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PAPEL DE LA HORMONA JUVENIL Y EL ENVEJECIMIENTO EN LA ASIGNACIÓN DE RECURSOS A DIFERENTES COMPONENTES DE ADECUACIÓN EN LIBÉLULAS (ODONATA)

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P r e s e n t e

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Papel de la hormona juvenil y el envejecimiento en la asignación de recursos a diferentes componentes de adecuación en libélulas (Odonata)

Daniel Matías González Tokman

Resumen

Los seres vivos deben utilizar una cantidad limitada de recursos para cubrir sus diversas funciones. La teoría evolutiva indica que cuando los recursos son limitados, como casi siempre en la naturaleza, la inversión a una función comprometerá la asignación de recursos a otra función. En los animales, esta asignación está mediada por hormonas. En mi tesis exploré experimentalmente el papel de la hormona juvenil (HJ) en la asignación de recursos a diversas funciones, principalmente al sistema inmune y supervivencia en libélulas territoriales. La HJ es conocida por favorecer la actividad sexual a costa de la respuesta inmune, pero la mayoría de los estudios se han hecho con hembras y en condiciones de laboratorio. Existe poca evidencia sobre el efecto de la HJ en machos y en condiciones naturales. Durante la etapa adulta de los insectos, la HJ promueve la senescencia (e. g. el deterioro fisiológico que ocurre con la edad) aunque, una vez más, aquello ocurre en condiciones controladas de laboratorio. En una primera parte de mi tesis exploré el papel de la HJ en la senescencia en condiciones naturales y en cautiverio. Una razón para suponer que la HJ determina las tasas de senescencia en condiciones naturales es que ésta podría modificar la estrategia reproductiva. La HJ podría fomentar la inversión terminal a la reproducción y, en consecuencia, tener distinto efecto sobre adultos jóvenes y viejos. Por esto, en una segunda parte evalué el efecto de la HJ sobre la senescencia en un escenario de inversión terminal en machos de distinta edad. Encontré que la HJ reduce la supervivencia pero sólo cuando los machos están infectados y en condiciones naturales (no en condiciones de laboratorio, donde a los machos no se les permitió reproducirse ni alimentarse). Encontré además que la HJ detiene la senescencia

en condiciones naturales, contrario a lo que sucede en el laboratorio en éste y otros insectos. Sin embargo, no encontré evidencia de que la HJ promueva la inversión terminal a costa de la supervivencia. Finalmente, encontré que los machos viejos utilizan una estrategia de inversión terminal a la reproducción cuando su esperanza de vida se reduce por un reto inmunológico. Los machos jóvenes, por el contrario, parecen ser cautelosos, aparentemente para no comprometer su condición fisiológica y poder reproducirse en el futuro. Los resultados de esta tesis resaltan la importancia de combinar estudios conductuales y fisiológicos tanto en condiciones controladas de laboratorio como en condiciones naturales.

Abstract

Living organisms have a limited amount of resources to cover different functions. Evolutionary theory indicates that when resources are limited, which is the most common situation in nature, investment in one function will take resources from another function. Resource allocation to different functions is mediated by hormones. In this work I explored the role of juvenile hormone (JH) on resource allocation to different functions, such as immune response and survival, in territorial damselflies. JH is known to promote sexual activity at the expense of immune response, but most evidence comes from females and laboratory conditions. Evidence on the effect of JH in males and in natural conditions is lacking. JH has different effects during an insect's life. During adulthood, JH promotes senescence (e. g. physiological deterioration with aging) but, again, that is known to occur under controlled conditions in the laboratory. Here I explored the role of JH in both natural and captive conditions. One main reason to suppose that JH shapes senescence in natural conditions is that it could alter reproductive strategies by, for example, promoting terminal investment in reproduction, thus having different effects in young and old adults. I thus assessed the effect of JH on senescence in a scenario of terminal investment in males of different age classes. JH drastically reduced male survival, but it occurred only in natural conditions and when males were infected. Such effect was not found in the lab, where males were not allowed to reproduce or eat. I also found that JH ameliorated senescence in natural conditions, contrary to what occurs in the lab in the same and other insect species. However, I did not find evidence that JH promotes terminal investment at the expense of survival. Finally, I found that old males

incur in terminal investment in reproduction when their life expectancy is experimentally reduced by an immune challenge. On the other hand, young males seem to be more cautious, apparently to avoid physiological deterioration and to increase their chances of future reproduction. These results highlight the importance of integrating behavioral and physiological studies in both laboratory controlled and natural conditions.

