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# Variación temporal en expresión de un ornamento, tamaño corporal, reservas energéticas, respuesta inmune y supervivencia en machos de una libélula territorial

# T E S I S

**QUE PARA OBTENER EL GRADO ACADÉMICO DE:**

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**P R E S E N T A**

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"Después viene la muerte de los resultados en las acciones
al igual que la sombra de un cuerpo
con esta empresa el conocimiento y la memoria
me lleve a ser extremadamente prudente
para mantener lo esencial en mi práctica" –**Filosofía budista**

**Pensamientos**

"Con la ciencia, las ideas germinan en un lecho de teoría, forma y práctica que impulsan su crecimiento, pero los jardineros deben tener cuidado...

...porque algunas semillas son las de la ruina...

...y las flores más bellas son en ocasiones las más peligrosas." – **"V"**

"Lo bello es siempre raro. Lo que no es ligeramente deforme presenta un aspecto inservible." – **C. Baudelaire**

"La vida de cada hombre es un camino hacia sí mismo, el ensayo de un camino, el boceto de un sendero**."– H. Hesse**

"La naturaleza es siempre demasiado fuerte para la teoría." **– D. Hume**

"Somos animales que nos arrastramos y lo único que tal vez nos podría salvar mínimamente es el cerebro" **–G. House**

"Dentro de nosotros existe algo que no tiene nombre y eso es lo que realmente somos." **–J. Saramago**

"Everything should be made as simple, as posible, but no simpler" **–Iwasa y Pomiankowski**

"If you think that you are strong enough

If you think that you belong enough

Nice dream, nice dream"… **–Radiohead**

**"**Vivimos en una plácida isla de ignorancia en medio de mares negros e infinitos, pero no fue concebido que llegáramos muy lejos" **–H.W. Love**craft

"The world, unfortunately, rarely matches our hopes and consistently refuse to behave in a reasonable manner" **–S. J. Gould**

**CONTENIDO**

**Resumen**

1- La variación estacional en la respuesta inmune es un tópico que pocas se ha veces investigado en invertebrados. En el presente trabajo la respuesta inmune fue estudiada usando varias generaciones de machos territoriales de la libélula *Hetaerina americana* (Fabricius) durante año y medio.

2- Se registró la variación estacional en el patrón de pigmentación de la mancha roja en la base de las alas, un carácter sexual secundario (CSS) y se relacionó con el tamaño del cuerpo, reservas de grasa del tórax y abdomen, tres componentes de la respuesta inmune (melanización, fenoloxidasa [FO] y óxido nítrico [ON]) y la concentración de proteína en hemolinfa (como un posible indicador de la condición inmune). También se midió la supervivencia de los individuos después de ser infectados con la bacteria *Serratia marcescens* durante dos épocas (octubre-2007 y enero-2008) que tuvieron valores contrastantes en el contenido de FO y mancha alar.

3- Se encontró una variación cíclica en el CSS, siendo más intenso en la época de lluvias (julio-octubre) y reducido en las épocas de secas (enero-abril). Un patrón similar se halló para el ON, mientras la melanización fue variando a lo largo del período de estudio. La actividad de la FO, contenido de proteína y las reservas de grasa no fue posible relacionarla con el patrón estacional de la mancha. La supervivencia fue significativamente más baja en el mes de enero que en octubre.

4- Animales con un ornamento y tamaño más grande tuvieron los valores más altos en melanización, actividad de la FO, ON y concentración de proteína. Sin embargo la relación entre la mancha y tamaño corporal con respecto a las reservas de grasa no fue clara.

5- A diferencia de otros estudios, el ornamento no estuvo correlacionado con todos los componentes inmunes (a excepción del ON).

6- De manera similar a lo que ocurre en vertebrados, la supervivencia fue más baja durante los meses de secas. En el caso de los estudios en vertebrados, los mismos individuos sufren estos cambios, mientras en *H. americana*, distintas cohortes mostraron diferencias estacionales.

**Palabras clave**: *Hetaerina americana*, variación estacional, ornamento, reservas de grasa, inmunidad, supervivencia.

**Abstract**

1- Seasonal variation in immune response has rarely been investigated in invertebrates. We have studied this using territorial adult males of the damselfly, *Hetaerina americana* (Fabricius), in several generations for a year and a half.

2- We investigated and related seasonal variation in a red pigmented wing spot size (an ornamental trait) and body size to fat reserves, three immunity components – melanization ability, phenoloxidase (PO) and nitric oxide (NO) activity, and haemolymph protein concentration (a possible indicator of immune condition)– and survival ability after a bacterial immune challenge.

3- There was seasonal variation in spot expression, being more intense in the non-winter months and less intense in winter months, and, to some extent, a similar pattern was found for NO. Although there was also variation in melanization, PO activity, protein concentration and fat reserves, this was not consistently related to variation in spot size. Survival was lower in the winter than in non-winter times.

4- Animals with larger spot and body size, had larger values of melanization, PO and NO activity and protein concentration. The relation of spot and body size with fat reserves is not clear.

5- Unlike other studies, ornament was not similarly correlated with all typical immune components (at best, mainly NO).

6- Similar to what occurs in vertebrates, survival is lower during winter. In the case of vertebrate studies, however, the same individual suffers these changes. In the American rubyspot, distinct cohorts showed the seasonal differences.

Key words: *Hetaerina americana*, seasonal variation, ornament, fat reserves, immunity, survival.

# 1. INTRODUCCIÓN

## 1.1 Selección sexual

Las diferencias que existen entre machos y hembras para Darwin podrían atribuirse a diferencias ecológicas o conductuales. Sin embargo los caracteres fenotípicos como cuernos, astas, plumajes vistosos y colores extravagantes no podían ser explicados por procesos que inmiscuyeran directamente a la selección natural. Aunque el tema fue explorado someramente en el "Origen de las especies", no fue sino 12 años después cuando Darwin expone extensamente su teoría de la selección sexual que define como el éxito que presentan ciertos individuos sobre otros del mismo sexo, exclusivamente en lo que a la reproducción se refiere (Darwin, 1871).

Mientras los caracteres sexuales primarios son todas aquellas estructuras directamente inmiscuidas en la reproducción, tales como glándulas u órganos copulatorios o abarcando incluso la propia formación de gametos; los caracteres sexuales secundarios (CSS) solo aparecen hasta alcanzar la madurez sexual. También puede mencionarse que un CSS es más exagerado en un sexo que en el otro, puede utilizarse para dominar al oponente en los enfrentamientos o se exhibe en el cortejo, no provee un beneficio inmediato en la supervivencia, es costoso de producir y posee una alometría positiva durante la ontogenia individual (varias referencias citadas en Senter, 2007). Como ejemplo de un CSS está la aleta caudal que desarrollan los machos de los peces dulceacuícolas conocidos como gupis cuando se encuentran maduros sexualmente y que puede alcanzar grandes proporciones en comparación con el resto del cuerpo. Se ha comprobado que el tamaño y el número de despliegues de la aleta están relacionados con la preferencia de las hembras y con el número de cópulas de los machos (Bischoff *et al*., 1985). La selección natural no podía explicar la aparición y evolución de los CSS que por su obviedad eran conspicuos y que por esto podrían reducir las oportunidades de supervivencia del organismo al ser fácilmente detectados por los depredadores (Darwin, 1871). Dentro de la teoría de la selección sexual explica que la aparición y mantenimiento de estos CSS, suponen una ventaja para sus portadores y donde los machos con mejores CSS tienen mayores ventajas reproductivas (Darwin, 1871).

Darwin describió dos mecanismos principales en la evolución y mantenimiento de los CSS, uno determinado por la elección de las hembras (también llamado intersexual) y otro dado por competencia entre machos (también conocido como intrasexual). En el primer mecanismo, generalmente las hembras evalúan distintos aspectos fenotípicos y/o conductuales de los machos antes de elegir a su pareja. En el

segundo mecanismo, los machos compiten por conseguir recursos o territorios con el fin de atraer a las hembras, o bien luchan directamente por ellas. La visión tradicional darwiniana concibe que mediante el enfrentamiento directo los machos, han podido desarrollar armas como cuernos o astas que aumentan la probabilidad de ganar una lucha a un coespecífico.

Recientemente, un tercer mecanismo ha cobrado importancia como una explicación alternativa a los dos anteriores. Basándose en el hecho de que los machos tienen una presión muy intensa por conseguir apareamientos, estos podrían evolucionar mecanismos para contrarrestar la presión femenina por limitar su acceso a la reproducción. Las hembras en este punto podrían aparearse a tasas subóptimas y, por lo tanto, ejercería una presión para hacer que en las hembras evolucionen mecanismos para contrarrestar tal presión. Esto generaría una especie de carrera armamentista antagónica cuya base sería un conflicto entre los sexos (Parker 1979, 1984; Arnqvist y Rowe, 2005).

### 1.2 Los CSS como indicadores de la condición

Entre los supuestos que se utilizan para entender la evolución de los CSS es la existencia de un vínculo directo entre la condición del individuo y los CSS. La condición hace referencia a la viabilidad que puede tener un organismo y que puede tener tanto componentes genéticos como ambientales (Iwasa, *et al*., 1991; Iwasa y Pomiankowski, 1994). En los últimos años se han recopilado suficientes evidencias empíricas que confirman lo anterior (Andersson, 1994; Johnstone, 1995; Kotiaho, 2001). Como factores ambientales que influyen en la condición se ha experimentado principalmente con el alimento y el parasitismo. En gallos silvestres se evaluó el efecto del nematodo *Ascaridia galli* en los CSS (ornamentación y elongación de las plumas y coloración facial) y en caracteres no sexuales (longitud del tarso, longitud y anchura del pico) entre un grupo experimental y un grupo control. Se encontró que las diferencias entre los dos grupos de machos solo se presentaron cuando se evaluaron los CSS, pero no así para los otros caracteres (Zuk, *et al*.,1991). Finalmente puesto que la condición está influida por la varianza ambiental o genética en la habilidad para adquirir o asimilar recursos, características dependientes de la condición como son los CSS, éstos pueden utilizarse como señales de calidad genética o fenotípica.

### 1.3 Los CSS como indicadores de la capacidad inmune

En 1982 Hamilton y Zuk, en un artículo ahora clásico, iniciaron las investigaciones entre la relación de la selección sexual y el parasitismo. Partiendo de algunos supuestos de genética de poblaciones, evaluaron el efecto de seis especies de protozoos y una especie de nematodo sanguíneo en el brillo, despliegue del cortejo y canto (los tres CSS) en 109 especies de aves paseriformes de Norteamérica. Los resultados mostraron en la mayoría de los casos correlaciones positivas (de débiles a fuertes) entre las variables, lo que sugirió que los CSS podrían funcionar como indicadores de resistencia contra patógenos.

