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No hay metas definitivas para el conocimiento; el progreso del conocimiento no es más que una diferenciación de los planteamientos

- Hermann Hesse-

A mi hermano chapis (1980-2008)…

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Resumen

La comprensión en la función y evolución de los caracteres sexuales secundarios (CSS) ha sido uno de los temas más controvertidos en la biología evolutiva. A lo largo de este proyecto planteé un enfoque integral como la vía para conocer y entender las implicaciones funcionales y ecológicas de un CSS, usando para ello a un grupo de libélulas del genero *Hetaerina* (Odonata:Calopterygidae) como modelo de estudio. Este enfoque se fundamenta en el supuesto de que cualquier característica morfológica compartirá un gran numero de interacciones con otros componentes dentro y fuera del individuo. Dadas estas relaciones, si estos caracteres se consideran de forma aislada podría arrojarnos resultados erróneos de su valor adaptativo real. Utilizando a la teoría de la inmunocompetencia como eje inicial para la explicar la presencia de los CSS, encontré que su expresión no tiene porqué reflejar o guardar relaciones positivas con la condición inmunológica del portador, y que es imprescindible tomar en cuenta factores externos como la estacionalidad, disponibilidad de alimento, interacciones intraespecíficas, etc. , e internos como las actividades metabólicas que promueve la formación del caracter ú otras funciones neuro-endocrinas que indirectamente influyen sobre el mismo. Por otro lado, dado lo conspicuo del caracter en cuestión, encontré que la expresión de una de sus características, el color rojo, tiene una importante papel sobre la conducta agonística en contiendas animales por la adquisición de un recurso, el cual influye fuertemente sobre el éxito reproductivo de la especie. En este caso, si el color no esta presente, los individuos disminuyen su éxito reproductivo y supervivencia producto de un mayor desgaste fisiológico originado por un aumento de exhaustivas y costosas contiendas entre co-específicos. En conclusión, tomando en cuenta este enfoque integral ahora sabemos que las características de este CSS pudieron haberse originado simplemente por una seríe de procesos metabólicos favorecidos parcialmente por presiones de seleccion natural y que ahora dichas características participan además en selección sexual, teniendo una fuerte influencia sobre la supervivencia y éxito reproductivo de la especie.

Abstract

The function and evolution of secondary sexual characteristics (SSC) has been one of the most controversial topics in evolutionary biology. During my PhD procject, I raised a holistic approach as the way to understand the functional and ecological implications of a SSC using dragonflies from Heaerina gender (Odonata: Calopterygidae) as study model. This approach is based on the assumption that any morphological trait shares a large number of interactions with other internal components and even outside the individual. Given these relationships, if these traits are considered in isolation, then could throw us erroneous results of the actual adaptive value. Using the "immunocompetence handicap theory" as initial axis to understand the presence of a SSC, I found that a SSC does not have to reflect or keep positive relations with immune status of the bearer. Besides, it is essential to consider other factors such as seasonality, or availability of food. On the other hand, I also found that the presence of red color in this SSC had a strong influence in animal contests for a resource that influences reproductive success the species. In this case, if red color is absent, individuals decrease their survival due to an increment in exhaustive and expensive agonistic encounters between conspecifics. In conclusion, using this holistic approach, we now know that this CSS may have originated simply by a series of metabolic processes partially favored by natural selection pressures, but now it is also participating in sexual selection, having a strong influence on survival and reproductive success of the species.

Introducción General.

La presencia de caracteres morfológicos, usualmente en los machos, conocidos en un principio como "rasgos exagerados" (e.g. colores intensos, largas plumas o aletas brillantes, cantos elaborados, cuernos, astas, danzas etc.) fue el punto central en una de las criticas más fuertes que recibió la teoría de la Selección Natural (Darwin, 1859). Esta crítica se basaba en los efectos deletéreos que estos rasgos producían sobre la supervivencia de sus portadores, dado que los convertía en presas más conspicuas y fáciles de depredar. Se argumentaba que por dichos efectos negativos estos individuos dejarían menos descendencia que aquellos que no portaban tales "rasgos" y con el paso del tiempo serían eliminados de la población, situación que evidentemente no sucedía en la naturaleza.

Tratar de explicar esta evidente incongruencia fue un gran reto y no fue hasta 1871 cuando en *"The Descent of Man, and Selection in Relation to Sex"* se propone una conciliación. Darwin consideró que la selección natural no era suficiente como única presión evolutiva sino que además otro tipo de selección podría estar involucrada. Esta selección denominada sexual, arguyó Darwin, era la responsable del mantenimiento de estos rasgos debido a que a pesar de ser desventajosos con respecto a la supervivencia, eran necesarios para lidiar exitosamente en lo que él llamó "Competencia Sexual". A partir de ese momento estos "rasgos exagerados" fueron agrupados dentro de los llamados carateres sexuales secundarios (CSS) nombrados por primera vez por Jonh Hunter (1780), para así poder diferenciarlos de los primarios (CSP), conformados por los genitales y gónadas (Darwin, 1871). El mecanismo esencial de este nuevo tipo de selección consistía en la competencia por obtener el mayor número de copulas y/o parejas y aumentar de este modo el número de descendientes. Por consiguiente, el poseedor del CSS haría despliegue del mismo para competir de forma directa a través de encuentros agonísticos

(usualmente entre machos) o indirecta para ser elegido como pareja (usualmente por las hembras).

Las réplicas a este nuevo tipo de selección no se hicieron esperar ya que los argumentos planteados en esta teoría se han vuelto igual o aún más controvertidos que la propia teoría de selección natural (Geddes y Thomson, 1908; Andersson, 1994). Sus primeras críticas fueron expuestas principalmente por dos de sus contemporáneos, Alfred Russel Wallace (1823-1913) y William Keith Brooks (1848-1908). Aunque Wallace propone de manera alterna una teoría de selección natural muy similar a la planteada por Darwin, en su libro *"Darwinism"* (1889) hace explícita su inconformidad sobre la existencia de esta nueva fuerza selectiva, argumentando que la selección natural es la única responsable de la presencia de estos rasgos, siendo innecesaria una "Competencia sexual". Su idea se basó principalmente en dos argumentos 1) Los "rasgos exagerados" son necesarios para el reconocimiento entre especies y 2) La razón del por qué las hembras no presentaban estos caracteres se debía al mayor riesgo de depredación que traía consigo el momento de la incubación y/o puesta de huevos. Este último argumento se basó en lo observado en aves e insectos en donde los machos por lo general no cuidaban de los huevos y no incurrían en dicho riesgo. Brooks por su parte no obstante que admitío la idea de selección sexual, plantea en su libro *"The Law of Heredity"* con una visión propia para su época, que los machos son los únicos que tienen la capacidad de trasmitir y expresar naturalmente las variaciones en los CSS gracias a la función única de sus gametos masculinos, dejando el papel de la hembra como mero ente pasivo de elección e incapaces de expresar estos caracteres. Estos ejemplos y otros tantos contemporáneos (Vease también Mivart, 1876 en Geddes y Thomson, 1908) fueron sólo el inicio de una historia compleja que ha estado detrás del "porque?" de la

presencia de los CSS, y que se ha convertido en uno de los tópicos más estudiados en biología evolutiva (Andersson y Simmons, 2006).

Similar a otros caracteres en los individuos, los CSS podrían estar influyendo o siendo influenciados por factores no solamente internos (i.e Estado nutrimental, sistema neuro-endocrino, etc.) sino también externos, tales como relaciones interespecificas o las presiones ambientales. Por lo tanto, siguiendo esta lógica sería plausible alcanzar una mayor comprensión de la presencia y función de estos caracteres si consideramos la mayor cantidad de conexiones posibles en las que este atributo está implicado. Para lograr tal acercamiento y haciendo alusión a uno de los creadores de la etología, Nikolaas Tinbergen (1963), es necesario tener una visión desde dos ejes explicativos, el proximal y el terminal, los cuales nos contestarían distintas preguntas aunque complementarias entre ellas. El eje proximal acometería al ¿Qué y cómo un sistema trabaja y responde ante factores extrínsecos? y al ¿Qué mecanismos del desarrollo explican que este sistema funcione de esta forma (y no de otra) en su estado de vida actual? Mientras que el eje terminal aludiría al ¿Cuál ha sido el valor adaptativo de estas características?, ¿Que fuerzas selectivas explican su expresión? y ¿Qué precursores filogenéticos dan cuenta del rasgo en esta época evolutiva?

Abordar una pregunta en un solo eje sin considerar la información que arroja su eje complementario puede dar respuestas erróneas o con muy poco contenido explicativo. En otras palabras, si la pregunta *¿Porqué está presente cierta característica?* es complementada con el conocimiento que se tiene de los mecanismos que la generan, cómo funciona, de dónde proviene y los vínculos que existen con otros organismos, entonces este "Porqué?" adquirirá un mayor sentido explicativo y se podrá por lo tanto, visualizar los patrones generales que rigen no solo al

organismo en cuestión, sino a todos aquellos que cumplen con tales requisitos, o al contrario, se podrá ver el patrón especial que separa a este organismo del resto.

Usando a un grupo de libélulas del genero *Hetaerina* (Odonata:Calopterygidae) como modelo de estudio, he dedicado mi proyecto doctoral a hacer un ejercicio de esta visión explicativa, intentando vincular la información obtenida a partir de estos dos ejes para abordar los posibles mecanismos funcionales y evolutivos que han dado lugar a la expresión y mantenimiento de un CSS que se encuentra presente en los machos.

Comenzaré planteando el problema desde un eje explicativo terminal, retomando algunas teorías que proponen las posibles funciones de estos CSS sobre los individuos y que dieron lugar a su expresión y evolución. Una de estas teorías, basándose en la propuesta Darwinista de la selección sexual o competencia por el apareamiento, plantea que durante dicha competencia (directa o indirecta) la expresión de los CSS aportaría información "honesta" sobre la condición física de un individuo a sus conspecíficos (Zahavi, 1975; Andersson, 1994; Savalli, 1994). Este planteamiento explica que la relación existente entre la expresión de un CSS y el reflejo de su condición se fundamenta sobre los costos tanto de producción como de supervivencia que implica la presencia de tales atributos. Por lo tanto, los machos en mejor estado físico tendrían los recursos para pagar dichos costos al momento de maximizar la expresión de sus CSS. En una competencia directa, esta función ¨honesta¨ sería muy útil en el contexto de un encuentro agresivo, donde los contendientes podrían obtener información acerca de la capacidad competitiva y/o condición de los contrincantes. Mientras que en una competencia indirecta podría ser útil en un caso donde las hembras evaluaran a los machos antes de aceptar copular. En este caso, los machos indicarían su "calidad" genética como padres a través del despliegue de estos CSS.

La idea de la honestidad de los CSS, a pesar de ser controversial, ha desencadenado un gran número de nuevas vertientes para explicar el papel de estos caracteres. Por ejemplo, hace tres décadas Hamilton y Zuk (1982) enriquecieron esta idea proponiendo que el grado de expresión de un rasgo (p.eg. tamaño, intensidad de color, etc.) dependería fuertemente de la capacidad del individuo para responder exitosamente ante un ataque por patógenos. Según esta idea, la expresión de los CSS es muy sensible a los efectos negativos de los patógenos sobre los hospederos, de tal manera que si el portador no es lo suficientemente bueno defendiéndose, la expresión reducida de sus caracteres lo reflejaría. Más recientemente Folstad y Karter (1992), basándose ahora en un eje explicativo funcional o proximal complementaron esta idea proponiendo que la expresión y desarrollo de los CSS representaba además altos costos fisiológicos para el individuo. Estos costos se basaban en los distintos mecanismos endócrinos que promovían la expresión de los CSS (p.eg. Testosterona) pero que a su vez generaban efectos antagónicos sobre el sistema inmune. Dicha idea conocida como la hipótesis de la inmuncompetencia (HIC) propone un conflicto (o "trade-off" por su nombre en ingles) en los individuos al momento de maximizar la función sexual (los rasgos) a costa de la inmunitaria, siendo esto, la base da la honestidad de los CSS, dado que solo los machos en mejor condición podrían pagar los costos de producir un CSS de forma enaltecida sin disminuir su capacidad inmune, la cual sería indispensable para su supervivencia (Sheldon y Verhulst, 1996).

Sin embargo, una critica a esta hipótesis de asumir que la intensidad en la expresión de los CSS está fuertemente relacionada con la capacidad inmune, es que resulta complicado predecir el tipo de relación existente entre ambas funciones (ya que pueden ser positivas, negativas o inexistentes; Westneat y Birkhead, 1998). Una relación negativa podría encontrarse por ejemplo, si un individuo en alguna etapa de su ciclo vital invierte la mayoría de sus recursos en la función

sexual y poco a la inmune, sin que esto afecte su adecuación (Getty, 2002). Dicho de otro modo y retomando la idea de la inminente conectividad que existe sobre cualquier característica en el individuo, la resistencia a enfermedades no es una entidad monolítica que puede ser medida como la longitud o el peso. Esta depende de muchos factores que entre ellos incluye la capacidad de reconocer y responder ante agentes extraños (Gillespie et al., 1997; Roitt et al., 2001). La compleja naturaleza de este sistema puede limitar la evolución de los CSS como entes informativos de la condición inmune del portador (Getty, 2002; Lawniczak et al., 2007). Inclusive distintas críticas a esta idea han sido planteadas entre otros por Adamo y Spiteri (2009), quienes concluyen que un CSS difícilmente podría comunicar la habilidad inmune. Esta falta de claridad en la hipótesis de HIC se ha originado en parte por la falta de integración de más de un factor en la compleja relación Reproduccion-Inmunidad.

Un buen modelo para solventar esta falta de claridad y promover un mejor acermaciento a la posible función de los CSS como indicadores de la capacidad inmune son los insectos, especialmente un grupo de libélulas de la familia Calopterygidae. Esta familia ha sido estudiada en profundidad en lo que a selección sexual e inmunidad respecta. Los machos, tienen el rasgo particular de poseer patrones específicos de pigmentación en sus alas, mismos que varían en su expresión dentro de una misma especie. Esta pigmentación está sexualmente seleccionada, ya sea en un contexto de elección femenina, como sucede en el genero *Calopteryx* (Siva-Jothy, 1999) o por competencia intrasexual entre machos como en el género *Hetaerina* (Grether, 1996). En el caso de este último, los patrones de pigmentación alar se presentan en forma de pequeñas zonas pigmentadas de color rojo (excepto *Hetaerina titia*, que tiene rojo y negro) en la base de sus cuatro alas (Grether, 1996a). En *Hetaerina* la pigmentación alar, la musculatura corporal y las reservas energéticas se desarrollan en los primeros 20 días de la fase adulta, tiempo en el cual el

animal se dedica a buscar y consumir alimento (Grether, 1996a). Una vez alcanzada la madurez sexual, la pigmentación alar es totalmente fijada y comienza la búsqueda del recurso reproductivo (Grether 1996b). Los machos en este genero, así como en la mayoría de los calopterígidos, compiten por la posesión y defensa de un territorio en las riveras de ríos. La posesión de estos territorios les permitirá aumentar la tasa de apareamiento y por ende la probabilidad de éxito reproductivo con respecto a los que no lograron defender un territorio (revisado por Suhonen et al., 2008). Estudios tanto experimentales como observacionales han encontrado una relación positiva entre el tamaño de la pigmentación alar y la posesión de dichos territorios (Grether, 1996a Serrano-Meneses et al., 2007; Raihani et al., 2008). Esto ocurre gracias a que este carácter puede tener la virtud de reflejar ciertos aspectos fisiológicos del individuo, es decir, es un carácter dependiente de la condición. Apoyando esta idea, en *Hetaerina americana* el tamaño de la pigmentación se relaciona positivamente con las reservas energéticas (Contreras-Garduño et al., 2008), la masa muscular utilizada para el vuelo (Contreras-Garduño et al., 2008; Serrano-Menesen et al., 2007), y en general con la capacidad inmunológica del individuo (e.g., Contreras-Garduño et al., 2006, 2007; Serrano-Meneses et al., 2007). Estos tres componentes son de suma importancia para la obtención de territorios. Por ejemplo, de manera directa quien tenga valores más altos de reservas energéticas y masa muscular tendrá más probabilidades de ganar un combate y, por lo tanto, un territorio (Marden y Waage, 1990; Plaistow y Siva-Jothy, 1996; Koskimäki et al., 2004; Contreras-Garduño et al., 2006; Serrano-Meneses et al., 2007). La condición inmunológica por su parte, también influye en las contiendas por territorios. Por ejemplo, individuos que han sido infectados artificialmente son menos capaces de conservar u obtener un territorio (González-Tokman et al., 2011).

De manera muy general la habilidad inmune dependerá de la cantidad, variedad y velocidad en la que los individuos puedan producir los componentes necesarios no solo para responder ante un patógeno, sino también para evitar su presencia (revisado en Schmid-Hempel, 2011). Con base en este concepto se ha visto que en *H. americana,* la actividad de una enzima llamada fenoloxidasa (FO), un importante componente del sistema inmune encargado de activar y liberar componentes citotóxicos contra patógenos (ver González-Santoyo y Córdoba-Aguilar, 2012), es mayor en los animales con pigmentación alar más grande cuando son sometidos a un reto inmunológico o inclusive en condiciones naturales (Contreras-Garduño et al., 2007). Esta relación positiva fue además encontrada en términos de supervivencia posterior a un reto inmune (Contreras-Garduño et al., 2007).

A pesar de la evidencia previa que corrobora la HIC al encontrar relaciones positivas entre la pigmentación alar y la "habilidad inmune", recientemente se han encontrado resultados contradictorios en una especie hermana, *Hetaerina titia*. Los machos de *H. titia* presentan un patrón muy peculiar de pigmentación alar, pigmento rojo en la base de sus alas y negro en el resto de las mismas (Córdoba-Aguilar et al., 2007). Los estudios realizados en esta especie sugirieron que el grado de melanización (i.e. otro tipo de defensa inmune en donde la FO también es necesaria, y que se activa en contra de macro-parásitos como metazoos o parasitoides como huevos de avispa e inclusive larvas de otros insecto; Beckage, 2008; González-Santoyo y Córdoba-Aguilar, 2012) estaba positivamente relacionado con el tamaño del pigmento negro pero no con el rojo (Córdoba-Aguilar et al., 2007). En un estudio posterior, tampoco se encontró relación alguna entre el grado de pigmentación (roja, negra o ambos) y la supervivencia en machos sometidos artificialmente a una infección bacteriana (González-Tokman y Córdoba-Aguilar 2010).

Otro resultado que no apoya la HIC según lo visto en *H. americana* fue encontrado en esta misma especie cuando se añadió otro factor: la estacionalidad. En este estudio encontraron que las relaciones mancha-habilidad inmune no se presentan en todas las épocas del año, sino únicamente en aquellas donde la disponibilidad de alimento era alta (de Agosto-Septiembre) (Córdoba-Aguilar et al., 2009a). Todos estos resultados hacen difícil extrapolar lo encontrado con *H. americana* y aún más difícil poder asumir que la expresión de la pigmentación alar va en la dirección prevista por la HIC. Sin embargo, los machos de las 37 especies del género *Hetaerina* poseen patrones de pigmentación similares y podría ser que *H. titia* sea la excepción en las relaciones de pigmentación y respuesta inmune. Para poner a prueba esta idea haría falta incluir más especies y conocer qué tanto es extrapolable lo encontrado en *H. americana (o H. titia)* . De igual forma habría que considerar también las variaciones estacionales como un factor que puede estar influyendo en la relación CSS-Inmunidad.

Por esto, en el primer capítulo de este proyecto me enfoqué a investigar si las correlaciones entre la "habilidad inmune" y la expresión de la pigmentación alar es un patrón general en este género y si estas son afectadas por factores externos estacionales. La hipótesis que se abordó en este primer capítulo fue que el tamaño de la pigmentación alar es un indicador de la capacidad inmunológica en los machos del genero *Hetaerina.* Para someter a prueba esto, la primera fase experimental en este capítulo consistió en la colecta de 5 de las 9 especies presentes en México (*H. americana, H. cruentata, H. occisa, H. titia y H. vulnerata)* para evaluarle la habilidad inmune, determinada a partir de la actividad enzimática de FO, y realizar correlatos entre la expresión de esta enzima y el tamaño de la pigmentación alar. En una segunda instancia, para evaluar el efecto de la estacionalidad como posible factor que afecta estas relaciones (Córdoba-Aguilar et al., 2009a), se comparó en las 5 especies la misma respuesta inmune en momentos

contrastantes del año (Julio-Octubre y Diciembre-Enero). Como fue mencionado anteriormente, la idea que predice relaciones positivas entre CSS e Inmunidad se ha visto contradecida por un gran numero de evidencia empírica que no ha encotrado dicho patrón. Dado que la mayoria de esta evidencia no ha puesto a prueba la HIC bajo un enfoque experimental, se ha argumentado que debido a los altos costos que se generan al montar una respuesta inmune, dicha relación puede estar presente solamente si el individuo es sometido a un reto que promueva la activación de su sistema inmunitario, en este caso los individuos se verían en la necesidad de evidenciar su capacidad de respuesta y no en condiciones donde el individuo no lo requiera (Zuk y Stoehr, 2002). Por esto, en una tercera etapa describo un experimento que me permitió retar inmunológicamente a 2 de las 5 especies estudiadas (*H. americana y H. vulnerata*) y evaluar si se presenta la relación mancha-habilidad inmune que apoya la HIC. Los datos que conforman este capítulo fueron ya publicados en la revista Behaviour, y la cita completa es: González-Santoyo I, González-Tokman DM, Córdoba-Aguilar A & Lanz-Mendoza H (2010) Phenoloxidase activity and melanization ability do not always correlate with wing pigmentation expression in *Hetaerina* damselflies (Zygoptera:Calopterygidae). Behaviour 147: 1285-1307.

Un problema importante que ocurre en general en los estudios inmuno-ecológicos tanto en vertebrados como en invertebrados es que el sistema inmune tienen una variedad impresionante de componentes que son utilizados en la defensa del organismo. Unos componentes son muy generalizados y colaboran en contra de un amplio rango de agentes patogénicos, usualmente clasificados dentro del sistema innato, mientras que otros son muy especializados ya que sólo se activan ante la presencia de un tipo muy especifico de patógenos (Abbas et al., 2002). Esta gran diversificación ha generado múltiples confusiones en este tipo de estudios, principalmente cuando se pretende definir los componentes inmunes más adecuados que brinden información de

la “habilidad inmune”. En la mayoría de los casos, se ha optado por el componente que metodológicamente sea el más sencillo, rápido o económico de evaluar. Esto ha generado resultados muy controvertidos y difícilmente repetibles en otros grupos bajo condiciones similares (Moreno-Garcia et al., *2012*). En insectos por ejemplo, incluyendo el primer capítulo de este proyecto, comúnmente se evalúa la “habilidad inmune” a través de la actividad enzimática de la FO. Sin embargo, aunque la FO es importante para la inmunidad de cualquier invertebrado, su activación se da bajo ciertas condiciones tanto del mismo individuo como del patógeno (Kanost y Gorman, 2008). El uso de esta enzima además de su importancia, se debe a su fácil determinación y los costos reducidos que esto implica, y ha generado una multidud de contradicciones en la inmunoecología de insectos. Por ejemplo, muchos estudios no han encontrado realmente que la FO refleje la “habilidad” de un individuo para defenderse de los patógenos. Por citar uno de ellos está el caso de *Gryllus texensis,* donde la actividad de FO no predijo la supervivencia de los individuos al ser infectados con tres distintos tipos de bacterias, *Serratia marcescens, Serratia liquefacien* y *Bacillus cereus* (Adamo, 2004). Esto sin embargo, no significa que la FO no esté participando en la defensa, sino que existe una gran inconsistencia metodológica en su uso. Un ejemplo de estas inconsistencias son las variaciones en el tiempo de incubación y medición de la enzíma, los distitos sustratos utilizados o el tipo de patógeno inoculado (Gonzalez-Santoyo y Cordoba-Aguilar 2012). Tales diferencias generan resultados igualmente inconsistentes con la posibilidad de cometer graves errores al momento de interpretarlos. Así, en el segundo capítulo me he enfocado a hacer una revisión extensa de esta importante enzima en el sistema inmune de los invertebrados. Esto era necesario ya que a pesar de que la última revisión fue realizada en 2008 (Kanost y Gorman, 2008), esta sólo abarca los nuevos descubrimientos relacionados a las vías de señalización de la enzima. En esta revisión en

cambio, además de presentar los estudios más recientes en términos de estos mecanismos funcionales, también aborda los posibles costos evolutivos tanto en términos fisiológicos como en las relaciones pleiotrópicas con otros caracteres, la evidencia empírica que pone a prueba el papel de la FO como una medida confiable de la "habilidad inmune", y planteo algunas líneas fundamentales para la investigación futura de esta enzima destinadas principalmente a explicar las nuevas moléculas encontradas en este sistema, la coordinación con otros componentes inmunes y las variaciones presentes con respecto al sexo, ciclos de vida, estacionalidad, tipo de patógeno y tipo de hospedero. Esta revisión fue ya publicada y la cita completa es: González-Santoyo, I., y A. Córdoba-Aguilar. 2012. Phenoloxidase: a key component of the insect immune system. Entomologia Experimentalis et Applicata. 142:1-16.