Introducción

La hormona juvenil (HJ) es una de las hormonas más versátiles del reino animal debido a que controla una gran cantidad de procesos fisiológicos y conductuales de los insectos, tales como desarrollo larvario, metamorfosis, maduración sexual, conducta locomotora y de cortejo, aprendizaje y respuesta inmune, entre otras (Flatt, Tu, & Tatar 2005). Aunque la HJ juega un papel fundamental durante la etapa larvaria de los insectos, regulando su desarrollo hasta la etapa adulta, el presente trabajo está enfocado a evaluar los efectos de esta hormona en insectos adultos, particularmente en machos de libélulas. Mi objetivo fue conocer el papel de la HJ como mediador en el desvío de recursos entre diferentes rasgos de historia de vida.

Para estudiar el efecto de la HJ sobre alguna característica conductual o fisiológica se puede (a) medir los niveles de HJ en la hemolinfa de un insecto y correlacionarlos con la expresión de ese carácter (Tobe & Chapman 1979), (b) remover quirúrgicamente la corpora allata, la glándula productora de HJ (Herman & Tatar 2001; Rolff & Siva-Jothy 2002), o (c) modificar experimentalmente los niveles de HJ con aplicaciones de HJ, de análogos a la HJ (i. e. ácido metoprénico), o de inhibidores de la síntesis de HJ (i. e. fluvastatina) y ver su efecto sobre la expresión del carácter (Rolff & Siva-Jothy 2002; Rantala, Vainikka, & Kortet 2003b; Contreras-Garduño *et al.* 2009). En el presente trabajo modifiqué los niveles de HJ mediante aplicaciones tópicas y vi su efecto en machos adultos tanto en condiciones naturales como en condiciones controladas de laboratorio.

El rol de la HJ en los insectos adultos se ha comparado con el que juega la testosterona en los vertebrados (Rantala *et al.* 2003b). La razón principal es que altos niveles de HJ (o de testosterona) se asocian con un incremento en la actividad reproductiva (i. e. agresividad, atractivo sexual), que al aumentar compromete otras características como la resistencia a patógenos (Rolff & Siva-Jothy 2002) o la longevidad (Herman & Tatar 2001). Estos compromisos se deben a que varias funciones de un individuo deben ser cubiertas por una cantidad limitada de recursos (Zera & Harshman 2001). Así, cuando el recurso es escaso, la inversión a una característica (i. e. reproducción) restará recurso a la otra (i. e. respuesta inmune) (Sheldon & Verhulst 1996; Zera & Harshman 2001). Por el contrario, dicho compromiso no debería ocurrir si el recurso es abundante (Zera & Harshman 2001). Una de las hipótesis más relevantes en selección sexual, la "hipótesis de la desventaja en la inmunocompetencia" (*immunocompetence handicap*, en inglés), establece que es la testosterona la responsable de provocar el compromiso entre la reproducción y la supervivencia porque favorece la asignación de recursos a la reproducción a costa de la respuesta inmune (Folstad & Karter 1992). En la presente tesis exploré si también es el caso de la HJ (Anexo 1; González-Tokman *et al.* 2012b; González-Tokman, Córdoba-Aguilar, & Forbes 2012a).

Los compromisos fisiológicos provocados por la testosterona (o la HJ) causan un deterioro en la fisiología de los organismos a medida que éstos envejecen, que culmina en una menor probabilidad de sobrevivir y reproducirse (Tatar, Chien, & Priest 2001; Flatt & Schmidt 2009). A este proceso se le llama senescencia, y también fue objeto de estudio

de la presente tesis. La evidencia del efecto de la HJ en la senescencia apunta a que altos niveles de esta hormona favorecen la senescencia, ya que están asociados con una menor longevidad y un deterioro fisiológico (Tatar *et al.* 2001; Herman & Tatar 2001), pero todos ellos se han basado en estudios en laboratorio realizados bajo condiciones controladas. En la presente tesis exploré el papel de la HJ sobre la senescencia en condiciones naturales.