Explorando la misma idea, Folstad y Karter (1992) después de revisar distintas pruebas empíricas, propusieron la hipótesis de la inmunocompetencia. Bajo esta hipótesis un macho que presenta un CSS intensamente expresado estaría comunicando a las hembras su capacidad de resistencia a los patógenos, una característica que puede ser heredada a la progenie. Folstad y Karter (1992) propusieron como mecanismo entre la función inmune y la capacidad inmunológica, la testosterona (o cualquier otra sustancia que cumpla esta función), que por un lado estimula el desarrollo de los CSS pero que también tiene un efecto inmunosupresor en vertebrados. Aunque algunos ejemplos parecen sustentar este principio (p. ej. Zuk *et al.,* 1990), el hecho es que el mecanismo mediado por la testosterona está en duda (Roberts *et al.,* 2004). Recientemente Peters (2007) explica que al considerar que los carotenos son una materia prima en la constitución de muchos CSS y además de cumplen con una función antioxidante en el organismo y teniendo en cuenta que las células del sistema inmune son de las principalmente afectadas por el estrés oxidativo podría existir una "competencia" entre los CSS y el sistema inmune por estos compuestos. A este esquema se agrega el reciente descubrimiento de la testosterona como regulador en el transporte de carotenos en la corriente sanguínea, lo que hace aun más complejo el conjunto de interacciones que puede existir entre sistema inmune y los CSS.

Sheldon y Verhulst (1996) al recopilar diferentes pruebas de las relaciones antagónicas entre la respuesta inmune y otros componentes de la adecuación, propusieron una relación diferente entre la capacidad inmunológica y la expresión de un CSS. Para ello partieron del punto que la respuesta inmune es costosa y que, por lo tanto, tiene una gran demanda de recursos, lo cual llevaría a posibles compromisos entre el sistema inmune y otros componentes asociados con la reproducción. Estos autores mencionan que la relación entre el sistema inmune y los CSS, más que ser un

mecanismo mediado por la testosterona u otras hormonas, debe verse en términos de recursos y gastos, donde dependiendo de la condición de un individuo, será la cantidad de recursos a invertir en ambas funciones. Dado que los organismos pueden entrar en conflictos en la distribución de recursos a distintas funciones, los organismos que mayores problemas enfrentarán son aquellos en peor condición. Este enfoque ha sido un buen marco de referencia sobretodo en insectos quienes carecen de testosterona (Schmid-Hempel, 2005).

### 1.4 Sistema inmune en insectos

Los insectos poseen un sistema inmune innato y al igual que en vertebrados tienen un componente celular y uno humoral (Levine y Strand, 2002). Esta división se realiza con fines prácticos, aunque se sabe que ambos contribuyen de manera complementaria al momento de identificar y eliminar un agente extraño. En adelante se describirá de manera resumida algunos de los principales mecanismos de defensa en insectos y los cuales utilicé en mi tesis.

Una vez que un patógeno ha podido pasar exitosamente el exoesqueleto o los epitelios que recubren al insecto, se activa la cascada de la fenoloxidasa (FO). Esta reacción compleja y emanada a partir de que los hemocitos reconocen a un agente extraño, da lugar a que distintos hemocitos se aglomeren y produzcan una serie de reacciones y compuestos encaminados a eliminar tal agente (Tzou *et al*., 2002; Cerenius y Söderhäll 2004). Algunos de estos compuestos son detallados a continuación.

La melanización es uno de los mecanismos de la respuesta inmune más utilizados contra agentes extraños de tamaño relativamente grande. La FO activa que se encuentra en la hemolinfa, convierte los fenoles a quinonas los cuales conducen a la formación de melanina (Cerenius y Söderhäll, 2004). Ésta se utiliza para recubrir el agente extraño mediante la formación de cápsulas celulares (Schmid-Hempel, 2005; Cerenius *et al*., 2008).

Otro componente de la respuesta inmune es el óxido nítrico (ON). Este es un gas inestable y altamente reactivo producido de la oxidación de la L-arginina+acitrulina mediante la óxido nítrico sintetasa. Se ha mostrado su toxicidad contra virus, hongos, bacterias, protozoos (intra y extracelulares), así como en algunos metazoos. (Rivero, 2006). También se ha encontrado que puede ser la primera línea de defensa contra oocinetos de *Plasmodium* en mosquitos y en *Rhadnius prolixus* en infecciones con *Tripanosoma rangeli* (Dimopoulos *et al*., 1998; Herrera-Ortiz *et al*., 2004).

Cabe mencionar que estos componentes (actividad de la FO, ON e intensidad de melanización) han sido estudiados o se han propuesto como indicadores de la capacidad de defensa en insectos (p. ej. Schmid-Hempel, 2005).

### 1.5 Sistema inmune y cambios estacionales

Hasta hace algunos años se manejaba un modelo estático de los hospederos para la respuesta inmune y el parasitismo. Sin embargo, si se considera que los organismos enfrentan variaciones ambientales, ya sean cíclicas o temporales, cuyos efecto principal es la variación en la disponibilidad de recursos (principalmente alimenticios), así como en la probabilidad de enfrentarse a ciertos patógenos (Møller *et al*., 2003; Martin *et al*., 2008), se espera que la respuesta inmune varíe.

Las aves y mamíferos son los grupos donde más se ha explorado la estacionalidad y la respuesta inmune (p. ej. Møller *et al*., 2003; Nelson, 2004; Martin *et al*., 2008). El patrón general que se ha encontrado es que la respuesta inmune, aumenta en invierno y disminuye en primavera. Martin *et al*., (2008) describieron dos modelos teóricos que explican dicho patrón. Por un lado, el incremento de la respuesta inmune invernal se debe a que existen mecanismos fisiológicos amortiguadores que aumentan la respuesta inmune en esta época, mientras que el segundo supone compromisos entre la respuesta inmune y diferentes componentes de la reproducción que explican la inmunosupresión en primavera. Puesto que en humanos y otros vertebrados se han detectado que ciertas enfermedades presentan patrones anuales consistentes, la respuesta inmune de los hospederos puede estarse viendo afectada por estas fluctuaciones temporales de los patógenos. La variación geográfica se agrega como otra variable que puede afectar a la inmunidad. En realidad este es un campo poco incursionado, aún en vertebrados, pero los resultados muestran hallazgos interesantes. Por ejemplo, en especies de aves que tienen una distribución tanto en regiones templadas como tropicales se ha encontrado que estas últimas invierten más en la defensa, que las poblaciones ubicadas más al norte (Martín *et al*., 2008). Finalmente puesto que las hormonas en vertebrados cumplen importantes funciones de comunicación entre el ambiente exterior e interior, se piensa que estos mensajeros químicos podrían también estar interviniendo en las variaciones estacionales de la respuesta inmune (Møller *et al*., 2003; Nelson, 2004; Martín *et al*., 2008).

Son sólo dos los estudios empíricos en insectos donde se ha cuantificado la variación estacional de la respuesta inmune. En un análisis reciente sobre este tópico,

Córdoba-Aguilar y Contreras-Garduño (2006) revisaron la literatura sobre las causas de la respuesta inmune y su relación con los CSS en libélulas y sugieren que, dado que la respuesta inmune está fuertemente ligada a componentes bióticos y abióticos de un ambiente, la respuesta inmune puede ser un buen indicador de la calidad ambiental. Ellos ponen énfasis a que esta calidad puede variar de acuerdo a la estacionalidad. En cuanto a las investigaciones empíricas, Yourth *et al*., (2002) encontraron que la capacidad de encapsulación por melanina de la libélula *Lestes forcipatus*, ante ataques por ácaros, fue más alta en individuos que emergieron tardíamente (agosto) en la estación, en comparación con los del inicio (junio) de la misma. Las causas de esta variación no fueron estudiadas. Por otra parte, Contreras-Garduño *et al*., (en prensa) compararon la capacidad de melanización, el área de la pigmentación alar (un CSS) y las tasas de alimentación (como una manera de saber la disposición de recursos alimenticios) en dos hábitats con valores diferentes en la precipitación total anual y el clima usando machos adultos de la libélula *H. americana*. Los autores encontraron que los animales de Xochitepec, Morelos (clima templado subhúmedo y precipitación total anual de 1000 mm) tuvieron valores más altos en melanización, mancha y tasa de alimentación que en Metztitlán, Hidalgo (clima semiseco y precipitación total anual entre 700- 800 mm). Además, compararon la actividad de la FO y de ON en dos momentos del año: (octubre y abril) en una tercera población: Tehuixtla, Morelos (mismo patrón climático que en Xochitepec) cuando las densidades de adultos son muy diferentes en donde existe una alta y baja densidad respectivamente. Encontraron que en octubre la respuesta inmune fue más alta. Los autores sugirieron que la actividad inmune y la mancha están fuertemente influidos por la disposición de alimento y que, aunque a mayor densidad, los costos de producción de ambas características son altos, los animales invierten en ambas funciones (Contreras-Garduño *et al.,* en prensa).

### 1.6 La libélula *Hetaerina americana* como sujeto de estudio

*H. americana* Fabricius es un caballito del diablo (Zygoptera:Calopterygidae) que se distribuye desde el norte de Estados Unidos de América, pasando por México y llegando hasta Honduras (Paulson, 2007). Los adultos tienen un marcado dimorfismo donde los machos presentan un color metálico iridiscente principalmente en el tórax, con una mancha roja en la base de las alas, mientras las hembras presentan variación en el color que puede ir de verde a café y con una incipiente mancha en la base de las alas (Grether, 1996a; Contreras-Garduño, 2007). Las larvas pasan una fase estrictamente

acuática para emerger como tenerales, una etapa donde el integumento es claro y blando, con una duración promedio de 24 horas (Corbet, 199). Poco a poco se van endureciendo las alas y ya entonces comienzan a alimentarse en su fase prereproductiva. En esta etapa, el organismo dedica gran parte del tiempo a alimentarse, recursos que se usarán durante la madurez (Corbet, 1999). La duración del teneral y del estadio prereproductivo puede variar entre 1 y 18 días, al término del cual el área y color de la mancha habrán terminado de formarse (Córdoba-Aguilar y Cordero-Rivera, 2005).

Durante este tiempo de maduración, otras características masculinas también culminan su desarrollo como son los músculos del tórax y las reservas de grasa (Córdoba-Aguilar y Cordero-Rivera, 2005). Estas características son muy importantes durante la competencia por apareamientos, ya que dado que *H. americana* es una especie territorial, la obtención de un territorio es esencial. Una vez maduro sexualmente y con las características musculares y energéticas, los machos se dirigen a los ríos para obtener un territorio. Las peleas por un territorio se dan en el aire, donde ambos contendientes se enfrentan en vuelos espirales, lo cual puede prolongarse por varios minutos o incluso horas (Johnson, 1962; Grether, 1996b). El éxito de estas peleas está relacionado con las reservas de grasa donde aquellos machos con mayor contenido de estas resultan ganadores (Contreras-Garduño *et al*., 2008). Los machos con manchas rojas más grandes también tienen más reservas de grasa, lo cual ha llevado a pensar que estas manchas son un indicador de la capacidad inmune (Contreras-Garduño *et al*., 2008).

Las peleas parecen ser la vía para obtener apareamientos, ya que no se ha observado que las hembras elijan a los machos (Johnson, 1962; Bick y Sulzbach, 1966; Grether, 1996b). De hecho, experimentos recientes cuyo objetivo fue modificar los sitios defendidos por los machos, no cambiaron las tasas de apareamiento (Córdoba-Aguilar *et al*., en prensa). Las hembras llegan a los territorios y, sin cortejo previo, son acosadas por los machos y tomadas en cópula (remoción de esperma de machos anteriores y transferencia del mismo) por los machos más grandes (Grether, 1996a; Serrano-Meneses *et al*., 2007; Córdoba-Aguilar, en prensa). Cabe hacer mención que anterior a la cópula propiamente se distinguen las etapas de enganche, vuelo precopulatorio en tándem e inicio de la cópula (aleteo del macho y atracción de la hembra hacia delante). Dado que las hembras no ponen huevos en el sitio defendido por los machos, la pareja se dirige en tándem a sitios donde esta puesta se da (Raihani *et al*., 2008; Córdoba-Aguilar *et al*., en prensa). Durante estas travesías en la búsqueda de

estos sitios, otros machos interceptan a la pareja, y, nuevamente, los machos más grandes son más victoriosos al desplazar a otros de menor tamaño (Córdoba-Aguilar *et al*., en prensa). El hecho de que en los territorios no existen recursos que las hembras puedan utilizar ni aparentes beneficios materiales aportados por los machos en general, ha llevado a pensar que el sistema de apareamiento de esta especie es un lek donde las hembras parecen tener pocas opciones de elección de pareja.