Retomando las ideas del conflicto entre la expresión de un CSS y la inmunidad, y dado los correlatos entre pigmento alar y actividad inmune encontrados en *H. americana*, me cuestioné sobre la base bioquímica de la pigmentación alar en *Hetaerina*. Esto, según Sheldon y Verhulst (1996) sería la base de los *trade-offs* en la maximización de las funciones sexuales e inmunológicas. Un ejemplo que parece apoyar este principio viene de otro calopterígido, *Mnais costalis*, donde se encontró que la melanina, producto final de la activación enzimática de la FO (Cerenius y Söderhäll, 2004) es el componente que forma la mancha en las alas masculinas (Hooper et al., 2002). Este mismo componente es utilizado por los insectos para responder ante agentes extraños (por ejemplo, vía encapsulación; Cerenius y Söderhäll, 2004; Gonzalez-Santoyo y Cordoba-Aguilar, 2012) y en *M. costalis*, la produccíon de melanina utilizada para encapsular un agente extraño se correlaciona positivamente con la intensidad de expresión de la mancha (Hooper et al., 2002). Con este ejemplo en particular se ha mencionado a la FO y por ende a la melanina como los recursos centrales en conflicto y base del *trade-off* entre la función sexual e

inmune en insectos (Hooper et al., 2002). Sin embargo en el caso de *Hetaerina* los componentes que forman la pigmentación alar no han sido aún identificados, y por tener un color rojo, es poco probable que la melanina sea el componente que forma tal pigmento. La pigmentación alar en los insectos usualmente se debe a 4 diferentes componentes: melaninas, omocromas, pterinas y flavonoides (Nijhout, 1991). Sin embargo, el posible candidato constituyente del pigmento rojo de *Hetaerina* es la omocroma xantomatina, debido a que este se asocia comúnmente con la coloración roja en varias especies de lepidópteros (e.g. Precis; Nijhout, 1997) y odonatos (e.g. Futahashi et al., 2012). Estos pigmentos son los últimos componentes que se sintetizan en la ruta metabólica del triptófano (Linzen, 1974) y aunque no parecen tener relación directa con el sistema inmune de los invertebrados, otras funciones en el organismo se le han atribuido. Así, para el tercer capítulo determiné los componentes bioquímicos que dan lugar a la coloración roja en las alas de *Hetaerina*.

Mi último capítulo versa sobre la función del pigmento alar rojo, en términos de comunicación. En animales, este color se asocia a aspectos de dominancia y/o capacidad de combate (artrópodos, e.g., Kaiser et al. 1990; peces, e.g., Dijkstra et al. 2010; reptiles, e.g., Olsson et al. 2007; aves, e.g., Pryke 2009; mamíferos, e.g., Setchell and Wickings 2005). Señalizar estos aspectos puede tener una función adaptativa muy importante ya que permiten a los actores resolver conflictos para prevenir contiendas física y energéticamente muy costosas (Johnstone y Norris 1993). Así, se ha propuesto que las señales rojas han evolucionado y mantenido por mecanismos honestos de señalización (Maynard-Smith et al. 1988; Johnstone y Norris 1993; Számadó 2000), es decir que aportan información de la condición del portador como es el nivel de agresión o capacidad fisiológica para mantener el estatus. La honestidad de estas señales se mantiene evolutivamente no solo por los costos que directamente se asocian a la agresión como

son los socialmente impuestos (Tibbetts e Izzo, 2010), sino también los costos indirectos asociados a esta conducta como es, por ejemplo, el gasto energético de producir la señal (Johnstone and Norris 1993). Amplia evidencia teórica ha explicado que la presencia de estas señales es necesaria en la comunicación referente a encuentros agonísticos (Rowher 1975, Jonsthone y Norris, 1993; Jonsthone 1997; Számado 2000; Szalai y Számado 2009). Incluso se ha propuesto que no se podría dar una población evolutivamente estable sin la presencia de estas insignias, dado los costos que traería consigo los encuentros agonísticos bajo cualquier circunstancia (Szalai y Számado 2009). Sin embargo, hay pocos estudios experimentales que han investigado los costos en adecuación y energéticos si no existe un sistema de señalización en contiendas animales (Pryke et al. 2002). Según la teoría, los efectos terminales o el costo evolutivo de no poseer una señal que refleje el estatus es una supervivencia reducida, ya que se elevaría el porcentaje o la intensidad de los encuentros agonísticos. Por otra parte, los mecanismos causales o proximales que desencadenaría esta menor supervivencia se podría dar a nivel energético. Así, en el cuarto y último capítulo de este proyecto me he dedicado a poner experimentalmente a prueba lo que estos modelos teóricos han propuesto. La hipótesis de este capítulo propone al color rojo en las alas de *H. americana* como una señal que comunica la habilidad de pelea o la posesión de un territorio, necesario para evitar encuentros agonísticos bajo cualquier circunstancia. Este capítulo está actualmente en revisión en la revista PlosOne.

Presento una discusión general al final de mi tesis que pretende integrar la parte proximal, los mecanismos de la relación inmunidad y CSS, con sus efectos evolutivos últimos, es decir la adecuación. Mi idea es usar mis hallazgos para ahondar en la teoría de la selección sexual, teniendo siempre presente la importancia de la fisiología y bioquímica de los CSS. No pierdo de

vista que mi tesis abre nuevas inquietudes, así es que igualmente uso esa última sección para proponer nuevas ideas por poner a prueba.

CAPITULO I

Phenoloxidase activity and melanization do not always covary with sexual trait expression in Hetaerina damselflies (Insecta: Calopterygidae)

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Phenoloxidase activity and melanization do not always covary with sexual trait expression in *Hetaerina* damselflies (Insecta: Calopterygidae)

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Summary

Sexual selection theory indicates that males use sexual traits to signal immune ability, a hypothesis known as the immunocompetence principle. A positive relationship between sexual traits and immune ability is not always present. Here we illustrate this pattern by using five damselfly species in the genus *Hetaerina*. Previous studies have documented a positive correlation between sexual trait expression (wing spot size) and immune ability in members of this genus. These studies have also documented that there are fitness and energetic costs of producing and bearing wing pigmentation. First we used five *Hetaerina* species to investigate the correlation between spot size and phenoloxidase (PO) activity (a key insect immune component) in two contrasting seasons. Second, we experimentally challenged males of two *Hetaerina* species and correlated spot size with PO activity and melanization ability. Results indicate either a positive relationship, a negative relationship or, more commonly, no relationship at all between immune components and wing pigmentation. Season did not predict any of these relationships or expression of spot size and PO activity. These results, although limited to two immune components, indicate that the relationship between sexual trait expression and immunity is not always consistent.

Keywords: sexual selection, damselflies, pigmentation, phenoloxidase, melanization.

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Introduction

One potential consequence of increasing sexual trait expression is the simultaneous suppression of immune function (Sheldon & Verhulst, 1996). Since both sexual trait expression and immune ability are energetically costly traits, an animal may face a resource allocation trade-off. According to the sexual selection theory, only males in better condition can produce both an intense sexual trait and a robust immune response. If there is a genetic basis for the production of sexual traits and immune ability, females may benefit by passing along these 'good genes' to their offspring by choosing to mate with showy males (reviewed by Andersson & Simmons, 2006). These ideas have traditionally been coined as the male immunocompetence principle hypothesis (Folstad & Karter, 1992). One problem, however, with the assumed relation 'higher sexual trait expression-higher immune ability' is that, rather than a positive relationship, a negative relationship can be also observed (Westneat & Birkhead, 1998). This negative relationship, although not as extensively documented as a positive relationship, may be found if, for example, an individual invests little in immunity, but more on a sexual trait without necessarily affecting its fitness (Getty, 2002). These positive and negative relationships, thus, do not allow establishing clear predictions based on the sexual trait expression-immune ability relationship (Westneat & Birkhead, 1998; Getty, 2002; Lawniczak et al., 2006). These problems were critically reviewed recently by Adamo & Spiteri (2005, 2009) who concluded that is unlikely that sexual traits can communicate immune ability. To our knowledge, however, apart from the theoretical approaches, empirical studies of the relationship between sexual trait expression and immunity are not common.

One animal group in which sexual selection and immunity ideas have been profusely tested is calopterygid damselflies. Males of most species are territorial and bear wing pigmentation patterns which are sexually selected either in the context of female choice (Siva-Jothy, 1999) or male competition (e.g., Grether, 1996a,b). In the case of the genus *Hetaerina*, wing pigmentation patterns are present in the form of a red spot at the base of four wings (Grether, 1996a,b; Córdoba-Aguilar et al., 2009a). Both experimental and observational studies have found evidence for a positive correlation between the expression of spots and mating success, via more successful territorial defense (Grether, 1996a; Serrano-Meneses et al., 2007). A number of studies in *H. americana* have found that spot size positively relates to muscular

fat reserves, muscle mass and several components of immune response (e.g., Contreras-Garduño et al., 2006, 2007, 2008a; Serrano-Meneses et al., 2007). These relationships hold only in males that are defending a territory. These condition-dependent traits become substantially impaired after losing a territory in an energy-consuming fight (Contreras-Garduño et al., 2006). Furthermore, the costs of spot production have been experimentally demonstrated; an immune challenge at the time of spot formation negatively affected spot size (Contreras-Garduño et al., 2008a). Since in *Hetaerina* damselflies, female choice does not seem to operate (Grether, 1996a; Córdoba-Aguilar et al., 2009a), the link between sexual trait expression and immune ability is not explained via female choice. Thus, it is accepted that a male in good condition means the ability to produce a number of costly traits which implies that immune ability is indirectly favoured (Contreras-Garduño et al., 2007). This is not surprising: it may occur that a number of physiological traits, including immune ability, may be indirectly favoured (Adamo & Spiteri, 2005). Thus, body condition is a mediator of the relationship between spot size and immune function.

Despite previous evidence supporting a positive correlation between ornament expression and immune ability in *Hetaerina*, mixed results for this correlation have recently been found. One case is that of *H. titia*. Unlike other family members, males of this species bear two pigmentation patches: the red spot on the wing basis, and a black spot on the rest of wings (Córdoba-Aguilar et al., 2007). Previous results in *H. titia* suggested that melanization ability (one form of immune defence against parasitic protozoans and metazoans, fungi and parasitoids such as wasp eggs or larvae; Gillespie et al., 1997; Lavine & Strand, 2002; Brennan & Anderson, 2004) was positively correlated with black, but not red, spot expression (Córdoba-Aguilar et al., 2007). In a subsequent study, *H. titia* males were immune challenged using the insect-specific bacteria *Serratia marcescens* and both black and red, spots were entered as predictors of survival (González-Tokman & Córdoba-Aguilar, 2010). Results indicated that black and red spot did not predict survival. Mixed results have also been found in *H. americana*. Contreras-Garduno et al. (2006, 2007) reported positive relationships between spot expression and immune components (melanization, phenoloxidase (PO) activity) and hydrolytic enzymes. *H. americana* reproduces the entire year in central Mexico, although it is also clear that there is a marked seasonality with larger densities from October to December (Córdoba-Aguilar, 2009).

A study carried out via collections throughout the year found that the positive relation between spot and immunity did not hold in all seasons, and that there was a high seasonal variation in spot size and immune ability (Córdoba-Aguilar et al., 2009b). For example, higher values of spot size and immune ability were found in August–September compared to January–March (Córdoba-Aguilar et al., 2009b). Further studies are needed to see whether there is variation in the correlation between spot and immune ability in other *Hetaerina* species.

Here we investigate whether the correlation between spot expression and immune ability holds in five *Hetaerina* species. Our general aim is to investigate whether such relationships follow a widespread pattern. First, using collected animals from the field we aimed to see whether the positive relationship between spot expression and PO activity holds in other species of *Hetaerina* by using five members of this genus (*H. americana*, *H. cruentata*, *H. occisa*, *H. titia* and *H. vulnerata*). In these data, we considered seasonality in our correlations between spot and immune expression. Second, since our animal collection was carried out in the absence of a pathogenic infection and this may affect estimates of immune ability (Zuk & Stoehr, 2002), we utilized an experimental approach. We immune challenged males of *H. americana* and *H. vulnerata* under controlled conditions and then correlated spot size expression with PO activity and melanization ability. Although previous studies had established that PO activity was a key indicator of immune strength not only in *Hetaerina* (e.g., Contreras-Garduño et al., 2007), but also in other insects (reviewed by Schmid-Hempel, 2005), some recent studies have not confirmed this (Yang et al., 2007). Unlike PO activity, however, melanization seems a better predictor of immune response and pathogen resistance (Rantala & Rolf, 2007). Furthermore, both immune components have been traditionally used in the studies of evolutionary ecology of immune response in insects (Schmid-Hempel, 2005).

Materials and methods

Study species

Recently emerged *Hetaerina* males take 4–7 days to reach sexual maturity. During this time internal organs complete development, and body and wing spot colouration become fixed (Córdoba-Aguilar, 1993). After maturation,

males return to riverine areas to fight for a territory. Males defend territories to secure reproductive access to females, and females receive no benefits from the territory other than mating (Córdoba-Aguilar et al., 2009a). Males may engage in either of two territorial tactics. Territorial males are those that have gained a territory and defend it against conspecifics while non-territorial males have not acquired a space and either fight for one or act as satellites usually in the territories' boundaries. These tactics are conditional (sensu Taborsky et al., 2008) in the sense that they are adopted depending on the male energetic condition (in this case, fat reserves). Territorial males (in prime energetic condition) are more likely to show a positive relation between ornament expression and immune ability than non-territorial males (Contreras-Garduño et al., 2006, 2007, 2008a). Males with larger spots have higher mating success in four (*H. americana*, *H. cruentata*, *H. titia* and *H. vulnerata*) out of the five species used in this study (Córdoba-Aguilar et al., 2009a).

Animal collection

Non-experimental animals were collected with a butterfly net in two different seasons, the first from December 2008 to January 2009, and the second in August 2009. Exact dates of collection were: *H. americana* January 23 and August 19 (both 2009), *H. cruentata* December 20 and August 15 (2008 and 2009, respectively), *H. occisa* December 21 and August 14 (2008 and 2009, respectively), *H. titia* December 23 and August 17 (2008 and 2009, respectively) and *H. vulnerata* January 24 and August 23 (both 2009). Previous studies have suggested that both seasons are likely to differ in food resources, which explains why animals show differences in nutritional and immunological condition in both time periods (Contreras-Garduño et al., 2009; Córdoba-Aguilar et al., 2009b). We limited our collection to a single day for all animals for each species in each season. Collection was made in the following locations in Mexico: *H. americana* in Tehuixtla, Morelos (18°33′26.14″N, 99°16′28.08″W); *H. cruentata* in Coatepec, Veracruz (19°27′52.84″N, 96°56′07.05″W); *H. occisa* in Plan del Río, Veracruz (19°25′23.08″N, 96°40′20.78″W); *H. titia* near La Mancha, Veracruz (19°31′03.24″N, 96°23′24.09″W) and *H. vulnerata* in Jiutepec, Morelos (18°52′50.22″N, 99°09′53.96″W). Since males may vary in their nutritional and immunological condition according to their age and territorial

status (i.e., territorial and non-territorial), only mature and territorial males were collected. For assessing male age, we followed but modified the criteria of Plaistow & Siva-Jothy (1996). Briefly, we classified adult individuals in three different categories: the first category is comprised of teneral, newly emerged males, with their wings and exoskeleton neither rigid nor pigmented; the second category included mature and sexually active males, with flexible wings, and abdominal, thoracic and wing pigmentation already fixed. The third category included old males, which show abundant pruinescence in the abdomen and thorax, and their wings are opaque, inflexible and frequently broken. We carried out behavioural observations to assess male territorial status: when males were observed fighting (i.e., directing flights towards conspecific males that flew nearby, and returning to the same perching spot repeatedly) for riparian territories for at least 20 min they were considered territorial (see Contreras-Garduño et al., 2006). Otherwise, males were considered non-territorial. In the present study, only territorial males of age category 2 were used. Previous studies have found that these middle-aged males show the highest levels of PO activity, melanization, fat muscular reserves and muscle mass compared to males of other ages (Contreras-Garduño et al., 2006, 2007, 2008a; Serrano-Meneses et al., 2007). Males in age categories 1 and 3 are either in the process of constructing the above physiological parameters (in the case of age category 1) or these have been depleted due to past reproductive activities (in the case of age category 3; Contreras-Garduño et al., 2008a). In fact, the significant relationships between immune ability and wing pigmentation were found using age category 2 males but not with the other age categories (Contreras-Garduño et al., 2008a). Thus, for the purposes of our study, using males of age category 2 are the best representatives to illustrate if there is a relationship between immune ability and wing spot size. Sample sizes in the first and second season, respectively, were: *H. americana* $N = 22, 24$; *H. cruentata* $N = 11, 37$; *H. occisa* $N = 18, 21$; *H. titia* $N = 11, 23$; and *H. vulnerata* $N = 14, 27$.

Experimental approaches to activate the immune system

Two experiments were conducted to induce the immune response. In the first experiment, bacteria were used and PO activity was recorded as a response variable. In the second experiment, a nylon filament implant was used and melanization and PO activity were recorded as response variables. In both

experiments, immune response was related to wing spot size. In March 2010, 120 *H. americana* and 75 *H. vulnerata* males were collected at the localities indicated above (Tehuixtla and Jiutepec for each species, respectively). Males of age category 2 were randomly assigned to one of three treatments: bacteria-infected ($N = 47$ for *H. americana* and $N = 25$ for *H. vulnerata*), control animals ($N = 42$ for *H. americana* and $N = 25$ for *H. vulnerata*) and sham ($N = 30$ for *H. americana* and $N = 25$ for *H. vulnerata*). *Serratia marcescens* was used as the bacterial agent for several reasons: (i) it is insect specific and has been detected in *Hetaerina* species (all authors' unpubl. data); and (ii) it not only induces PO activation (Contreras-Garduño et al., 2007) but at a high dosage, it may cause death (all authors' unpubl. data). Approximately 700 colony forming units of bacteria were suspended in 1 μl phosphate buffer (PBS 1×, pH 7.0). This 1-μl amount was then injected in the dorsal area of the thorax using a 10-μl Hamilton syringe. The sham group was injected with 1 μl of bacteria-free PBS while the control group was not manipulated at all. In both the experimental and sham groups, the syringe penetrated just beneath the cuticle to avoid damage of internal organs. After manipulation, each male was placed in an individual closed container (4.5 cm height × 1.4 cm width) with a stick for perching and a moist cotton ball to avoid dehydration. All containers were placed in a dark box to reduce animal movement that could lead to energetic exhaustion. No food or additional water was provided during this time. Twenty-four h after manipulation (while all animals were still alive), animals were retrieved from their containers and haemolymph was extracted by perfusion for PO activity readings (see below).

In March 2010, 22 *H. americana* and 14 *H. vulnerata* males were collected from the same places as indicated above for use in the second experiment. A previously disinfected (one hour in 70% ethanol) nylon filament (2 mm length, 0.2 mm diameter) was completely inserted (using fine dissection forceps) in the 4th thoracic pleura of the mid-ventral region of all animals. The implant induces a melanization response by the insect which resembles what occurs with infection by natural parasites (Rantala & Roff, 2007). Soon after filament insertion, each male was placed in a closed plastic container (4.5 cm height × 1.4 cm width) with a stick for perching and a moist cotton ball to avoid dehydration. For the same reasons as indicated above in the bacterial infections, animals were left in a dark box. No food or additional water was provided during this time. Twenty-four h later (while all animals

were still alive), animals were retrieved from their containers and the filament was carefully retrieved using fine forceps by removing the exoskeleton surrounding the area where the filament was introduced under a dissecting microscope. Each filament was preserved in 70% ethanol for melanization recording. During filament extraction, haemolymph was extracted by perfusion for PO activity readings (see below).

PO activity in haemolymph

Haemolymph was extracted by perfusion 24 h after animal collection or experimental manipulation. Since all samples were analysed in the same laboratory and travelling times between sites and this site were variable, we used 24 h as the best logistic compromise for animals to be alive between collection and immune assessment. Soon after collection, animals were individually placed in a glassine envelope in a dark box at approx. 22°C with no food. Under these conditions, animals reduce their activity so that they do not die of energetic exhaustion (all authors' unpubl. data). We could have provided food to them to keep their initial collecting body condition; however, the number of prey damselflies accept is highly variable among individuals (unpubl. data), which may also have affected our immune estimates. We were confident that animal manipulation still would provide trustable immunity measures as previous studies in *Hetaerina* (where originally spot size and immunity have been correlated) have relied on similar procedures (Contreras-Garduño et al., 2006, 2007). We injected 200 μl PBS 1× (pH 7) with a 1.5-ml insulin syringe in the ventral portion of the male's thorax, at the level of the second pair of legs. A mixture of PBS and haemolymph was then recovered from a small incision made at the tip of the last abdominal segment. About 100 μl (five drops) of this mixture was recovered for each male for measurement of PO activity.

PO activity was measured spectrophotometrically by recording the formation of dopachrome from L-dihydrophenylalanine (L-DOPA, Sigma; Contreras-Garduño et al., 2006), in propylene 96 Microwell® plates (Eppendorf). Sample volumes corresponding to 10 μg protein (see below) were mixed with PBS to reach a total volume of 150 μl in each well. L-DOPA (50 μl of a 3 mg/ml PBS solution) was added to each well as a substrate for triggering PO activity. The plate was incubated for 10 min at 30°C to initiate the reaction between PO and L-DOPA. After incubation, PO activity was recorded every 10 min for 1 h using a spectrophotometer (ELx800

microwell reader) at 490 nm. A mixture of 150 μl PBS and 50 μl L-DOPA was used as blank. PO activity is expressed as absorbance units (change in absorbance/min per mg protein).

Protein determination

Since haemolymph extraction is not homogeneous between individuals, PO activity cannot be measured per volume of haemolymph. Thus, protein concentration needs to be measured and corrected (Contreras-Garduño et al., 2007). Protein amount in the sample was determined using a colorimetric method (bicichronic acid assay (BCA), using the Pierce protein assay kit). Briefly, each well was filled with 10 μl haemolymph sample plus 40 μl PBS and 150 μl BCA reagent. The plate was incubated for 20 min at 37°C and absorbance was recorded in a spectrophotometer at 560 nm. For determining the amount of protein in each sample, we compared each absorbance value with a standard curve that contained seven albumin concentrations (0, 2.5, 5, 7.5, 10, 12.5, 20 and 30 μg albumin/ml).

Filament melanization response

Each melanized filament was preserved in 70% ethanol and then placed in a Petri dish, also in 70% ethanol. Filaments were then photographed under a dissecting microscope (Olympus S2H-ILLK) equipped with a digital camera (Cannon Power Shot G6). Three pictures were taken each with a different position of the filament. This was done as the distribution of melanine around the filament is highly irregular (Contreras-Garduño et al., 2006). The size of melanized areas (i.e., the melanization response) around the filament were measured using Corel Photoshop C2 image analysis software. A relative value was obtained in relation to the whole implant area, and an average was used from the three pictures.

Wing and spot size measurement

Using a digital caliper, wing size in all animals was measured in the right anterior wing as the distance from the distal tip to the site of wing insertion to the thorax. Spot was measured as the proportion of the total wing area that was covered by pigment (Contreras-Garduño et al., 2007). This relative value controls for allometric relationships with body size (Córdoba-Aguilar

et al., 2003). Such measurements were obtained from digital images of the four wings, where the number of pixels of the entire wing and the pigmented portion were counted with Adobe Photoshop 7.0. The average value of all wings was used as the measure of wing pigmentation proportion. For *H. titia*, the only species with two coloured patterns in the wings, both the red and black pigmentation areas were measured.

Parasitism levels

One source that can influence immunity values is parasitism levels. For example, a high parasitism level can be related to high immune activity (Sheldon & Verhulst, 1996). To see whether this was the case of our animals, we checked parasite absence/presence and intensity levels. Two predominant parasites in odonates are intestinal gregarine protozoans and ectoparasitic mites (Forbes & Robb, 2008). The former were looked for by dissecting the intestinal tract using fine forceps. When present, gregarines are clearly visible by their whitish and oval appearance. In the case of ectoparasitic mites, we inspected the exoskeleton for mites or for scars left by mite feeding tubes if mites were present in the past. This method has been proven effective in damselflies (Forbes, 1991).

Consistency of measures

To avoid possible observer bias, the same person (IG-S) recorded all measurements. Furthermore, to assess consistency, replicate measures (the same structures or parts were measured twice) were recorded for the following traits: (i) nylon filament melanization; (ii) wing spot size; and (iii) body size. PO and protein quantification were not repeated as only one extraction was possible due to the fact that the animal did not have additional haemolymph after one perfusion. To alleviate this situation, PO activity and protein were recorded twice in 15 house cricket (*Acheta domesticus*) males obtained from a pet shop. These animals were treated exactly the same as the damselflies but rather than 100 μl haemolymph, 200 μl was gathered. This allowed division into two parts (100 μl each) to record PO activity and protein amount in each.

Statistical analyses

Spot size proportions were arcsin transformed prior to analyses. If PO activity values were not normally distributed, data were log transformed. If data did not satisfy normality or homogeneity of variances assumptions, nonparametric tests were used. For comparing PO activity or spot size between seasons, t-tests or Mann–Whitney U-tests were carried out. Relationships between PO activity and spot expression were analyzed with linear regression models. The dependent variable for the linear regression model was PO activity. A spearman correlation between PO activity and spot size was carried out in August for *H. titia.* PO activity was regressed against or correlated (Spearman correlations following Bonferroni corrections) with body size for all species within each season to see how much variation in immune ability was explained by body size. Correlations were used when data were not amenable for transformation to normal distribution. For the bacteria-based experiments, two general linear models (GLMs) were constructed. In the first GLM, we investigated whether PO values were explained by treatment (infected, control and sham groups) and wing pigmentation in *H. americana*. A second GLM using the same factors was carried out for *H. vulnerata*. In both GLMs, body size was entered as a covariate. These two GLMs were carried out separately, as a GLM does not allow having two fixed factors (in this case species and treatment). For the nylon filament-based experiments, a multiple regression analysis was carried out in which PO activity and melanization were entered as dependent variables while wing pigmentation and body size were entered as independent variables in both *H. americana* and *H. vulnerata*. Consistency of measures was tested using Pearson correlations following Bonferroni adjustments. Analyses were carried out in SPSS 15.0.

Results

H. americana

Males collected in January showed higher PO activity than males collected in August (Table 1, Figure 1). Wing spot size was higher in August than in January (Table 2, Figure 2). Wing spot size was negatively correlated with PO activity in January ($r^2 = -0.327$, $p = 0.005$) but there was not a

Table 1. Seasonal differences in PO activity in males of five *Hetaerina* species.

Species	Mean ± SD (Units/μg protein)		t-value (p)
	Dec–Jan (N)	August (N)	
H. americana	0.123 ± 0.106 (22)	0.010 ± 0.010 (24)	23.637, 38.686 (<0.001)
H. cruentata	0.169 ± 0.102 (11)	0.229 ± 0.150 (37)	−1.502, 24.193 (0.146)
H. occisa	0.089 ± 0.053 (18)	0.269 ± 0.175 (21)	−0.447, 24.227 (<0.001)
H. titia	0.231 ± 0.152 (11)	0.091 ± 0.126 (23)	U = 35.000 (<0.001*)
H. vulnerata	0.065 ± 0.013 (14)	0.011 ± 0.004 (27)	9.130, 44 (<0.001)

*Mann–Whitney U-test.