Cuando los individuos son viejos o están en un mal estado fisiológico (i. e. están enfermos) pueden incrementar su inversión al que posiblemente sea su último evento reproductivo, a pesar de que ello resulte en una muerte más rápida. Esta estrategia se conoce como inversión terminal (Williams 1966; Clutton-Brock 1984), y ha sido ampliamente estudiada en vertebrados y en hembras (Cotter, Ward, & Kilner 2010; Hoffman *et al.* 2010), pero poco en invertebrados y en machos. De forma alterna, los individuos podrían ser cautelosos y esperar un momento preciso para reproducirse en el futuro, cuando las condiciones sean más favorables (McNamara *et al.* 2009). Existe poca evidencia sobre este tema, que es relevante en la evolución de las estrategias reproductivas. En la presente tesis exploré cuál es la estrategia adoptada por machos de libélulas (González-Tokman, González-Santoyo, & Córdoba-Aguilar 2013), y cómo la HJ regula este proceso en condiciones naturales.

El contenido de esta tesis es novedoso en gran medida porque la mayor parte del estudio fue realizada con insectos en el campo, bajo condiciones naturales. La mayoría de la investigación enfocada a aspectos fisiológicos de la biología evolutiva de insectos se hace

en condiciones de laboratorio, lo que permite controlar con precisión los efectos de las variaciones ambientales (i. e. temperatura, disponibilidad de alimento, infecciones, competencia sexual) sobre alguna característica o sobre el resultado de algún experimento. Sin embargo, las variaciones ambientales son fundamentales en el proceso evolutivo, y por lo tanto la evidencia de laboratorio debe complementarse con evidencia de lo que sucede en la naturaleza.

El estudio de aspectos fisiológicos y evolutivos en insectos en condiciones naturales no ha recibido tanta atención en gran medida porque resulta difícil seguir individuos durante una etapa importante de su vida. En el presente trabajo he utilizado técnicas de captura-marcaje-recaptura (CMR) de individuos modificados experimentalmente para estimar las probabilidades de supervivencia en condiciones naturales, con lo cual se puede tener un buen estimado de su adecuación (medida como supervivencia) en la naturaleza (Lebreton *et al.* 1992; Nussey *et al.* 2008). El principal sujeto de estudio utilizado en la presente tesis es la libélula *Hetaerina americana* (Odonata: Zygoptera), aunque en el anexo 1 se estudió a *Celithemis eponina* (Odonata: Anisoptera). *H. americana* es un excelente modelo de estudio debido a su sistema de apareamiento semejante a un lek (e. g. competencia por hembras sin defensa de recursos; Córdoba-Aguilar *et al.* 2009), con tácticas alternativas en machos que dependen de su calidad: son territoriales los machos de alta condición fisiológica, y no territoriales los de peor condición (Contreras-Garduño, Canales-Lazcano, & Córdoba-Aguilar 2006; Contreras-Garduño *et al.* 2008). Aunado a que es una especie abundante, estas cualidades permiten abordar preguntas muy variadas en el estudio de la selección sexual y la teoría de historias de vida. Al ser territoriales, los

machos son fácilmente detectables y presentan altas tasas de recaptura, lo que permite implementar diseños experimentales complejos en estudios con CMR en condiciones naturales. Sobre la biología de *C. eponina* se conoce menos, pero es un buen modelo de estudio por estar altamente parasitado por ácaros, por su abundancia y por su disponibilidad a ser alimentado en cautiverio una vez colectado.

El objetivo principal de la presente tesis fue evaluar el papel de la hormona juvenil y el envejecimiento en las estrategias de asignación de recursos a diferentes componentes de adecuación, tanto en condiciones naturales como en condiciones controladas. La hipótesis de trabajo es que la hormona juvenil es un regulador fundamental de procesos conductuales y fisiológicos en los insectos y, por lo tanto, interviene en gran medida en su adecuación.

Capítulo 1.

Prueba de la hipótesis de la desventaja en la inmunocompetencia en el campo: la manipulación hormonal compromete la supervivencia en libélulas infectadas

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SUPPORT FOR THE IMMUNOCOMPETENCE HANDICAP HYPOTHESIS IN THE WILD: HORMONAL MANIPULATION DECREASES SURVIVAL IN SICK DAMSELFLIES

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The immunocompetence handicap hypothesis (ICHH) states that hormones enhance sexual trait expression but impair immunity. Previous tests of the ICHH have been hampered by experimental design problems. Here, we report on an experimental test of the ICHH that includes manipulations of both hormones and infections in males of the territorial damselfly, *Hetaerina americana*, with accurate survival measurements. We conducted a fully factorial experiment subjecting each individual to one of three topical treatments: methoprene (a juvenile hormone analog), acetone, or control, and one of three injection treatments: bacteria, PBS, or control. We measured survival of manipulated males in both the wild and in captivity. As predicted, survival was most heavily impaired in methoprene-bacteria males than in the other groups in the wild, and no survival differences emerged in captive animals. This result confirms that survival is one cost an animal pays for increased hormonal levels. This corroborates theoretical predictions of the ICHH.

