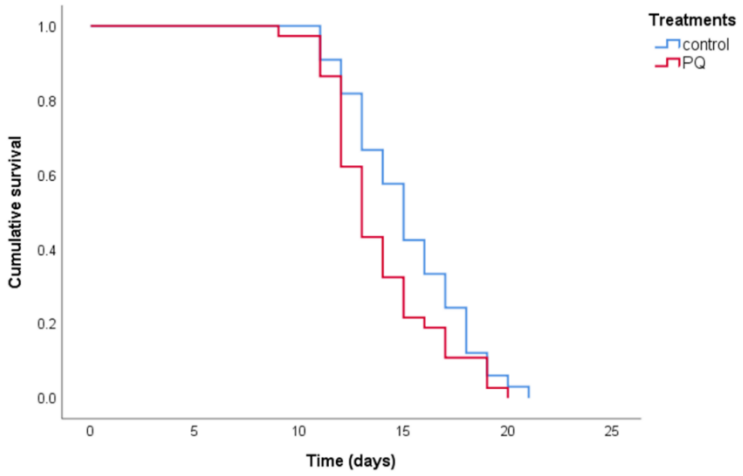

Figure 4. Survival test when males mate 3 hours after facing oxidative stress treatment (PQ 40mM) *vs*. Control. Breslow test: Control (n = 43) vs. PQ 40 mM (n = 48) (X^2_1= 4.67; p = 0.03)

1.3 Survival after oxidative stress and full reproductive investment (courtship plus mating)

Survival results after mating are shown in Figure 4. Males with 40 mM dosage that courted and mated 3h after PQ administration carried out terminal investment since they exhibited higher pheromone production to attract mates and, after mating (Figure 2), they displayed lower survival rates compared to control males (Breslow: $X^2_1 = 4.67$; p = 0.03; control n = 43; PQ n = 48; Figure 4). Nonetheless, there were no significant differences in survival when courtship and the mating event occurred 24 hours after PQ administration (Breslow: $X^2_1 = 0.09$; p = 0.75; control n = 36; PQ n = 44).

DISCUSSION

Terminal investment is a strategy employed by organisms when biotic or abiotic factors become unfavorable for survival, thus posing a risk to future reproductive success (Clutton-Brook, 1984; Sadd et al., 2006; Kivleniece et al., 2010).

This study adds to our understanding by demonstrating that oxidative stress, immune challenges (Nielsen & Holman, 2012) and nutrient deficiencies (Krams et al., 2015), favor resource allocation towards sexual signal production during courtship (in the case of *T. molitor*, pheromone production) and mating, at the cost of survival. Notably, this terminal investment response is triggered by the doses of pro-oxidants and the investment in both courtship and mating, rather than solely in courtship. The mate choice trials provide evidence suggesting that the production of pheromones in *T. molitor* is likely regulated by the risk of mortality resulting from oxidative stress. They exhibit an increase in pheromone production when the level of pro-oxidants reaches a critical threshold. This parallels the

observation of increased reproductive activity in female aphids (*Acyrthosiphon pisum*) at a critical threshold of ingested pathogens (Hendry et al., 2016).

Only males exposed to a moderate dose of PQ (40 mM) exhibited higher attractiveness to females compared to control males. In contrast, lower doses (5 and 20 mM) or higher doses (80 mM) failed to elicit a similar terminal investment response. This suggests that when the dose remains below the threshold, as in the case of lower doses, males prioritize future reproduction over immediate reproduction by allocating significant resources towards recovery. Conversely, when exposed to an elevated dose, males were unable to invest in courtship, indicating the presence of a terminal investment ceiling beyond which resource allocation towards immediate reproduction becomes unfeasible. Similar findings were reported by Hendry et al. (2016), where the highest doses of pathogens did not enhance the reproduction of *A. pisum* females. Although the terminal investment hypothesis proposes a trade-off between survival and traits associated with immediate reproductive success, clear evidence of this trade-off, and subsequent testing, remains limited (Table 1). Previous studies on terminal investment in *T. molitor* have primarily focused on indirect measures of survival, such as assessing humoral and cellular immune responses (Sadd et al., 2006; Krams et al., 2011) and metabolic costs (Krams et al., 2014). However, these parameters do not directly reflect survival costs. Considering the current results, both survival and reproduction were contingent upon the extent of damage incurred. On the one hand, males exposed to the low dose of 20 mM did not experience a survival cost, potentially employing their resources to trigger an antioxidant response and prioritize future reproductive output over immediate gains. This response aligns with the trade-off between survival and reproduction proposed by life history theory (Stearns, 1989) and suggests an honest signal of condition (Zahavi, 1975). On the other hand, males exposed

to the highest dose (80 mM) likely faced such severe damage that they were unable to recover, resulting in their premature demise and failure to attract females, thus serving as an honest indicator of compromised condition.

Males exposed to the medium dose of PQ (40 mM) underwent courtship and immediate mating following the challenge, which negatively impacted their survival. However, they were found to be more attractive to females compared to the control group. This aligns with the terminal investment hypothesis, indicating that these males that were prompt to die invested more resources in pheromone production. Understanding the costs and benefits associated with regulating reproductive investment over the short, medium, and long term would be valuable. Notably, the results obtained with the 5 mM dose were intriguing. Although the emitted pheromone levels were lower compared to the control group, these males exhibited longer survival, indicating a toxicological effect known as hormesis. Hormesis occurs when low doses of a toxic substance elicit a beneficial response (Calabrese et al., 2007). This phenomenon has been observed in various taxa, including insects (Stebbing in 1982). It is proposed that low levels of reactive oxygen species can stimulate a favorable antioxidant response, potentially increasing longevity (Radak et al., 2008; Ristow & Zarse, 2010). Furthermore, pro-oxidants and free radicals derived from nitrogen can trigger the immune response, leading to the activation of microbial peptide production (Herrera-Ortiz et al., 2011). Future research should test this hypothesis.