El tamaño de la mancha, aunque no así características del color (Contreras-Garduño *et al*., 2007b), parecen reflejar la capacidad energética del individuo. Los experimentos con machos territoriales con manchas más grandes y machos no territoriales (machos que no pudieron conseguir un territorio) con manchas más pequeñas han indicado que los primeros tienen mejores capacidades inmunológicas en términos de melanización, FO, enzimas proteolíticas y mayor supervivencia ante retos inmunológicos artificiales (inserción de implantes de nailon) y vivos (bacterias como *Serratia marcencens*, una bacteria típica de insectos) (Adamo, 2004b; Contreras-Garduño *et al*., 2006; Contreras-Garduño *et al*, 2007a).

Aunque está claro que es poco probable que los machos indiquen a otros machos acerca de sus capacidades inmunológicas, estas características podrían ser seleccionadas indirectamente por las mayores reservas de grasa (Contreras-Garduño *et al*., 2008). Un experimento reciente, ha corroborado esta idea: ante un reto artificial durante el desarrollo de la mancha, este carácter resultó de tamaño más pequeño comparado con machos que no fueron retados (Contreras-Garduño *et al*., 2008).

La libélula *H. americana* es una especie ideal para estudiar la temporalidad de la respuesta inmune y su relación con la mancha (el CSS) por varias razones: i) Como lo demostraron Contreras-Garduño *et al*. (en prensa), estos animales están presentes como adultos durante todo el año en las zonas del centro de México. Dado que la vida como adulto no dura más de 40 días (Serrano-Meneses *et al.,* 2007), existen varias generaciones en un año; ii) A pesar de que la relación entre la respuesta inmune y el CSS (la mancha alar) es clara, esta no se ha seguido a lo largo de un año. Uno esperaría que si el componente alimenticio varía en diferentes estaciones (ver Contreras-Garduño *et al*., en prensa), no siempre existirá una relación estrecha entre estos dos caracteres; iii) En los machos de estos insectos una de las principales funciones donde las reservas de lípidos son utilizadas en la búsqueda y defensa de un territorio (Plaistow y Siva-Jothy, 1996). Si se considera que las densidades poblacionales en el sitio de estudio fluctúan en el año, se podría esperar que las reservas no se mantengan constantes,

partiendo de que mayores densidades conllevan a mayores encuentros agonísticos y por ende un mayor gasto energético. Finalmente Contreras-Garduño *et al*., (en prensa) usaron individuos de sólo dos meses diferentes para la comparación estacional y sólo dos componentes inmunológicos (FO y ON). Esto, aunque es un acercamiento previo al problema de la estacionalidad, no refleja en su totalidad la capacidad de defenderse contra patógenos vivos como las críticas recientes en insectos han sugerido (ver Adamo, 2004).

En esta tesis presento los resultados de comparar la expresión de la mancha alar, reservas de grasa, melanización, FO, ON y supervivencia ante retos con la bacteria *S. marcescens* (esto en dos meses de actividad inmunológica contrastante y para poner a prueba si en meses donde la actividad inmunológica es alta, existe mayor supervivencia ante ataques patogénicos) en machos adultos territoriales de *H. americana* a lo largo de año y medio. En un segundo punto, he comparado las relaciones entre la expresión de la mancha con respecto a los componentes de la respuesta inmune, reservas de grasa y tamaño corporal entre meses contrastantes o para todos los meses del estudio. Presento el contenido central de mi tesis a manera de artículo para revisar, dado que este fue recién aceptado para publicación en la revista *Ecological Entomology*.

### 1.7 Hipótesis

a) La estacionalidad influirá en el CSS, reservas de grasa, respuesta inmune (fenoloxidasa, óxido nítrico y melanización), concentración de proteínas en hemolinfa y supervivencia posterior a un reto bacteriano en machos territoriales de la libélula *H. americana.*
b) La relación entre el CSS y los componentes de la respuesta inmune no siempre será similar.
c) La supervivencia después de ser infectados con *S. marcencens* será diferente en los individuos que se encuentran en una época donde la expresión de la respuesta inmune sea más robusta comparados con la de individuos de una época con una respuesta inmune menos robusta.

### 1.8 Objetivo general

Conocer si la pigmentación, respuesta inmune (fenoloxidasa, óxido nítrico y melanización), grasa y tamaño corporal, concentración de proteínas y resistencia a patógenos varían en distintas épocas en el año en machos adultos territoriales de *H. americana*.

#### 1.8.1 Objetivos particulares

a) Registrar la pigmentación alar, respuesta inmune (fenoloxidasa, óxido nítrico y melanización), concentración de proteínas, tamaño corporal y reservas de grasa en machos adultos territoriales en seis diferentes épocas del año.
b) Comparar la supervivencia de los machos adultos territoriales después ser inoculados con la bacteria *Serratia marcescens* en dos épocas donde la condición masculina es distinta.
c) Registrar si el tamaño de la mancha covaría en el tiempo con la respuesta inmune (fenoloxidasa, óxido nítrico y melanización), la concentración de proteínas y las reservas de grasa.
d) Registrar si el tamaño corporal se correlaciona con el ornamento, respuesta inmune (fenoloxidasa, óxido nítrico y melanización), la concentración de proteínas y reservas de grasa.

# Seasonal variation in ornament expression, body size, energetic reserves, immune response, and survival in males of a territorial insect.

## 2. Introduction

One emphasis of ecological and evolutionary immunity studies is to see immune response as an evolving trait and thus analyse how its expression will ultimately affect host's fitness (Schmid-Hempel, 2003, 2005). One area where this emphasis has been applied is sexual selection. Male ornament expression has been positively associated with immune ability (e.g. Aguilera & Amat, 2007; Kurtz *et al.,* 2007; Cotter *et al.,* 2008). Given the constant pressure that pathogens exert on hosts, the latter are facing a regular energetic demand to invest in immunity. Since not all host individuals are able to deal successfully with such high energetic standards, only those males in better condition will produce a more robust immune response and also more elaborate ornaments (Sheldon & Verhulst, 1996). One interpretation of this relationship is that immune ability is one of the different aspects of condition that ornaments communicate to conspecifics (e.g. Neff & Pitcher, 2005; Andersson & Simmons, 2006). The idea that ornaments and immunity are energetically coupled goes well with a resource allocation conflict perspective (Roff, 1992) in which both traits share composition and/or manufacture resources (Sheldon & Verhulst, 1996).

A common implication in ecological immunity studies is that all immunity components are costly to produce and, thus, may be equally reflected by ornament expression. This assumption may be far from true for the following reasons (Adamo, 2004a; Adamo & Spiteri, 2005): each pathogen may elicit species-specific immune responses, the immune system can change its responses to optimize its strength against different pathogens, and there is no *a priori* basis to assume that all immune components are related with each other. Despite this, very few tests have been done for such implication (but see, for example, Keil *et al.,* 2001). One approach to this is that rather than measuring an infinite number of immune metrics, immune components that have been previously shown to be related to ornament expression should be simultaneously analysed to see how they co-vary with the ornamental trait in question. This analysis should be done including different generations to see whether a positive correlation still holds. Such analysis should be preferably accompanied by pathogen challenges to see which immune trait correlates with host survival, this given that correlation between immune traits and disease resistance is usually poor (Adamo, 2004a).

Seasonal variation in immune response has been documented in vertebrates (for fish see Bowden *et al.,* 2007; for mammals and birds see Martin *et al.,* 2008; for all these taxa and humans see Altizer *et al.,* 2006). In general, it has been shown that the same individual experiences a reduction in immune ability in winter as compared to summer (reviewed by Hillgarth & Wingfield, 1997). Winter is when weather conditions are harsh and/or food shortage is more likely (Lloyd, 1995; Nelson *et al.,* 2002). These vertebrate studies are in sharp contrast with research in invertebrates as our knowledge in this group is reduced to a handful of works. The few pieces of information have indeed uncovered that there is variation in immunity in individuals emerging in different times during the same season (i.e. variation in immunity does not occur in the same individual; e.g. Smith & McIver, 1984a and b; Yourth *et al.,* 2001, 2002) which may be associated to variation in parasite impact along the season (Yourth *et al.,* 2001, 2002). These studies have not even been carried out in different seasons (e.g. winter *vs* non-winter times) as the species studied have time-limited breeding seasons (e.g. no more than three months in summer). The closest approach to such aim were two studies carried out in summer in a territorial calopterygid which suggested that PO levels may be different between years possibly due to yearly variation in gregarine parasite impact (Siva-Jothy, 2000; Rolff & Siva-Jothy, 2004).

In this paper we have investigated the seasonal change and its possible effects on a male ornament, body size, energetic reserves, three immune components and survival ability after a bacterial challenge using adult males of a territorial damselfly, the American rubyspot (*Hetaerina americana*). The conditions in which our study was done allow us answering the questions implied above: we have used more than one generation (as larvae and adults live approximately two months and one month respectively; all authors unpub. data), several immune parameters were measured and survival after a pathogen attack was recorded.

After emergence, American rubyspot males feed extensively to construct muscular fat reserves. During this time, males also construct a red pigmented spot located at the basis of each wing and whose area (but not other aspects of it, such as colour; Contreras-Garduño *et al.,* 2007a) correlates with fat muscular reserves at the time that the animal competes for a territory or is defending one (Contreras-Garduño *et al.,* 2006, 2008; Serrano-Meneses *et al.,* 2007; Raihani *et al.,* 2008). The size of the spot is considered an ornament as those males with larger spots and, therefore, more fat resources are more likely to defend a territory and gain more matings (Grether 1996a;

Serrano-Meneses *et al.,* 2007). A second reason is that, according to a resource allocation conflict perspective, a recent experiment confirmed that spot size and immune ability are energetically coupled: at the time of spot formation, immune-challenged males ended up with a smaller spot compared to control males (Contreras-Garduño *et al.,* 2008). Spot size positively correlates with immune and survival ability after a bacterial challenge (Contreras-Garduño *et al.,* 2006, 2007b, in press a). For example, territorial males (which have larger wing spots) produced higher levels of melanization (the act of melanine covering of foreign targets of relatively large size that have penetrated the exoskeleton; Gillespie *et al.,* 1997), phenoloxidase (PO) activity (PO gets activated once a pathogen has entered the host, allowing for a plethora of immune responses to act and resulting in the death and melanization of pathogens; Tzou *et al.,* 2002, Cerenius & Söderhäll, 2004) and nitric oxide (NO) activity (NO is activated when the host is exposed to different micro-organisms such as Gram-negative and Gram-positive bacteria, and gives a signal to the occurrence of infection to haemocytes; Foley & O'Farrell, 2003; Herrera-Ortíz *et al.,* 2004; Leclerc & Reichhart, 2004) compared to non-territorial males (which have smaller spots). Furthermore, these two male statuses have been challenged with *Serratia marcescens*, a highly pathogenic bacteria that typically infects this (Córdoba-Aguilar & Lanz-Mendoza, unpub. data) and other insect species (e.g. Matsumoto *et al.,* 1998; Adamo, 2004b), and the results have indicated that territorial males survived for longer than non-territorial males (Contreras-Garduño *et al.,* 2007b).