KEY WORDS: **Infection, insect, juvenile hormone analog, mark-recapture, Odonata, survival, sexual selection, trade-off.**

Fitness depends, to a large extent, on how an individual optimizes resource allocation to reproduction and survival (Stearns 1992). To this end, animals have evolved physiological means to shift resource allocation to these functions as environmental conditions change (Stearns 1992; Flatt et al. 2005). Hormones are key mediators of communication between environmental cues and the organism's internal state and therefore natural subjects for investigations of plastic responses to exogenous cues (Flatt et al. 2005; Hau 2007). The immunocompetence handicap hypothesis (ICHH) indicates that hormones are responsible for resource allocation between sexual and immune functions (Folstad and Karter 1992). According to the ICHH, sexual expression and immune function will be traded-off, that is, animals whose hormonal levels promote sexual trait expression will become immune-depressed.

A number of studies have tested the ICHH in both vertebrates and invertebrates (reviewed by Jacobs and Zuk 2010). The usual approach is to experimentally manipulate hormonal levels (via castration, direct hormone implantation, or using endocrine antagonists that suppress hormonal action), and then assess changes in immune function via measurements of immune component levels (e.g., Belliure et al. 2004; Ros et al. 2006; Ashley et al. 2009) and/or intensity or prevalence of pathogens (e.g., Uller and Olsson 2003; Deviche and Parris 2006; González-Tokman et al. in press). Support for the ICHH has been mixed: increased hormonal levels have not always led to a weakening of the immune system (Roberts et al. 2004; Hasselquist 2007). One major drawback of many studies is that although hormonal level is experimentally manipulated, infection usually is not manipulated. There may be

additional noncontrolled factors (e.g., risk of exposure to infection) that may arise as confounding factors after hormonal levels have been manipulated. One example of a confounding factor is that encounters with pathogens depend on a number of factors that are not necessarily driven by testosterone levels. If the assumption that hormonally increased and control animals are encountering pathogens with the same probability is violated, then differences between experimental groups may arise for reasons other than the factor of interest (e.g., due to contact among cospecifics or aerial transmission; reviewed by Rudolf and Antonovics 2005). Therefore, it is important to experimentally manipulate both hormonal levels and immune challenges. In the only two studies that have achieved this, both have provided support for the ICHH (Lindstrom et al. 2001; Mougeot et al. 2006). A second shortcoming of many prior studies that investigate the ICHH is that survival has only been rarely studied; instead proxies for survival have been used (reviewed by Kotiaho 2001). A key prediction of the ICHH is that increased hormonal levels lead to reduced survival (Kotiaho 2001). Related to this, survival in the wild has to be assessed using modern techniques that can distinguish between survival and recapture rates. Such techniques are essential especially for groups that are likely to have biases in longevity, which may be the case for experimental individuals whose longevity is usually underestimated (Lebreton et al. 1992; Williams et al. 2002). In this article, we have solved the above issues using a territorial calopterygid damselfly as a study subject.

Previous findings in males of territorial calopterygid damselflies have provided evidence that increased levels of juvenile hormone (JH) lead to an increase in territorial aggression and wing pigmentation, two key sexual traits used during mate acquisition, with a reduction in immunocompetence (Contreras-Garduño et al. 2009, 2011). However, such hormonal manipulation did not show convincingly that survival was impaired (Contreras-Garduño et al. 2009). Here, we manipulate both hormonal levels (with methoprene acid, an analog of JH, JHa) and infection in the American rubyspot (*Hetaerina americana* Fabricius) and assess survival, predicting it to decrease in methoprene acid treated and infected animals. Because our second prediction is that decreased survival is explained by an increase in territorial behavior, we assess this indirectly by manipulating hormonal level and infection using animals whose territorial behavior was prevented. Our working hypothesis is that elevated hormonal levels will negatively affect survival.