The second hypothesis focuses on the time interval between the induction of oxidative stress and reproductive investment, specifically courtship and mating. Among males exposed to the 40 mM dose, individuals that only engaged in courtship behavior did not experience a reduction in survival. However, when the mating event occurred 3 hours

after the administration of the pro-oxidant PQ, shorter survival was observed. This finding supports the notion that mating carries an energetic cost (Harshman & Zera, 2007). Additionally, reproduction has been reported to increase vulnerability to oxidative stress (Salmon et al., 2001), suggesting a trade-off between these two components (Speakman & Garratt, 2014; Blount et al., 2016). Therefore, we emphasize the significance of including mating events in the experimental design when evaluating terminal investment. Our results suggest that the apparent contradiction in studies that did not detect survival costs may be because they did not incorporate mating events following the assessment of investment in SSCs and they did not use different doses of stressors. Duffield et al. (2018) investigated the role of age as a modulator of the pathogen threshold required to promote terminal investment in the calling behavior of male *Gryllodes sigillatus* crickets. Although no costs were observed in immune response or insect survival following investment in calling behavior, the crickets were not allowed to reproduce or invest in mate attraction. This may have led to the conclusion of a lack of terminal investment. However, considering the complete reproductive investment, including courtship, and mating, could potentially support the presence of a terminal investment strategy.

Our findings suggest that the mating strategy of males is influenced not only by the severity of damage caused by a dose of stressor (in this case, the pro-oxidant PQ) but also by the occurrence of mating itself, beyond just courtship. Interestingly, when males in our study experienced an oxidative challenge followed by a reproductive encounter after 24 hours, no immediate survival cost was observed. This implies that a 24-hour period allows for sufficient recovery from the oxidative challenge. Following the expenditure of energy for both recovery and the reproductive event, these males survived similarly to healthy individuals. To the best of our knowledge, this result represents the first evidence

highlighting the significance of reproductive investment as a mediator of the terminal investment response. Our results support a recent proposal which state that the doses of stressors and reproductive investment may favor or impede the outcome of the terminal investment strategy (Foo et al., 2023) and that the variation in the amount of stressors activate the terminal investment strategy after a umbral threshold (Duffield et al., 2017).

It is important to assess whether there are any costs to the overall reproductive success of males, despite their unaffected mating performance in a suboptimal condition for terminal investment. Evaluating factors such as sperm and offspring quality, as observed in other species exposed to oxidative and pro-oxidative substances (Garrat et al., 2013; Sun et al., 2016; Kamkar et al., 2018), would be crucial. Studies have reported a decrease in insect sperm count following exposure to PQ (Lacoume et al., 2009), and cytotoxic and genotoxic effects on the male germline cells of vertebrates (D'Souza et al., 2006; Lacoume et al., 2009). Future research should investigate the oxidative stress levels in males preferred by females to determine if there are costs associated with their reproductive success and the condition of their offspring. Additionally, a relatively unexplored area of investigation in relation to male terminal investment focuses on the costs incurred by females. It is possible that the most attractive males may exhibit reduced fertility or transfer oxidative molecules in their ejaculate, as has been demonstrated with other seminal components (Mueller et al., 2007; Avila et al., 2011). Understanding these aspects would provide valuable insights into the broader implications and trade-offs associated with male terminal investment.

Finally, we acknowledge that our experimental design does not eliminate the possibility that variation in male quality could influence the level of costs they experience.

Controlling for individual genetic quality is an essential step in understanding the costs associated with reproductive investment, such as the production of SSCs (Achorn & Rosenthal, 2020). Based on our review of the literature, studies addressing this specific question have mainly focused on controlling the condition of organisms rather than their genetic quality. Nevertheless, our findings demonstrate that the reproductive strategies of males in response to different levels of environmental challenges (oxidative damage caused by different PQ doses) and the timing between the stressor and mating (at 3- or 24-hours post-challenge) may shape the behavior and physiology of males, leading to a reproductive strategy of terminal investment or the production of honest signals.

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CAPÍTULO VI.

Cordero-Molina, S. & Contreras-Garduño, J. Short communication: He is a ten, but…Dishonest males decrease antioxidant protection in ejaculate. (submitted).

Abstract

The direct benefits a female can obtain from mating may vary according to the males' condition, which often correlates positively with the exaggeration of male secondary sexual characteristics (SSC). However, some males produce deceptive signals when they are prompt to die, a phenomenon known as terminal investment (TI). We investigated whether there is a trade-off between SSC and ejaculate quality when males engage in TI. We used *Tenebrio molitor* beetles and a pro-oxidant to induce TI in males. We measured the levels of prooxidants, antioxidants, and protein levels in the ejaculate, as well as how time between pro-oxidant ingestion and copulation affects the ejaculate quality. Although the levels of pro-oxidants and protein in the ejaculate, as well as the quantity of offspring, do not differ from healthy males, we observed a significant decrease in the level of peroxidase in the spermatophores of dishonest males. Furthermore, we noticed that antioxidant protection in the ejaculate could be restored and even increased if males delay mating after being challenged.

During female choice, females seek to obtain the maximum benefits from the chosen male (Andersson, 1994). These benefits are categorized into indirect and direct benefits. Indirect benefits are those that the offspring inherit through the genes of the chosen partner, as these genes could increase the viability and fitness of the offspring (Zahavi, 1975; Hamilton and Zuk, 1982). However, observing and measuring indirect benefits is experimentally challenging (Achorn and Rosenthal, 2020). Direct benefits are acquired directly from the male and potentially favoring female's condition and fertility (Eberhard, 1996; Møller and Jennions, 2001). Direct benefits include parental care, food, territory, or ejaculate (Andersson, 1994; Eberhard, 1996). The ejaculate contains immune protection molecules (Lung et al., 2001), nutrients (Fedorka and Mousseau, 2002), and antioxidant molecules (Chargé et al., 2010). In this sense, the quality of the ejaculate is referred as its fertilization potential due to its components (Chargé et al., 2010). A positive correlation between SSC and fertilization capacity has been demonstrated in insects (Polak et al., 2021), suggesting that SCC are honest signals of ejaculate traits (Pomiankowski and Wedell, 2021).