Spot and the aspects of immune condition it reflects are variable along the year in the American rubyspot. In a population in central Mexico and in only two time points measured, males that emerged in October produced larger spots and higher immune response (in terms of PO and NO activity) than males that emerged in April (Contreras-Garduño *et al.,* in press a). Here we have extended this study by measuring melanization ability, PO and NO activity, haemolymph protein concentration (a possible indicator of immune condition; Adamo, 2004b), fat reserves and survival after a bacterial challenge for a year and a half. Our first aim was to see whether there was seasonal variation in spot size, immune and energetic components. We predicted that any variation in all these components in the first year will coincide with variation in the following year. As for the bacterial challenge, our prediction was that seasonal variation in immune ability will correlate with seasonal variation in survival after the challenge. Our second aim was to see how much spot size covaries with energetic and immune components in this

seasonal comparison. We expected that independently of how much spot size varies, such variation will correlate with that of fat reserves and immunity values. We have introduced body size for both aims to see whether this trait correlates with all variables, including spot size for two reasons: a) previous examinations of body and spot size in the American rubyspot have provided non-consistent patterns, although all studies have been carried out for a relatively short time period (Grether, 1996b; Contreras-Garduño *et al.,* 2006; Serrano-Meneses *et al.,* 2007; Raihani *et al.,* 2008); and, b) including body size in our comparisons allow us seeing to what extent this and not only spot size, explains variation in immune ability.

## 3. Material and Methods

### 3.1 Study area and male collection

We carried out collections in an approximate 1200m stretch of the river Amacuzac (18º 36' 39'' N, 99º 10' 52'' W, 890 altitude) located in Morelos, Mexico. This is a semi-tropical area with rains in March-April and July-August and a fluctuating temperature that goes from 24 to 36 ºC. We carried our six collections to cover a total of 15 months: October (2006), January, April, July, October (2007) and January (2008). Previous to collection and on the first day, males were netted and numbered using a permanent ink marker on its right forewing. Collection for morphological and physiological measures was carried out the following two to three days between 1000 to 1500 hrs, the hours of maximum sexual activity (Serrano-Meneses *et al.,* 2007). Only territorial males were collected for two reasons: a) at times in the year when male density is relatively low (e.g. January to April), the competition for territories and male density is so low that only territorial males are found (all authors, unpub. data); and, b) a significant correlation between spot size and immune and energetic components is present only for territorial males; this is because non-territorial males are usually too young (in the process of manufacturing such components) or too old (energetically exhausted due to past intense sexual competition) which gives extremely low energetic and immunity values (Contreras-Garduño *et al.,* 2006; in press a). Thus, to accomplish the second aim of our study - to see whether there is correlated variation in spot size and immune and energetic components - using non-territorial males was not logical. We distinguished territorial status using the rationale that territorial males remain in the same site where they were previously captured for marking. Non-territorial males did not do this. This requisite has been successfully used and validated with previous

records of territorial and non-territorial behaviour in this species (e.g. Raihani *et al.,* 2008). Since even within territorial males, males vary in age and, therefore, condition (Contreras-Garduño *et al.,* 2008), we controlled for this by using only animals whose body and spot colour was shiny, and transparent and undamaged wings. Males with these characteristics have roughly the same age (for a rationale of this see Contreras-Garduño *et al.,* 2006; for other closely related species see, for example, Plaistow & Siva-Jothy, 1996). During collection and prior to morphological and physiological measurements, each male was placed within an assay tube with a wood piece (to serve as a perching piece) and humid cotton (to avoid dehydration) in a dark box (to reduce the animal's activity so that the male would not die due to energetic exhaustion). For each collection, three independent male groups were used: one first group for melanization ability (N for all sampled months = 133); a second group for PO activity (N for all sampled months = 142); and a third group for NO activity (N for all sampled months = 143). Protein quantification was obtained for the second and third groups (thus N = 285). Spot and body sizes were measured in all groups. Fat reserves were quantified for the second and third group as it was not clear, in the case of the first group, whether implanting a nylon filament for melanization recording (see below) could have a negative effect on fat reserves (see Contreras-Garduño *et al.,* 2006). Manipulations during recordings of immune response and survival ability were carried out at an approximate temperature of 25 ºC.

**3.2 Spot and body size**

Each wing was removed from its site of insertion to the thorax. A digital picture of each wing was taken. Each picture was then visualized on a computer screen and the relative size (with respect to total wing area) was measured (in pixels) using the software Image Tool for Windows® (version 3.0). An average of all four wings was obtained. Using the same images, the length of the right forewing was also measured (in mm), which was used as an indicator of body size (see Serrano-Meneses *et al.,* 2007 for the rationale of this).

**3.3 Fat reserves**

We used the method of Marden (1989). The thorax and abdomen were separated from the other body parts, and placed in a desiccator at 28 ºC for 24 hrs. These structures not only have the highest fat content but such content is used during flight (Marden, 1989). The desiccator eliminated humidity from samples which were then weighed in a semianalytic balance (Scientech SA120). The samples were placed within

an eppendorf tube with 0.5 ml of chloroform and left in the same desiccator also at 28 ºC for 24 hrs. After this time the chloroform has been extracted and evaporated along with the fat content. Samples were re-weighed. The weight difference in both recordings represents the total fat the individual had.

### 3.4 Immune response

#### 3.4.1 Melanization ability

We used the protocol of inducing a host's cellular immune response via an artificial nylon challenge (e.g. Rantala & Roff, 2007). This technique is based on inserting a nylon piece within the insect body and then quantifying the area of cells that melanized the implant. It has to be mentioned that this protocol does not distinguish between melanization and encapsulation (which have actually led some authors to say that it was encapsulation what they measured; e.g. Rantala & Roff, 2007; Ryder, 2007). We used a 2 mm length and 0.1 mm width nylon which was previously disinfected in 96% ethanol. The nylon piece was manipulated with dissection forceps and inserted in the ventral side of the fourth abdominal pleura, parallel to the body plan. Animals were then returned to their assay tubes in a dark box (to reduce insect activity) for 24 hrs. The implants were carefully retrieved by first removing the surrounding exoskeleton (so that the melanized cells did not become adhered to the exoskeleton when the implant was retired) and then withdrawing the nylon. Each implant was stored in 70 % ethanol but was rehydrated during 24 hrs previous to melanization quantification. Each nylon piece was placed under a stereoscopic microscope (Olympus S2H-ILLK) and three pictures were taken using a digital camera (Cannon Power Shot G6) attached to the microscope, varying the position of the nylon piece for each picture. Given the irregular distribution of melanized cells on the implant, the three pictures can provide a more complete view of melanization ability (Contreras-Garduño *et al.* 2006). The relative area of melanization in relation to the whole nylon area was measured in pixels using the software Image Tool® (version 3.0) for windows. An average of each of three pictures for each implant was obtained. This methodology has proven to be repeatable in this species (Contreras-Garduño *et al.,* 2006).

#### 3.4.2 PO and protein quantification

Each animal was inoculated with 7 µl of a mix of phosphate buffer saline and protease inhibitors (PBS-PI) in the thorax region where heads are inserted. After one minute, the head was removed and the thorax was gently pushed to get 2 µl of haemolymph plus PBS-IP. 100 µl of phosphate buffer saline and protease inhibitors

were added to this 2 µl and the sample was kept at 0 °C. Before PO activity recordings, protein content was quantified not only because it was going to be used for the two aims of this study but also to control for individual differences. Related to this latter issue, previous studies in this species have revealed that such control is necessary as, during haemolymph extraction, more protein could be obtained from some individuals than others (Contreras-Garduño *et al.,* 2007b). These differences may provide unrealistic PO activity differences among individuals. We controlled for this using the Pierce method (using the BCA$^{TM}$ protein assay commercial kit). From each sample, 10 µl were taken to which we added 40 µl of phosphate buffer saline and protease inhibitors plus 150 µl of pierce reagent. A known concentration of albumin was used as a standard curve (provided in the kit) which was compared to our sample. Once the protein content of each of our samples was known, we adjusted them to 10 U/mg of protein. Then we were able to record PO activity which was done (in duplicate) spectrophotometrically (Model 350 BioRad) at 490 nm via recording dopachrome formation from L-dihydroxyphenylalanine (L-DOPA Sigma). As blanks, 175 µl of phosphate buffer saline was mixed with 25 µl of L-DOPA and the optical density was also measured at 490 nm. Three PO lectures were taken every 15 min (15, 30 and 45 min) and an average was taken. Enzyme activity was expressed as units with each representing an absorbance change per minute (Söderhäll & Häll, 1984).

**3.4. 3 Nitric oxide quantification**

The Griess reaction was used. For this, 50 µl of each haemolymph sample was mixed with 50 µl of sulfanilamide and 50 µl of 0.1 % naphthylethylenediamine (Sigma, St. Louis, MO, USA). This mixture was incubated for 10 min at room temperature. Using a plate reader and at 540 nm, absorbance was recorded after 15 and 30 minutes but an average was obtained for both recordings. NO was quantified using a $NaNO_2$ (1-100 µM) standard reference curve for each assay. Similar to PO quantification, protein content was quantified (as indicated above) to be used for the aims of this study and also to control for individual differences (for the same reasons indicated above for PO recordings). Results are provided as nitrite and nitrate concentration.

**3.4.4 Survival after a bacterial challenge**

Our results indicated that October and January exhibited extreme values in the expression of the immune components we measured (see results section). Given this, we used these two time points for challenging territorial males with *S. marcescens*: October 2007 and January 2008. Using an initial stock of 37.08 x $10^5$ colony forming units

(CFU) of this bacterium, we prepared and used four dilutions (100, 75, 50 and 25%) in a culture media. We used these four concentrations as we did not have *a priori* information of the dosage that could kill these animals. Using a microsyringe, we inoculated 3 μl of each of these different concentrations in the dorsal side of the thoracic region where the head is inserted. We had four male groups: a) a group inoculated with bacteria ("bacterial challenge"); b) a group inoculated only with culture media (with the same volume as the first group with bacteria; "culture media control"); c) a group that was only pinched with the syringe ("syringe control"); and, d) a control group manipulated as the other three but in which no inoculation or pinching was produced ("sham"). Each individual was left in an assay tube with humid cotton and a wooden piece (for perching) at 25 °C in a dark box. Every hour we checked whether animals were still alive. An animal was considered dead when it did not show any movement activity even when manually retrieved from the tube. No food or water was provided during the experiment.