Materials and methods

The study was carried out in Tetlama River, Morelos, Mexico (18°45′ 55″N, 99°14′ 45″W) in November–December 2010. Our study site (500-m long) was a sunny area delimited by large, shaded parts, (damselfly density and activity are extremely low in shady areas; all González-Tokman et al., pers. obs.). Animals were captured with a butterfly net. Captures and surveys were carried out in the sunny portion of the river. To avoid potentially confounding effects of the age on the measured variables, we only used young mature males that can be distinguished from younger or older males because of the texture and appearance of their wings and thorax (age class 3 according to Plaistow and Siva-Jothy 1996). At this stage, males show the highest immunity and territorial activity (Contreras-Garduño et al. 2008). Using an indelible marker, we marked each male by writing a three-digit number on the clear part (i.e., not the wing spot) of his left anterior wing. Also, we measured the left anterior wing with a digital caliper (± 0.01 mm) as an approximation of body size. After experimental manipulation (see below), animals were released where they were originally collected.

TREATMENTS

Each male received a topical application and an injection. Topical applications consisted of one of three treatments: hormonal increase (experimental), sham, or control. The hormonal-increase treatment consisted of methoprene acid, a JHa, which is known to modify the behavior and physiology of odonates and their parasites at the same dose used in the present study (Contreras-Garduño et al. 2009, 2011; González-Tokman et al. in press). From a dilution of 5 mg of methoprene acid mL^{-1} distilled water, we took 1 μl and diluted it 1:1000 in acetone. Using a micropipette, we took a 3 μl drop (15 ng methoprene acid) of the methoprene + acetone treatment (Met) and applied it topically on the dorsal part of the head. We used 3 μl of acetone (Ac) as sham treatment. Met and Ac treatments penetrate rapidly the cuticle near the corpora allata (Contreras-Garduño et al. 2009), the organ where JH is naturally synthesized (Flatt et al. 2005). Finally, individuals were handled but not given a topical application for the control group. All individuals received an injection immediately following the topical application. Injections consisted of one of three treatments: infection, sham, or control. Individuals in the infection group were infected with the gram-negative bacterium *Serratia marcescens.* This bacterium is common and highly lethal in wild American rubyspot populations in Central Mexico (González-Tokman et al. 2011). We resuspended bacteria from a laboratory culture (Instituto Nacional de Salud Pública, Cuernavaca, México) in phosphate buffer saline (PBS 1×, pH = 7) in a concentration of 700 colony formation units (CFU) μl^{-1}. We injected 1 μl of the mixture of bacteria + PBS (Bac) in the dorsal thorax at the location where wings are inserted and the exoskeleton is not rigid. We injected 1 μl of PBS as sham treatment. Finally, individuals were handled but not injected for the control group. Topical treatments never made direct contact with the injury caused by the injection.

SURVIVAL IN THE FIELD AFTER METHOPRENE AND BACTERIAL TREATMENTS

We determined whether Met had different effects on the survival of infected (Bac) and healthy (PBS, Control) animals under natural conditions. To estimate survival in the field, we used MARK 6.1 software (White and Burnham 1999). We used a capture–recapture approach (for similar approaches see Munguía-Steyer et al. 2010; Buzatto et al. 2011) that allows dissociation of survival (φ) and recapture (p) probabilities by calculating maximum likelihood estimates from encounter histories of regular surveys (Lebreton et al. 1992; Williams et al. 2002). These methods have rarely been applied to experimental data despite the fact that they allow comparing survival of individuals of different treatments. In our study case, where treatments could modify not only survival but also recapture probabilities (i.e., due to dispersal), capture–recapture approach is a correct method for estimating survival accurately. We tested 61 different models (Table S1) that included the different combinations of predictors of survival and recapture: Top treatment, Inj treatment and time, plus additive and interactive models (Table S1).

From November 18–25, we collected 476 males and assigned them to any of the nine different combinations of one topical (Top) treatment (Met, Ac, Control) and one injected (Inj) treatment (Bac, PBS, Control). Sample sizes were as follows: Met–Bac = 54, Met–PBS = 52, Met–Control = 53, Ac–Bac = 52, Ac–PBS = 53, Ac–Control = 53, Control–Bac = 52, Control–PBS = 53, and Control–Control = 54. We marked each individual and released it to the river after no longer than 2 min of manipulation. After manipulation and marking, we recorded the presence of adult marked males during 21 consecutive days, from November 24 to December 15. We used this 21-day period given that residual longevity of males we used was about 15 days (González-Tokman et al., unpubl. data). Detection of individuals was based on surveys done by three observers, from 1100 to 1400 h, the time at which animals are more active (Contreras-Garduño et al. 2008).