The honesty of the SSC allows females to distinguish between attractive healthy males and unattractive, poorly condition males (Cotton et al., 2004). Since the production of an ejaculate is energetically costly, a male with compromised resources will produce a low-quality ejaculate (Fitzpatrick and Lüpold, 2014). Therefore, mate selection should enable females to avoid low-quality ejaculates. A low-quality ejaculate can bring costs, such as reduced offspring viability (D'Souza et al., 2006), low fertilization (Deepananda and De Silva, 2013), and decreased levels of beneficial substances for the female's condition (Avila et al., 2011).

However, females are in risk of obtain low-quality ejaculates when males in suboptimal

condition produce dishonest signals through their SSC (Copeland and Fedorka, 2012; Duffield, 2015). This phenomenon, identified as terminal investment (TI), promotes reproduction by allowing individuals with low condition to enhance traits, such as SSC (Clutton-Brock, 1984). Thus, we aimed to investigate whether male dishonesty leads to a decrease in ejaculate quality. We hypothesized a trade-off between dishonest SSC due to TI and ejaculate quality in terms of oxidative stress protection. Accordingly, we predicted that males undergoing TI due to oxidative stress will have elevated levels of pro-oxidants and reduced levels of antioxidants and protein in their ejaculate compared to control males. Additionally, this may lead to reduced offspring production.

We addressed this hypothesis in the beetle *Tenebrio molitor*. Males of this species produce more sexual pheromones to attract females when they are in good condition (Rantala et al., 2003). Also, sexual pheromones of *T. molitor* provide honest information regarding their oxidative stress status (Ruiz-Guzman et al., 2021). However, compromised survival results in greater attractiveness than healthy males (Sadd et al., 2006). For example, exposure to high doses of the herbicide paraquat (PQ) causes males to emit deceptive signals and appear more attractive than healthy males (Cordero-Molina et al., 2023). Additionally, TI by oxidative stress is dependent on the time elapsed between administration of PQ and mating. Males do not trigger a TI response and recover from oxidative stress when the time between PQ ingestion and mating is 24 hours (Cordero-Molina et al., 2023). Therefore, we investigated how the time lapse impacts ejaculate content. Our second hypothesis is that males who have recovered from ingesting PQ may not exhibit a trade-off between SSC and ejaculate quality. We predict that a time of 24 hours will favor the males repair the damage received by PQ and the levels of pro-oxidant, antioxidant and protein in ejaculate should be restored.

To test our hypothesis, we used 240 insects, 30 males and 30 females assigned to each

treatment group. The insects were individually housed in 6-well Corning* boxes. After reaching adult stage at 11-13 days of age, the males were treated with either the control or the experimental treatment. The experimental treatment involved orally administering 1 µl of a 40 mM paraquat solution (PQ; SIGMA) to induce TI (Cordero-Molina et al. 2023). The control treatment involved administering 1 µl of sterile distilled water. After treatment administration, pairs were formed 3 or 24 hours later as follows: a) PQ-3h males with females, b) control-3h males with females, c) PQ-24h males with females, and d) control-24h males with females. Each pair was placed in a glass Petri dish with a filter paper base and the occurrence of copulations was recorded for 10 minutes.

After copulations, females were anesthetized on ice and sacrificed within the first hour. The females were dissected to extract the spermatophore. Each spermatophore was individually placed in a vial containing 200 µl of cold PBS. We aimed to use a sample size of 30 for each group. However, some of the spermatophores were broken during extraction, resulting in a reduced sample size for ejaculate analysis. The final sample sizes are presented in Table 1.

Spermatophores were disrupted using an automatic macerator (3 cycles of 10 seconds), then centrifuged at 17000x*g* for 10 min at 4°C. Later, the supernatant were taken and distributed for analysis according to the specifications of each commercial kit used. For hydrogen peroxide (H_2O_2) analysis, the “Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit” kit was used. To measure non-enzymatic antioxidants, we used the “Total Antioxidant Capacity (TAC) Colorimetric Assay Kit. Biovision™” kit, we used the Protein Mask reagent to enable the measurement of antioxidant capacity by only small non-proteic molecules. To determine the amount of protein, the "Pierce™ BCA Protein Assay Kit" kit was used.

A different group of males received the PQ or control treatments and mated 3 hours after as

described before. Once the copulations occurred, the females were transferred individually in a Petri dish with food *ad libitum* for 5 days. Subsequently, the number of eggs laid was recorded.

Given that each experimental condition was paired with a respective control, we analyzed prooxidants, antioxidants, protein, and egg count using U Mann-Whitney comparations between experimental condition against its respective control. We performed all tests with the program SPSS Statistics, 22.0.0.0.ts

The results show that males mated 3 hours after receiving the oxidative challenge (oxidized males) present a state of oxidative stress caused by an imbalance between oxidant and antioxidant molecules in their bodies (Finkel and Holbrook, 2000). Although there are no changes in H_2O_2 levels in the ejaculate ($U = 150$; $p = 0.231$), the level of peroxidase decreased ($U = 61$; $p < 0.002$; Fig 1). This imbalance suggests that males mounted an antioxidant defense to mitigate the increase in oxidants caused by PQ, thus ensuring that H_2O_2 levels resembled those of the control group (Table 1). Transferring a spermatophore with a low level of peroxidase could compromise the integrity of the gametes. Oxidative stress is an etiological factor of male infertility (Aitken et al., 2012) due to the damaging effects of reactive oxygen species at a cytotoxic and genotoxic level in gonads and gametes (D'Souza et al. 2006; Lacoume et al., 2009). In addition, antioxidant protection in the ejaculate is important because spermatozoa produce high amounts of ATP, leading to a high generation of reactive oxygen species that can damage the cell membrane (Aitken et al., 2012; Blount et al., 2001).