**3.5 Statistical analyses**

Seasonal variation of each variable was compared for all months. If necessary, data were transformed to use parametric tests but this was the case only for PO (transformations based on $\log_{10}$). When data showed normal distributions and homogeneity of variances, one way analyses of variance (ANOVA) were used, and Tukey tests for post hoc comparisons. However, when data were not amenable for transformation, a non-parametrical ANOVA (Kruskal Wallis test) was used applying the false discovery rate procedure for post-hoc multiple comparisons to control for the expected proportion of null hypotheses that are falsely rejected (Benjamini & Hochberg, 1995). In all post-hoc comparisons, we were interested in, first, whether the changes of immunity values were similar to those of spot and body and, second, whether the same month comparisons (October 2006 and 2007, and June 2007 and 2008) did not change (as would be expected). Survival data were analysed using the non-parametrical model of Kaplan-Meier and the Logrank (Mantel-Cox) statistic (Moenchow, 1986; Pike & Thompson, 1986). In these analyses, however, body size was compared among treatments within each month to see whether survival differences were not affected by this trait. To see the relation between spot and body size with fat reserves, melanization, PO and NO activity and protein concentration, a regression analysis was done for each relation in which spot and body size were the independent variables. Two types of regressions were done. The first for all sampled months and the second for those two

months that showed the more extremes values for both spot size and the dependent variable in question. The second type was used as data were not amenable for transformation for some relations in the case of all sampled months. The two-month comparison provided, at least for some cases, data that could be analysed and, therefore, interpreted. For those cases in which variables were not normally distributed, the data distribution type was indicated in the model and the regression was carried out using a generalized linear model. Results are indicated as mean ± STD unless otherwise stated. All analyses were performed using SPSS (ver. 15; Chicago, Illinois, USA).

## 4. Results

### 4.1 Seasonal variation in spot size, body size, fat reserves and immune response

There was variation in spot (ANOVA, $F_{5,171} = 139.88$, $P = 0.0001$; Fig. 1) and body size (ANOVA, $F_{5,173} = 32.877$, $P = 0.0001$; Fig. 2). Months when spot size was at its highest level were July and October of both years while January (both years) and April had the lowest values (Fig. 1). There were no differences when the same months (October 2006 and 2007, and June 2007 and 2008) were compared (Table 1). Body size had corresponding changes to spot size (Fig. 2; Table 1). For example, non-winter times had higher values than winter times, and October 2006 and January 2007 were not different to October 2007 and January 2008 respectively (Table 1).

Fat reserves were also different for all months (Kruskal-Wallis test = 19.075, $P = 0.02$; Fig. 3). To some extent, similar patterns to spot changes were obtained in which October was higher than January but only in the 2007-2008 comparison but not for the 2006-2007 comparison (Table 1). However, in the same month comparison, despite October of both years had similar values, January of both years were not the same (Table 1).

Melanization values showed statistical differences (ANOVA, $F_{5,171} = 139.88$, $P = 0.0001$; Fig. 4). There was no close association with spot size changes. For example, October was not consistently higher than January (Table 1). Furthermore, both October months were significantly different while January months were not (Table 1). PO was also different among months (Kruskal-Wallis test = 63.465, $P = 0.0001$; Fig. 5). Again, there was not clear association with spot size changes: October was higher than January in only one out of three comparisons (the other two were not different). The same-month comparison was not significant for October 2006 and 2007 but it was for January 2007 and 2008 (Table 1). Protein concentration was also different among months

(Kruskal-Wallis test = 47.370, $P < 0.0001$; Fig. 6). However, October had higher values than January only in one out of three comparisons (Table 1). October 2006 and January 2007 had higher protein values than the same months in 2007 and 2008 respectively (Table 1). These patterns are different to those observed for spot size changes. NO varied across months (Kruskal-Wallis test = 31.114, $P = 0.0001$; Fig. 7) and there was a clearer pattern of seasonality and changes according to spot changes: October was higher than January in two out of three comparisons (Table 1). The data of both October years were significantly different but January data were not (Table 1).

**4.2 Survival after a bacterial challenge**

Although there were differences in body size for all groups for both months ($F_{7, 93} = 14.688$, $P = 0.0001$), within each month, there were no differences in size among treatments (all Tukey tests $P > 0.05$). There were no difference in survival among the "culture media control", syringe control" and "sham" groups (all $P > 0.05$) in both October (2007) and January (2008) which indicates that our manipulation had no effect. In October (2007), any of the four dosages of the "bacterial challenge" group had a significantly reduced survival compared to "culture media control", "syringe control" and "sham" groups (Table 2). The same occurred for January (2008) (Table 2).

Comparing the survival of each dosage of the "bacterial challenge" with its corresponding dosage of the same group of the two months, animals from October had a higher survival than those from January for all bacterial concentrations except for 100 % (Table 3).

**4.3 Relations of spot and wing size with fat reserves and immunity**

All results appear in table 4. For the two-month comparison, spot size was positively related to fat reserves which was not the case when all months were compared. For all months, spot size was positively related to PO activity, melanization, NO and protein concentration. However, only for PO, NO and protein concentration, but not for melanization, the relation held for the two-month comparison.

When body size was used for all sampled months, no relation was found for fat reserves and melanization, but a positive relation was found with PO and NO activity, and protein concentration. For the two-month comparisons, regressions with PO, melanization, NO and protein concentration were significant. Wing and spot size were significantly and positively related for all months (Fig. 8) and the two-month comparison.

## 5. Discussion

We detected seasonal variation in spot size expression in which the second half of the year (the non-winter times) had the highest values of this trait which is coherent with previous results in this species (Contreras-Garduño *et al.,* in press a). At this time of the year, male density and territorial competition are high (Serrano-Meneses *et al.,* 2007). Since spot size seems to communicate the energetic status of males (Contreras-Garduño *et al.,* 2008), possibly at this time males invest considerably in spot production to make this communication more effective. Such exaggeration in spot expression would seem unnecessary in winter times as there are relatively few males to compete with (in fact, several territories remain vacant at this time; all authors, unpub. data). Another hypothesis is that food shortage can negatively affect spot production (Contreras-Garduño *et al.,* in press a). Interestingly, spot values were similar in the same months between years reflecting how predictable this trait is (which is in sharp contrast with the other traits we measured). It seems that, whatever the explanation for the seasonal changes in this trait, spot size is important enough to become highly consistent through years no matter what occurs with its correlated traits. If spot and the other aspects of condition we measured here share the same resources, according to a resource allocation scenario, spot does not seem to be resource-compromised or it is well buffered against any possible resource allocation conflict. Previous results have indicated that spot expression correlates with fat reserves (Contreras-Garduño *et al.,* 2006; Serrano-Meneses *et al.,* 2007; Raihani *et al.,* 2008) but despite this, we did not find that in the second half of the year, fat reserves showed the highest values. As for immune components, although variation was also detected, this was not in the direction spot size had. In fact, only NO showed variation that was close to spot size variation. In general, all energetic and immunity components seem so much variable that several disparate hypotheses can be put forward to explain them. One first hypothesis is that of male competition intensity. It is known that exhaustive and energetically demanding behaviours, such as fighting, negatively affect immunity (reviewed by Schmid-Hempel, 2005). In damselflies, territorial contests have a negative effect on fat reserves and immunity (reviewed by Suhonen *et al.,* 2008). Although such contests do not result in injuries, research has shown that at least melanization (Koskimäki *et al.,* 2004; Contreras-Garduño *et al.,* 2006; however, see Siva-Jothy *et al.,* 1998) and PO activity (Contreras-Garduño *et al.,* in press b) decrease perhaps as a correlated response due to energetic exhaustion. Given this evidence, one would expect that if male competition

intensifies at some time points, this will equally affect all physiological traits we measured here which was not the case. A second hypothesis is food shortage. Since immune ability is highly and immediately affected by food (Siva-Jothy & Thompson, 2002), this can be a source of variation. However, this factor, similar to the first hypothesis, does not seem to operate at the same intensity for fat reserves and all immunity traits (for example, unlike PO which was very variable, NO was not). A third hypothesis is pathogen-specific immune responses assuming changes in pathogen pressure in different seasons. Different immune components will not be expressed at the same level for the following reasons: a) not all immune responses are used for the same pathogens (Adamo, 2004a); b) there may be more than one pathogen simultaneously attacking a host (Adamo, 2004a); c) pathogen pressure may vary with time; and, d) not all immune components are related among them (e.g. Keil *et al.,* 2001). Such combination of factors may impede that a host can fully co-evolve with its pathogens, and, actually, evidence of such diffuse co-evolution has been detected in other odonates (reviewed by Forbes & Robb, 2008). Whether this is the case for our results we do not know.

Survival differences were detected at dosages lower than 100 % between seasons which suggest time-based differences in immune ability. It has to be mentioned that using a naturally-occurring pathogen bear some problems which are relevant to explain our results. For example, it may be that animals show seasonal variation in resistance due to seasonal differences in bacterial pressure. If in January, for example, bacteria are rare, damselflies would not be expected to allocate resources to immune components against such pathogenic threat and, in the face of an infection in this time, they could be more likely to die. Despite this drawback, our results are in support of seasonal mortality differences after an infection in months where harsh conditions occur due to a weakened immune ability, as has been detected in vertebrates (Lloyd, 1995; Nelson *et al.,* 2002; Altizer *et al.,* 2006). In vertebrates, it has been shown that the same animal is able to shift its investment of resources to immunity in response to trade-offs with reproduction (see Martin *et al.,* 2008). In the case of our study animal this is clearly not the explanation as we used different cohorts. NO values, but not melanization and PO, showed its lowest levels during winter months. As indicated above, one should not expect, however, that all immune parameters can be predictors of survival ability. That only NO was a closer indicator of immune ability in relation to bacterial challenge does not make sense as both NO and PO are potent immune arms used against a wide variety

of pathogens including bacteria (for NO see reviews by Bogdan *et al.,* 2000; Rivero, 2006; Carton *et al.,* 2008; for PO see reviews by Cerenius & Söderhäll, 2004; Lemaitre & Hoffmann, 2007; Kanost & Gorman, 2008). Two questions in relation to how insect immunity works are difficult to explain. The first is that melanization scores should be related to PO as PO activity actually precedes melanization activity during a pathogen attack (e.g. Siva-Jothy, 2000). One explanation is that we quantified PO in animals that were not immune challenged. Possibly, animals that are nylon-implanted will be able to produce higher PO values, more closely related to melanization scores. The second question is why melanization and NO do not exhibit similar seasonal differences despite previous evidence and theory suggesting that both are condition dependent (e.g. Siva-Jothy, 2000; Rivero, 2006). This may be related to the actual mechanisms the insect uses to deal with bacterial infections. For example, the targets that melanization is used against (e.g. metazoans of large size and bacteria; Gillespie *et al.,* 1997), seems more limited than those for which NO is used (e.g. virus, bacteria, protozoan and metazoan parasites; Bogdan *et al.,* 2000; Rivero, 2006; Carton *et al.,* 2008). It may well be that despite our experiment, bacteria could not be an important pathogenic agent in our damselfly populations so that our manipulation only simulated a non-existent situation during which animals were not necessarily prepared. In fact, only the October and January comparison when NO scores were not different was in 2007 and 2008 respectively, which is actually when the bacterial challenge was carried out. If NO had been the underlying factor acting against bacteria, the October and January difference should have been found in these years. Despite this, our unpublished results indicate that *S. marcescens* indeed is a common bacterium in our study population. In fact, this bacterium is more common in this population than intestinal gregarines and haemolymph-sucking mites (which are highly detrimental on adult odonate fitness; reviewed by Forbes & Robb, 2008) which were extremely rare both in previous years (Córdoba-Aguilar *et al.,* 2007) and in the years we sampled for the present study (all authors, unpub. data). NO results are similar to previous research in a close American rubyspot population which indicated that NO (and PO) was higher in October than in April (Contreras-Garduño *et al.,* in press a). As for PO results, it should not be expected that PO activity is related to bacterial infections as studies in *Drosophila* indicate (Kanost & Gorman, 2008). It may be that NO is not necessarily coupled with PO activity and that it was used to deal with pathogens other than *S. marcescens*. It may also be that given that NO participates in other non-immune functions (e.g. flight;

reviewed by Rivero, 2006), these functions may be more important in the American rubyspot. As for protein concentration, Adamo (2004b) observed that this was related to bacterial resistance in crickets which is not apparently the case of our animals. Not surprisingly, larger animals and with larger spots, had more protein in their haemolymph (see also Contreras-Garduño *et al.,* 2007b). Of course, the proteins we measured are a general set that may be used for immune and non-immune functions (see also Adamo, 2004b) so that further research disentangling this should be done.