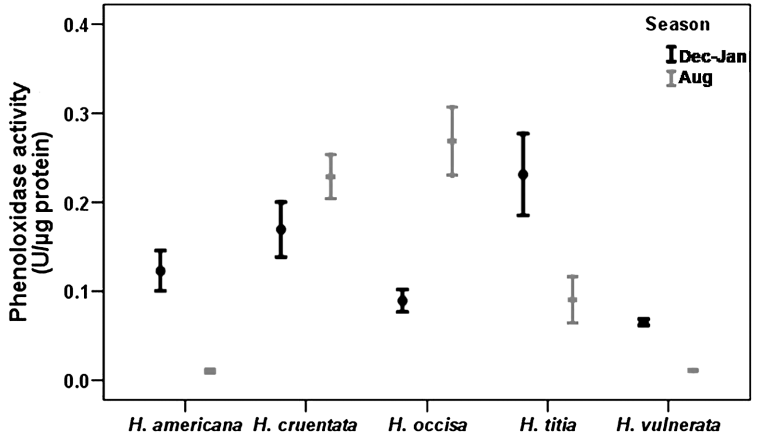

Figure 1. PO activity in males of five different *Hetaerina* species according to season. Bars represent standard errors.

significant correlation in August ($r^2 = -0.0368$, $p = 0.368$; Figure 3a). There was no correlation between PO activity and body size in January ($r_{\text{Spearman}} = -0.020$, $p = 0.930$, $N = 22$) and August ($r_{\text{Spearman}} = 0.202$, $p = 0.345$, $N = 24$).

H. cruentata

PO activity did not differ between males captured in December and males captured in August (Table 1, Figure 1). However, wing spot size was higher in December than in August (Table 2, Figure 2). The relation between wing pigmentation and PO activity was negative in December ($r^2 = -0.429$,

Table 2. Seasonal differences in spot values in males of five *Hetaerina* species.

Species	Mean ± SD		*t*-value (*p*)
	Dec–Jan (*N*)	August (*N*)	
H. americana	14.38 ± 2.41 (22)	20.72 ± 2.19 (24)	−9.338, 44 (<0.001)
H. cruentata	9.50 ± 0.69 (11)	10.31 ± 0.84 (37)	−2.944, 46 (0.005)
H. occisa	11.50 ± 2.03 (18)	15.88 ± 0.88 (21)	−8.024, 20.970 (<0.001)
H. titia			
Red spot	6.62 ± 0.50 (11)	6.05 ± 1.44 (23)	U = 117.000 (0.482*)
Black spot	0	51.48 ± 17.11 (23)	
H. vulnerata	10.35 ± 0.56 (14)	12.44 ± 0.78 (27)	−9.850, 39 (<0.001)

*Mann–Whitney U-test.

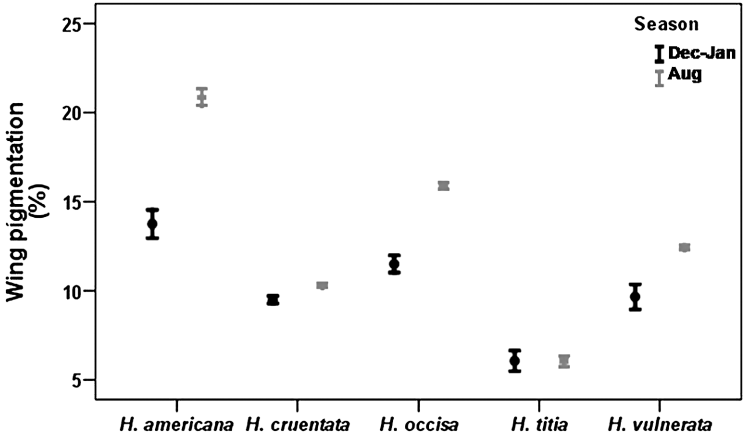

Figure 2. Variation in red wing spot size in males of five *Hetaerina* species in two seasons. Bars represent standard errors.

$p = 0.029$) but non-significant in August ($r^2 = -0.004$, $p = 0.703$; Figure 3b). PO activity was not predicted by body size neither in December ($r^2 = 0.122$, $p = 0.291$, $N = 11$) nor August ($r^2 = 0.258$, $p = 0.238$, $N = 37$).

H. occisa

PO activity was higher in August than in December (Table 1, Figure 1), and so were wing spot size values (Table 2, Figure 2). Wing spot size and PO

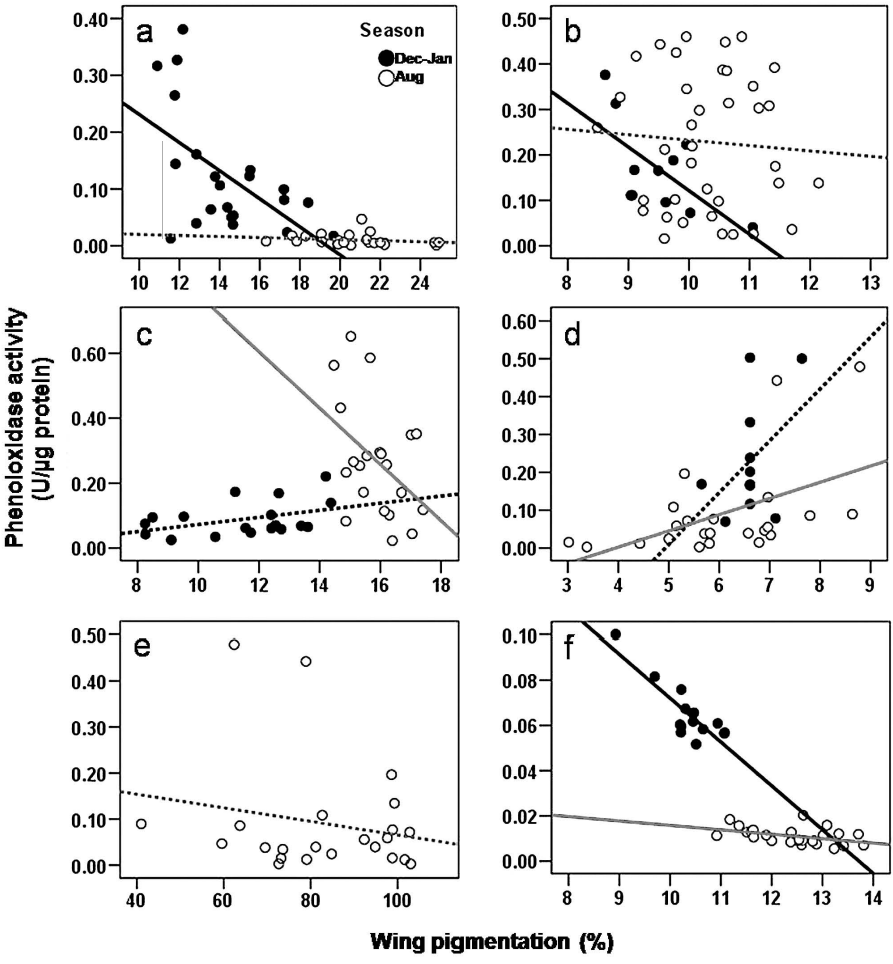

Figure 3. Linear regressions between spot size and phenoloxidase activity in two different seasons in *Hetaerina*: (a) *H. americana*, (b) *H. cruentata*, (c) *H. occisa*, (d, e) *H. titia* and (f) *H. vulnerata*. For *H. titia*, the relation of PO activity and wing spot size is given for (d) red and (e) black pigmentation. Black lines fit full circles (December) and grey lines fit open circles (July). Full lines represent significant relations, while dotted lines are non-significant relations.

activity were negatively correlated in August ($r^2 = -0.190$, $p = 0.048$), but no significant relation appeared in December ($r^2 = 0.1656$, $p = 0.094$; Figure 3c). PO activity was predicted by body size in August ($r^2 = 0.392$, $p = 0.004$, $N = 20$) but not in December ($r^2 = 0.193$, $p = 0.07$, $N = 17$).

H. titia

PO activity was significantly higher in December than in August (Table 1, Figure 1), and wing red spot size did not differ between seasons (Table 2, Figure 2). Wing red spot and PO activity were positively correlated in August ($r^2 = 0.341$, $p = 0.003$) while they were not correlated in December ($r_{\text{Spearman}} = 0.253$, $p = 0.453$; Figure 3d). Wing black spot was absent in December, while in August the proportion of wing black pigmentation was $51.48 \pm 17.11\%$ (Table 2). Wing black spot and PO activity were not correlated in August ($r^2 = -0.031$, $p = 0.418$; Figure 3e). PO activity was not explained by body size in December ($r^2 = 0.008$, $p = 0.791$, $N = 11$) nor was correlated with body size in August ($r_{\text{Spearman}} = 0.041$, $p = 0.852$, $N = 23$).

H. vulnerata

PO activity was higher in January than in August (Table 1, Figure 1). Also, wing spot size was higher in August than in January (Table 2, Figure 2). The relation between wing spot size and PO activity was negative in both seasons (January $r^2 = -0.7225$, $p < 0.001$; August $r^2 = -0.1789$, $p = 0.028$; Figure 3f). There was no correlation between PO activity and body size in January ($r_{\text{Spearman}} = -0.256$, $p = 0.398$, $N = 14$) and in August ($r_{\text{Spearman}} = -0.251$, $p = 0.206$, $N = 27$).

Bacterial challenge experiment

PO activity in *H. americana* was not explained by treatment ($F_{2,33} = 2.122$, $p = 0.136$), wing spot ($F_{1,33} = 0.152$, $p = 0.699$) nor body size ($F_{1,33} = 0.464$, $p = 0.5$). As for *H. vulnerata*, PO activity was different according to treatment ($F_{2,24} = 7.477$, $p = 0.003$). A Tukey test revealed that the bacteria-treated group had higher PO activity values compared to control ($p = 0.007$) and sham ($p = 0.02$) groups. Sham and control groups were not different ($p = 0.763$). Neither wing spot size ($F_{1,24} = 0.521$, $p = 0.471$) nor body size ($F_{1,24} = 1.376$, $p = 0.252$) predicted melanization.

Nylon filament challenge experiment

PO activity ($r^2 = 0.123$, $p = 0.961$) and melanization ($r^2 = 0.021$, $p = 0.895$) were not explained by wing spot size in *H. americana*. Similarly,

PO activity ($r^2 = 0.123$, $p = 0.444$) and melanization ($r^2 = 0.021$, $p = 0.767$) were not explained by body size. As for *H. vulnerata*, PO activity ($r^2 = 0.204$, $p = 0.229$) and melanization ($r^2 = 0.300$, $p = 0.295$) were not explained by wing spot size. PO activity ($r^2 = 0.204$, $p = 0.213$) and melanization ($r^2 = 0.300$, $p = 0.128$) were not explained by body size.

There was no correlation between PO activity and melanization for *H. americana* ($r_{\text{Pearson}} = 0.454$, $p = 0.258$) and *H. vulnerata* ($r_{\text{Pearson}} = -0.051$, $p = 0.889$).

Parasitism levels

No gregarine, mite or mite scars were found in all damselflies except for *H. titia*, in which 22 males (out of 35) had gregarines in August.

Consistency of measures

All repeated measures were highly consistent with each other (Table 3).

Table 3. Consistency tests of wing pigmentation, body size, melanization, PO activity and protein quantification among species and experimental treatments.

Species and variable	r_{Pearson}	p	N
Wing pigmentation			
H. americana	0.991	<0.001	11
H. cruentata	0.955	0.003	11
H. occisa	0.841	0.018	11
H. titian	0.985	<0.001	11
H. vulnerata	0.980	<0.001	11
Body size			
H. americana	0.990	<0.001	11
H. cruentata	0.952	0.001	11
H. occisa	0.952	<0.001	11
H. titia	0.916	0.001	11
H. vulnerata	0.981	<0.001	11
Melanization: *H. americana* ($N = 7$) and *H. vulnerata* ($N = 8$)	0.749	0.001	15
PO activity: House cricket	0.992	<0.001	15
Protein amount: House cricket	0.993	<0.001	15

In the case of melanization, males of both species (*H. americana* and *H. vulnerata*) were analyzed as a single group.

Discussion

Previous studies in *Hetaerina* species have suggested different associations between wing pigmentation and immune ability: In *H. titia* no relation was found (González-Tokman & Córdoba-Aguilar, 2010), while in *H. americana* the relation was at times positive (Córdoba-Aguilar et al., 2009b). Despite the limitation of using two immune components (PO activity and melanization ability) our present results also show inconsistent patterns (i.e., negative, positive or, more commonly, null) and interspecific and seasonal differences. Furthermore, a given season did not predict that a particular relation (i.e., negative or positive) would be found for all species.

We have five hypotheses to explain why PO activity and melanization ability were not related to wing pigmentation in *Hetaerina*. One is that immune ability (and body condition, in general) is rapidly affected by stressful conditions, an issue that has been supported by a number of studies (reviewed by Schmid-Hempel, 2005) and which could apply to those non-experimental animals we collected. The fact that spot is fixed a few days after adult emergence, while immune ability changes during adulthood (Contreras-Garduño et al., 2006, 2007, 2008a), means that there is a limited time in the adult's life when there will be a positive correlation between spot size and immune ability (i.e., when the animal is fighting for or defending a territory). Such positive correlation would also apply for all body condition indicators (e.g., muscle mass, fat reserves). There are at least two stressors that can negatively affect immune ability: poor diet (i.e., Siva-Jothy & Thompson, 2002) and aggression. Support for negative effects of aggression has been found in *H. americana* (Contreras-Garduño et al., 2006). In case any of these two stressors, food or aggression, prevails then immune ability may be negatively expressed affecting its relationship with ornament expression. However, one would expect that food shortage will coincide with a season with fewer males and weaker individual condition. If this were the case, one should expect consistent patterns in the same season across species. A similar logic should apply for aggression. Since this is not the case, there is little room for the operation of the above stressors especially given the case that we controlled these in our experiments. Of course, food and aggression may have other effects on aspects of body condition different to immunity which may affect the relation between this latter variable and spot size. It may be that still within a season, there are subtle individual differences in body condition

that may affect immunity. Unfortunately, a fine control of body condition is not likely. A second hypothesis is related to trade-offs between different immunity components (Rantala & Roff, 2007). If the relative benefits of each immune component for a given host vary between seasons, there may be different relationships between immune components and sexual trait expression. A third hypothesis is that, at least in the populations or species concerned here, they may have evolved towards uncoupled ornament-immunity relations. Previous studies have suggested that there is no direct selection acting on immune ability via selection on spot size in *Hetaerina* because spot is favoured only via male competition. The fact that females do not choose males in these species (Grether, 1996a; Córdoba-Aguilar et al., 2009a), suggests that only male competition, in which spot size is key, indirectly selects for immune ability. If females had a role, then one may argue that an indirect benefit for them is that offspring would inherit immune ability. Since male competition selects for better energetic condition, immune ability may be selected especially if both energy- and immune-related elements are shared. However, the relation fighting-immune ability may break if their constitutive elements are no longer shared. Related to these arguments, one piece of evidence suggests that both spot size and immunity may become decoupled. A calopterygid study in which selection acting on both traits was measured, found that selection favoured PO expression but not spot size (Rolff & Siva-Jothy, 2002). What leads to such decoupling is unclear especially assuming that for being a male in good condition, immunity is implied (Siva-Jothy, 2000). A fourth hypothesis is that PO and melanization activity are not good indicators of male condition, immune ability and/or pathogen resistance. Apart from the evidence we have discussed above in *Hetaerina*, other studies also support our claim. For example, in the geometrid *Epirrita autumnata*, starvation periods impaired melanization but, paradoxically, PO activity increased (Yang et al., 2007). Furthermore, no evidence that PO activity predicts pathogen resistance has been found in some studies (e.g., Adamo, 2004; Leclerc et al., 2006; Schwarzenbach & Ward, 2007; Moreno-García et al., 2010). The experimental results we obtained from our challenge experiment actually support the claim that PO may not be used to infer pathogen resistance since infected *H. americana* males did not show higher PO activity values compared to control males. In fact, there was no correlation between both immune components in the two species. This is consistent with a previous result which detected no relation between parasite burden

and PO activity in *H. americana* (Contreras-Garduño et al., 2008b). A final fifth hypothesis is that rather than PO activity, pro-phenoloxidase (proPO) could be related to wing spot size. The inactive precursor of PO, pro-PO is activated by a number of pathogens and during melanization and encapsulation (e.g., Sugumaran & Kanost, 1993; Söderhäll & Cerenius, 1998). Pro-PO has been alternatively used as an indicator of immune strength but only in the absence of a pathogenic challenge (during an immune challenge, only PO activity is increased; e.g., Adamo, 2004; Jacot et al., 2005). Although this is the case for our collected, non-manipulated males, this does not apply to those challenged animals. Still, it remains unclear whether pro-PO could be a useful immunity variable to be used in these animals.

There were important differences in the results obtained from field collected animals and experimental animals. We found negative correlations between spot size and PO activity in three of the four collections from the field collected individuals for *H. americana* (August) and (both seasons *H. vulnerata*). In contrast, experimental males did not show a similar pattern. These results indicate that comparing PO and the expression of a sexual trait show different results depending on whether a challenge is employed. Even when there is a relation when no challenge is present, such relation disappears when the animal is challenged. Furthermore, the fact that a nylon implant induces a lower PO activity compared to bacteria may suggest that the animal is producing a quantitatively different immune response via the ability to discriminate between both challenge types by the host.

Previous research in *Hetaerina* compared immune ability between territorial and nonterritorial males (Contreras-Garduño et al., 2006, 2007). Given that males of both statuses differ in spot size, no within-status correlations were carried out to see whether the relation spot size-immune ability held. Such correlation is valid for territorial males as the condition of nonterritorial males is so poor that no relation will be found. Together, these results suggest that the differences in spot size and immune ability between males of different status are so high, that even when within each status, there is enormous variation in these traits but which do not necessarily correlate with each other. In other words, the comparison between both status is valid but this does not mean that spot size can always correlate with PO and melanization.

Our results differ from previous findings in the sister genus *Calopteryx*. In *C. splendens*, wing pigmentation has been found to correlate with melaniza-

tion ability (Rantala et al., 2000), PO activity and parasite resistance (Siva-Jothy, 1999). It is hard to reconcile these differences especially given that we have supported our approach using non-activated and activated immune responses. Our results do not obscure the fact that positive relationships between ornament expression and immunity may arise but that at times, such relation may differ from what is theoretically expected. Ornaments are costly per se, although there are few direct tests that show evidence for this (Kotiaho, 2001). In *H. americana*, there is evidence that an immune challenge (in the form of nylon filament insertion) during spot formation, negatively affects spot size (Contreras-Garduño et al., 2008a). Ornaments are costly in the sense that they must impair fitness (i.e., survival; Kotiaho, 2000). Still, evidence for this has been also provided in *Hetaerina americana*: Grether & Grey (1996) experimentally showed that more highly ornamented males were less successful in capturing prey. Thus, fitness arguments are clear that spots are costly and still they do not correlate with immunity values. Therefore, even in a well known system such as the *Hetaerina* complex, the immunocompetence principle does not seem to apply. Of course, one has to bear in mind that our critical position is based on two immune parameters. What is important, however, is that such parameters have been key in previous immunocompetence studies and during the formation of the immunocompetence principle in insects (Lawniczak et al., 2006).

Our results, therefore, uncover a more general pattern in sexual traits and immunity: the presence of mixed results of the relationship between these two variables. In general, negative or positive relationships have been found (Lawniczak et al., 2006) but, to our knowledge, mixed results have neither been reported for the same species nor for different species from the same genus nor in different seasons as we report here. Clearly, the relation between male condition and immunity is far more complex than initially thought (Adamo & Spiteri, 2005, 2009).

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CAPITULO II

Phenoloxidase: a key component of the insect immune system

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MINI REVIEW

Phenoloxidase: a key component of the insect immune system

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Abstract

The innate immune system in insects is composed of a large variety of specific and non-specific responses that are activated in response to the presence of foreign agents. One important element in such responses is the enzyme phenoloxidase (PO). Here, we review recent progress in PO research and discuss new applications in the emerging field of ecological immunology. Phenoloxidase produces indole groups, which are subsequently polymerized to melanin. The enzymatic reactions in turn produce a set of intermediate products such as quinones, diphenols, superoxide, hydrogen peroxide, and reactive nitrogen intermediates, which are important during defense against bacterial (gram+ and −), fungal, and viral agents. Phenoloxidase requires a complex system of activation and inhibition that involves various cell types, PO zymogens, inhibitor enzymes, and signaling molecules. Finally, research in evolutionary ecology has studied the costs of PO in terms of resource use and pleiotropic relations with other key traits and functions. These studies indicate that PO is a costly trait, whose production and maintenance have fitness costs for hosts. Phenoloxidase does not seem to be an indicator of resistance but rather of host condition. Finally, we put forward some basic directions for future investigation of PO aimed at explaining its activating system, its substrates, its coordination with other immune components to fight off pathogens, and variation in PO in relation to gender, life stages, seasonality, and across different host species.

Introduction

Recent advances in invertebrate immunology have documented a complex array of host defenses. These defenses include phagocytosis, melanization (i.e., synthesis and deposition of melanin around the pathogen), synthesis of extracellular matrix, adhesion cells, recognition molecules, reactive intermediates of oxygen and nitrogen, proapoptotic molecules, pro-inflammatory cytokines, and antimicrobial peptides (Nappi & Vass, 2001; Tunaz et al., 2003; Bulet et al., 2004; Nappi & Christensen, 2005). Within this broad range of immune responses, an important immune component used by arthropods is melanogenesis (Asada et al., 1999; Eleftherianos and Revenis 2011; Amparyup et al., 2009).

Enzymatic and non-enzymatic reactions play an important role in melanogenesis. This process is responsible for encapsulating multicellular pathogens, repairing tissues, and defending against other pathogens such as bacteria (gram+ and −), fungi, and even viral agents (Boman, 1986; Ashida & Brey, 1997; Nappi & Christensen, 2005). Both intermediate products (quinones, diphenols, superoxide, hydrogen peroxide, and reactive nitrogen intermediates) and their activating enzymes [phenoloxidase (PO) and dopa decarboxylase] play important roles. Among the most important enzymes is PO. This enzyme is responsible for the activation of melanogenesis in invertebrates (Sussman, 1949; Wyatt, 1961). Furthermore, recent research has documented PO as an important tool used against several pathogens (Cerenius & Söderhäll, 2004).

Invertebrate PO requires a complex system of activation and inhibition. Activation and inhibition involve different cell types (hemocytes, plasmatocytes, and crystal cells), PO zymogens (i.e., the inactive form of PO: proPO), proPO inhibitor enzymes (serpins), signaling molecules (peptidoglycan, membrane lipids, and viral protein segments), and even PO itself. The role of PO in the immune system has

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been subject to review in the past (Ashida & Brey, 1997; Söderhäll & Cerenius, 1998; Sugumaran, 2002; Cerenius & Söderhäll, 2004; Kanost & Gorman, 2008). Nevertheless, as there is constant discovery and modification of components involved in this complex system, our aim is to integrate old and new information. Our review mainly focuses on (1) an integration of information regarding PO participation in melanogenesis along with regulatory mechanisms of the PO-activating system, and (2) present and future applications of PO knowledge in the field of ecological immunology.

Our review is divided into three sections. In the first section, we discuss the role of PO in melanogenesis. Here, we describe the general properties of melanin, discuss the role of melanin in invertebrate immunity, and review the site of melanin synthesis and the regulatory mechanisms that control melanin action. The second section focuses on the PO enzyme as a key component in the invertebrate immune system, its chemistry and physical properties, the types and structures of invertebrate POs, the site of PO synthesis, the regulatory genes, and the components that promote or inhibit the PO system. Finally, the last section describes new applications of the PO system in the emerging field of ecological immunology.

PO and melanogenesis

The main role of PO in melanogenesis is to convert phenols to quinones, which subsequently polymerize to form melanin (Figure 1; Söderhäll & Cerenius, 1998). The most commonly described melanization pathway is the melanization process against parasitoids eggs in fruit flies (reviewed in Nappi & Vass, 1993; Figure 2). Melanin is formed from the amino acid phenylalanine, which first is hydroxylated to tyrosine by phenylalanine hydroxylase. Tyrosine is then hydroxylated by the active PO to produce DOPA. DOPA is then oxidized to dopaquinone and which is immediately converted to dopachrome by a spontaneous and non-enzymatic reaction. A non-enzymatic structural rearrangement of dopachrome, followed by decarboxylation generates 5-6 dihydroxyindole (DHI). Dihydroxyindole is then oxidized by PO to form 5-6 indolequinones. Finally, indolequinones are polymerized to eumelanin or, in the presence of thiol compounds, pheomelanins (Nappi & Christensen, 2005). In addition, DHI can be produced by an alternative pathway where DOPA decarboxylase removes CO_2 from DOPA to form dopamine (Figure 3; Beerntsen et al., 2000; Christensen et al., 2005).