Figure 1.

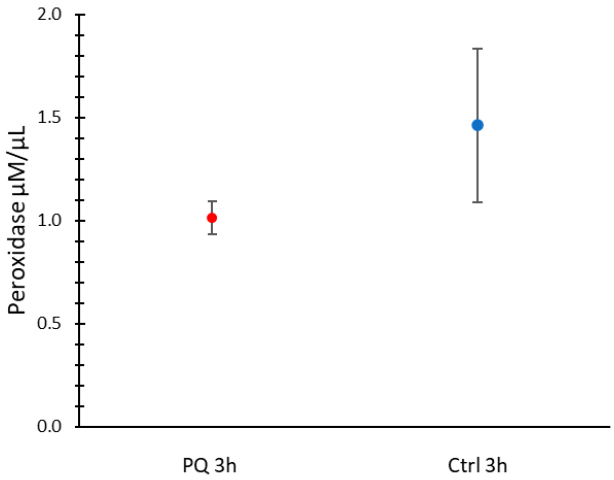

Figure 1: Concentration of peroxidase in spermatophores of dishonest males (PQ) and healthy males (Ctrl). Spermatophores were transferred to females 3 hours after males received treatment. Error bars: CI 95%

Table 1. Summary of results from the analysis of ejaculate quality of oxidized males with paraquat (PQ) compared to their control at 3 or 24 hours after ingesting PQ. n.s.d., no significant differences; TAC, total antioxidant capacity.

Parameter	3 h	24 h
Hydrogen peroxide	n.s.d. U = 150; p = 0.231 PQ N= 26; Ctrl N= 15	n.s.d. U = 139; n = 17; p= 0.66 PQ N= 17; Ctrl N= 18
Peroxidase	PQ < Control U = 61; p < 0.002 PQ N= 26; Ctrl N= 15	n.s.d. U = 107.0; p= 0.205 PQ N= 17; Ctrl N= 17
TAC	n.s.d. U = 181; p = 0.705 PQ N= 26; Ctrl N= 15	PQ > Control U = 80; p = 0.016 PQ N= 17; Ctrl N= 18
Protein	n.s.d. U = 171; p = 0.919 PQ N= 26; Ctrl N= 15	n.s.d. U = 127; p = 0.985 PQ N= 17; Ctrl N= 17

The stress faced by oxidized males did not affect the quantity of other non-protein antioxidants (U = 181; p = 0.705). However, the ejaculate of males mated 24 hours after the oxidative challenge (recovered males) showed no differences in H_2O_2 (U = 139; n = 17; p= 0.66) or peroxidase levels (U = 107.0; p= 0.205) compared to the control, but they had more

non-protein antioxidants than healthy males (U = 80, p = 0.016; Fig 2). Non-protein antioxidants such as carotenoids are components of the ejaculate (Heller et al., 2000) but unlike protein antioxidants, there is less information about their role in protecting gametes. However, there is evidence indicating that these antioxidants enhance fertility (Andrabi et al., 2008; Simmons et al., 2018). The increased antioxidant protection in recovered males suggests that they are the best choice because they provide more direct benefits. However, *T. molitor* is characterized as a gregarious and promiscuous species (Worden and Parker, 2001), making it unlikely for dishonest males that produce high levels of pheromones to go unnoticed by females or delay mating to recover.

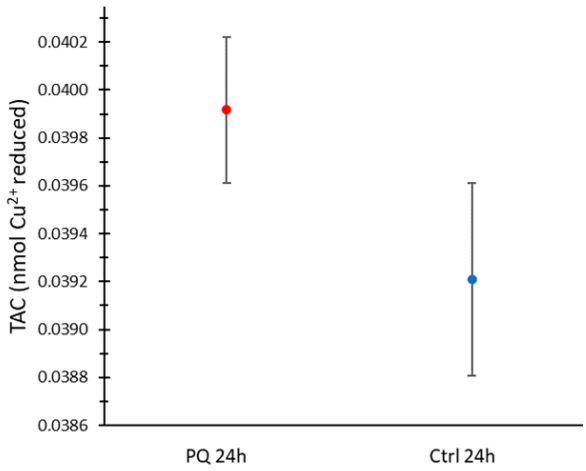

Figure 2: Measurement of total antioxidant capacity (TAC) in spermatophores of oxidized males (PQ) and control males (Ctrl). Spermatophores were transferred to females 24 hours after males received treatment. Error bars: CI 95%

No differences were observed in the protein levels of any of the groups compared to their respective controls (Table 1). Furthermore, the results indicate that neither copulation nor the seminal products of oxidized males influence the number of offspring that a female can

produce (U = 185, n = 40, p = 0.681). We acknowledge that our design does not address whether paternal oxidative stress negatively affects the condition of the offspring. Furthermore, it is important to mention that our study do not explore the effects of cryptic female choice. It has been proposed that some molecules in the ejaculate could serve as honest signals of the male's condition or genetic quality to females, allowing them to bias paternity (Cordero, 1995). Another aspect deserving attention in the future is how females may exert control over fertility by shaping the environment provided to sperm in the female reproductive tract. A hostile environment to sperm with lower antioxidant protection could negatively affect reproductive success. Further exploration of these mechanisms could provide valuable insights into female reproductive strategies and their effects on male reproductive success.