Spot size shows positive relations with all immune variables, but this was depended on the time frame used. Of course, one criticism of our analysis is that only territorial animals were used. As we mentioned earlier, non-territorial males are literally non-existent in those months around winter when male density is too low and when they do occur, their relation spot size-energetic and immunity values is null. Despite this shortcoming, spot size seemed to be a consistent predictor of animal condition which is in agreement with previous results in this (Contreras-Garduño *et al.,* 2007) and other calopterygid species (Siva-Jothy, 2000 but see Rolff & Siva-Jothy, 2004). This was not strictly the case for fat reserves for all sampled months but it was for the two-month comparison. Spot size has been experimentally shown to be an indicator of fat reserves in males that have the age to compete for territories (Contreras-Garduño *et al.,* 2008), a relation that was interpreted as the spot being an honest indicator of male energetic condition. The only explanation is that such relationship does not hold consistently perhaps due to variable expenditure of fat during male competition for territories in some periods. Fat can be spent in a short time during fights (e.g. Contreras-Garduño *et al.,* 2006; for other damselfly species see, for example, Plaistow & Siva-Jothy, 1996) and since it is constructed from food, there may be times at which the combination of these two factors may provide too much noise for the relationship between this trait and spot size to hold. Body size was not a good indicator of fat reserves but it was for immune ability. Perhaps due to allometric relationships, larger animals can have better immune responses, a situation that will hold at any time. However, this does not seem to be the case for fat reserves. A study in another territorial calopterygid, *Calopteryx splendens xanthostoma*, whose breeding season is limited to summer and which also bears a wing ornament, has found no relationship between size and PO (Rolff & Siva-Jothy, 2004). This study, however, was done in a single month. Why there is a relationship of PO and body size in the American rubyspot but not in *C. s. xanthostoma* is difficult to explain. One factor is that the fact that our population is present for the

whole year may make that the relationship between body size and correlated values could be easier to find. For example, the fact that the "bad" season lasts several months, may produce animals with extremely bad condition, clearly different from those animals emerging in the "good" season. Finally, with respect to these comparisons, spot and body size were closely related. Despite this, both traits show variable relationships with the other components of condition we measured. Several authors have called the attention that not necessarily all immune traits will be directly related to ornaments and that not all immune components will be related with each other (Keil *et al.,* 2001; Adamo, 2004a; Adamo & Spiteri, 2005). Our results support this argument. It seems that, despite the advances in ecological immunity studies, immune responses have to be carefully selected to be linked to ornaments. In the case of the American rubyspot, previous studies had found that spot size was related to all immune traits we measured here. However, according to our present results, NO and survival were perhaps the best surrogates of immune condition.

The typical seasonal-based collection of immunity data includes mainly comparisons between a breeding and a non-breeding period in the same animals in which it is expected that animals will increase their investment to immunity during the breeding period (e.g. Møller *et al.,* 2003). In our study, we quantitatively assessed changes in immunity across a number of generations and when animals were sexually active. At this time, a high investment to reproduction is expected and is when trade-offs with immune function are more likely (Schmid-Hempel, 2005). Using the survival results, it can be said that at some points of the year, such trade-offs will be more acute than in other times. This would speak about possible fluctuating selection and immune investment across generations. Studies in other damselflies in temperate environments have pointed out important annual and/or seasonal variation in parasite pressure which would select for increased investment to immunity when parasites may be more common (Yourth *et al.,* 2002; Rolff & Siva-Jothy, 2004). Since we have not been able to assign particular pathogens that could cause such trade-offs, the possibility exists that other factors may explain our seasonal differences in spot size and NO expression and survival. Two of these factors are energetic exhaustion via male competition and food availability. These two hypotheses and that of pathogen pressure, are currently under assessment.

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## 7. Annexes (Tables y Figures)

Table 1. Probability values from between-month comparisons for all traits measured in adult males of the American rubyspot. Spot size, body size and melanization were compared following Tukey tests, while fat reserves, PO, NO and protein activity were analysed using the false discovery rate procedure.

| *Months and years compared* | *Spot size* | *Body size* | *Fat reserves* | *Melanization* | *PO* | *NO* | *Protein* |
|---|---|---|---|---|---|---|---|
| Oct 06-Jan 07 | 0.0001 | 0.008 | 0.485 | 0.098 | 0.0001 | 0.001 | 0.198 |
| Oct 06-Apr 07 | 0.0001 | 0.0001 | 0.025 | 0.999 | 0.0001 | 0.0001 | 0.0001 |
| Oct 06-Jul 07 | 0.0001 | 0.01 | 0.796 | 0.014 | 0.025 | 0.0001 | 0.0001 |
| Oct 06-Oct 07 | 0.579 | 0.359 | 0.707 | 0.004 | 0.149 | 0.0001 | 0.0004 |
| Oct-06-Jan 08 | 0.0001 | 0.001 | 0.023 | 0.023 | 0.544 | 0.0001 | 0.0001 |
| Jan 07-Apr 07 | 0.0001 | 0.129 | 0.001 | 0.048 | 0.074 | 0.122 | 0.0001 |
| Jan 07-Jul 07 | 0.0001 | 0.0001 | 0.908 | 0.956 | 0.0001 | 0.152 | 0.0001 |
| Jan 07-Oct 07 | 0.0001 | 0.0001 | 0.755 | 0.741 | 0.0001 | 0.160 | 0.0001 |
| Jan 07-Jan 08 | 0.928 | 0.982 | 0.002 | 0.922 | 0.0001 | 0.321 | 0.0001 |
| Apr 07-Jul 07 | 0.0001 | 0.0001 | 0.011 | 0.006 | 0.0001 | 0.830 | 0.671 |
| Apr 07-Oct 07 | 0.0001 | 0.0001 | 0.007 | 0.002 | 0.0001 | 0.904 | 0.849 |
| Apr 07-Jan 08 | 0.0001 | 0.471 | 0.572 | 0.011 | 0.005 | 0.526 | 0.159 |
| Jul 07-Oct 07 | 0.0001 | 0.697 | 0.817 | 0.994 | 0.378 | 0.822 | 0.831 |
| Jul 07–Jan 08 | 0.0001 | 0.0001 | 0.007 | 1.0 | 0.038 | 0.682 | 0.247 |
| Oct 07–Jan 08 | 0.0001 | 0.0001 | 0.005 | 1.0 | 0.070 | 0.549 | 0.315 |

Table 2. $\chi^2$ and probability (*P*) values from comparing the "bacterial challenge" (four dosages) with the other experimental groups using the Kaplan-Meier (Mantel-Cox Logrank) survival analysis in two different seasonal times. Direction of difference refers to which group had a lower survival. All comparisons with 1 degree of freedom.

| | | October | | | January | | |
|---|---|---|---|---|---|---|---|
| *Bacterial challenge concentration* | *Group* | $\chi^2$ | *P* | *Direction of difference* | $\chi^2$ | *P* | *Direction of difference* |
| 25 | Culture media control | 24.75 | <0.0001 | 25 | 20.09 | 0.0017 | 25 |
| | Syringe control | 26.48 | <0.0001 | 25 | 10.57 | 0.0011 | 25 |
| | Sham | 24.71 | <0.0001 | 25 | 9.83 | <0.0001 | 25 |
| 50 | Culture media control | 28.14 | <0.0001 | 50 | 20.54 | 0.0023 | 50 |
| | Syringe control | 26.69 | <0.0001 | 50 | 10.80 | 0.001 | 50 |
| | Sham | 24.91 | <0.0001 | 50 | 9.32 | <0.0001 | 50 |
| 75 | Culture media control | 29.30 | <0.0001 | 75 | 19.77 | 0.0026 | 75 |
| | Syringe control | 25.97 | <0.0001 | 75 | 10.35 | 0.0013 | 75 |
| | Sham | 24.28 | <0.0001 | 75 | 9.044 | <0.0001 | 75 |
| 100 | Culture media control | 29.87 | <0.0001 | 100 | 19.14 | 0.0025 | 100 |
| | Syringe control | 26.55 | <0.0001 | 100 | 10.08 | 0.0015 | 100 |
| | Sham | 24.87 | <0.0001 | 100 | 9.16 | <0.0001 | 100 |

Table 3. $\chi^2$ and probability (*P*) values from comparing the same bacterial concentration within the "bacterial challenge" group between October and January using the Kaplan-Meier (Mantel-Cox Logrank) survival analysis. Direction of difference refers to which group had a lower survival. All comparisons with 1 degree of freedom.

| *Bacterial challenge concentration* | $\chi^2$ | *P* | *Direction of difference* |
|---|---|---|---|
| 25 | 21.937 | < 0.0001 | January |
| 50 | 12.946 | 0.003 | January |
| 75 | 6.969 | 0.0083 | January |
| 100 | 2.781 | 0.0954 | None |

Table 4. Regression coefficients and values ($r^2$ and $X^2$ respectively) and associated probabilities (*P*) in different comparisons of male traits in the American rubyspot for all sampled months and for month pairs that differed extremely in the values of such traits. The first column specifies which months for the two-month comparisons were used.

| *Variables (months and years)* | *All months* | | *Both months* | |
|---|---|---|---|---|
| | *Regression* | *P* | $r^2$ | *P* |
| Spot size *vs* fat reserves (Jan 07-Apr 07) | 0.007 ( $r^2$) | 0.377 | 0.137 | 0.021 |
| Spot size *vs* PO (Jan 07-Jul 07) | 0.199 ( $r^2$) | 0.0001 | 0.424 | 0.0001 |
| Spot size *vs* melanization (Apr 07-Oct 07) | 154.376 ($X^2$) | <0.0001 | 0.126 | 0.059 |
| Spot size *vs.* NO (Oct 06-Jan 08) | 39.025 ($X^2$) | <0.0001 | 10.203 ($X^2$) | 0.001 |
| Spot size *vs* protein concentration (Jan 07-Oct 07) | 78.549 ($X^2$) | <0.0001 | 0.147 | 0.023 |
| Body size *vs* fat reserves (Jan 07-Apr 07) | 0.01 ( $r^2$) | 0.236 | 0.001 | 0.869 |
| Body size *vs* PO (Apr 08-Jul 07) | 359.793 ($X^2$) | <0.0001 | 0.224 | 0.0001 |
| Body size *vs* melanization (Apr 07-Oct 07) | 0.001 ( $r^2$) | 0.736 | 0.199 | 0.005 |
| Body size *vs.* NO (Oct 06-Jan 08) | 31.850 ($X^2$) | <0.0001 | 10.121 ($X^2$) | 0.001 |
| Body size *vs* protein concentration (Jan 07-Oct 07) | 27.713 ($X^2$) | <0.0001 | 0.256 | 0.002 |
| Body size *vs* spot size (Ene 07-Jul 07) | 0.308 ( $r^2$) | 0.0001 | 0.396 | 0.0001 |

Fig. 1. Seasonal changes in relative pigmentation of spot size in adult males of the American rubyspot. Sample sizes for each month are shown on the top.