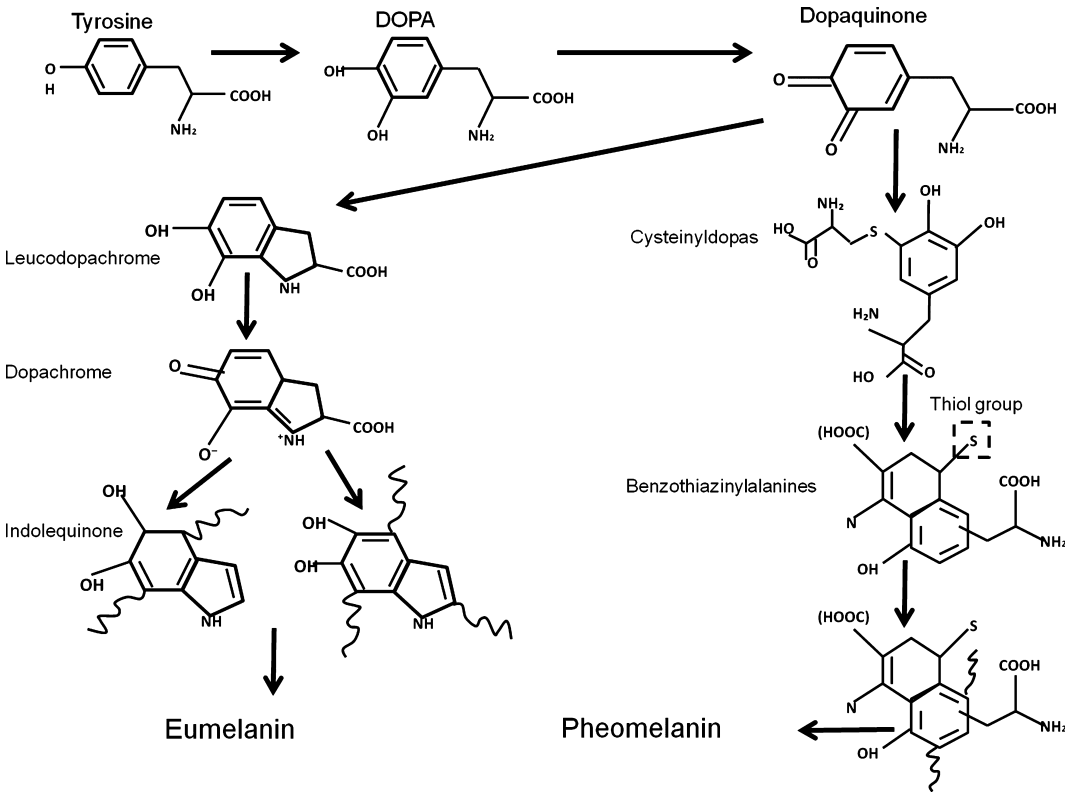

Figure 1 Types of melanins and their precursors. Left: Eumelanin by dopachrome. Right: Pheomelanin by benzothiazine.

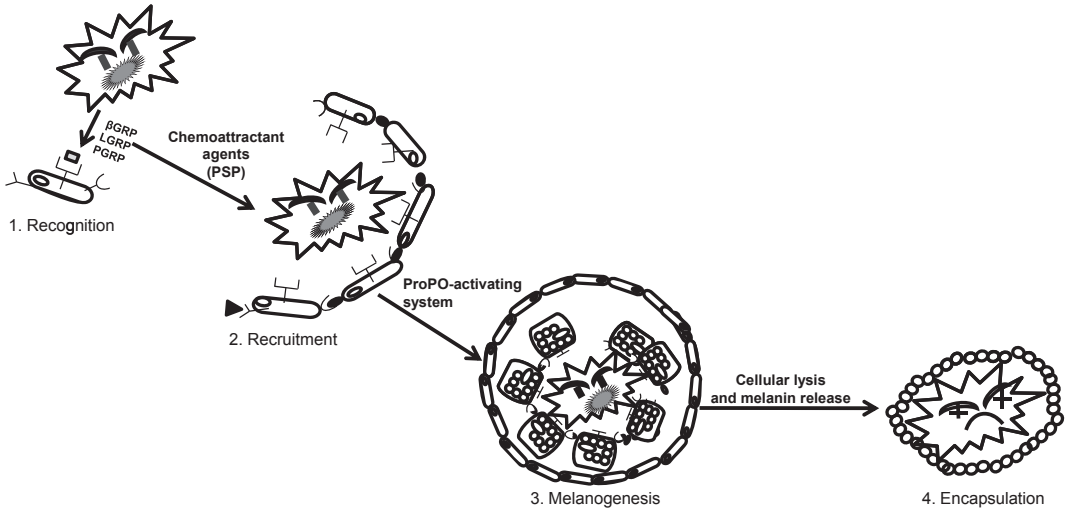

Figure 2 Melanization includes four steps: (1) the recognition of pathogens through specific molecules [e.g., lipopolysacharides (LPSs), LGRP, PGRP]; (2) the recruitment of hemocytes to surround the pathogen by chemoattractant proteins (e.g., plasmatocyte spreading peptide, PSP); (3) melanogenesis within hemocytes; (4) melanin release and pathogen encapsulation.

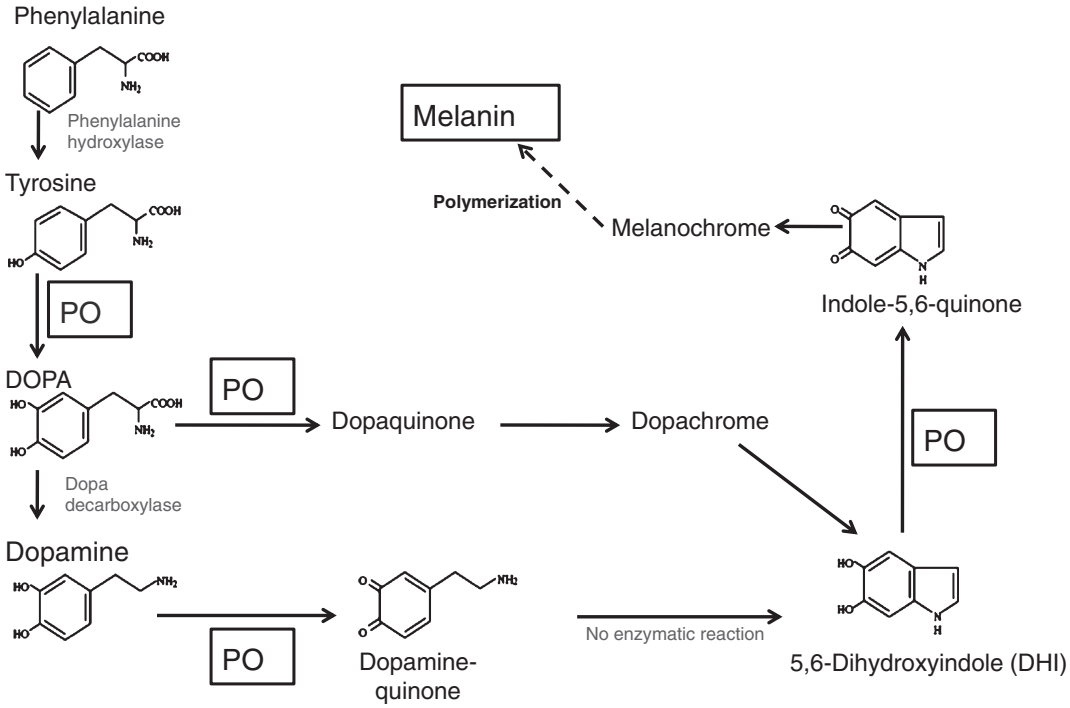

Figure 3 Phenoloxidase role in melanin synthesis. Phenoloxidase participates in the formation of DOPA from Tyrosine (Tyr). PO also convert DOPA to dopaquinone, and 5,6-dihydroxyindole (DHI) to indole-5,6-quinone. Note the alternative way to obtain DHI from dopamine, in which PO is also involved.

Melanins are a group of pigment-forming chemical compounds that are widely distributed in nature. They are found in plants, animals, and protists. The most common melanin is eumelanin which is formed by a dihydroxyndol polymer which comes from dopaquinone (dopachrome), and produces a dark brown or blackish color (Napolitano

et al., 2000). Another common type of melanin is pheomelanin, which is formed by the polymerization of bezothiazine and produces reddish or brownish colors (Figure 1; Napolitano et al., 2000)

Melanin fills many roles in invertebrates besides pigmentation. Several studies have shown that mechanical injury or the presence of macropathogens (e.g., nematodes or parasitoids) results in melanin deposition around the damaged tissue or foreign object. This process is commonly known as melanization (Ashida & Brey, 1997; Sugumaran, 2002; Cerenius & Söderhäll, 2004; Kanost et al., 2004; Nappi & Christensen, 2005). In arthropods, melanization starts when a foreign target is located by hemocytes via a signaling cascade involving lipopolysaccharides, peptidoglycans, glucans, and their recognition proteins - the beta-1,3-glucan recognition protein (βGRP) (Figure 2; reviewed by Strand, 2008). Hemocytes surround the foreign body and release chemoattractant proteins (e.g., plasmatocyte spreading peptide or PSP). These proteins attract plasmatocytes (Lavine & Strand, 2002), which form a multicellular plasmatocyte wall (Wood et al., 2006). The internal layers of the plasmatocyte wall increase and become thick and dark due to melanin production. Melanin is eventually deposited onto the foreign target. The melanin capsule prevents the growth and reproduction of the pathogen and eventually leads to its death, mainly by starvation (Gillespie et al., 1997).

The role of the PO activating system in invertebrate immunity

ProPO and PO, structures and types

Phenoloxidase is a member of the tyrosinase group, whose main function is to oxidize phenols. Tyrosinase proteins from different species are diverse in terms of their structural properties, distribution, and cellular location. It has even been suggested that there is no one common tyrosinase protein structure (Mayer, 2006). All tyrosinases, however, have in common a binuclear type 3 copper center within their active site. This copper center is surrounded by three histidine residues (Jaenicke & Decker, 2003).

Substrate binding in PO occurs at a site containing a series of hydrophobic amino acids with benzene rings (Nappi & Christensen, 2005). The copper center reacts with dioxygen (O_2) and a highly chemically reactive intermediate is obtained by oxidizing the substrate.

Phenoloxidases are expressed as inactive zymogens (proPOs) in all insects and are converted to active PO when required. ProPOs are polypeptides that contain two copper atoms per protein molecule, with a total weight of 50–60 and 70–80 kDa in their active and inactive forms, respectively (Ashida & Brey, 1997). In arthropods, the proPO amino acid sequences are homologous with hemocyanins (an oxygen-transport protein in invertebrates) and hexamerins (storage proteins). ProPO and hemocyanin contain two well-conserved copper atoms that form an oxygen-binding site, whereas hexamerin lacks copper atoms and therefore does not recruit oxygen. Recent studies support the idea that POs are the ancestral enzyme, that hemocyanins evolved secondarily, and finally that hexamerins diverged from hemocyanins (Decker & Terwilliger, 2000; Burmester, 2001; Burmester, 2002).

Gene expression of proPO

Researchers have found two or more proPO genes in most insect species, although there are a few cases, such as the honeybee, with only one proPO gene (Evans et al., 2006). ProPOs are sometimes highly conserved. For example, *Drosophila melanogaster* Meigen and *Tribolium castaneum* (Herbst) share two of three proPO genes (Waterhouse et al., 2007). In other groups, there has been a radiation of the proPO gene family – for example, mosquitoes have nine proPO genes in *Anopheles gambiae* Giles and 10 in *Aedes aegypti* (L.) (Waterhouse et al., 2007). It is not clear why PO is highly conserved in some clades and diversified in others. One possibility is that a higher number of pathogen species may lead to increased specialization, via a higher gene number in host species (Waterhouse et al., 2007). A second possibility is that a coevolutionary arms race between hosts and pathogens may lead the former to evolve specific regulator mechanisms. In mosquitoes, for example, the same genes that are involved in the melanization process against *Plasmodium berghei* Vincke and Lips, do not offer any protection against *Plasmodium falciparum* Welch (Cohuet et al., 2006; Michel et al., 2006). One last explanation is that there is differential expression of proPO in relation to distinct insect life-history stages, so that different genes are involved in the same process, but at different ontogenetic times (Li et al., 2005). Thus, if life history stages are different between host species, different gene numbers may appear.

The site of PO synthesis

Synthesis of proPO occurs mostly in hemocytes (Figure 4; Cerenius & Söderhäll, 2004) with species-specific variation in relation to hemocyte type. In lepidopterans, a type of hemocyte, known as oenocytoids, functions as the site of proPO synthesis (Jiang et al., 1997). In *D. melanogaster*, crystal cells, another class of hemocytes present in larval stage, provide a site for proPO synthesis (Rizki et al., 1985; Williams, 2007). In mosquitoes, proPO is synthesized in granular hemocytes and oenocytoids (Hillyer et al., 2003;

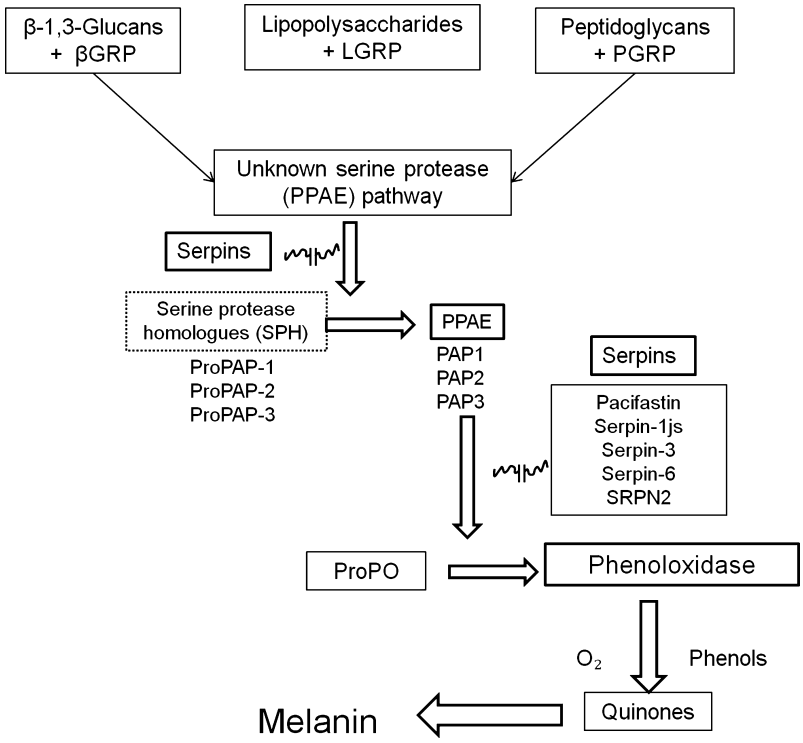

Figure 4 General model for proPO activation system in insects. The system starts when hemolymph recognition proteins (βGRP, LGRP, or PGRP) bind to β-1,3-glucans, lipopolysaccharides, or peptidoglycans situated on the surface of the pathogen. The interaction will activate an unknown serine protease pathway. The activation of a serine protease pathway leads to prophenoloxidase activation and active PO forms. Phenoloxidase catalyzes the oxidation of phenols to form quinines, which then are polymerized to obtain melanin. A regulatory mechanism is present, consisting of serine protease inhibitors (serpins) such as pacifastin, serpin-1, and serpin-3.

Castillo et al., 2006). The fat body, however, is definitively not a place where proPO synthesis takes place.

ProPO secretion

Arthropod proPOs lack a peptide signal for their secretion; therefore the presence of proPO in hemolymph probably results from hemocyte lysis (Kanost & Gorman, 2008). The hemocytes in which proPO synthesis takes place (oenocytoids, crystal cells, and granular hemocytes) vary in robustness. Some sites are extremely fragile, and hemocyte lysis occurs immediately after an injury or infection. In these sites, secretion can occur at a low rate even in the absence of injury or infection, perhaps to maintain standard levels of proPO in hemolymph. As a result, it is difficult to evaluate baseline levels of proPO in insects that have fragile oenocytoids (Ashida & Brey, 1997).

In lepidopterans, proPO mostly appears in hemolymph. In other groups such as locusts and cockroaches, proPO appears mainly stored in hemocytes until a pathogen induces its release (Brehélin et al., 1989; Durrant et al., 1993).

ProPO-activating system in invertebrates

ProPO can be activated by several factors. In the laboratory, activation of proPO in hemolymph can occur via detergents, fatty acids, or alcohols. Researchers have activated proPO purified from *D. melanogaster* by 2-propanol (Asada, 1998). ProPO from *Sarcophaga bullata* Parker may be activated by the cationic detergent cetyl pyridinium chloride (Xie et al., 2007). In nature, insect proPOs can be activated by amphiphilic lipids such as lysolecithin and perhaps by the presence of damaged cells (Sugumaran & Nellaiappan, 1991). Other natural proPO activators are β-1,3-glucans, lipopolysaccharides, and peptidoglycans. Natural proPO activators have been identified in several insect pathogens such as gram+ bacteria, gram− bacteria, and fungi (Cerenius & Söderhäll, 2004). All of these activation agents could trigger a conformational change in the protein, resulting in an accessible binding site for the substrate (Kanost & Gorman, 2008). Experiments with proPO purified from silkworm identified the presence of a serine protease cascade as an intermediate step between natural proPO activators and the production of active proPO (Ashida et al., 1974; Ashida & Dohke, 1980). Serine proteases are hydrolases with a serine amino acid in their active center (Hedstrom, 2002). Hydrolases include trypsin, chymotrypsin, and subtilisin. Serine proteases cut the polypeptide chain in the carboxyl side of specific amino acids, resulting in peptide bond degradation (Hedstrom, 2002). Studies in the laboratory have generally used chymotrypsin as a proPO activator. This activator has become a useful experimental tool to assay both PO in the hemolymph and total PO in the whole insect (Sugumaran et al., 1985; Saul & Sugumaran, 1987; Adamo, 2004).

The first proPO activating serine protease (PAP), also known as prophenoloxidase-activating enzyme (PPAE), to be purified was extracted from cuticle (Aso et al., 1985), and perhaps has a role in proPO activation upon wounding (Kanost & Gorman, 2008). In addition, PAPs have been purified in hemolymph from *Manduca sexta* L. (Jiang et al., 1998, 2003), *Bombyx mori* L. (Satoh et al., 1999), *Holotrichia diomphalia* Bates (Lee et al., 1998), and crayfish *Pacifastacus leniusculus* (Dana) (Wang et al., 2001). Three types of PAPs have been identified, PAP-1, PAP-2, and PAP-3 (Figure 4).

ProPO activating serine proteases can be found before and during an infection depending on the animal and the PAP type. For example, PAP from *B. mori* is expressed in integument, hemocytes, and salivary glands, but not in the fat body (Satoh et al., 1999). Its putative *M. sexta* ortholog, PAP-1, is expressed in the larval fat body, tracheae, and nerve tissue, and is up-regulated in the fat body and hemocytes after injection of bacteria (Zou et al., 2005). ProPO activating serine protease-2 expression was detected in the fat body and hemocytes only after *M. sexta* larvae were injected with bacteria (Jiang et al., 2003). ProPO activating serine protease-3 mRNA is present at a low level in the fat body and hemocytes of naïve *M. sexta* larvae, and its expression is significantly upregulated in those tissues after injection of bacteria (Jiang et al., 2003). At the prepupal stage, it is highly expressed in integument, the fat body, and hemocytes (Zou & Jiang, 2005). Finally, in some cases, PAPs require the presence of protein cofactors or serine protease homologs (SPH) for their activation (Figure 4; Jiang et al., 1998; Kwon et al., 2000). These SPHs have been found in genomes such as *D. melanogaster*, *A. aegypti*, *A. gambiae*, and *Apis mellifera* L. (reviewed in Zou et al., 2006; Waterhouse et al., 2007; Kanost & Gorman, 2008).

PO activity in insect hemolymph

Mammalian tyrosinases and insect POs catalyze three types of reactions in the melanogenesis process: (1) hydroxylation of a monophenol, (2) oxidation of an *o*-diphenol and (3) dehydrogenation of a dihydroxyindole (Korner & Pawelek, 1982). However, insect POs and mammalian tyrosinases have different amino acids sequences in their protein structures (van Holde et al., 2001).

The insect ability to produce multiple enzymes with similar functions was a major challenge for PO activity studies. For example, laccase can directly oxidize both *o*- and *p*-diphenols, but not monophenols (Solomon et al., 1996). Similarly, tyrosine hydroxylase can hydroxylate monophenols, but does not oxidize diphenols. Another enzyme produced by insects, peroxidase, can oxidize both monophenols and diphenols (Nappi & Vass, 1993; Okun, 1996; Vie et al., 1999). Later studies have shown that these enzymes have different substrate specificity. In addition, several chemical inhibitors can be used to isolate the enzyme activity specifically (Barrett, 1991). Although insect POs can potentially oxidize monophenol groups, there are only a few isolated POs from *S. bullata* and *M. sexta* documented with this function (Chase et al., 2000).

Phenoloxidase substrate

There are two classes of compounds that have been documented as substrates for insect POs: (1) monophenols, such as tyrosine or tyramine and (2) catechol substrates, including 1,2-dihydroxybenzene (catechol), 4-methyl catechol, DOPA, dopamine, *N*-acetyldopamine (NADA), *N*-ß-alanyldopamine (NBAD), *N*-acetylnonepinephrine (NBANE), 3,4-dihydroxyphenylacetic acid (DOPAC), norepinephrine, 3,4-dihydroxymandelic acid, and 3,4-dihydroxybenzoic acid (Chase et al., 2000). Of these catechols, DOPA has been most frequently used to assay PO activity experimentally. Based on solubility and oxidative properties, dopamine, NADA, or NBAD could be the natural substrates for PO (Sugumaran, 2002). Nonetheless, tyrosine and most of the diphenols mentioned above have been detected in the hemolymph of one or more insect species, but typically in their non-substrate form. These compounds are stored in hemolymph until needed (Zhao et al., 1995).

Regulatory mechanisms of PO activity

Phenoloxidases are expressed as inactive zymogens (proPOs) in all insects and are converted to active PO by PPAEs. Nevertheless, PO also produces several molecules such as proteases that could degrade host proteins, cytotoxic quinines, reactive oxygen and nitrogen intermediates. These molecules could harm the host if produced in excess. Therefore, the presence of regulatory mechanisms of PO activity is necessary to produce an optimal response localized to a specific place and for a specific duration (Cerenius & Söderhäll, 2004; Nappi & Christensen, 2005; Kanost & Gorman, 2008).

In insect hemolymph, several serine protease inhibitors have been identified that inhibit PO activation (Kanost & Jiang, 1996; Kanost, 1999). One such serine protease inhibitor is pacifastin, which may be obtained from the crayfish *P. leniusculus* Dana (Hergenhahn et al., 1987; Liang et al., 1997). Other groups of serin proteases inhibitors are serpins (Silverman et al., 2001; Gettins, 2002). Several types of serpins (serpin-1j, serpin-3, serpin-6) have been indentified in *M. sexta* (Jiang et al., 2003; Kanost, 2007). A serpin-3 ortholog has been found in *Hyphantria cunea* Drury (Park et al., 2000). Studies in *A. gambiae* have shown that

decreased expression of a serpin-3 ortholog (SRPN2) results in the formation of melanotic pseudotumors and increases lysis of ookynetes. It is likely that all of these effects are caused by excessive synthesis of melanin (Michel et al., 2005). Studies in *Musca domestica* L. also found a 4 kDa peptide as an efficient PO inhibitor. This peptipe directly inhibits the PO activity rather than PPAEs (Tsukamoto et al., 1992). Finally, studies in *M. sexta* showed that dopachrome isomerase is also a PO regulator. Because dopachrome isomerase is activated by the insect's own presence of PO, this leads to down-regulation (Sugumaran et al., 2000).

Phenoloxidase in an evolutionary ecology context

Recently, evolutionary biologists and immunologists alike have studied how physiology and ecology interact to drive the evolution of immune defense systems (reviewed by Schulenburg et al., 2009). A key assumption in these studies is that there are individual differences in immune ability. Variation in immune ability may be maintained via costs that emerge either through limited resource availability and/or pleiotropic effects on non-immune related, genetically linked traits (Schmid-Hempel, 2005). In the following section, we review studies of PO activity in the context of pathogen defense, their costs and the environment*gene interaction to explain PO activity.

Just how useful is PO against pathogens?

Table 1 summarizes studies in which a variety of immune challenges were used and an association was examined between proPO and/or PO activity and indicators of individual success against any pathogen agent. We did not find broad support for a positive relationship between PO activity and successful pathogen defense (Table 1). A number of reasons can be put forward to explain this unexpected finding before accepting the idea that PO is not useful against pathogens. First, many studies failed to estimate the severity of the pathogen challenge used (e.g., failed to measure the median lethal dose LD_{50}). Thus, some studies used challenges that far from represented a real threat when too low a dose was used, or else overwhelmed any differential individual immune response and induced antiseptic shock when too high a dose was used. A second reason is that many studies did not consider that PO may be used for other functions besides immunity (i.e., egg production, pigment synthesis, molting, or sclerotization), and hence measuring total PO activity in a sample cannot be considered as a good indicator of immunocompetence. This can be solved by finding out which PO deals with immunity and then to quantify its activity. Third, PO may only be activated for short periods of time in specific tissues and also acts in different ways depending on the immune challenge (Nappi & Christensen, 2005). As an alternative, immunoecological studies could measure proPO activators such as serine proteases, activation of proPO genes, or even proPO itself for more accurate measurements of immunity (Cerenius et al., 2008). One final explanation is that the use of dead pathogens may trigger different responses than real pathogens (e.g., Contreras-Garduño et al., 2007). This has to be properly controlled once such variation in response is known.

Resource-based costs of PO production

The cost of production and maintenance of the PO system (including the proPO-activating system) is likely to be high for two reasons. First, the main compound of the proPO-activating system – tyrosine – is obtained from phenylalanine, which can only be obtained from ingested food (Chapman, 1998). Second, melanin, a final product of proPO-activating system, is a nitrogen-rich compound, which may require substantial nitrogen or protein investment for its synthesis (Blois, 1978; Lee et al., 2008). Thus, production and maintenance of the proPO-activating system is dietary-dependent. Several laboratory studies have supported this. For example, the mealworm beetle, *Tenebrio molitor* L., and the damselfly *Hetaerina americana* Fabricius that were fed ad libitum showed higher PO values than males that were non-fed (Siva-Jothy & Thompson, 2002; Rantala et al., 2003; González-Tokman et al., 2011). Other studies have also found that a protein-based diet is needed for an optimal proPO-activating system. For example, in the Mormon cricket, *Anabrus simplex* Haldeman, animals fed a high-protein diet showed higher values of total PO activity than animals fed a high-carbohydrate diet (Srygley et al., 2009). Related results were found in the caterpillars *Spodoptera exemptra* Walker and *Spodoptera littoralis* Boisduval: infected animals fed a higher protein:carbohydrate ratio had more PO activity and survived for longer than those fed a lower protein:carbohydrate ratio (Lee et al., 2006; Povey et al., 2009). A possible explanation for these results is that protein content directly affects the production of amino acids, which can be used for the synthesis of several compounds of the proPO-activating system, including PO itself (Abisgold & Simpson, 1987).