In conclusion, choosing dishonest males can be costly for females because the production of SSC during TI may be traded-off with the antioxidant protection in the ejaculate. Our results also reveal costs for males that massively invest in SSC when their condition is low, as their level of attractiveness exposes them to rapid copulation instead of having the opportunity to recover and avoid the compromise in ejaculate quality.

Acknowledgments

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CAPÍTULO VII.

Discusión general

Discusión general

El estudio de los mecanismos neuronales implicados en la elección de pareja es fundamental para tener una visión integral de los comportamientos observados en la naturaleza. A pesar del creciente interés en estos mecanismos, los enfoques dirigidos a la elección femenina son menos comunes y frecuentemente adoptan una perspectiva descriptiva y fisiológica, en lugar de situarse dentro del marco teórico de la selección sexual. Las hembras de diversas especies exhiben una amplia gama de mecanismos para elegir pareja, y esto está relacionado con las diferencias en sus sistemas de apareamiento, historias de vida y evolutivas. Por ello, es necesario que los modelos utilizados para investigar los mecanismos neuronales de la elección femenina sean igualmente diversos.

Debido a su diversidad, el estudio de la elección femenina en los invertebrados resulta especialmente interesante. En la revisión presentada en el capítulo II, se resalta el empleo de enfoques neurobiológicos en el análisis de la elección femenina, con el fin de profundizar en sus mecanismos desde diferentes niveles de estudio. Un ejemplo es explorar las regiones cerebrales implicadas en la etapa precopulatoria con el propósito de entender cómo se integran las señales masculinas (por ejemplo, rasgos visuales, olfativos sonoros, etc.), las cuales influyen en la receptividad de la hembra o en el rechazo a los machos. Además, se propone estudiar el papel de los neurotransmisores y neurohormonas en el proceso de toma de decisiones al elegir una pareja. Por ejemplo, se plantea investigar si los neuropéptidos parecidos a la oxitocina presentes en los invertebrados también regulan comportamientos como el apego a la pareja o la agresividad, y si estos mecanismos pueden moldear los sistemas de apareamiento, tal como se ha observado en vertebrados.

Adicionalmente a comprender la fisiología detrás de la atracción, el apareamiento y la toma de decisiones durante la elección femenina, la revisión destaca la importancia de investigar los mecanismos también durante la etapa postcopulatoria y en el contexto del conflicto sexual.

Posteriormente, estudié la elección femenina del escarabajo *Tenebrio molitor*. Examiné la respuesta del cerebro de las hembras durante la elección de pareja, así como el comportamiento de los machos, y propuse explicaciones sobre la función de las preferencias de las hembras por machos conocidos. A continuación, resumiré y discutiré mis resultados.

Las hembras de *T. molitor* tratadas con inotocina se aparearon menos que las hembras control. Es posible que la inotocina haya incrementado la sensibilidad olfativa en las hembras, volviéndolas más selectivas cuando eligen pareja. Estudios en mamíferos han mostrado que la administración de oxitocina puede influir positivamente en el procesamiento de los olores, y alterar temporalmente los circuitos neuronales entre el bulbo olfatorio y la zona cortical de procesamiento olfativo (Woolley et al., 2015; Oettl et al., 2016); Resultaría de gran interés investigar si la inotocina afecta los circuitos olfativos utilizados en la comunicación social en insectos.

En cuanto a la localización de la inotocina en el cerebro, observé que las células secretoras de este péptido proyectan hacía los cuerpos fungiformes, que constituyen la principal área de aprendizaje y memoria en insectos (Zars, 2000; Hourcade et al., 2010; Menzel, 2014). Aunque estudios previos han identificado la presencia de inotocina en los cuerpos fungiformes, no abordan la ruta que conecta las neuronas secretoras con estas

zonas (Aikins et al., 2008; Binzer et al., 2014; Koto et al., 2019). Por tanto, dichos trabajos en conjunto con mis resultados sugieren que la inotocina desempeña un papel en el procesamiento de información durante la formación de memoria, lo cual hasta ahora no se había planteado en insectos.

Basándome en la hipótesis de que la inotocina incrementa la selectividad de las hembras, evalué si las hembras preferirían a parejas conocidas en un encuentro futuro. Encontré que la inotocina tiene un efecto similar al de la oxitocina en potenciar la memoria social, ya que las hembras tratadas con inotocina mostraron una tendencia significativa a aparearse nuevamente con el mismo macho con el que se habían apareado cinco días antes, en comparación con las hembras control. Este resultado es consistente con estudios en roedores y mamíferos que han demostrado que la administración de oxitocina durante un primer encuentro social promueve la memoria y facilita el reconocimiento de individuos en encuentros subsecuentes (Ferguson et al., 2000; 2001; Rimmele et al., 2009). Además, investigaciones realizadas en humanos involucrados en relaciones románticas heterosexuales, han revelado que la oxitocina promueve la preferencia por la pareja al tiempo que induce un distanciamiento social frente a nuevas posibles parejas (Scheele et al., 2012; Freeman et al., 2021).

Además de los numerosos estudios sobre el papel de la oxitocina en la memoria social de sistemas monógamos, también se ha encontrado que este péptido puede promover la preferencia y el reconocimiento de la pareja en especies con sistemas de apareamiento flexibles, como el pez medaka (Yokoi et al., 2020). Esto sugiere que la conservación de los genes homólogos al gen de la oxitocina puede afectar la preferencia

de pareja en los sistemas de apareamiento, ya sea que estos sean estrictamente monógamos, polígamos, o plásticos como ocurre en varias especies (Gowaty, 2013; Kvarnemo, 2018).