Fig. 2. Seasonal changes in body size in adult males of the American rubyspot. Sample sizes for each month are shown on the top.

Fig. 3 Seasonal changes in fat reserves in adult males of the American rubyspot. Sample sizes for each month are shown on the top.

Fig. 4. Seasonal changes in melanization in adult males of the American rubyspot. Sample sizes for each month are shown on the top.

Fig. 5. Seasonal changes in PO activity in adult males of the American rubyspot. Sample sizes for each month are shown on the top.

Fig. 6. Seasonal changes in protein concentration in adult males of the American rubyspot. Sample sizes for each month are shown on the top.

Fig, 7. Seasonal changes in NO activity in adult males of the American rubyspot. Sample sizes for each month are shown on the top.

Fig. 8. Relation between relative pigmentation of spot size and wing length in all sampled months in males of the American rubyspot.

Fig. 1.

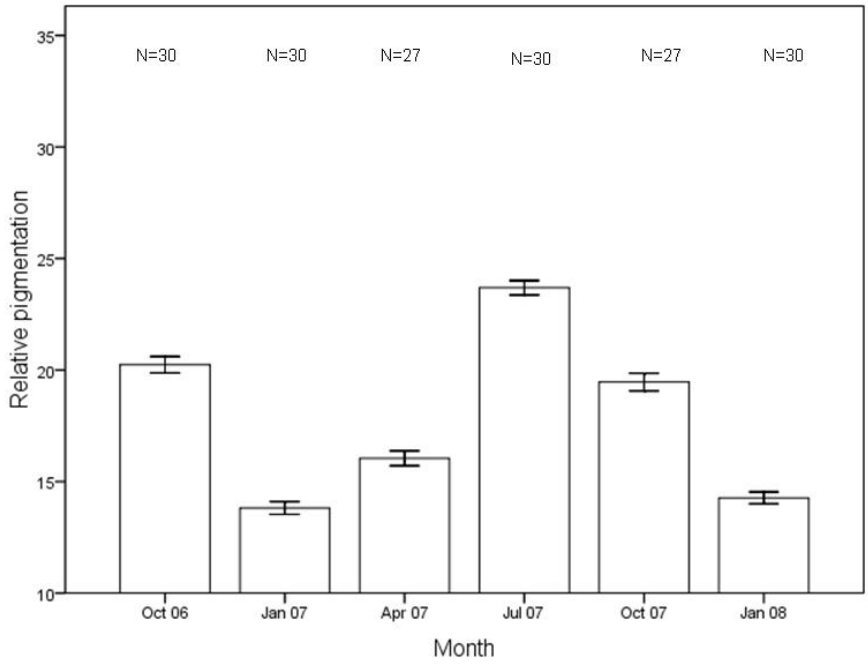

N=30
N=30
N=27
N=30
N=27
N=30
35
30
25
20
15
10
Relative pigmentation
Oct 06
Jan 07
Apr 07
Jul 07
Oct 07
Jan 08
Month

Fig. 2

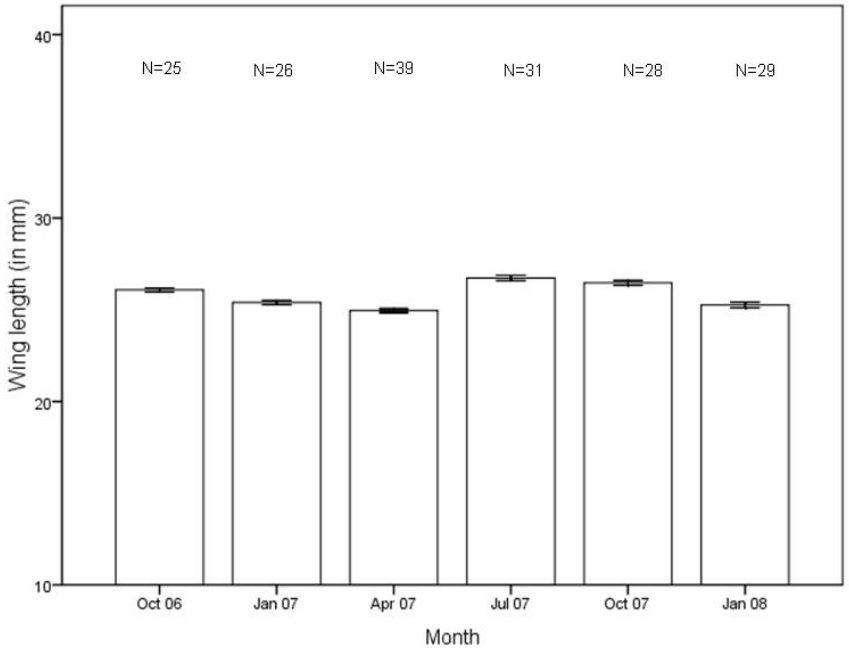

40
30
20
10
Wing length (in mm)
N=25
N=26
N=39
N=31
N=28
N=29
Oct 06
Jan 07
Apr 07
Jul 07
Oct 07
Jan 08
Month

Fig. 3

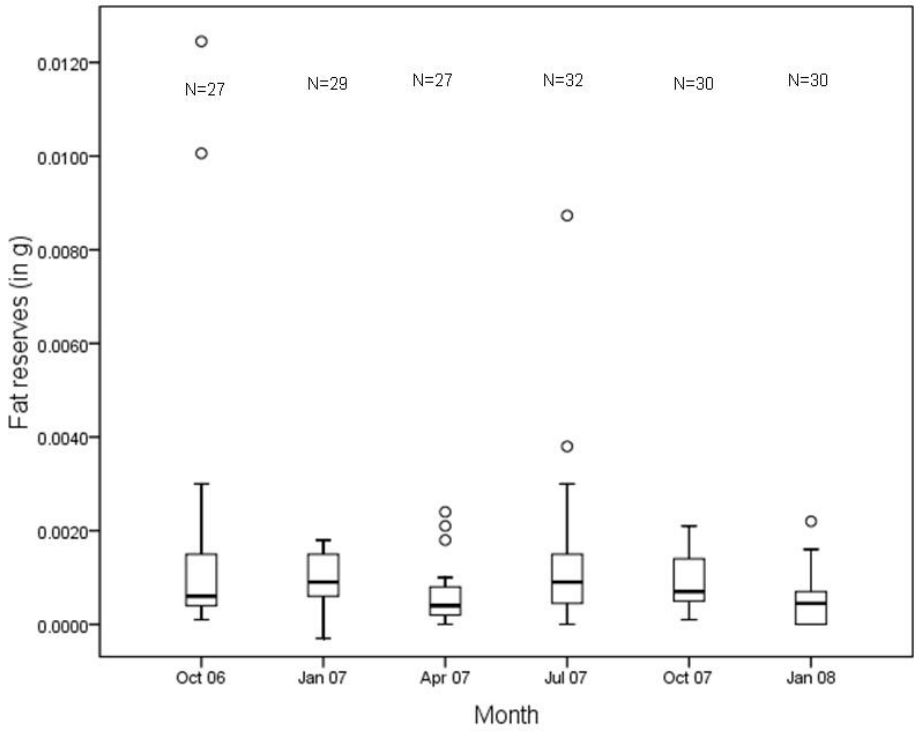

Fat reserves (in g)
0.0120
0.0100
0.0080
0.0060
0.0040
0.0020
0.0000
N=27
N=29
N=27
N=32
N=30
N=30
Oct 06
Jan 07
Apr 07
Jul 07
Oct 07
Jan 08
Month

Fig. 4

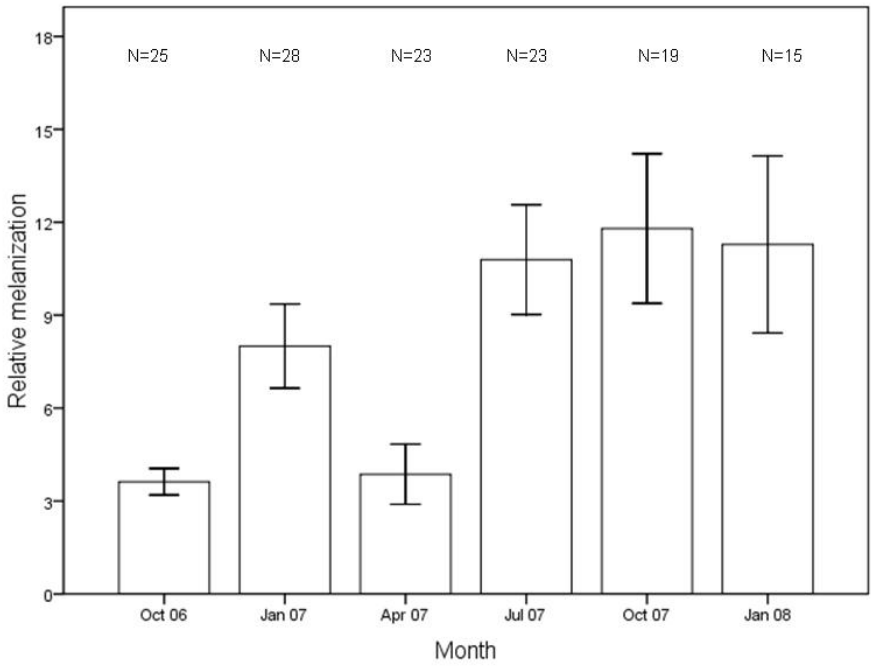

N=25
N=28
N=23
N=23
N=19
N=15
Relative melanization
0
3
6
9
12
15
18
Oct 06
Jan 07
Apr 07
Jul 07
Oct 07
Jan 08
Month

Fig. 5

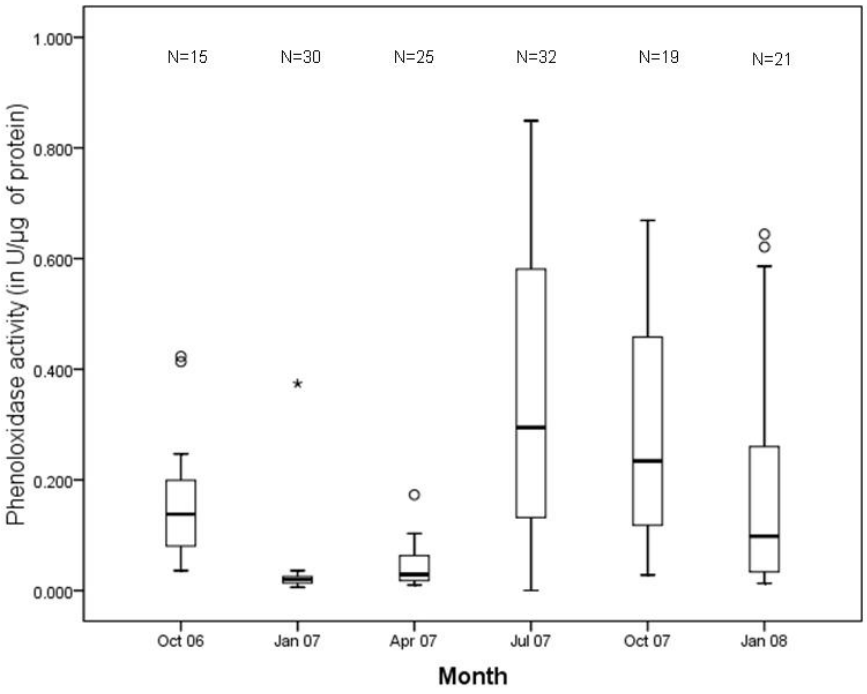

Phenoloxidase activity (in U/µg of protein)
1.000
0.800
0.600
0.400
0.200
0.000
N=15
N=30
N=25
N=32
N=19
N=21
Oct 06
Jan 07
Apr 07
Jul 07
Oct 07
Jan 08
Month