If PO production is diet-dependent, then it follows that individuals in good condition [condition defined as any trait closely linked to viability (Iwasa et al., 1991; Iwasa & Pomiankowski, 1994)] ought to have high levels of proPO and PO production. We have reviewed the existing evidence testing this prediction by filtering studies in which different sources of stress were studied in relation to their

Table 1 Phenoloxidase as an indicator of strength against pathogens

Species	Immune challenge	Relation PO/proPO activity and pathogen defense	Basis of relation	References
Spodoptera exempta	Contaminated diet with nuclear polyhedrosis virus (NPV)	Positive	Larvae with higher survival after an NPV infection showed higher PO activity in hemolymph	Reeson et al. (1998)
Calopteryx splendens xantostoma	Eugregarine parasite burden	Negative	Males with higher capacity to fight off eugregarines had lower PO levels	Siva-Jothy (2000)
Gryllus texensis	Inoculation with *Serratia marcescens*, *Serratia liquefaciens*, and *Bacillus cereus*	Non-significant	Total PO levels did not predict male survival after three bacterial challenges	Adamo (2004)
Daphnia magna	Infection with one bacterial species (*Pasteurina ramosa*) and three microsporidia species (*Octosporea bayeri*, *Ordospora colligate*, *Glugoides intestinalis*)	Non-significant	PO activity in hemolymph did not predict immunocompetence in any immune-challenged subjects	Mucklow et al. (2004)
Drosophila melanogaster	Natural infection with fungus *Beauveria bassiana* and challenge with a needle contaminated with the yeast *Candida albicans*, the gram− bacteria *Agrobacterium tumefaciens* and *Escherichia coli* or the gram+ bacteria *Enterococcus faecalis* and *Staphylococcus aureus*	Non-significant	*Drosophila* mutants that fail to activate PO in the hemolymph following microbial challenge were as resistant to infection as wild-type flies	Leclerc et al. (2006)
Hetaerina americana	Inoculation with *S. marcescens*	Positive	Territorial males subjected to bacterial inoculation and nylon monofilament implants had higher PO activity and survival for longer than non-territorial males	Contreras-Garduño et al. (2007)
Litopenaeus vanamei	Challenge with white spot syndrome virus	Negative	Down regulation of proPO	Ai et al. (2008)
Plodia interpunctella	Oral inoculation and direct intrahaemocoelic injection of naturally occurring granulosis virus (PiGV)	Non-significant	No elevated hemolymph PO activity during the early stage of infection. Hemolymph PO activity increased in the susceptible (infected) larvae only at a later stage of the infection	Saejeng et al. (2010)
Hetaerina vulnerata	Inoculation with *S. marcescens*	Non-significant	Males subjected to bacterial inoculation had higher PO activity after 24 h, but survival was not related	González-Santoyo et al. (2010)
Daphnia magna	Spores of *P. ramosa*	Positive	PO levels in infected hosts did predict spore load	Pauwels et al. (2011)

effect on animal condition. These studies appear in Table 2. From this review, there is clear evidence that individuals in better condition produce higher levels of proPO and/or PO. The fact that stressors across these studies varied so considerably suggests that proPO and PO are shaped by a suite of environmental factors.

Table 2 Relationship between phenoloxidase and individual condition

Species	Stressful source affecting condition	Relation condition-proPO/PO	Basis of relation	References
Spodoptera exempta	Population density	Positive	Larvae reared at high density had significantly higher PO levels	Reeson et al. (1998)
Gryllus texensis	Aging	Negative	Old males showed lower PO values than mature males	Adamo et al. (2001)
Schistocerca gregaria	Population density	No relation	Animals in *solitaria* and *gregaria* phase did not differ in PO activity	Wilson et al. (2002)
Tenebrio molitor	Nutritional status	Positive	Starvation reduced the PO activity	Siva-Jothy & Thompson (2002)
S. exempta	Population density	Positive	Animals kept under crowded conditions showed higher PO values in hemolymph, midgut, and cuticle compared to animals kept in solitary conditions	Wilson et al. (2001)
Daphnia magna	Nutritional status	Positive	Well-fed *Daphnia* showed higher PO activity than *Daphnia* kept at a low food level and wounding provoked a higher level of PO activity	Mucklow & Ebert (2003)
T. molitor	Nutritional status and attractiveness	Positive	Males in better nutritional conditions had more PO activity and were more attractive for females	Rantala et al. (2003)
T. molitor	Attractiveness	Positive	More attractive males had higher PO levels after an immune challenge by a nylon monofilament insertion	Sadd & Schmid-Hempel (2006)
Lestes viridis	Predation risk	Negative	The presence of a predator (fish) reduced PO and proPO activity in early hatched larvae	Stocks et al. (2006)
Hetaerina americana	Territoriality	Positive	Territorial males had higher PO activity levels than non-territorial males	Contreras-Garduño et al. (2007)
Epirrita autumnata	Nutritional status	Negative	Starvation during the final phase of larval development increased both the absolute and specific PO activity	Yang et al. (2007)
Bombus terrestris	Aging	Negative	PO activity decreased with age	Moret & Schmid-Hempel (2009)
Aedes aegypti	Nutritional status	No relation	No direction for males and females	Moreno-García et al. (2010)
H. americana	Nutritional status	Positive	Males under starvation showed lower PO values than ad libitum fed males	González-Tokman et al. (2011)

Life-history theory predicts that competition for finite resources leads to resource allocation conflicts among different functions (Stearns, 1992). Two relevant questions in this context are: (1) which functions become impaired when insects are immune challenged and (2) what physiological mechanisms mediate resource allocation in insects? As for the first question, various immunoecological studies have provided direct and indirect evidence that resources allocated to proPO and/or PO production affect a plethora of functions. Some examples of traits affected by immune function resource allocation trade-offs are production of sexual traits (e.g., Siva-Jothy, 2000; Rantala et al., 2003; Pomfret & Knell, 2006; Sadd et al., 2006), mating (Rolff & Siva-Jothy, 2004), aggressive behavior (Contreras-Garduño et al., 2009), and larval development (Cotter et al., 2004). The wide array of affected functions suggests that there are common pathways in the utilization of resources. The presence of these common pathways also suggests that in many life stages, insect hosts are subject to unavoidable risks of infection. As for the second question, juvenile hormone (JH) is a strong candidate as a physiological mechanism to regulate insect resource allocation. Juvenile hormone is a hormone produced by the corpora allata that affects a number of developmental and life history functions in insects. For example, the presence of JH favors sexual maturation, vitellogenesis, reproduction, and metabolism, but its absence leads to a reduction in lifespan, survival, diapause, stress resistance, and immunity (Flatt et al., 2005). One example that shows not only JH control but also the effects of JH on very different functions is the case of calopterygid damselflies. In these animals, experimental increase of a JH analog (methoprene) led to increased aggression during territorial behavior at the expense of PO activity in males (Contreras-Garduño et al., 2008). Similarly, the JH analog also led males to develop increased wing pigmentation, a sexual trait used to court females, at the cost of survival (Contreras-Garduño et al., 2011).

Pleiotropic effects of PO activity

Studies of narrow-sense heritability (h^2) have concluded that PO is a heritable trait (h^2>0.8) in *S. littoralis* (Cotter & Wilson, 2002; Cotter et al., 2004), in the yellow dung fly, *Scathophaga stercoraria* L. (Schwarzenbach et al., 2005; Schwarzenbach & Ward, 2007), the mealworm beetle *T. molitor* (Armitage & Siva-Jothy, 2005), and the cricket *Gryllodes sigillatus* Walker (Gershman et al., 2010a,b). This is not surprising, given the strong relationship of PO with fitness (reviewed by Schulenburg et al., 2009). However, due to this relationship, it is also expected that natural selection will erode additive genetic variation (Stearns, 1992). It has been suggested that one possible way to maintain such high genetic variance is via antagonistic pleiotropy between PO and other traits (Cotter et al., 2004), that is, a gene or set of genes with positive effects on one component of fitness, but a negative effect on another component (Roff, 1992). Probably, the most common example of such a genetic trade-off comes from a study of experimental selection in the yellow dung fly, *S. stercoraria* (Hosken, 2001). After 12 generations of enforced monogamy or polyandry, PO values in polyandric lines were lower than in monogamous lines. However, polyandric flies also evolved larger reproductive organs. These results indicate a potential evolutionary trade-off between investment in reproductive tissues and immunity (Hosken, 2001). More recent studies have found genetic trade-offs between PO activity and cuticular darkness (Rolff et al., 2005), development rate (Cotter et al., 2004, 2008b), survival (Schwarzenbach & Ward, 2006), and pupal mass (Klemola et al., 2007). Interestingly, antagonistic pleiotropy has been detected between PO and lysozyme-like activity, another key immune component (Moret & Siva-Jothy, 2003; Cotter et al., 2004, 2008a; Bailey & Zuk, 2008). All these pieces of evidence strongly indicate that increases in PO-based immune ability may not outweigh its benefits. Therefore, selection should favor individuals that are more prudent in their investment into PO. For example, if pathogen action is not as challenging in a certain environment, selection may act to favor a PO reduction so that fitness can be maximized. One example of a genotype*environment interaction is the negative effect on survival in *S. stercoraria* shown by Schwarzenbach & Ward (2006), which only occurs when animals are not provided with food.

Finally, PO has positive genetic correlations with functions that seem costly. For example, selection on PO activity led to larger first clutches (Schwarzenbach & Ward, 2006), pupal weight, and adult longevity (Cotter et al., 2004).

Gene*environment interaction in the study of PO

A genetic background that allows different phenotypes is likely to have an advantage in heterogeneous environments (Zhivotovsky et al., 1996). The ability of a genotype to give rise to distinct phenotypes when exposed to different environments is known as phenotypic plasticity (Roff, 1997; Nylin & Gotthard, 1998; Schlichting & Pigliucci, 1998). Given the role of environment and the genetic basis of PO, it is not surprising that this trait is phenotypically highly plastic. Phenotypic plasticity in PO production has been investigated in lepidopterans (Cotter et al., 2008a), beetles (Barnes & Siva-Jothy, 2000), and mosquitoes (Moreno-García et al., 2010). These studies indicate the extent of the variance in PO activity caused by environmental influences. For example, and contrary to what has

been determined for other insects (Siva-Jothy & Thompson, 2002; Rantala et al., 2003; González-Tokman et al., 2011), PO was not food dependent in mosquitoes (Moreno-García et al., 2010). Whether other insects also show this and whether this is an adaptive response remain to be seen.

Some remarks for future development

In the last decades, there has been growing knowledge about PO activating systems, especially regarding the components that activate or inhibit PO activity in insect immune systems. However, there are still gaps in our understanding of the PO activating system. Some of these gaps are a consequence of the intrinsic properties of PO. For example, most cells that synthesize PO are fragile hemocytes that are difficult to study under controlled conditions (i.e., in culture). Also, intermediate molecules involved in the PO activating system are rapidly activated and degraded, which makes their isolation and recognition difficult. Due to these logistical challenges, some aspects remain to be elucidated. For example, although it is known how quinones are polymerized to melanin in vitro, it remains unknown how this takes places in vivo. Also, despite the discovery that some molecules that act as protein cofactors of serine proteases (i.e., SPH1, SPH3) are involved in PO activation by gram+ bacteria or fungi, it is still unclear whether there are analog cofactors acting in the face of infections by gram− bacteria.

Another important gap is that the natural substrates of PO are unknown. Under laboratory conditions, DOPA has been used as the main PO substrate in in vitro studies, but cathecols such as NADA and NBAD are also present in insect hemolymph and could be used as natural substrates of PO in insect immune response. Indeed, PO has higher affinity to NADA and NBAD than to DOPA (Asano & Ashida, 2001). Furthermore, more research is needed to determine which substrates are used by PO in response to infections with different pathogens.

Immunoecological studies are still in their infancy. A basic issue is to resolve the extent to which PO is the immune parameter against pathogens. The insect immune system is composed of a number of other components in addition to PO which have received relatively less attention. These other components, along with PO, should be examined in a more coordinated manner to form an integrated view of the evolutionary ecology of PO. One example is the detected negative genetic correlation between PO and lytic activity (Cotter et al., 2004), which constrains the host response to immune challenges. In relation to the environmental action, future studies should address PO-related questions with more realistic scenarios of natural interaction between hosts and their pathogens to explain coevolutionary scenarios. In this respect, it remains unclear, for example, why both sexes (Moreno-García et al., 2010), different stages of the same individual (Eleftherianos & Revenis, 2011), different seasonal times of the same species (Córdoba-Aguilar et al., 2009), and different but closely related species (Córdoba-Aguilar et al., 2009 et al., 2006) show differences in PO activity. These differences may be related to differential exposure to distinct pathogens. Moreover, not only gene*environment but also gene*gene interactions of hosts and their pathogens can explain susceptibility to infection (Lazzaro & Little, 2009), which has not been studied for PO. One final issue is to relate PO activity with selection strength, which will show which PO phenotypes are favored (e.g., Rolff & Siva-Jothy, 2004). As with a number of physiological traits, little is known about the strength of natural selection, and given the importance of PO for survival, future studies should address this.

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CAPITULO III

Identificación de los pigmentos de la mancha roja de las alas en la libélula *Hetaerina americana*

Isaac González-Santoyo, Daniel González-Tokman & Alex Córdoba-Aguilar

Capitulo III
Identificación de los pigmentos de la mancha roja de las alas en la libélula *Hetaerina americana*

Resumen.

La pigmentación alar en los machos de *H. americana* ha sido definida como un carácter sexual secundario seleccionado vía competencia intrasexual. Dicho atributo ha sido motivo de diversos estudios ecológicos en donde se ha determinado que su tamaño está asociado con la capacidad de defensa de un territorio, oportunidades de apareamiento, supervivencia y en general, la condición fisiológica del portador. Sin embargo, una parte clave en el entendimiento del porqué de estas relaciones, es el conocer la base bioquímica de la pigmentación alar, la cual aún no ha sido explorada. En este capítulo presento los resultados de indagar la naturaleza bioquímica del pigmento rojo en *H. americana* usando para ello diferentes pruebas de identificación basadas en las propiedades fisicoquímicas de las omocromas, uno de los grupos de pigmentos que comúnmente se asocia al color rojo en insectos. Encontré que la pigmentación alar roja es producto de la presencia de la forma reducida de la omocroma xantomatina. Dado que estos pigmentos se forman a partir de la ruta metabólica del triptófano, un aminoácido esencial que se obtiene únicamente por la dieta, es posible que la xantomatina depositada en las alas puede fungir como un indicador de qué tan bien alimentado esté un individuo. De ser cierto, la mancha sería una señal que refleja la condición fisiológica del portador.

Introducción

H. americana, una especie de odonato que ha sido motivo de estudios ecológicos exhaustivos (Grether 1996; Contreras-Garduño et al., 2007; González-Santoyo et al., 2010; Anderson y Grether, 2010) presentan un peculiar atributo en sus alas, una pigmentación de color roja en los machos, la cual ha sido propuesta como un caracter sexual secundario (CSS) seleccionado gracias a la competencia intrasexual de territorios (Grether, 1996).

Los estudios arriba citados en *H. americana* han determinado que el tamaño de la pigmentación alar en los machos se asocia con la capacidad de defensa de un territorio, siendo el territorio la vía de acceso a las hembras y por ende de su éxito de apareamiento (Grether 1996; Contreras-Garduño et al., 2007; Serrano-Meneses et al., 2007). El tamaño de la mancha, además, se correlaciona positivamente con una serie de aspectos de la condición del macho como es las reservas de grasa, la respuesta inmune, y la supervivencia (Contreras-Garduño et al., 2007; Fig. 1).

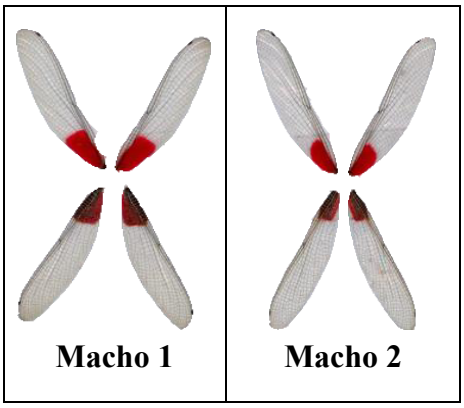

Fig 1. Pigmentación alar roja de dos machos de *H. americana*. Se puede observar que el individuo 1, el cual presentaba una conducta territorial, muestra una mayor pigmentación alar que el individuo 2, siendo este último un macho no territorial.

Una parte clave en el entendimiento del porqué de la relación entre la condición del individuo, su éxito de apareamiento, supervivencia y el tamaño de la mancha, es conocer la naturaleza bioquímica que da lugar a la pigmentación en las alas, aspecto que no ha sido explorado y que es necesario para la interpretación correcta de dichas asociaciones.

La pigmentación alar en los insectos usualmente se debe a 4 diferentes componentes: melaninas, omocromas, pterinas y flavonoides (Nijhout, 1991; Champan, 1998). Sin embargo, la coloración roja esta asociada principalmente al grupo de las omocromas, las cuales han sido reportadas en varias especies de lepidópteros (e.g. *Precis*; Nijhout, 1997), e inclusive odonatos (e.g. *C. servilia, S. darwinianum y S. frequens*, Futahashi et al., 2012). Este grupo de pigmentos son el resultado de la ruta metabólica del triptófano en donde las omatinas más comunes son la xantomatina y la rodomatina (Linzen, 1974). Debido a sus principales propiedades fisicoquímicas tales como la cualidad de oxido-reducción, absorción en luz ultravioleta y visible y baja solubilidad, las omocromas pueden actuar como receptores y donadores de electrones, lo que las convierte en pigmentos dinámicos que pueden modificar la coloración de los individuos que los posean. Por ejemplo la xamtomatina es ampliamente utilizada en el mimetismo de muchos grupos de arácnidos (e.g Oxford y Gillespie, 1998), en cambios de coloración entrada la madurez sexual o causantes del dimorfismo sexual en algunos odonatos (eg. Futahasi et al., 2012). Otra función muy importante de las omocromas es que son el grupo de pigmentos oculares mas importes y de mayor presencia en los ojos compuestos de todos los insectos, esto gracias a su capacidad de reducir la energía de luz que incide sobre los receptores oculares para lograr un incremento en la agudeza visual (Linzen, 1974). Finalmente, una función que ha sido poco explorada es su posible papel como productos finales del metabolismo. Se ha reportado que el color rojo-carmín presente en el meconio de un gran numero de lepidópteros se debe a la presencia de omocromas

(Chapman, 1998), las cuales se originan posiblemente a causa de funciones metabólicas normales en la metamorfosis. El proceso catabólico y anabólico que ocurre durante la metamorfosis origina que los individuos tomen sus reservas proteicas como un recurso energético y como nuevas cadenas polipeptídicas para la construcción de otras proteínas; esto puede generar un exceso de triptófano o sus metabolitos, los cuales son altamente tóxicos en etapas adultas (Cerstiaens et al., 2003). Por lo tanto, se ha propuesto que otra función de las omocromas es la inactivación de los efectos tóxicos de sus precursores a través de la formación de las mismas (Linzen, 1974; Chapman, 1998).

En el presente capítulo puse a prueba la hipótesis de que el grupo de las omocromas son los pigmentos que dan lugar a la coloración roja en las alas de los machos de *Hetaerina americana*. Durante mi estudio utilicé 4 pruebas que se basan en las principales propiedades fisicoquímicas de dichos pigmentos, la solubilidad, reacción óxido-reducción, absorbancia en el espectro UV-Visible y cromatografía de capa fina. Mis resultados los discuto en el marco de las relaciones entre el tamaño de la mancha, la adecuación y la condición de los machos de *H. americana*.

Materiales y Métodos

1. **Animales y preparación del pigmento**

Para la extracción y determinación de las omocromas se utilizaron 30 machos adultos. Los individuos fueron capturados en noviembre de 2009 en las riveras del rio Tetlama ubicado en el estado de Morelos, México. Poco después de su colecta los machos fueron sacrificados por congelación a -20°C durante 20 minutos. Este método de sacrificio evita los posibles efectos de algún componente químico en la determinación del pigmento. Posterior a su muerte, todos los individuos fueron secados, colocándolos en una estufa de cultivo (BG, E-41) a 40°C durante 24 horas. A cada individuo se le extrajeron los 2 pares de alas, de las cuales se recortó la mancha roja para los análisis.

2. **Extracción del pigmento**

La extracción de las omocromas ubicadas en zona alar pigmentada se basó en la técnica descrita por Nijhout en 1997 con algunas modificaciones tomadas de Umebachi y Uchida, 1970.
Las manchas fueron trituradas y homogenizadas utilizando un mortero de porcelana y metanol al 99% a una temperatura de 4°C. El homogenado fue entonces centrifugado durante 5 minutos a 14,000 rpm y el sobrenadante fue desechado. Posterior a la centrifugación, el precipitado fue lavado por dos tiempos con metanol 99% y tres con éter etílico. Cada lavado fue acompañado por sucesivas suspensiones y centrifugados de 5 minutos a 14,000 g. En el último lavado (con éter etílico), el extracto fue colocado en la campana de cultivo a 40°C hasta que el solvente se evaporó completamente (alrededor de 15 minutos). Al residuo seco obtenido de los lavados se le adicionó 4ml de ácido clorhídrico (HCl) al 2% disuelto en 99% de metanol. La solución obtenida fue centrifugada durante 10 minutos a 14,000 rpm y el sobrenadante fue recuperado. El sobrenadante fue concentrado a un cuarto de su volumen utilizando un concentrador SpeedVac

(Thermo®, Mod. ISS110) y se mezcló con 1.5 ml de agua destilada, adicionándole además un poco del gas monóxido de di-azufre (SO) para dejarlo reposar 24 horas a 4°C. El pigmento rojo resultante fue precipitado por centrifugación (10 min, 14,000 rpm) y se desechó el sobrenadante. Este nuevo precipitado fue disuelto en una pequeña cantidad de buffer de fosfatos sorensen (pH 8.5) y 2 ml de agua destilada fría, para volver a centrifugarlo por 10 minutos (14,000 rpm). Después del centrifugado, una pequeña cantidad de SO fue introducida al sobrenadante y la solución se dejó reposar a 4 °C durante 24 horas. Finalmente, la solución obtenida fue lavada con una pequeña cantidad de agua fría, centrifugada (10 min, 14,000 rpm) y el sobrenadante fue decantado y desechado. En este último lavado se obtuvo como precipitado pequeñas cantidades de un polvo color parduzco o rojizo, el cual fue utilizado para la identificación de las omocromas

3. Pruebas para la identificación de las omocromas

a) Solubilidad

La solubilidad es una de las propiedades principales de las omocromas ya que son insolubles en todos los solventes orgánicos neutrales, agua y ácidos diluidos (Linzen, 1974). Variando en su grado de solubilidad, las omocromas pueden diluirse en soluciones altamente alcalinas o ácidos fuertemente concentrados. Para mi estudio seleccioné 8 tipos de solventes, que se dividieron en dos grupos dependientes de su grado solubilidad sobre las omocromas; los solventes y los no solventes. El pigmento purificado fue diluido en cada uno de los distintos compuestos y se comparó su solubilidad entre estos grupos. Para los compuestos solventes se utilizó metanol ácido (HCl al 2% disuelto en metanol 99%), HCl 2N y buffer sorensen a pH 8.5, mientras que para los no solventes, se usó agua destilada, éter etílico, acetona, HCl 0.01N y buffer sorensen a pH 5.5.

b) Reacción Óxido-Reducción

La propiedad química mas conspicua de las omocromas es el cambio de coloración gracias a su capacidad de oxidación y reducción reversible o reacción REDOX (Linzen, 1974). En un estado reducido las omocromas forman coloraciones rojas mientras que en un estado oxidado la coloración originada es amarilla. Para examinar esta propiedad el pigmento purificado se disolvió en un buffer de fosfato sorensen (0.067M, pH = 8.5) al cual se le adicionó 10 µl de ácido ascórbico como componente reductor y se observó el color de pigmento formado. Posteriormente a dicha solución se le agregó 10 µl de nitrito de sodio ($NaNO_2$) como componente oxidativo y se observó el cambio de coloración. Por último, se volvió a adicionar 10 µl de ácido ascórbico para observar el cambio a la coloración original.

c) Absorbancia en el espectro ultravioleta y visible

Dado que todos los metabolitos del triptófano retienen la fluorescencia del anillo aromático (Linzen, 1974), las omocromas presenta una conducta espectral muy peculiar (Bolognese et al., 1988), la cual se caracteriza por la presencia de 2 a 4 picos de absorbancia distribuidos en los rangos UV y visible.

Los picos espectrales de las omocromas han sido tradicionalmente caracterizados en presencia de solventes como buffer sorensen a pH neutro y con HCl al 5N (Umebachi y Uchida, 1970; Linzen, 1974). Para esta prueba, disolví el pigmento purificado utilizando los mismos solventes y obtuve los picos de absorbancia con el uso de un espectrofotómetro (Hitachi, Mod. U-3900) a lo largo de una longitud de onda de 230 nm a 600 nm. La omocroma xantomatina ha sido ampliamente caracterizada con lo que respecta a sus picos de absorbancia, por lo que en este estudio se convirtieron todas las posibles omocromas presentes en el pigmento, como omatina-D, en xantomatina y de esta manera caracterizar óptimamente los picos de absorbancia que

presentaba el pigmento purificado. Para esto, el pigmento disuelto con HCl 5N se dejó reposar durante 5 días (Umebachi y Uchida, 1970) y se observaron los picos de absorbancia a las longitudes mencionadas anteriormente. Finalmente, los picos obtenidos fueron comparados con los encontrados por Umebachi y Uchida (1970), al igual que los observados por Nijhout (1997).

d) Cromatografía de capa fina

Por último, para determinar el o los tipos de omocromas contenida en la pigmentación alar de *H. americana*, separé los compuestos presente en el pigmento purificado a través de una cromatografía de capa fina (CCF) siguiendo la metodología descrita por Nijhout (1997).