SURVIVAL IN CAPTIVITY AFTER METHOPRENE AND BACTERIAL TREATMENTS

In November 18, additional 156 males were captured and manipulated with the same treatments as above (Top + Inj). After manipulation, males were kept in captivity in 5-mL assay tubes with a perch and a cap of humid cotton for keeping a temperature of about 26°C. During the experiment males were not fed. The experiment ended when the last male died. Males were monitored every 4 h to record the time to death. Sample sizes were as follows: Met–Bac = 16, Met–PBS = 15, Met–Control = 17, Ac–Bac = 17, Ac–PBS = 18, Ac–Control = 17, Control–Bac = 15, Control–PBS = 14, and Control–Control = 27. Although starvation can have different effects in infected and noninfected insects (González-Tokman et al. 2011), and in animals that differ in parasite resistance (Valtonen et al. 2010), it helps to homogenize individual resource availability and to avoid confounding effects of adopting different feeding strategies when infected (e.g., Adamo et al. 2010; González-Tokman et al. 2011).

STATISTICS

For analyzing survival under natural conditions, we employed Comarck–Jolly–Seber (CJS) capture–recapture models that estimate survival and recapture parameters based on encounter histories (Lebreton et al. 1992). The global model included time, Top treatment, Inj treatment, and the interaction between both treatments: φ(Top × Inj + t) P(Top × Inj + t). We tested the goodness of fit of the global model assessing if there is overdispersion estimating the c-hat using the median c-hat approach (White and Burnham 1999; Buzatto et al. 2011). Overdispersion factors greater than 3 indicate structural deficiencies in the model. Our global model had slight overdispersion (c-hat = 1.093). For this reason, we employed the Akaike Information Criteria for overdispersed data (QAIC; Burnham and Anderson 2002) to select the best of the competing models (i.e., the model with the lowest QAIC value). Given that two models had similar QAIC values (ΔQAIC = 0.85, see results), we used likelihood ratio tests (LRTs) to determine significant differences among models. Specifically, we tested the significance of the interaction Top × Inj present in the global model compared with a reduced model that was an additive model (Top + Inj). Because there was variation in survival along time, we estimated the mean survival parameters for the combinations of Top and Inj treatments using a variance component approach (Williams et al. 2002).

Survival in captivity was analyzed with a proportional hazard Cox regression model that included Top + Inj + Body size + Top:Inj. The best model was selected based on AIC.

Prior to parametric tests, homogeneity of variances was tested with the Fligner–Killeen test (Crawley 2007). The presence of outliers was explored with Cook's distance, but no outlier was detected (Cook's distance < 1). Analyses were done in R (R Core Development Team 2009, version 2.10.0) and MARK 6.1 software (White and Burnham 1999).

Results

SURVIVAL IN THE FIELD AFTER METHOPRENE AND BACTERIAL TREATMENTS

Survival parameters differed along time, Top, Inj treatments, and the interaction of Top × Inj treatments (Table 1). Male damselflies treated with the combination of methoprene and bacteria (Met–Bac) had a significantly lower probability of surviving in the field than males with any other combination of treatments (Table 1, Fig. 1). The bacteria groups had a decreased survival when compared with control treatment groups. However there

Table 1. Estimates of daily survival (φ) and recapture (*P*) probabilities of American rubyspot males exposed to one topical (Top) and one injected (Inj) treatment. Estimates were calculated from a variance components approach of the best fitted model φ(Top × Inj + t) *P*(Top + Inj).