Fig. 6

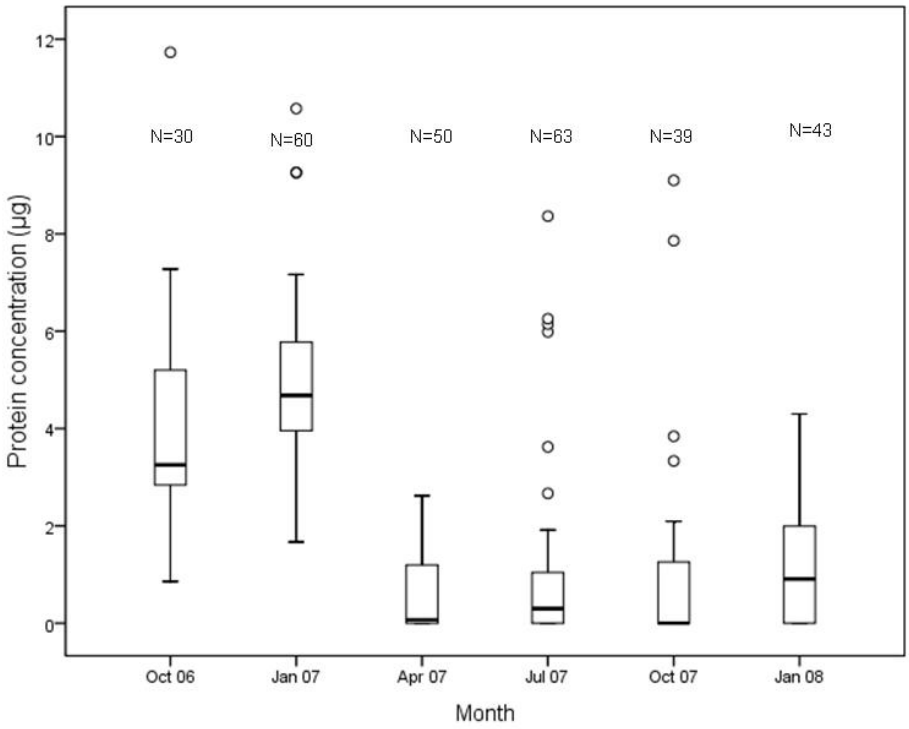

Protein concentration (µg)
12
10
8
6
4
2
0
N=30
N=60
N=50
N=63
N=39
N=43
Oct 06
Jan 07
Apr 07
Jul 07
Oct 07
Jan 08
Month

Fig. 7

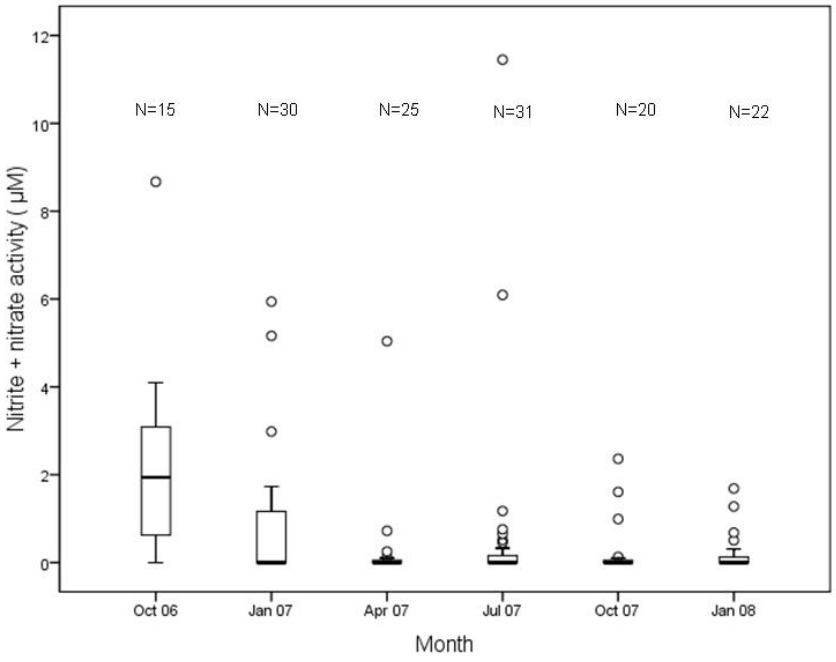
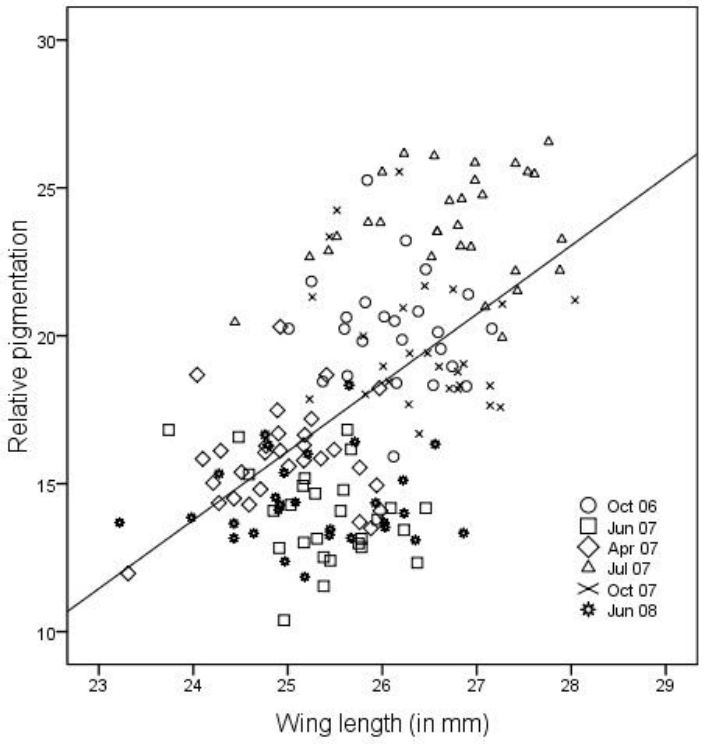

Nitrite + nitrate activity ( µM)
12
10
8
6
4
2
0
N=15
N=30
N=25
N=31
N=20
N=22
Oct 06
Jan 07
Apr 07
Jul 07
Oct 07
Jan 08
Month

Fig. 8.


Relative pigmentation
Wing length (in mm)
30
25
20
15
10
23
24
25
26
27
28
29
Oct 06
Jun 07
Apr 07
Jul 07
Oct 07
Jun 08

## 8. CONCLUSIONES

- La pigmentación alar mostró un claro patrón estacional, con valores más altos en julio, disminuyendo paulatinamente hacia octubre. Algunas probables explicaciones no excluyentes entre sí podrían ser un incremento de recursos alimenticios durante la temporada de lluvias y/o un aumento en la competencia intraespecífica, siendo la selección sexual la fuerza que estuviera favoreciendo a los machos con manchas más grandes.

- Aunque el contenido de grasa mostró variación durante el trabajo, no fue posible vislumbrar un patrón como el de la pigmentación. La intensidad en la defensa de los territorios y/o aumento-decremento de recursos alimenticios podría ser extremadamente variable inclusive cuando se trate de los mismos meses entre diferentes años, lo que habrá contribuido a que no se encontrará la relación entre mancha-grasa descrita en anteriores trabajos.

- La melanización y la actividad de la fenoloxidasa manifestaron una variación temporal, en tanto que el óxido nítrico fue el único parámetro inmune que mostró una variación más estrecha con la mancha. Esto probablemente es consecuencia de la combinación de los factores ya mencionados anteriormente: variabilidad temporal en la intensidad de defensa de los territorios, variación en la disposición de recursos alimenticios e influencia por patógenos.

- A pesar de que se detectó variación en la concentración de proteínas en hemolinfa a través de los muestreos, no se encontró una relación en el tiempo con la pigmentación. Tampoco se encontró una correspondencia entre la concentración de proteínas y alguno de los componentes de la respuesta inmune

- Los machos de octubre tratados con *S. marcencens* en concentraciones de 25% y 50% tuvieron una supervivencia más alta, en contraste con los machos de enero. Esto está de acuerdo con los resultados de óxido nítrico. Esto sugiere que este componente inmunológico pudiera sería un indicador fiel de capacidad inmune en estos animales ante ataques bacterianos.

- La pigmentación no fue un buen indicador de las reservas de grasa, cuando se compararon meses extremos, aunque el análisis global mostró una débil relación entre estas variables. Esto probablemente es consecuencia de que la defensa de los territorios y/o las restricciones ambientales son más intensas en ciertas épocas del año.

- El tamaño del ala se relacionó positiva y significativamente con la fenoloxidasa, la melanización y la pigmentación, pero no con las reservas de grasa. Lo anterior indica que individuos más grandes podrían desplegar una respuesta inmune más alta en estos dos componentes aunque resulta paradójico que esto no sea el caso para las reservas de grasa, lo cual contradice otros estudios con libélulas.

## 9. Perspectivas

- Dentro de los factores ambientales, en este estudio y el llevado a cabo por Contreras-Garduño *et al*. (en prensa) se manejó el alimento como la principal variable que pudiera estar afectando al CSS, reservas de grasa y la respuesta inmune. En la fase teneral se ha comprobado que periodos de inanición repercuten negativamente en las reservas de grasa y en la mancha (Contreras-Garduño *et al*., 2008). Sin embargo, la etapa de larva puede resultar igualmente importante. Con este panorama resulta primordial realizar un experimento en larvas que sean expuestas a distintos niveles de estrés alimenticio y cuantificar las variables medidas en el presente trabajo en los adultos.

- Investigaciones previas con *H. americana* indican que la tasa de parasitismo por gregarinas y ácaros en el sitio de muestreo, es muy baja durante todo el año (Córdoba-Aguilar *et al*., 2006). No obstante poco se sabe respecto a otros patógenos (p. ej. bacterias y hongos) los cuales no tan sólo podrían estar presentes, sino también fluctuar a lo largo del año. Esto debería investigarse.

- La cuantificación de grasa podría realizarse de manera más fina. En este caso enfocándose en la medición de lípidos que están relacionados con la respuesta inmune (ver por ej. Hernández-Hernández *et al*., 2003).

- Las fluctuaciones en las variables medidas a lo largo del año tienen repercusiones en términos de selección sexual. Dado que el tamaño de la mancha puede advertir a los contrincantes acerca de las capacidades de lucha de un macho en particular (Contreras-Garduño *et al*., 2008), sería interesante saber si esta comunicación cambia a lo largo del año. Otro punto relacionado es saber si existe variación en la prioridad que los machos de una determinada época asignan a la mancha, comprometiendo la FO, si es que la mancha está sujeta a mayor intensidad de selección sexual (p. ej. mayor competencia por territorios).

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