Para la CCF se utilizó como fase estacionaria polar una placa cromatografica de sílica gel (Merck, Darmstadt, Germany, Silica Gel 60 F254) y una solución de fenol: agua al 3:1 como el eluyente líquido apolar. La placa cromatrográfica se dividió en 4 canales a 2 cm de distancia entre ellos. En cada canal se colocaron 10µl del pigmento purificado disuelto en metanol ácido (HCl 2% disuelto en metanol 99%). La placa fue colocada en posición vertical sobre una cámara cromatográfica rectangular (Aldrich) saturada por el eluyente (fenol: agua) utilizando papel filtro alrededor de la misma. Esta placa se dejó reposar dentro de la cámara durante 2 horas para permitir tanto la completa separación de los compuestos como el desplazamiento final del eluyente a través de la placa. Terminado este tiempo se extrajo la placa y se identificaron todas las bandas presentes en el espectro de visible y en el ultravioleta con el uso de un transiluminador UV/Blanca (a 312nm para UV). A cada banda identificada se le determinó su valor constante o valor de referencia (Rf), dividiendo la distancia recorrida por el compuesto separado (cm) entre la distancia recorrida del eluyente. Los valores Rf de los compuestos encontrados en cada banda fueron promediados y comparados con los valores Rf encontrados por Nijhout (1997) en omocromas purificadas y caracterizadas.

Resultados

Se encontró que la pigmentación alar roja en la alas de *H.americana* es producto de la presencia de la omocroma xantomatina y de su estado reducido la dihidro-xantomatina. Esta conclusión se baso en las cuatro pruebas realizadas:

a) Solubilidad

El pigmento rojo purificado fue altamente soluble en los compuestos seleccionados como solventes de omocromas (Linzen, 1974), metanol ácido, buffer sorensen a pH = 8.5 y ligeramente soluble en HCl 2N. Mientras que fue insoluble en los solventes de agua, acetona, éter etílico, HCl 0.01N y buffer sorensen a pH = 5.5.

b) Oxido-reducción

Al disolver el pigmento purificado en un buffer de fosfatos (.067M, pH = 8.5) la solución presentaba una coloración amarilla lo cual sugiere la posible presencia de omocromas. Esta solución se tornó roja al adicionar ácido ascórbico al 1% (señal de la reducción de las omocromas), y nuevamente amarilla al adicionar $NaNO_2$ al 1%. Dicha reacción REDOX mostró la presencia de omocromas en el pigmento purificado.

c) Absorbancia en el espectro UV-Visible

Los picos de absorbancia mostrados para el pigmento disuelto en buffer de fosfatos (pH=7.0) fueron de 227-236 nm y de 439-445 nm (Fig. 2a), mientras que el pigmento disuelto en HCl fueron de 243nm, 370-375nm y 475nm (Fig. 2b). Tanto los valores encontrados en la dilución con buffer de fosfatos como en HCl coinciden con los observados previamente para xantomatina descritos inicialmente por Umebachi y Uchida (1970), Linzen (1974) y finalmente por Nijhout (1997).

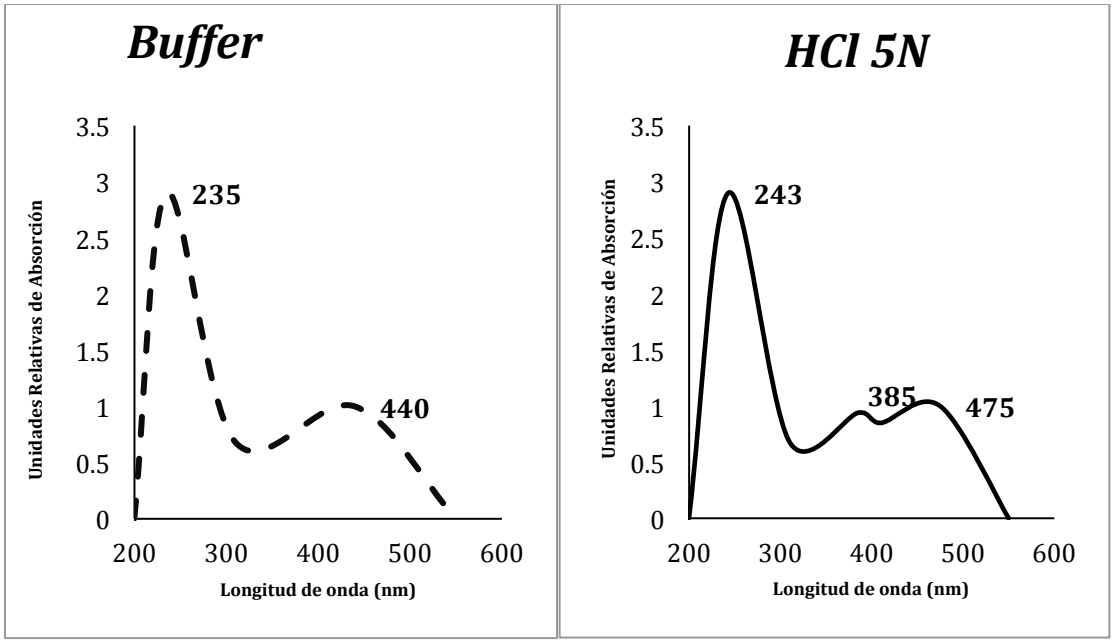

Figura 2. Picos de absorbancia utilizando buffer de fosfatos (a) y HCl (b) como solventes del pigmento rojo en las alas de machos de *H . americana*.

d) Cromatografía de capa fina

El análisis de CCF sobre el extracto de pigmento alar produjo tres diferentes bandas en los cuatro canales colocados. La primera banda de color parduzco-rojizo en luz visible y observable en UV

obtuvo un Rf promedio de 0.15 ± 0.015. Estos valores coinciden con la omocroma dihidro-xantomatina (Rf = 0.13; Nijhout, 1997). La segunda banda de color amarillo y observable también en UV obtuvo un valor Rf de 0.36 ± 0.012 el cual coincidió con el Rf que previamente fue reportado para la omocroma xantomatina purificada de *Precis coenia* (Rf= 0.36; Nijhout, 1997). La ultima banda de un tenue color amarillo-café y ligeramente observable en UV obtuvo un Rf de 0.53 ± 0.018, la cual presumiblemente pertenece a la 3-hidroxikinurenina, precursor de las omocromas y reportada por Nijouht (1997) con un Rf = 0.56 (Fig. 3).

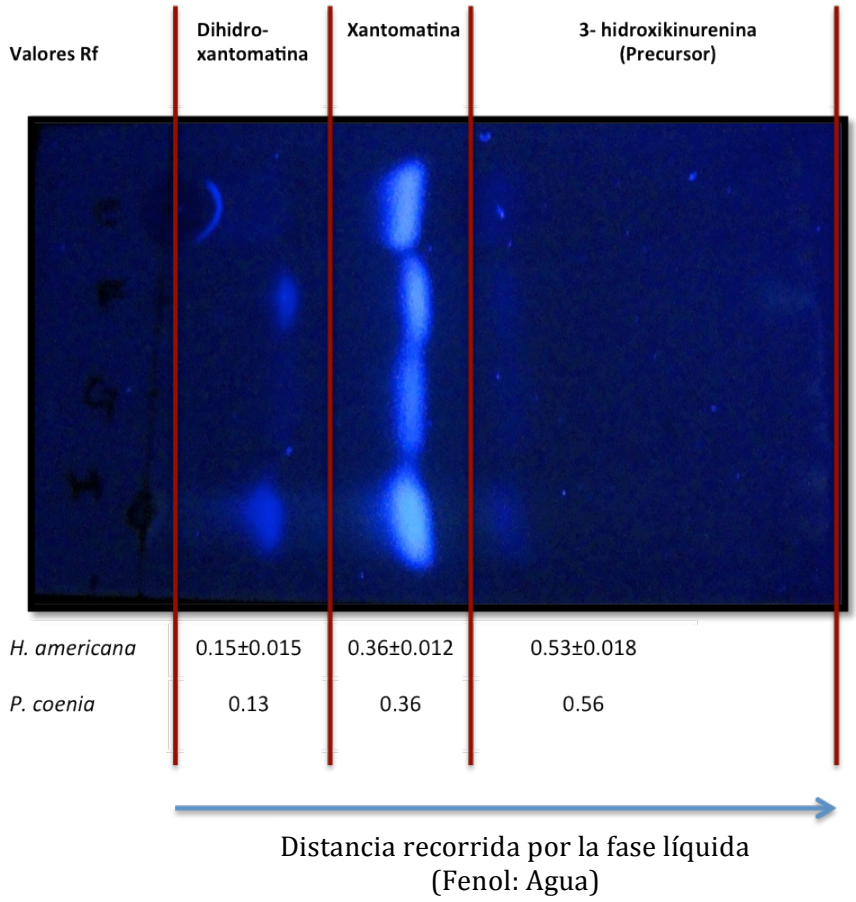

Fig. 3 Fotografía en luz UV y valores Rf de las omocromas xantomatina, dihidro-xantomatina y su precursor 3- hidroxikinurenina extraídas del pigmento purificado de las alas de machos de *H. americana*. Los valores Rf obtenidos fueron comparados con los Rf de omocromas purificadas de *Precis coenia* por Nijhout en 1997.

Discusión

Se determinó que la pigmentación alar roja en *H. americana* es producto de la presencia de la forma reducida de la omocroma xantomatina. Esta omocroma es depositada a manera de gránulos pigmentarios en la células alares, las cuales son formadas por dos capas de tegumento estrechamente sobrepuestas y unidas por un conjunto de venaciones que se conectan con el hemocele para que la hemolinfa pueda fluir libremente hacia las alas (Chapman, 1998). La síntesis de xantomatina puede ocurrir dentro del citoplasma de la células epidermales tomando su precursor la 3-Hidroxikinurenina de la hemolinfa (Linzen, 1974), mientras que la estabilización en su forma reducida a manera de dihidro-xantomatina, necesaria para la formación de la coloración roja, puede darse gracias a la unión de este pigmento con una serie de proteínas que la protegen de la oxidación. Apoyando esta idea se ha encontrado que las omocromas presentes en la forma estacionaria de color rojo-parduzco en la especie polimórfica del lepidóptero *Precis coenia* poseen un único sitio de anclaje proteico el cual no está presente en la forma de color pálido (Rountree y Nijhout, 1995).

El tamaño de la mancha roja en *H. americana* se ha asociado positivamente con capacidades inmunológicas, reservas energéticas y supervivencia tanto en el campo como en condiciones controladas (Contreras-Garduño et al., 2006, 2007). La identificación química de los pigmentos de la mancha es de suma importancia para estudios futuros que ayuden a entender los vínculos con funciones fisiológicas. En adelante expongo un marco teórico para entender el mecanismos funcional de la mancha roja dada la identificación de sus pigmentos.

Puesto que la xantomatina es producto de la ruta metabólica del triptófano (Linzen, 1974), la cantidad de pigmento sintetizado dependerá en gran medida de la cantidad y capacidad para

adquirir este aminoácido esencial, el cual se obtiene únicamente a través de la alimentación (Champan, 1998). En *H. americana* es ampliamente conocido que la pigmentación alar es un CSS utilizado para definir contiendas macho-macho por la adquisición de territorios (Serrano-Meneses et al., 2007), siendo el individuo con mayor pigmentación alar el que tendrá mayor posibilidad de obtener el territorio (Grether, 1996; Contreras-Garduño et al., 2007). Sin embargo, poco se había conocido acerca de la base fisiológica de esta relación, una hipótesis es que el pigmento xantomatina depositado en las alas puede fungir como un indicador de qué tan bien alimentado esté un individuo, convirtiéndose así, en una señal honesta de la condición fisiológica del portador. Este tipo de señales son de suma importancia para evaluar la condición del contrincante previa a un encuentro agonístico y evitar encuentros costosos e innecesarios (Jonhstone y Norris, 1993).

De manera interesante, el precursor de la xantomatina, la 3-hidroxikinurenina, es un metabolito con propiedades neurodegenerativas en fases adultas de insectos, el cual puede ocasionar desde parálisis temporal hasta la muerte (Linzen, 1974; Cerstiaens et al., 2003). Dicho metabolito fue encontrado también en el extracto del pigmento alar obtenido de *H. americana*. Por lo tanto, el color rojo como sucede en otros grupos, podría también servir para disuadir depredadores dado el posible carácter tóxico de la mancha. Por ejemplo, podría ser que los machos advierten su naturaleza tóxica con una coloración tipo aposemática, de tal forma que un depredador asocie el color rojo con aspectos negativos como mal sabor o veneno (Svádová et al., 2009).

La naturaleza química del pigmento rojo sirve de base para nuevas preguntas en un contexto evolutivo y ecológico. Por ejemplo, la presumible toxicidad del pigmento de la mancha podría ser seleccionado positivamente en *H. americana*. Un ejemplo es el de la polilla *Utetheisa ornatrix* donde los machos presentan un mecanismo que convierte una parte de los alcaloides

tóxicos obtenidos en la dieta por feromonas que son utilizadas para atraer hembras (Eisner y Meinwald, 1995). El resto de los alcaloides son transferidos a las hembras para utilizarse en los huevos y evitar su depredación (Eisner et al., 2000). En este sistema el macho evolucionó mecanismos para evitar los efectos tóxicos de los alcaloides, al igual que para ser utilizados a nivel de selección sexual en un contexto de elección femenina. En el caso de *Hetaerina* sería fascinante explorar si existe algo parecido. Es decir, una posible ventaja femenina de aparearse con machos con manchas más grandes, es que las hembras asegurarían a sus hijos genes con similar capacidad de lidiar con compuestos tóxicos.

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CAPITULO IV

When a signal is missing: survival and physiological costs of a manipulated fighting signal

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When a signal is missing: survival and physiological costs of a manipulated fighting signal

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Abstract

Signals of fighting indicate an high animal's pronbability to attack and so they serve to prevent costly aggressive encounters. However, according to theory, a discrepancy between the signal and the ensuing attacking behavior, will imply negative fitness consequences for the signaler in terms of survival and reproductive success. To our knowledge these consequences are poorly studied. Here we used males of the territorial damselfly *Hetaerina americana*, which bear a red wing spot during territory defense that has evolved as a fighting ability signal. After modifying the red color of the spot (covering with blue ink) in territory owners, we looked at: a) the behavioral responses by conspecific males; b) survival cost (using proper and modern statistical techniques) and c) three physiological mediators of impaired survival: muscular fat reserves, muscle mass and immune ability. Our predictions were that males with a modified red spot were more attacked by conspecifics resulting in lower survival and physiological condition. Compared to control males (males whose red spot was not changed), experimental males had reduced survival, were less able to hold a territory, and had a reduced muscle mass. It seems that spot-modified males were not able to communicate their territory tenancy, which explains why they lost their defended sites after exhaustive aggressive encounters. This is the first study that provides support for theoretical models using robust survival statistical analyses and physiological indicators of condition, that a modified signal of fighting ability predicts a fitness cost for bearers.

Key words: Signaling theory, fighting signals, red, territoriality, survival costs, muscle mass, damselfly

Introduction

Animal signals used in constest convey information that frequently is effective to mediate aggressive conflicts. In particular, signals used to indicate fighting ability are interpreted to communicate reliably an individual's probability of attacking (reviewed by [1], [2]). One example is the use of signals during territorial defense: a resident animal will use a signal to communicate its ability to displace a conspecific intruder. In case the intruder persists on staying in or approaching the resident's territory, then the resident will attack. This way of communication supposes that a discrepancy between a signal and the ensuing behavior should lead to a fitness cost for the signaler via enhanced aggression by conspecifics [3], [1], [4]. Thus, such cost makes the relation between a signal and its ensuing aggressive behavior reliable. This fitness cost is the basis for costly signaling theory [5].

Studies supporting aggression by conspecifics and fitness costs that emerge from a failed relation between a signal that reflects fighting ability by the signaler are practically inexistent but badly needed [1]. One reason for this is that this would require detailed experiments that manipulate either the signal or the subsequent aggression by the signaler, which is not easy. In this paper, we have experimentally covered a signal of fighting ability so that an animal does not communicate shuch ability adequately, and have measured both ultimate (survival) and underlying proximate (physiological) costs.

Red coloration is frequently associated to different aspects of fighting ability across animal taxa (e.g. arthropods [6]; fish [7]; reptiles [8]; birds [9] and mammals [10]). One case is that of the rubyspot damselflies, more specifically the red wing spots that male adults bear. When a nonterritorial male is looking for a riverine territory, it wanders through territories and in most cases leaves a place if a territorial male faces and shows the red spots to the former [e.g. 11]. For

those rare occasions when a true contest takes place, males with larger spots displace males with smaller spots [12]-[14]. The size of the red spot is selected via intrasexual competition [14] and correlates with several physiological components such as lipid-based fat reserves, muscle mass used for flight and immune ability [12], [15], [16]. These physiological components are related with condition and fitness for the following reasons. On one hand, fat reserves and muscle mass are used when a male disputes a territory, which explains their relation with territory tenancy and mating success [17]. On the other hand, immune ability (in the form of phenoloxidase, a key component in insect immunity) [18] and fat reserves would be strongly related with survival [19], [20]. These different pieces of evidence support the notion for a fighting signal function for wing spot.

We have used the American rubyspot damselfly, *Hetaerina americana,* as a model to look at the aggressive responses by conspecifics, and physiological and survival costs of not displaying the red aspect of the wing spot. More specifically, we covered the red spot with a blue ink in male territorial holders and evaluated the behavioral responses of their competing conspecific intruders, field survival of experimental animals (using adequate, modern capture-recapture techniques) [21], [22], and three components of physiological condition that are causative factors in both, contests for territorial tenure and survival: fat reserves, thoracic muscle mass, and immune ability. According to signaling theory, we have three predictions: compared to control males, males with covered wing spots would be more heavily attacked by conspecifics (1) which would lead experimental males to a lower survival (2) due to a poorer physiological condition (3). We think this the first test that a disruption between a signal of fighting ability and aggression leads to fitness costs for the signaler evaluating proximal and terminal causative factors.

Materials and Methods

Study subject

Recently emerged *H. americana* damselflies take 4–7 days to achieve sexual maturity (all authors' pers. obs). During this time the animals forage to develop muscle mass, accumulate fat body reserves, and develop and fix their red wings patches, which do not subsequently change further after sexual maturity is reached [23], [24]. Once mature, males return to riverine areas where they compete for mating territories [25]. Males may adopt two conditional reproductive tactics (*sensu* in [26]), depending on variables related to their physiological condition (i.e. fat reserves and muscle mass) [11], [12]: territorial and non-territorial. Territorial males are in better energetic (i.e. higher fat reserves and muscle mass) condition and they defend territories against conspecifics. Alternatively, non-territorial males are in worse physiological condition, are not able to acquire a space and either fight for one or act as satellites usually in neighboring territories [16], [17]. Fort he present study we only used mature males defending territories. Male maturity criteria were based on the classification of Plaistow & Siva-Jothy [27]. These authors separated adult males into four age-based classes: the first age comprises newly emerged, non-pigmented males, with soft flexible wings and exoskeleton and a zigzag erratic flight. A second category includes males with undamaged flexible wings and thorax, the red wing spot is about to be fully colored, and the main male behavior is foraging. A third age category comprises sexually active males (as judged by their continuous fighting activity on territories), which bear fully developed and conspicuous red wing patches [16]. A last category includes old males, which have abundant pruinescence upon the abdomen and thorax, and opaque, inflexible, and sometimes broken wings [27]. To distinguish a male that are defending a territory from we carried out behavioral observations. Territorial males were those animals that fought conspecific

males to defend a small riparian area (vegetation or rocks in a ratio of 1-3 m^2) and were not displaced by conspecific males for at least 20 min [12]. Males that did not hold territories were considered non-territorial.

For all experiments we only used territorial and mature males of age class 3. After capture with an insect net, each damselfly was individually marked on the left posterior wing with a unique combination of three digits using a permanent black marker. These numbers are readable at a distance of 3-4 meters. Following marking, individuals were photographed using a digital camera (Nikon P90 24x). From these pictures, we measured and then averaged the spot area of the four wings to obtain the proportion of the wing covered by the red pigment using Adobe Photoshop (version CS2). After marking, animals were immediately returned to their original capture sites. Manipulation did not last longer than two minutes.

Study site

Field work was carried out in the Tetlama River, Morelos, Mexico (18º 45' 55''N, 99º 14' 45'' W) from September to December 2010. For all experiments in this study, we chose three different sites of 200 m length along the river margins, each site separated by 800 meters. *H. americana* is unusual among odonates in that both sexes spend most of their adult life within a few hundred meters of their mating areas [14, all authors' pers. obs.], thus the distance between sites was used to avoid possible migration among them. The first site was used to determine territorial and mating behavior, the second site to determine survival in the field while the last site was used to determine predation rate (see below).

General procedure of experimental manipulation of wing spot color

Males were randomly assigned to three treatments. Males in the first treatment (hereafter, the "non-manipulated" treatment), were captured, marked, photographed and returned to their original capture site. Males in the second treatment (hereafter, the "sham" treatment) received the same manipulation but their wing-pigmented area was entirely covered with a transparent marker (COPIC sketch®, Colorless Blender 0). Males in the third treatment (hereafter, the "blue" treatment) had their red wing pigmented area entirely covered with a blue coloration using a pigment marker (Bic Mark-It®, color deep sea blue). Blue color was selected because its wavelength differs strikingly from the natural red color and because adult odonates show color-specific responses to colors that differ in spectral peaks [29].

To ensure that conspecifics perceive color different from blue-manipulated males but not from sham, we compared three color properties - brightness, red chroma and hue - among experimental treatments. In September 7, 2010, we used 16 non-manipulated males, 11 sham males and 13 blue-manipulated and measured their wing color properties at the site of the spot. Color properties were measured from the values of reflectance (R), obtained by the light reflected off the measured red wing spot compared with a white standard (Spectralon™), at 10-nm intervals across the range from 360 to 740 nm using a spectrophotometer (MINOLTA CR-200, Konica Sensing Inc., Osaka, Japan). Brightness was calculated by $[\Sigma_{R360nm+\ldots+R740nm}]$, red chroma by $[\Sigma_{R600nm+\ldots+R700nm}$/brightness] and hue by [wavelength where $R = [R_{max} + R_{min}]/2$] [30].

Effects of wing color manipulation on behavior

Along site one, we captured 120 males and randomly allocated them to the treatments mentioned above: non-manipulated (N = 40), sham (N = 40), and blue (N = 40). The study was carried out from November 2 to November 10, 2010. We made focal observations of each resighted marked male for 20 minutes the day after manipulation. The behavioral variables recorded during focal observations were the number of encounters when the territory holder remained in his site after being faced by a conspecific competitor and the number of territorial intrusions. For this study, we only considered animals that presented any kind of agonistic behavior. Sample sizes of observed animals were as follows: 7 non-manipulated males, 12 sham males and 12 blue.

Survival in the field

In the second site, we captured 278 males and randomly assigned them to the different experimental treatments explained above; non-manipulated (N = 92), sham (N = 93) and blue (N = 93). We marked and then recorded the survival of these three treatments from September 25 to October 28, 2010. One day after manipulating a we began daily visits to the river to record and construct a binary dataset of daily encounter history for each individual (0 for non-observed and 1 for observed).

We assessed survival using Cormarck-Jolly-Seber models (CJS) [21], as these can dissociate survival (ϕ) from recapture (p) probabilities [21], [22], [31]. In the construction of models we considered the inclusion of survival and recapture predictors, the experimental treatments as a predictor variable and red spot proportion (RSP) as an individual covariate.

Capture-recapture models were analyzed using MARK 6.0 software [32] in the framework of maximum likelihood estimation methods [21], [22]. Hence, no frequentist statistical tests (i.e. those based on P values) were performed for analyzing survival in the field (for a similar rationale see [33]). The procedure consisted in creating different models with treatment and RSP as predictors of survival and recapture probabilities. A goodness-of-fit was performed considering $\phi_{(g)}$, $p_{(g)}$ as the global model. An important assumption for model selection and parameter estimation is that data should not have severe over-dispersion (i.e. variance inflation factor, $\hat{c} > 3.0$) [21], [22]. The $\hat{c}$ value was obtained from the global model after performing 1000 bootstrap simulations. A perfect fit of the model would have a $\hat{c} = 1$. Greater values are interpreted as over-dispersed data. The global model presented slight over-dispersion ($\hat{c} = 1.107$), which is far from serious structural problems or violation of assumptions [21], [22]. Nevertheless, we corrected for over-dispersion using the $\hat{c}$ obtained from global model, and employed the Akaike Information Criterion for over-dispersion QAICc for model selection and ranking of competing models [34]. Since there was not a single model with clear support over the others we performed model averaging with the models that represented at least 0.001 of QAICc weight. Hence, we employed model averaging to estimate the effect of experimental manipulation and RSP on survival [34].

Physiological costs of lacking red

Individuals used in behavioural observations (i.e. from the site one) were recaptured either 1, 3 or 6 days after manipulation, had their haemolymph extracted (see below), and then stored in ethanol for quantification of fat reserves and muscle mass in the laboratory. The samples sizes

for day 1 were: non-manipulated = 10, sham = 8, blue = 7; the sample sizes for day 3 were non-manipulated = 8, sham = 4, blue = 14; and the sample sizes for day 6 were non-manipulated = 15, sham = 9, blue = 7.

Fat reserves and muscle mass quantification

Fat reserves and muscle mass from individuals used in behavioral observations were obtained from the thorax and abdomen since the rest of the body contains insignificant quantities of these compounds [27]. Procedures for measuring fat reserves and muscle mass are as follows. After removing the head and the legs, we dried thoraces and abdomens by placing them in a desiccator (for 24 h) and obtained their dry weight (to nearest 0.1 mg). Then we placed samples in 100% chloroform for 24 h for fat extraction (for similar procedures see [12]). Samples were then re-desiccated for 24 hours and re-weighed. Fat content was calculated as the difference between initial weight and final weight. After fat extraction, we submerged samples in 0.8 M potassium hydroxide for 48 h to extract the muscle. Again, we dried bodies for 24 hours, re-weighed them and calculated muscle mass as the difference between the pre-potassium hydroxide treatment weight and final weight (for a similar procedure see [16]).