En el cuarto capítulo encontré que las hembras prefirieron a las parejas conocidas en comparación con las desconocidas, incluso cuando ambos machos compartían características similares en términos de condición, atractividad, tamaño, edad y experiencia sexual. Esta preferencia se mantuvo cuando las hembras elegían únicamente entre los olores de ambos machos, sugiriendo que los rasgos recordados de ellos son señales químicas. Además, la administración del amnésico Zebularina provocó la pérdida de la preferencia por el macho conocido. Este resultado respalda la existencia de un mecanismo de memoria. Por otro lado, los resultados de los análisis de proteómica también respaldan la hipótesis de la memoria al revelar un perfil de proteínas en el cerebro de las hembras con preferencia por la pareja conocida, con funciones relacionadas a la formación de memoria. A continuación, mencionaré algunas de las proteínas que encontré desreguladas entre hembras con y sin preferencia por el macho conocido. Una de las proteínas desreguladas más interesantes fue la sinapsina, que se encontró en mayor cantidad en el cerebro de las hembras que prefieren al macho conocido. La sinapsina tiene un papel fundamental en el establecimiento de nuevas sinapsis, plasticidad neuronal y potenciamiento de la memoria (Cesca et al., 2010; Niewalda et al., 2015; Fahrbach & Van Nest, 2016; Kleber et al., 2016). Mientras que las hembras que eligieron al macho desconocido mostraron un mayor aumento en proteínas relacionadas a la remodelación del citoesqueleto. Posiblemente en el cerebro de las hembras que prefieren a la pareja conocida se establecen sinapsis que facilitan una evocación rápida de la memoria, mientras que, las hembras que prefieren al macho desconocido tienen otro arreglo en las conexiones neuronales que las conduce a

elegir de manera diferente. Otro resultado interesante fue el incremento en la cantidad del receptor ionotrópico de glutamato (iGluR) en las hembras que eligieron al macho conocido. En insectos, los iGluRs intervienen en la detección de señales químicas (Rytz et al., 2013), y también participan en la regulación de la síntesis de la hormona juvenil (HJ; Chiang et al., 2002). La HJ regula diversos comportamientos reproductivos como vitelogénesis (Li et al., 2019) y el procesamiento de feromonas sexuales (Anton & Gadenne, 1999). El incremento de este receptor podría significar que las hembras que prefieren al macho conocido tienen un umbral de respuesta más bajo a las feromonas de los machos, quizás debido a una memoria consolidada. Otras diferencias en el perfil proteico de ambos grupos indicaron que las hembras con memoria exhiben una mayor activación del metabolismo de glucógeno en el cerebro y, a su vez, se observó una disminución en algunas proteínas antioxidantes, como la peroxiredoxina 5 que promueve la longevidad en *Drosophila* (Radyuk et al., 2009). Estos cambios concuerdan con la idea de que el procesamiento de información en el cerebro y el uso de la memoria conllevan una alta demanda energética (Laughlin, 2001; Burns et al., 2011), lo que podría acarrear compromisos y costos ecológicos (Laughlin, 2001; Mery & Kawecki, 2005). La posibilidad de que existan costos oxidativos en el cerebro de las hembras con memoria enfatiza la importancia de investigar más a fondo la función de la memoria y sus costos en esta especie.

En otras especies con preferencia por parejas anteriores, se ha observado que el beneficio de la memoria radica en la formación de un vínculo de pareja para facilitar el cuidado parental (Emlen & Oring,1977; Schrader et al., 2020). Sin embargo, *T. molitor* no exhibe comportamientos de cuidado parental ni establece vínculos de largo plazo entre los sexos. Por lo que, mi hipótesis plantea que la variación constante en la condición de los machos y el continuo despliegue de señales deshonestas (Rantala et al., 2003; Sadd et al.,

2006; Kivleniece et al., 2010; Krams et al., 2011; 2015; Nielsen & Holman, 2012), han ejercido presión en la evolución de la memoria en las hembras. Con relación a esto, encontré que el macho conocido dejó de ser preferido cuando su condición disminuyó en el segundo encuentro con la hembra. Sin embargo, es interesante que el macho nuevo, a pesar de que era considerablemente más atractivo que el conocido, no fue el preferido. En resumen, a pesar de ser poliándricas, las hembras de *T. molitor* recuerdan y prefieren a sus parejas anteriores. Este comportamiento podría permitirles distinguir entre machos que pueden mantener constante su condición y su atractivo a lo largo del tiempo, y aquellos cuya condición disminuye con el tiempo; pero principalmente, les brinda la ventaja de asegurar el apareamiento con machos de buena condición y reducir el riesgo de elegir a machos desconocidos que podrían ser deshonestos. Aunque esta interpretación tiene ciertas limitaciones, dado que no se puede descartar la posibilidad de que las hembras hayan rechazado al macho conocido debido a que emitían menos feromonas, el conjunto de todos los resultados hasta aquí discutidos respalda la hipótesis planteada. No obstante, es necesario realizar experimentos que consideren este punto.

En la comparación de los perfiles de proteínas de las hembras apareadas y no apareadas, observé que el apareamiento provoca cambios en el cerebro en términos de remodelación de citoesqueleto, proliferación celular, transporte celular, síntesis y modificación de proteínas, remodelación de la cromatina y respuesta al estrés. En cuanto a las proteínas del citoesqueleto, cuatro de las siete desreguladas aumentaron debido al apareamiento, indicando que después de la cópula se establecen se establecen nuevas conexiones neuronales y se modifican las existentes. Posiblemente, impulsado por las nuevas conexiones neuronales, también hubo un aumento en proteínas con funciones de transporte y comunicación celular. Entre estas se encontró la proteína Wnt, cuya presencia