Phenoloxidase activity in haemolymph

From the same pool of individuals we measured phenoloxidase (PO) activity from the haemolymph following the perfusion technique used by González-Santoyo et al. [35]. Since haemolymph samples obtained by perfusion are a mixture of haemolymph and phosphate buffered saline solution in unknown proportions (i.e. some haemolymph samples can be more diluted than others), PO activity was standardized for the total protein mass in the sample.

Protein mass was determined using the method of the bicinchoninic acid assay with the PIERCE® protein assay kit. Following determination of protein concentration, PO activity was measured in volumes that contained 20 μg of protein per sample. PO activity in extracted haemolymph was measured by quantifying the formation of dopachrome from L-dihydrophenylalanine (L-DOPA, Sigma) [18].

Potential negative effects caused by color manipulation

In addition to disrupting the conspecific communication system, we also considered three other negative effects potentially caused by color manipulation that could influence survival, territorial behavior, and physiological condition. The color manipulation itself could cause negative effects via: a) increased wing weights; b) toxic effects of pigment components, and c) predation.

To determine whether wing weight was increased, we painted one anterior wing with either blue or transparent marker and left its corresponding anterior side-pair without manipulation. This was done for eight "blue" and eight "transparent" animals. Each wing was cut at its site of insertion to the body and was weighted twice (in mg) to obtain mean values for the analysis. We compared the wing weight according to treatment.

For testing potential toxic effects of pigments, we kept 10 animals of each treatment in captivity (i.e. in 5 mL essay tubes with a perching wooden piece and a cap of humid cotton at ca. 26 °C, without food) in a 12/12 hrs light-dark regime and with no food. Animals were kept for 36 hours, after which we checked for surviving animals (for similar approach see [36]). Two likely predators of odonates that use visual means are birds and other odonates [37], [38]. Therefore, we carried out an experiment on the third site to see whether there were differences in predation-

based survival between experimental treatments. This experiment was conducted from November 15 to November 20, 2010. 75 specimens of *H. americana* males were captured and were allowed to die after being placed in a glassine envelope and exposed to direct sunlight. On the following day, these animals were glued to the top of a 30 cm length wooden stick, which was placed in small container filled with water at 8 cm from its base, to avoid predation by ants. Specimens were glued into a natural perching posture at sites where territorial males usually defend territories. A total of 25 triads (each triad consisted of one male for each treatment) were placed along site with a separation of 4 m between triads. Animals were left for a total of 4 days, but they were inspected daily for evidence of damage and/or predation. Damaged and/or absent animals were not replaced with new dried specimens. Predation events were inferred by major injury to the specimen (wings, head, thorax or abdomen completely missing).

Statistical analysis

For testing color property (brightness, chroma and hue) differences among experimental treatments due to manipulation, we used analyses of variance and Tukey tests for post hoc comparisons.

As for behavioral data, given the small samples obtained and that sham and non-manipulated treatments did not differ significantly in the proportion of encounters where males remained in their original site after an intruder's invasion (General Linear Models [GLM] binomial: treatment: Likelihood ratio test $\chi^2 = 1.9695$, $df = 1$, $p = 0.1605$), proportion of aggression received (GLM binomial: treatment : Likelihood ratio test $\chi^2 = 1.7015$, $df = 1$, $p = 0.1921$), muscle mass (ANOVA: treatment: $F_{(1,49)} = 1.2097$, $p = 0.2768$), fat reserves (ANOVA: treatment: $F_{(1,50)} =$

0.7621, $p = 0.3808$) and PO activity (ANOVA: treatment: $F_{(1,51)} = 0.1388, p = 0.711$) we pooled them into a single treatment now called control.

To assess the effect of red spot manipulation on territorial behavior, all statistical tests included experimental treatment and RSP as predictor variables. For each response variable (e.g. proportion of encounters when territorial males remained in their original site or proportion of aggression received, etc.) we fitted GLMs of the binomial family (with logit link function). When residual models were over-dispersed we used a quasi-binomial correction [39]. In models with over-dispersion, we used Likelihood Ratio Test (LRT) for obtaining the simplest appropriate model. LRT is frequently used to determine whether or not data support a full model over a reduced model [33]. We searched for the simplest model by beginning with a global model that contained the most parameters and then compared it to a simpler model with one fewer parameter. The global model was Treatment:RSP, following comparisons by Treatment+RSP and finally with Treatment. When LRT of the most complex model was significantly greater than the simplest model (with a p-value < 0.05) then the complex model was chosen, and vice versa. Selection of the most complex model indicates that the benefit of the improved model fit outweighs the cost of higher model complexity [33].

We checked for the presence of influential observations in each selected model by measuring the Cook's distance of each observation (values greater than 1 are considered influential) [40]. However, we did not detect any outliers in any model tested.

To evaluate the effect of wing color manipulation on muscle mass, fat reserves, and PO activity, we fit linear models (LM), now adding the number of days between wing manipulation and recapture as an additional predictor variable. The best model (with and without interactions) was chosen based on the lowest AIC value [41]. When the new predictor variable (days of recapture)

significantly influenced any response variable we used Tukey tests for post hoc comparisons [39]. We used Fligner-Killeen tests to assess homogeneity of variance in the residuals and visual observations to assess normality [39].

For weight differences in wing pairs (one of them covered with ink), we used paired *t* tests. For toxic effects of blue and predation rate, the rates of survival among individuals in each treatment treatment were compared using χ^2 tests. Analyses were made in R software (R Development Core Team 2009, v.2.1.3.2) and SPSS (v. 15.0).

Animals were collected using a permit from the Secretary of Environment and Natural Resources (SEMARNAT) issued to AC-A. However, our study does not involve endangered or protected species. During manipulation, every effort was made to reduce animal suffering.

Results

Color properties among experimental treatments

Blue animals showed significant differences in brightness (ANOVA: $F_{2,37} = 17.34, P < 0.001$), chroma (ANOVA: $F_{2,37} = 32.747, P < 0.001$) and hue (ANOVA: $F_{2,37} = 38.155, P < 0.001$) when compared with no-manipulated (brightness: $t_{2,37} = 5.478, P < 0.001$; chroma: $t_{2,37} = 6.717, P < 0.001$; hue: $t_{2,37} = 7.363, P < 0.001$) and sham animals (brightness: $t_{2,37} = 4.624, p < 0.001$; chroma: $t_{2,37} = 7.280, P < 0.001$; hue: $t_{2,37} = 7.766, P < 0.001$). There were not differences between non-manipulated and sham males (brightness: $t_{2,37} = -0.385, P = 0.921$; chroma: $t_{2,37} = 1.211, P = 0.454$; hue: $t_{2,37} = 18.33, P = 0.518$).

Effects of wing color manipulation on behavior

For the number of encounters where the holder remained in its site after an intruder's invasion, the simplest model selected only considered experimental treatment as predictor variable. According to this, treatment had a significant effect on the probability of remaining on a territory following territorial intrusions (GLM: Treatment: LRT = 14.316, $df = 1$, $P = 0.031$; Table 1). Blue males were less likely to remain on defended sites following territorial intrusions (38.1 ± 37.9 %) than control males (66.0 ± 34.4 %; Table 2; Fig. 1). In the number of aggression received, the simplest model selected did not include any predictor variable (i.e. the addition of covariates did not improve model fit; Table 1). That is, all treatments received similar number of aggresions. Finally, there were no differences in the total number of encounters during the period (20 minutes) of focal observations (blue: 6.084 ± 1.12; control: 5.210 ± 1.436; $F_{1,29} = 0.369$, $P = 0.548$).

Survival cost of lacking red

Cormarck-Jolly-Seber models indicated that survival rates were best predicted by a model that included the main effects of both, treatment and RSP. Recapture rates were best predicted by a model that included the interaction between treatment and RSP, as well as the main effects of each (Table 3). Blue males had lower survival compared to sham and non-manipulated males, but there were no differences in survival between sham and non-manipulated males (Table 4). Additionally, in all treatments males with larger wing spot survived for longer as indicated by the slopes in blue ($\beta = 0.567$, CI 95 %: 0.524 to 0.609), sham ($\beta = 0.237$, CI 95 %: 0.219 to 0.254) and non-manipulated males ($\beta = 0.205$, CI 95 %: 0.189 to 0.220). Sham and non-manipulated males did no differ in their slopes (Fig. 2a). The same relation was also observed for recapture

probability in blue (β = 0.786, C.I 95 %: 0.688 to 0.884), non-manipulated (β = 0.169, C.I 95 %: 0.081 to 0.257) and sham males (β = 0.035, C.I 95 %: -0.041 to 0.111; Fig 2b).

Physiological cost of lacking red

Fat reserves were affected by treatment and recapture day (RD) but not by RSP ($F_{2,69} = 3.158$, $P = 0.0301$; Table 5). 6 days after manipulation, fat reserves were lower than after 3 days ($t_{55} = -2.864$, $P < 0.01$; Table 6), but did not differ 1 day after manipulation ($t_{54} = -1.309$, $P = 0.195$) or between days 1 and 3 ($t_{49} = 1.468$, $P = 0.147$; Fig. 3a; Table 2). Fligner-Killeen tests confirmed homogeneity of variance ($\chi^2 = 4.5608$, $df = 2$, $P = 0.102$). Muscle mass was only affected by treatment ($F_{1,74} = 4.740$, $P = 0.032$; Table 5): blue males had lower muscle mass than control males ($t_{73} = -2.178$, $P = 0.0326$; Fig. 3b; Table 5). Fligner-Killeen tests confirmed homogeneity of variance ($\chi^2 = 0.014$, $df = 1$, $P = 0.906$).

PO activity was best explained by treatment+RD+RSP+treatment:RSP+RD:RSP ($F_{7,68} = 4.635$, $P < 0.001$; Tables 5 and 6). In all treatments, PO activity was lower 1 day after manipulation than after 3 days ($t_{49} = 4.635$, $P < 0.001$) and 6 days ($t_{54} = 4.043$, $P < 0.001$). There were no differences between 3 and 6 days after manipulation ($t_{55} = 0.116$, $P = 0.998$; Table 4). RSP also showed a significant effect in PO activity (β = 4.815, $t_{68} = 2.316$, $P = 0.023$; Table 5) but not in the interaction with treatments. Fligner-Killeen confirmed homogeneity of variance ($\chi^2 = 3.749$, $df = 2$, $P = 0.154$).

Potential negative effects caused by color manipulation

Blue and transparent pigments did not differ significantly in the weight that they added to male wings (blue: paired *t* test, $t_7 = 0.428$, $P = 0.681$; weight added in each pair of wings: 0.045 ± 0.056 mg; clear: paired *t* test, $t_7 = 1.374$, $P = 0.21$; weight added in each pair of wings: 0.054 ± 0.043 mg) with its corresponding side-pair wing. We did not detect any toxic effects of pigment components since numbers of surviving animals held in captivity among treatments were not significantly different ($\chi^2 = 0.765$, N = 30, $df = 2$, $P = 0.681$). Four days after dead animals were placed in triads for predation testing, there were no significant differences in the rate of removal of animals across treatment treatments (GLM: binomial:Treatment: LRT $\chi^2 = 0.101$, $df = 2$, N = 75, $P = 0.950$).

Discussion and Conclusions

After no detecting potential negative effects of our manipulation, we have shown that blue males had reduced survival compared to control males, and that such negative effect was mainly explained by physiological condition and, to a lesser extent, behavior. That a reduced survival is not explained by aggression by conspecifics contradicts theory of signals in animal contests, which explain that a disrruptive comunication in those signals would lead an increased in agonistic encounters that result in high costs for signallers and receivers [1], [3], [5]. Note, however, that although all treatments were similarly attacked, blue males were less able to defend their territories successfully. However, we were unable to measure other contest-related variables that may also explain enhanced aggression by conspecifics following an inadequate signaling. One first example is contest duration: contests with blue males may have lasted longer than with other males, which led the former males to become more exhausted and more likely to

lose a territory. A second variable is that is not the duration of contests per se but the complexity of the flight what could be correlated with energetic costs. If this is true, then possibly blue males were induced to perform more complex flights than the other experimental males. A recent review has established that both possibilities are viable [42] and so remain to be investigated in our study subject. In functional terms, the reduced survival in blue males seems explained by a negative effect on muscle mass. Fat reserves followed the same trend despite being non-significant. Although both muscle mass and fat reserves are predictors of territorial roles (territorial and non-territorial behavior) and fighting success in the American rubyspot [16], [17] their relation is complex [43]. For example, in adults fat reserves seem more immediately affected by long-lasting contests compared to muscle mass, although after fighting males usually end up with a considerable reduction in muscle mass [16], [37]. Interestingly, while fat reserves and PO levels can be re-established, muscle mass cannot [43] which is what may have occurred to blue individuals. Related evidence for this has indicated that feeding may restore fat reserves and PO levels but it is not the case for muscle mass [36]. Thus, perhaps after a number of fights for a number of days, blue individuals were no longer able to defend their sites because their muscle mass tissue was too deteriorated to sustain flight and compete for territories.

One may wonder, in behavioral terms, what conspecifics perceived when males were unable to display the red spot in our study subject. Encounters between conspecific males are very common in the American rubyspot. Non-territorial males frequently fly over territories and, after facing an opponent for a fraction of second, leave immediately which has been interpreted as a respect for the "residency" asymmetry in calopterygids [42]. In fact, encounters that result in a change of the original territory roles are relatively rare [16]. Escalation occurs only if territory availability becomes too scarce [27] [44]. We believe that one function of short-lasting

encounters by non-territorial males is to "check" whether a territory is available, which is communicated by a territorial male that shows his red spot to non-territorial males. This level of communication may not only be used with conspecific males but also heterospecific males, given the interspecific territorial competition that has been detected in the *Hetaerina* genus [28]. In fact, we cannot discard that blue males may have been mistakenly identified given that even less drastic wing color manipulation can cause *Hetaerina* males to be classified as heterospecifics [28]. Thus, selection to avoid intra- and interspecific physical aggression may explain maintenance of red spots in this genus.

Previous studies in birds have indicated that aspects of red coloration (i.e. redness) may also be a signal component along with badge size [45] [46]. These studies have found that since redness is an indicator of male condition [47], [48], this color aspect may be taken as an indicator of condition. In the American rubyspot, the better the diet a larva has, the larger the spot its corresponding adult produces [16]. However, when long-lasting contest takes place, spot chroma, hue and brightness do not predict contest outcome but wing spot size does [13], [16]. In fact, these three aspects are relatively invariable thus they are not predicted by male diet or territorial status [13]. Spot size, however, does not seem selected during that first level of communicating residency according to our observations of aggressions. This suggests that, at this first step of communication, a large spot size is not needed but, possibly, the red nature only.

Interestingly, our results showed that spot size covaried closely with survival in all treatments, indicating that fitness costs are higher for males with small spot sizes than those with large spot sizes. That spot size correlates with survival supports a similar finding in the same species [49]. Furthermore, this previous finding detected a reduction in survival when the spot was increased [49]. This along with the cost of production of the spot based in terms of diet indicated above [16]

provides strong support that the spots are condition-dependent traits. Putting all these ideas and findings together, it seems that in American rubyspots, the communication system is reliable given that levels of signal intensity accurately accompany levels of signaler quality [14]. This goes well with theory which indicates that the survival costs of signaling are higher for a poor quality individual than for a high quality individual [50].

In summary, theoretical studies for the evolution of aggression have indicated that there must exist a signaling system that prevents unnecessary agonistic encounters [1], [3], [2], [5]. Such signaling system must reflect animal intention for attacking. An inappropriate signalling trait should thus lead to fitness costs based on enhanced aggression by conspecifics, [5]. Although our results do not provide strong evidence towards higher aggression for signalers whose signal is not able to communicate fighting intentions, they lend support to a survival cost mediated by physiological mechanisms.

Authors' Contribution

Conceived and designed the experiments: IG-S, DMG-T, REM-S, AC-A. Performed the experiments: IG-S, DG-T, REM-S, AC-A. Data analysis: IG-S, DMG-T, REM-S. Contributed reagents/ materials/analysis tools: IG-S, DMG-T, REM-S, AC-A. Article critical revision: IG-S, DMG-T, REM-S Article drafting: IG-S, AC-A. Thanks to Greg Grether and an anonymous reviewer for key suggestions on a first draft.

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Figures.

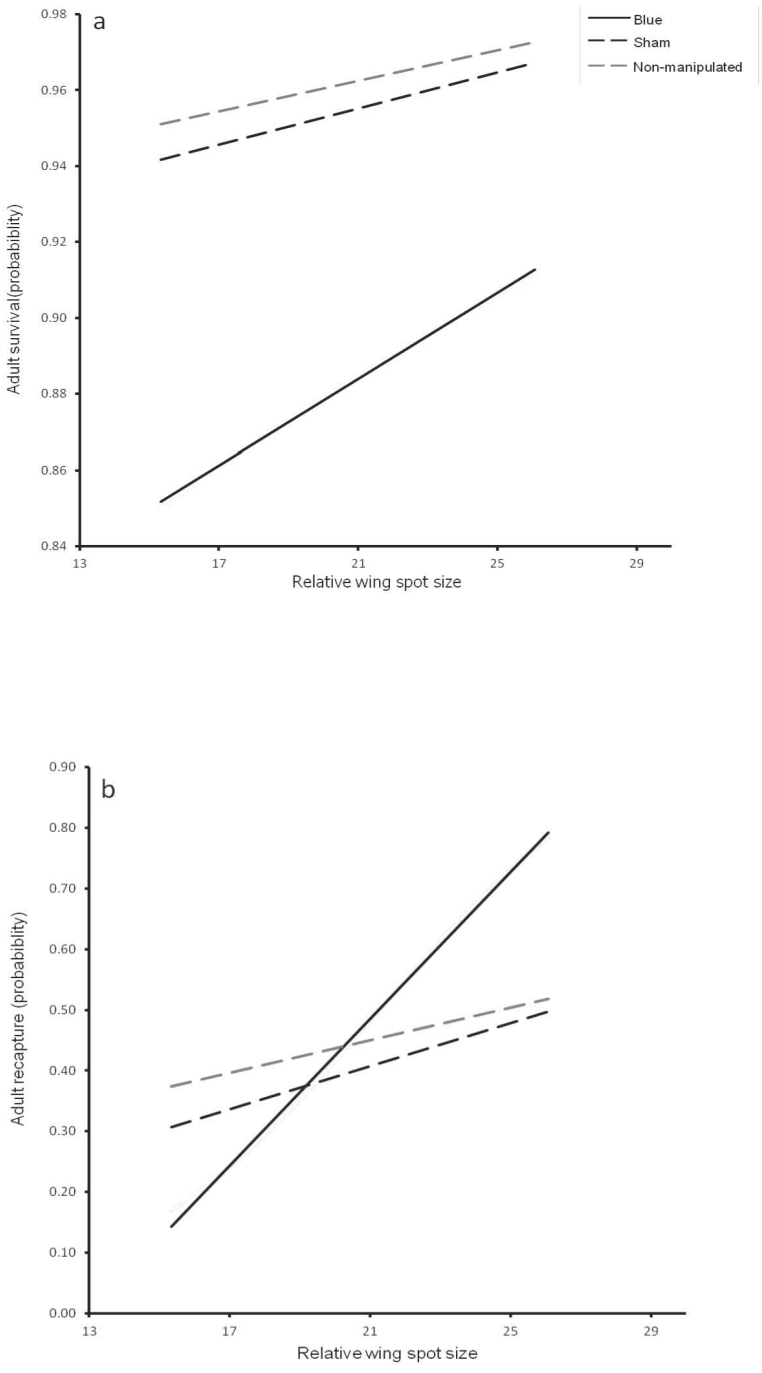

Figure 1. Predicted survival (a) and recapture (b) values according to red spot proportion and experimental treatment.

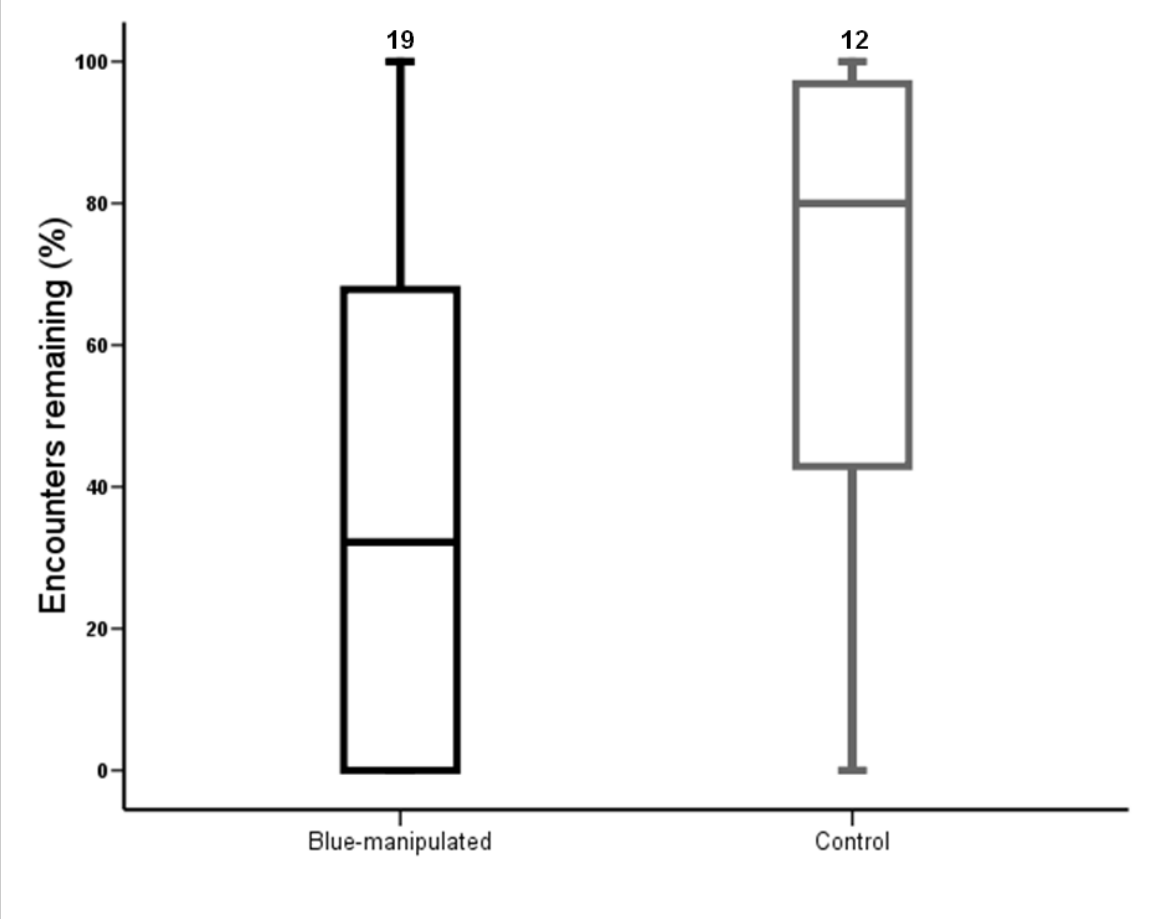

Figure 2. The proportion of encounters when the holder remained in the site after an intrusion according to experimental treatment. Boxes represent first, second and third quartiles; whiskers are the sample maximum and minimum observations; sample sizes are shown above each plot.

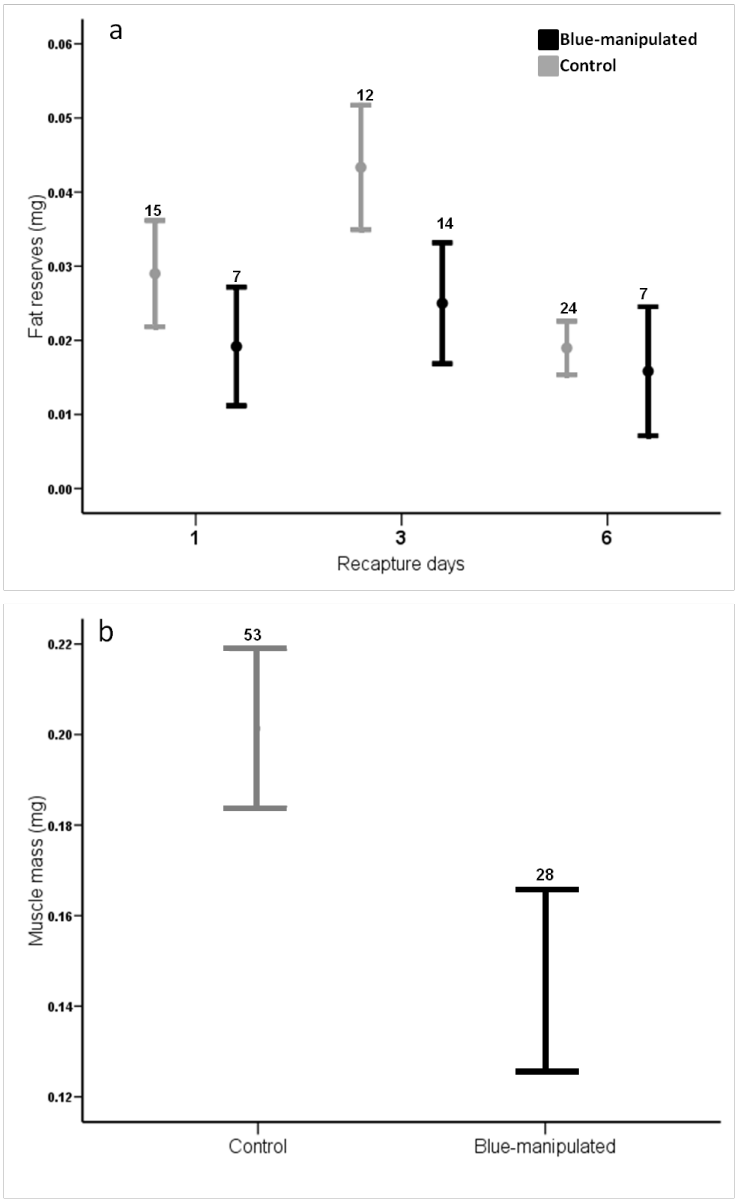

Figure 3. (a) Fat reserves in relation to recapture day, and (b) muscle mass according to experimental treatment. Lines show mean ± Standard errors. Sample sizes are shown above each plot

Tables

Table 1. Selection of higher supported models (AICc weight) whose survival (ϕ) and recapture (p) parameters include wing spot proportion (RSP) and experimental treatments.