está vinculada a la regulación de apoptosis, movilidad celular, movilidad de axones y establecimiento de sinapsis (Murat et al., 2010; Salinas, 2012). Otras proteínas que aumentaron fueron un transportador de colina, y colina es un precursor de la acetilcolina que regula comportamientos autónomos, motores y cognitivos (Picciotto et al., 2012). También encontré que de las once proteínas desreguladas relacionadas al transporte celular, diez de ellas aumentaron en las hembras apareadas. Algunas de estas proteínas fueron una proteína de membrana asociada a acarreadores secretores (Secretory carrier-associated membrane protein) y al tráfico vesicular en las neuronas (t-SNARE coiled-coil protein), lo que sugiere un incremento en el intercambio de neurotransmisores (Hubbard et al., 2000; Ramakrishnan et al., 2012; Cmarko et al., 2022). Aunque es previsible que el apareamiento induzca modificaciones en las conexiones neuronales y aumente el transporte celular debido a las necesidades del organismo para procesos posteriores, como la puesta de huevos, es importante destacar estos cambios, ya que el efecto del apareamiento en la estructura cerebral suele ser subestimado.

El cerebro de las hembras apareadas también presentó un aumento significativo en la concentración de vitelogenina en comparación con las hembras vírgenes. La vitelogenina, se ha encontrado en el cerebro de insectos eusociales (Wheeler et al., 2013; Münch et al., 2015; Kohlmeier et al., 2018), escarabajos con cuidado parental (Roy-Zokan et al., 2015) y también en *T. molitor* (Hernández-Villanueva et al., 2023), y no sólo está involucrada en la vitelogénesis, sino que también participa en la defensa antioxidante (Corona et al., 2007; Sun & Zhang, 2015). Se ha sugerido que la función de la vitelogenina en el cerebro es la amortiguación del daño durante procesos inflamatorios u oxidativos propios del envejecimiento y el metabolismo (Münch et al., 2015). La proteína Quiver, también conocida como proteína SSS también aumentó en las hembras apareadas. En

Drosophila, SSS regula la excitabilidad neuronal y promueve el sueño (Koh et al., 2018). Se ha demostrado que, después del apareamiento, las hembras de *Drosophila* reducen sus periodos de sueño a causa de péptidos en el eyaculado del macho (Garbe et al., 2016). Sin embargo, el aumento de SSS en *T. molitor* sugiere que las hembras podrían experimentar un aumento de sueño en lugar de una reducción. A su vez, también incrementó la proteína Indy, que se expresa mayormente en tejidos asociados con el metabolismo. La reducción de Indy extiende la longevidad en *Drosophila* (Neretti et al., 2009; Wang et al., 2009). Estos cambios en el cerebro de las hembras apareadas revelan aspectos poco explorados de la respuesta al apareamiento y sugieren que al igual que la elección de pareja puede ser una tarea costosa para el cerebro, el apareamiento también podría serlo. En futuros trabajos, se podría investigar si realmente existe estrés y daño oxidante en el cerebro de las hembras después del apareamiento y en aquellas que recuerdan a su pareja porque quizás, esto sea tan costoso que disminuya su supervivencia.

Algunas de las proteínas encontradas son particularmente interesantes debido a la escasa información sobre su función en el cerebro. Por ejemplo, la proteína SPATA20, que es conocida por su papel en la espermatogénesis y como factor de fertilidad (Sujit et al., 2020; Wang et al., 2023). SPATA20 posee un dominio tipo tioredoxina (Shi et al., 2004), y responde al daño al ADN (Sujit et al., 2020), lo que podría implicar una posible conexión con la respuesta al estrés oxidativo en el cerebro, pero este aspecto necesita de mayor investigación. Otras proteínas localizadas atípicamente en el cerebro fueron la quitinasa y proteínas parecidas a la luciferina monooxigenasa. Su presencia genera interrogantes sobre su multifuncionalidad y su papel en la elección de pareja.

Después de analizar el cerebro durante la elección de pareja, examiné cómo los machos responden y se ven afectados por las señales deshonestas que producen y envían a las hembras, al enfrentarse a un estresor oxidante. Con mis resultados, describí una curva que muestra la inversión reproductiva que realizan los machos en respuesta al nivel de la amenaza a la supervivencia que enfrentan. También determiné la dosis necesaria de un herbicida prooxidante para inducirles un estado de inversión terminal. Al igual que la inanición y los retos inmmunitarios (Sadd, et al., 2006; Kivleniece, et al., 2010; Krams et al., 2015), encontré que el estrés oxidante induce inversión terminal: los machos oxidados fueron más atractivos para las hembras que el grupo control.

Después de establecer cómo los machos varían la inversión reproductiva con respecto al estrés en el ambiente, analicé los costos asociados a la estrategia de la inversión terminal en los machos. La mayoría de los estudios de la estrategia de inversión terminal en *T. molitor* sugieren que el uso de señales deshonestas es una solución óptima para los machos de baja condición, ya que les permite maximizar su éxito reproductivo, a pesar de los costos de supervivencia asociados. Sin embargo, los machos que no se aparearon inmediatamente después de sufrir estrés oxidante se recuperaron y vivieron tanto como el grupo control, a diferencia de aquellos que sí se aparearon anticipadamente. Este hallazgo resalta que los machos enfrentan costos importantes al optar por iniciar la inversión terminal cuando podrían llevar a cabo una solución alternativa menos costosa. No obstante, quizás el impulso sexual de los machos en inversión terminal, junto con la notable preferencia femenina por ellos, les impide elegir no reproducirse y recuperarse. La respuesta registrada en estos experimentos sigue la idea planteada de que la inversión terminal es una estrategia dinámica, no solo en relación con el daño recibido, sino también en respuesta a otros factores ambientales como el ambiente social (Duffield et al., 2017),

que en este caso podría tratarse de la disponibilidad de parejas. Dado que el estrés oxidante es una amenaza ubicua para todos los organismos, el hecho de que también sea un factor que induce inversión terminal en *T. molitor,* sugiere que esta estrategia es común entre los machos de esta especie. Esto subraya la frecuencia de la deshonestidad masculina en la elección de pareja, lo que podría haber favorecido el surgimiento de una respuesta adaptativa en la población femenina para mitigar sus efectos.

Para establecer si realmente las hembras se beneficiarían de evitar a machos deshonestos, busqué los costos que podría implicar aparearse a un macho de baja condición. Encontré que los machos deshonestos que enfrentaron estrés oxidante disminuyeron la calidad del contenido de su eyaculado, y transfirieron espermatóforos con menos peroxidasa, un antioxidante enzimático. El estrés oxidante es un factor etiológico de infertilidad en machos (Aitken & De Iuliis, 2009; Aitken et al., 2012), por lo que la disminución de peroxidasa en el eyaculado podría poner en riesgo el material genético de los espermatozoides. Los espermatozoides habitan un ambiente altamente oxidante debido a que su movilidad genera niveles altos de especies reactivas de oxígeno (D'Souza et al., 2006; Koppers et al., 2008; Lacoume et al., 2009). La teoría de selección sexual plantea que las hembras prefieren los CSS exagerados de los machos debido a que estos rasgos son indicadores de los beneficios que pueden otorgar a la hembra (Møller & Jennions, 2001; Cotton et al., 2004). Estos beneficios se clasifican en beneficios indirectos y beneficios directos. Los indirectos se refieren a genes que aumentan la adecuación de la progenie (Zahavi, 1975; Hamilton & Zuk, 1982; Andersson, 1994). Mientras que los directos, son beneficios obtenidos inmediatamente del macho, como el cuidado parental, protección, alimento y moléculas en el eyaculado que pueden aumentar la viabilidad de la progenie y de la hembra (Eberhard, 1996; Blount et al., 2001; Møller & Jennions, 2001). Por lo tanto,

cuando las hembras de *T. molitor* son engañadas por una señalización deshonesta, no obtienen un eyaculado de calidad y en su lugar, enfrentan un riesgo potencial para la progenie. Además de no reducir su supervivencia, los machos recuperados que retrasaron su apareamiento incrementaron la cantidad de antioxidantes no enzimáticos en su eyaculado en comparación con el grupo control. Aunque hay poca evidencia, los antioxidantes no enzimáticos del eyaculado, como los carotenoides, podrían ser incorporados a los huevos (Heller et al., 2000), o ser aprovechados directamente por las hembras, lo cual no ha sido investigado. En futuros estudios sería interesante averiguar si las hembras que se encuentran en un estado de estrés oxidante podrían beneficiarse de los antioxidantes obtenidos de los machos. A pesar del potencial beneficio de aparearse con los machos que se han recuperado, en particular para *T. molitor,* parece poco probable que este escenario ocurra debido a la preferencia de los machos por invertir en la reproducción actual a través de la inversión terminal.

En este trabajo he planteado que la memoria de las hembras constituye una ventaja adaptativa que previene la manipulación masculina y los potenciales costos asociados. Sin embargo, una explicación alternativa basada en la explotación sensorial de las hembras sugeriría que la capacidad de memorizar a las parejas podría ser un rasgo no adaptativo para ellas. En este escenario, los machos podrían explotar la capacidad de las hembras de recordar y preferir a los machos conocidos, con el fin de reaparearse con ellas y aumentar su éxito reproductivo. Aunque esta hipótesis requiere de un diseño experimental que proporcione evidencias concluyentes, los resultados observados en el capítulo III podrían apoyar esta posibilidad. En dicho capítulo, observé que, a pesar de que el macho desconocido era más atractivo, las hembras no lo eligieron preferentemente en comparación con el macho conocido, cuyo nivel de atractividad era menor. Es posible que

en ocasiones, la elección femenina en *Tenebrio* se vea influenciada por una tendencia a preferir lo conocido.

Ambas alternativas podrían esclarecerse mediante el estudio de 1) otras especies poliándricas en las que los machos también se encuentren en ambientes que afecten la producción de CSS modulables como las feromonas (por ejemplo, en especies consideradas plaga); y 2) las poblaciones naturales de *T. molitor* para determinar si el rasgo de memoria ha estado presente bajo otras condiciones ambientales. Sin embargo, la información sobre las poblaciones naturales de *T. molitor* es limitada, y no se dispone de información que indiquen si la aparición de la estrategia de deshonestidad fue una novedad en el cambio de modo de vida a plaga, en la cual la especie frecuentemente tiene contacto con xenobióticos, plaguicidas y medidas de control biológico que desencadenarían la inversión terminal. Otra posibilidad es que el comportamiento de las hembras y el sustrato neuronal que les permite a recordar las parejas conocidas, sean hasta ahora solamente el resultado de la variación genética, a la espera de ser seleccionados en un ambiente donde la población masculina experimente una presión suficientemente perjudicial como para colocar a la mayoría en una condición costosa para la hembra.

Finalmente, los resultados que presento sobre el comportamiento de las hembras y el funcionamiento de su cerebro ofrecen una explicación alternativa al punto de vista del conflicto sexual antagonista que implican los CSS deshonestos de *T. molitor.* Este punto de vista asume que las hembras acceden a la manipulación masculina que favorecerá, preferencialmente, la adecuación del macho, y anulará la elección de las hembras (Holland & Rice, 1998; Chapman et al., 2003; Arnqvist, 2006). Mi trabajo destaca la importancia de

observar el cerebro de las hembras durante la elección de pareja debido a que en otras especies se podrían identificar señales de que la hembra no solo es manipulada, sino que, también poseen estrategias para eludir a machos manipulativos, y que incluso su cerebro puede imponer filtros que favorezcan la elección femenina por las mejores parejas.

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