Model						
Survival	Recapture	AICc	ΔAICc	AICc weight	Parameters	Deviance
$\varphi_{(treatment+RSP)}$	$p_{(treatment*RSP)}$	2894.401	0.000	0.249	10	2874.17
$\varphi_{(treatment * RSP)}$	$p_{(treatment*RSP)}$	2894.444	0.043	0.243	12	2870.11
$\varphi_{(treatment* RSP)}$	$p_{(treatment)}$	2895.081	0.680	0.177	8	2878.93
$\varphi_{(treatment* RSP)}$	$p_{(treatment+RSP)}$	2895.551	1.150	0.140	10	2875.32
$\varphi_{(treatment+ RSP)}$	$p_{(RSP)}$	2896.083	1.682	0.107	6	2883.99
$\varphi_{(treatment+ RSP)}$	$p_{(treatment+RSP)}$	2896.610	2.207	0.082	8	2880.45
$\varphi_{(treatment* RSP)}$	$p_{(.)}$	2904.701	10.300	0.001	7	2890.58

Table 2. Daily estimation of survival and recapture probabilities according to experimental treatment. Estimation was done according to the weight of the highest supported models.

	Treatment	Parameter	Estimate	Standard error	95% confidence interval	
					Lower	Upper
Survival	Blue-manipulated	φ_B	0.871	0.017	0.835	0.900
	Sham	φ_S	0.952	0.007	0.936	0.964
	Non-manipulated	φ_N	0.960	0.006	0.946	0.971
Recapture	Blue-manipulated	p_B	0.401	0.033	0.339	0.468
	Sham	p_s	0.388	0.023	0.343	0.434
	Non-manipulated	p_N	0.424	0.022	0.383	0.467

Table 3

Comparisons between paired models by the Likelihood Ratio Test for (a) proportion of encounters that the holder remained after an intrusion and (b) the proportion of territorial intrusions divided by the total number of encounters. The model comparison started with the most complex (i.e. the highest number of parameters) model versus the model with one parameter less until the simplest appropriated model is found.

	Encounters remaining	LRT	Df	P
	Compared with			
~Treatment+RSP+Treatment:RSP	~Treatment+RSP	93.38	1	0.448
~Treatment+RSP	~Treatment	95.14	1	0.798
~Treatment	~1	**95.33**	**1**	**0.031***
	Aggression received			
~ Treatment+RSP+Treatment:RSP	~Treatment+RSP	87.67	1	0.902
~Treatment+RSP	~Treatment	87.71	1	0.869
~Treatment	**~1**	87.77	1	0.318

The simplest appropriated models are in bold

*Significance of compared model with $P < 0.05$

"~1" without predictor variable

Table 4

Descriptive statistics (mean± SD) for physiological and behavioral variables of experimental treatments according to their recording time.

Variable	1day		3 days		6 days	
	Control (N=15)	Blue-Manipulated (N=7)	Control (N=12)	Blue-Manipulated (N=14)	Control (N=24)	Blue-Manipulated (N=7)
Fat reserves (mg)	0.29 ± 0.27	0.19 ± 0.19	0.43 ± 0.29	0.25 ± 0.25	0.19 ± 0.17	0.15 ± 0.20
Muscle mass (mg)	2.1 ± 0.80	2.0 ± 0.90	1.83 ± 1.79	1.09 ± 0.85	2.03 ± 1.2	1.1 ± 1.1
PO activity (OD/µg protein)	0.55 ± 0.31	0.40 ± 0.34	0.76 ± 0.21	0.76 ± 0.19	0.80 ± 0.23	0.78 ± 0.22

Behavior	Control (N=12)	Blue-Manipulated (N=19)
Encounters remaining (%)	66.03 ± 34.33	38.14 ± 37.90
Aggression received (%)	50.54 ± 36.21	67.00 ± 35.62

Table 5.Model selection for each physiological variable obtained from the lowest AIC value. Model selection started with the global model that contained Treatment (G), recapture days (RD), wing spot proportion (RSP) and their interactions (:).

Variable	Models	Df	Deviance	AIC
Fat reserves				
	G+RD+RSP+G:RD+G:RSP+RD:RSP+**G:RD:RSP**			-1208.19
	G+RD+RSP+ G:RSP+RD:RSP+**G:RD**	2	1.1e-07	-1209.84
	G+RD+RSP+G:RSP+**RD:RSP**	2	5.1e-08	-1212.78
	G+RD+RSP+**G:RSP**	2	0.4e-07	-1213.92
	G+RD+**RSP**	1	7.5e-08	-1214.45
	G+RD	1	1.9e-08	-1216.06*
Muscle mass	G+RD+RSP+G:RD+G:RSP+RD:RSP+**G:RSP:RD**			-1013.21
	G+RD+RSP+ G:RSP+RD:RSP+**G:RD**	2	1.1e-07	-1017.11
	G+RD+RSP+ RD:RSP+**G:RSP**	2	9.1e-05	-1019.69
	G+RD+RSP+**RD:RSP**	1	9.2e-05	-1021.42
	G+ RSP+ **RD**	2	9.5e-05	-1022.95
	G+**RSP**	2	9.7e-05	-1024.75
	G	1	1.0e-04	-1024.93*
PO Activity	G+RD+RSP+G:RD+G:RSP+RD:RSP+**G:RSP:RD**			-204.11
	G+RD+RSP+G:RSP+RD:RSP+**G:RD**	2	0.033	-207.44
	G+RD+RSP+G:RSP+RD:RSP	2	0.056	-210.34*

Removed parameters for the consequent comparison model are in bold

*Selected model with lowest AIC value

Table 6. Main effects of selected linear models over physiological variables.

Variable	Model selected	Estimate	SE	T	P
Fat load	Treatment+RD				
	Treatment				0.084
	RD				0.**047***
	Day 1 vs Day 3	-1.063e-04	7.25e-05	1.468	0.147
	Day 1 vs Day 6	-8.783e-05	6.71e-05	-1.309	0.195
	Day 3 vs Day 6	-1.942e-04	6.79e-05	-2.864	**0.006***
Muscle mass	Treatment	-6e-04	-3e-04	-2.178	**0.032***
PO activity	Treatment+RD+RSP+G:RSP+RD:RSP				
	Treatment				0.51
	G:RSP				0.292
	RD:RSP				0.072
	RSP				**0.02***
	RD				**0.00***
	Day 1 vs Day 3	1.854	0.737	2.518	**0.01***
	Day 1 vs Day 6	0.923	0.463	1.992	**0.05***
	Day 3 vs Day 6	-0.931	0.758	-1.230	0.22

Parameters with P <0.05 are in bold.

* Significance of the main effects at $p < 0.05$

DISCUSIÓN GENERAL

Discusión General

En mi proyecto doctoral planteé un enfoque integral como la vía para conocer y entender las implicaciones funcionales y ecológicas de un CSS. Este enfoque se fundamenta en el supuesto de que cualquier característica morfológica en los individuos compartirá un gran numero de interacciones con otros componentes dentro y fuera del individuo. Dadas estas relaciones, si estos caracteres se consideran de forma aislada podría arrojarnos resultados erróneos de su valor adaptativo real.

En el caso de *H. americana* encontré, por ejemplo, que la expresión de un CSS puede o nó reflejar o guardar relaciones positivas con la condición inmunológica del portador, y que es imprescindible tomar en cuenta una serie de factores tanto internos como externos cuando se pone a prueba este tipo de ideas. En contraste con la hipótesis propuesta en el primer capítulo de este proyecto, nuestros resultados mostraron patrones inconsistentes (i.e. relaciones positivas, negativas e incluso nulas) entre la condición inmune, evaluada a través de la actividad de FO y/o la capacidad de melanización (evaluada en 2 de las 5 especies), y el grado de expresión de un CSS (i.e. tamaño de pigmentación alar). Tales inconsistencias mostraron fuertes diferencias interspecíficas y estacionales. Estos resultados pueden deberse en parte al tipo de presiones selectivas que actuaron para promover la expresión de este CSS en los machos. Por ejemplo, si este CSS evolucionó vía elección de pareja, entonces la cualidad del mismo como indicador de la condición inmune tendría un fundamento adaptativo en los dos sexos. En el caso de las hembras, dicha información les generaría beneficios directos, al seleccionar individuos más inmunocompetentes y por ende con una menor probabilidad de transmitir patógenos al momento

de la cópula, y/o indirectos al poder heredar la capacidad inmune del padre a la descendencia. En el caso de los machos esta cualidad seria adaptativa ya que al transmitir la información de su capacidad inmune a través de su CSS aumentaría la posibilidad de ser seleccionado. Sin embargo, si el CSS se originó vía competencia intrasexual, como sucede en *H. americana* (Grether, 1996a), es muy posible que informar a los contrincantes acerca de la condición inmune no tenga efecto en el resultado de los encuentros, ni es probable que los machos muestren a través de sus manchas la capacidad inmunitaria a sus contrincantes (Contreras-Garduño et al. 2006; Lawniczak et al. 2007), a diferencia de lo que ocurriría al informar acerca de su capacidad competitiva. Por lo tanto, las asociaciones CSS-inmunidad serían indirectas y como consecuencia de la condición "per se" del portador (i.e. Contreras-Garduño et al., 2008).

Aunado a esto, existe una mayor probabilidad de asociación entre la función sexual (i.e expresión del CSS) y la función inmune si ciertos recursos están siendo compartidos entre ellas (Keymer y Read, 1991). De esta manera la expresión de un CSS (i.e. funcion sexual) podría reflejar ya sea el efecto negativo que tiene sobre la función inmune o la capacidad del individuo para solventar ambas funciones (Sheldon y Verhulst, 1996). De manera contraria, si estas características no comparten recursos, entonces será menos probable esperar alguna asociación entre ellas, situación que discutiré mas adelante.

Una tercera explicación se encontró a raíz de las manipulaciones experimentales realizadas en este estudio, donde se observó que la expresión de FO no fue un buen predictor de la capacidad inmune. En este caso, solo los machos de *H. vulnerata* y no en *H. americana* mostraron una mayor actividad de FO cuando fueron expuestos a una infección bacteriana (con *Serratia marcesens*). Esto sugiere que al menos para la interacción parásito-hospedero entre *S. marcecens* y *Hetaerina*, la enzima FO podría no ser un buen indicador de la respuesta inmune ante este

patógeno. Un candidato mas allá de la actividad de FO pudiera ser su propio cimógeno (proFO; González-Santoyo y Córdoba-Aguilar, 2012; Adamo, 2004). Dicho componente es sintetizado por el individuo a partir del aminoácido fenilalanina y es almacenado en grandes cantidades en los hemocitos de los insectos, específicamente en los granulocitos (Strand, 2008). De manera general, la proFO será nuevamente sintetizada hasta que esta se haya utilizado en su totalidad (en forma de FO) y, debido a los costos fisiológicos que implica su síntesis y activación, se ha observado que la cantidad total de este cimógeno puede variar de forma importante entre individuos no solo de diferente condición sino de diferente sexo, edad, entre otros factores que involucren diferencias en condición (Adamo, 2004; Kanost y Gorman, 2008; González-Santoyo y Córdoba-Aguilar, 2012; Kanost y Gorman, 2008). Dada esta capacidad diferencial para producir proPO, este cimógeno podría ser propuesto como otro indicador de la condición inmune en insectos (Schimid-Hempel, 2005).

La plasticidad y variedad en el sistema inmune ejemplifica claramente que las interacciones parásito-hospedero son mas complejas que la simple evaluación de un solo componente y que, por lo tanto, las relaciones CSS-inmunidad pueden verse afectadas por diversos factores externos. Apoyando esta idea se observó que aunque en algunas especies se encontró una relación entre estas dos características (inmunidad-pigmento), dicha relación no se dio cuando el individuo fue retado inmunológicamente. Además de esto, el hecho de que un implante de nylon induzca una actividad menor de FO comparado con un reto bacteriano, puede sugerir que el individuo está produciendo diferentes respuestas como un posible mecanismo de discriminación entre diferentes retos inmune, uno séptico y otro aséptico o de cuerpo extraño. En este caso en particular se activó una respuesta inmune de menor intensidad sobre retos asépticos (i.e filamentos de nylon) comparada con retos sépticos (bacterias).

Mis resultados, sin embargo, no pretenden descartar la HIC; más bien, quisiera usar estos resultados para abrir un panorama diferente a lo que teóricamente es esperado. Por las diferentes especies evaluadas, los diversos tipos de retos inmunes y los posibles efectos estacionales, se descubre un patrón más general en las relaciones reproducción-inmunidad. La presencia de resultados mixtos hace evidente que la relación entre condición individual, expresión de CSS e inmunidad es mucho mas compleja que lo que en un principio se planteó (Adamo y Spiteri, 2005, 2009). Si a esta complejidad se le suma la limitación de evaluar solo uno o pocos componentes de la inmunidad, entonces las predicciones teóricas pueden ser aún menos corroboradas en una observación experimental y empírica (Adamo, 2004; Jacobs ay Zuk, 2012; Adamo, 2012). Por ejemplo, durante el segundo capítulo de esta tesis se dejó en claro que a pesar del amplio conocimiento que se tiene sobre los mecanismos referentes a la activación y expresión de la enzima FO en insectos y su uso como indicador de la capacidad inmune, existe aún diferentes aspectos de este sistema que se encuentran inexplorados, lo que sugiere por lo tanto el uso con cautela de este tipo de herramientas.

Dada las propiedades intrínsecas del sitio de síntesis de la FO, un primer aspecto inexplorado es el estudio bajo condiciones controladas de los grupos celulares (hemocitos) donde es sintetizada la FO ya que son células sumamente frágiles y difíciles de reproducir en cultivos celulares (Strand, 2008). De la misma forma, varias moléculas intermediarias son rápidamente activadas y degradadas, lo cual genera que su aislamiento y caracterización sea muy complejo, de aquí que se desconozca por ejemplo como las quinonas son polimerizadas a melanina en condiciones *in vivo*. Otro punto importante es que se desconoce el sustrato natural de la FO. Bajo condiciones de laboratorio, como muchos estudios en ecoinmunología, se ha utilizado a L-Dopa descaboxilasa (L-DOPA) como el principal sustrato de activación, sin embargo otros sustratos

como es el caso del catecol N-acetil dompamina (NADA), el cual tiene una mayor afinidad y se encuentra presente en la hemolinfa del insecto, pudiese ser el sustrato natural. Dilucidar este punto puede ser de suma importancia para conocer la capacidad máxima de actividad de FO en respuesta a infecciones con diferentes patógenos.

En lo que respecta al área ecoinmunológica, el uso de la FO se encuentra aún en sus primeros pasos. Un punto básico a resolver es determinar si esta enzima es realmente un indicador de la habilidad inmune. A pesar de que la FO en insectos ha sido usada ampliamente y que tiene un estatus del mejor indicador inmune en estos estudios, mi revisión y otros trabajos ya publicados (González-Santoyo y Córdoba-Aguilar, 2012) en el capítulo 2, sugiere que estamos lejos de que esto sea correcto. El sistema inmune en los insectos está compuesto por un gran número de otros componentes además de la FO, los cuales han recibido muy poca atención. Estos componentes en conjunto con la FO deberían ser examinados de manera coordinada para formar una visión más integral de este sistema tan complejo. Un ejemplo de esto es que se ha detectado algunas pleiotropías antagónicas entre la FO y el sistema de lisoenzimas, lo cual genera un especialización en la respuesta que dependerá fuertemente del tipo de patógeno (Cotter et al, 2004) . En relación a los efectos ambientales sobre este componente, es necesario realizar estudios futuros en escenarios más realistas con interacciones naturales entre los hospederos y sus patógenos para entender de mejor manera los aspectos co-evolutivos que están detrás de estas interacciones. Relacionado con el punto anterior, aún no se conoce cual es el fundamento de las diferencias en la actividad de FO entre sexos, etapas de vida, estacionalidad, etc. y aunque tales diferencias pueden estar relacionadas a una variación en los tipos de patógenos a los que son expuestos durante los diferentes escenarios mencionados, esta idea aun no ha sido explorada. Dadas estas críticas, cabe preguntarse que llevó a los ecoinmunólogos a basar sus mediciones a

sólo FO y parte de la respuesta es una especie de herencia cultural tal como ocurre con la elección de sujetos de estudio (Owens, 2006).

Los estudios inmunoecológicos son un ejemplo de ecología funcional. Usando este principio y retomando la base fisiológica de las asociaciones entre la función sexual y la inmune, una posibilidad para estas asicoaciones se presenta cuando estas dos funciones comparten algún recurso. De aquí que en el tercer capítulo me haya cuestionado la base bioquímica de la pigmentación alar en *Hetaerina americana*. En esta exploración encontré que la pigmentación alar roja era consecuencia de la presencia de un pigmento llamado xantomatina, perteneciente al grupo de las omocromas. La xantomatina es sintetizada a partir de los metabolitos secundarios kinurenina y 3-hidroxikinurenina, y ha sido reportada en varios grupos de invertebrados (Oxford y Gillespie, 1998) incluyendo algunas especies de odonatos (i.e. Futahashi et al., 2012). El grupo de las omocromas son las responsables de una amplia variedad de colores (amarillo, rojo, café y blanco) producidos por diferentes combinaciones tanto de omatinas como de ominas (Seligy, 1972). La presencia de esta omocroma así como de su metabolito secundario la 3-Hidroxikinurenina (3-HK) en la pigmentación alar de *H. americana* me llevo a plantear una idea alterna a la relación CSS-inmunidad. Apoyando una de las hipótesis que explica la falta de relación entre pigmento-inmunidad encontrada en el genero *Hetaerina* expuesta en el primer capitulo, se encontró que dichas asociaciones eran poco probables que sucedieran, dado que a nivel bioquímico, estos componentes derivados de la ruta metabólica del triptófano, no guardan una relación directa con los recursos que utiliza el individuo para montar una respuesta inmune (Beckage et al., 1990). Sin embargo, dado que la xantomatina es producto de la ruta metabólica del triptófano, la cantidad de pigmento sintetizado podría depender en gran medida de la cantidad y capacidad para adquirir alimento. En este caso, dicha coloración podría estar

fungiendo como un indicador directo de qué tan bien alimentado esté un individuo e indirectamente estar indicando otro componente que requiera de recursos como es el inmunológico (Schild-hempel, 2005).

De manera interesante, el precursor de la xantomatina, la 3-HK, encontrado también en las alas de *H. americana* es un metabolito con propiedades neurodegenerativas en fases adultas de insectos, el cual puede ocasionar desde parálisis temporal hasta la muerte (Linzen, 1974; Cerstiaens et al., 2003). Un aspecto que no ha sido explorado es el de la función de las omocromas como productos finales del metabolismo y que servirían para eliminar los efectos tóxicos de los metabolitos secundarios del triptófano (i.e. la 3-HK), originados por el proceso catabólico y anabólico que ocurre durante periodos de fuertes cambios morfológicos, tales como metamorfosis en organismos holometábolos o el cambio de larva a adulto en hemimetábolos (Linzen, 1974; Chapman, 1998).

Planteo aquí por lo tanto, que en *H. americana* pudiera estar ocurriendo este fenómeno, en donde la xantomatina acumulada en las alas formaría parte de un mecanismo para lidiar con los efectos neurodegenerativos de lo metabolitos tóxicos del triptófano. Para poner a prueba esta idea se podría administrar a individuos machos el metabolito tóxico precursor de la xantomatina, la 3-HK y se observaría tanto la deposición de este pigmento en sus alas como la eliminación de los efectos neurodegenerativos de dicho metabolito.

Por último, tomando en cuenta el enfoque integral que he planteado a lo largo de este proyecto, otro punto que aún estaba sin explorar fue cómo la pigmentación alar y sus componentes, incluida la coloración roja, podían influir en el éxito reproductivo del portador para que estuviese sexualmente seleccionado. Se sabe ahora que el tamaño de pigmentación predice la posesión de

territorios y el éxito reproductivo del portador (Grether, 1996a; Contreras-Garduño et al., 2008). Sin embargo, en esta ultima sección propuse que el color rojo se ha fijado gracias a la función que tiene sobre las redes de comunicación generadas dentro de una población y hacia otras especies que estén ocurriendo en simpatría, y cuya característica es que compartan recursos. En este caso expliqué que el color rojo se ha mantenido como una señal honesta que indica la capacidad intrínseca de obtener un territorio. Estos territorios son prácticamente sitios de encuentro donde los machos no ofrecen ningún beneficio a las hembras (Córdoba-Aguilar et al., 2009b). Señalizar la capacidad de defensa de un lugar puede tener una función adaptativa muy importante ya que permite a los actores resolver conflictos para prevenir contiendas física y energéticamente muy costosas (Johnstone and Norris 1993). De manera general si esta idea es correcta, entonces carecer de dicha señal generará altos costos energéticos que terminaran repercutiendo sobre la supervivencia y adecuación del individuo.

En lo que a *H. americana* respecta encontré que los individuos manipulados en la pigmentación alar (de rojo original a azul) mostraron una menor supervivencia, la cual fue explicada gracias a los cambios en la condición fisiológica (menor musculatura torácica y menores reservas energéticas) y en la conducta territorial (menor capacidad de defender territorios). Estos resultados sugieren parcialmente que la mera presencia del color rojo en *H.americana* advierte la habilidad de pelea y la capacidad de defender un territorio "per se" de la especie, por lo que eliminarlo genera altos costos fisiológicos que resultan en efectos negativos sobre la supervivencia y en el éxito reproductivo (Grether, 1996a; Serrano-Meneses et al., 2007).

La interpretación evolutiva de mis resultados y en la ecología funcional es que la pigmentación alar, puede funcionar a un primer nivel para alertar de la presencia de un macho defendiendo un territorio. En un escenario donde existe una fuerte competencia intra e inter especifica por los

mismos recursos, esta señal seria favorecida por evitar agresiones físicas innecesarias (Anderson y Grether 2010). Apoyando esta idea, estudios en aves han indicado que el rojo puede señalar varios aspectos sobre la capacidad territorial, como el nivel de agresión, la condición fisiológica, etc. y aquellos individuos que portan esta señal son menos agredidos y presentan dominancia sobre otros colores (Pryke et al. 2001a; Pryke et al. 2001b; Pryke et al. 2002). Este primer nivel explicaría porque la gran mayoría de las peleas en *Hetaerina* y Calopterygidae en general, se dirimen en cuestión de segundos. Estos encuentros tan cortos se deberían a que un macho que no posee un territorio, simplemente llega a uno y necesita "cerciorarse" de que no está ocupado (Waage, 1988). Esta "revisión" de sitios funcionaría especialmente bien para aquellos machos con una condición energética que tienda a ser pobre ya que no emplearán sus recursos en pelearse con cada individuo que se encuentre ocupando un territorio. Pero es ampliamente adaptativo para el poseedor de un sitio, el advertir su presencia y estatus ya que los intrusos se retiran casi inmediatamente. Existen otras propiedades de la mancha que podrían evaluarse en caso que un macho intruso se enfrente al defensor. Estas propiedades asociadas frecuentemente con el tamaño de la pigmentación supondría un segundo nivel de comunicación. Este nuevo nivel sería de gran utilidad una vez que los individuos hayan decidido competir por el recurso, en este caso, el tamaño del carácter. En este segundo nivel propongo que son sólo aquellos individuos de mayor calidad energética los que se enfrentarían o buscarían enfrascarse en peleas largas y exhaustivas. Nótese que no estoy hablando de que estos encuentros sean sólo entre individuos de la misma especie, dado los hallazgos del grupo de Grether sobre los errores en el reconocimiento entre competidores entre diferentes especies de Hetaerina (Anderson y Grether, 2010). Estos errores producto de un sistema neuronal y cognoscitivo muy particular (Grether, 2011) promoverían que todas las especie de *Hetaerina* encontradas simpatria, usen los dos niveles que

propongo arriba. Mi planteamiento abre diferentes incógnitas. Una de ellas es por qué se selecciona sólo ser rojo en el primer nivel de comunicación y el tamaño del pigmento en el segundo. El primer punto es muy paradójico considerando que en otras especies no tan sólo el rojo sino la intensidad de éste se asocia a la capacidad de defensa de un sitio. Esto no parece ser el caso para *Hetaerina* por una restricción importante, y es que el rojo podría tener otra función eliminando componente tóxicos como fue planteado anteriormente.

Esta dependencia de la condición pero contraria a lo que usualmente se ha encontrado en otros caracteres cuya expresión se asocia positivamente con, por ejemplo, la dieta, supone que un macho de *Hetaerina* necesita tan sólo un toque de rojo para anunciar su estatus. Esto es de alguna manera coherente con los datos de Contreras-Garduño et al. 2008 en donde no encontró diferencias en aspectos del rojo entre machos territoriales y no territoriales en *Hetaerina americana*. Es conveniente añadir que también el rojo en aquellas otras especies donde este color conlleva un mensaje de estatus, son vertebrados donde el mecanismo de producción del rojo probablemente no surja de principios de toxicidad como aquí he planteado.

Otra incógnita tiene que ver con el tamaño de la mancha roja como un atributo sexual, lo cual supone que es la cantidad de rojo lo cual también podría ser seleccionado a favor. Estudios previos han encontrado que el tamaño de la mancha es dependiente de la condición, es decir la dieta, en *Hetaerina americana* (Jiménez-Cortés et al. 2012). Esto no tiene que ver con los atributos del rojo, sino con cuánto de rojo. En otras palabras, si algo se selecciona a favor es cuánto de omocroma es ubicada en las alas lo cual puede ser una medida indirecta de qué tan bien se alimentó un macho en su vida larvaria y/o adulta. Por último, aunque sabemos que los machos territoriales tienen manchas más grandes que los que no son territoriales, nunca se ha analizado si la intensidad de las peleas es mediada por el tamaño y los atributos del color de la

mancha. Un ejemplo de por qué necesitamos esta información es que según los modelos de "medición mutua" (mutual assessment) en las contiendas, el grado de escalamiento de una pelea depende de pequeños detalles que durante la pelea surjan y que reflejen la capacidad de lucha. Si, por ejemplo, el tamaño y la intensidad de rojo son caracteres usados para dirimir peleas en diferentes momentos de esta, no lo sabremos si solo se toman machos territoriales y no territoriales y se les mide la mancha o atributos de esta.

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