	Treatment				95% CI	
Parameter	Topical	Injected	Mean Estimate	Error	Lower	Upper
φ	Met	Bac	0.5439	0.0611	0.4242	0.6636
φ	Met	PBS	0.8372	0.0330	0.7726	0.9018
φ	Met	Control	0.9203	0.0191	0.8828	0.9578
φ	Ac	Bac	0.7900	0.0411	0.7094	0.8706
φ	Ac	PBS	0.8068	0.0378	0.7327	0.8810
φ	Ac	Control	0.9277	0.0176	0.8931	0.9623
φ	Control	Bac	0.7662	0.0445	0.6789	0.8534
φ	Control	PBS	0.8645	0.0290	0.8077	0.9213
φ	Control	Control	0.9076	0.0211	0.8662	0.9489
P	Met	Bac	0.5858	0.0481	0.4914	0.6802
P	Met	PBS	0.6020	0.0345	0.5342	0.6697
P	Met	Control	0.6944	0.0257	0.6440	0.7449
P	Ac	Bac	0.4462	0.0440	0.3600	0.5324
P	Ac	PBS	0.4628	0.0363	0.3916	0.5340
P	Ac	Control	0.5642	0.0273	0.5107	0.6177
P	Control	Bac	0.4421	0.0445	0.3549	0.5293
P	Control	PBS	0.4587	0.0337	0.3927	0.5247
P	Control	Control	0.5601	0.0283	0.5047	0.6156

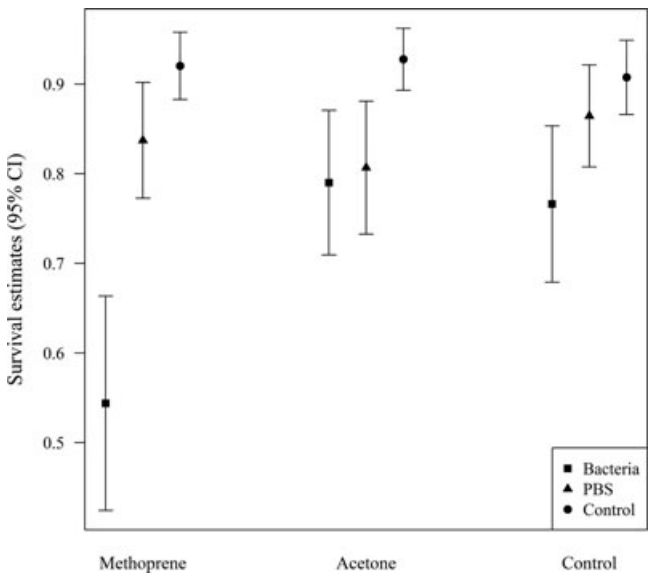

Figure 1. Estimates of mean daily survival probabilities of wild American rubyspot males exposed to one Top and one Inj treatment. Estimates were calculated from a variance components approach of the best fitted model φ(Top × Inj + t) *P*(Top + Inj).

was no difference between bacteria and PBS treatments with the exception of when Met was also applied (Table 1, Fig. 1).

The LRT comparing the model with an additive survival term: φ(Top + Inj + t) and the model with an interactive survival term φ(Top × Inj + t), both with *P*(Top + Inj), was significant ($\chi^2 = 10.747$, df = 4, $P = 0.030$), which means that adding the interaction term to the model resulted in a better fit. In the most supported model, time was a good predictor of survival (Table 2), which means that probability of survival is not constant across time. Recapture probabilities were best explained by the additive effects of Top and Inj treatments (Tables 1 and 2, Fig. 2). In general, the Met treatment is associated with higher recapture probabilities (Table 1, Fig. 2)

SURVIVAL IN CAPTIVITY AFTER METHOPRENE AND BACTERIAL TREATMENTS

Survival of males in captivity was not explained by any of the treatments or covariates included in the model. The model selection procedure excluded Top ($\chi^2 = 0.856$, $P = 0.652$) and Inj ($\chi^2 = 1.376$, $P = 0.503$) treatments and body size ($\chi^2 = 1.279$, $P = 0.258$) as well.

Discussion

Our results strongly support the ICHH, as males that were experimentally infected with bacteria (Bac) died faster than noninfected animals when supplemented with a JH analog (Met), but not under natural hormonal levels. Thus, our results indicate a fitness cost of hormones when animals are sick. Our results have important implications for future evaluations of the ICHH. Manipulation of both infection and hormonal levels is needed but had not been

Table 2. Summary of the model selection process to test the effect of one topically applied treatment and one injected treatment on daily survival and recapture probabilities of marked American rubyspot males in the wild.

Model description							
Survival components	Recapture components	QAICc	Δ QAICc	QAICc weight	Model likelihood	No. of parameters	*Q* deviance
φ(Top×Inj+t)	***P*(Top+Inj)**	**3280.851**	**0**	**0.45260**	**1**	**33**	**3213.246**
φ(Inj+t)	*P*(Top+Inj)	3281.706	0.85	0.29518	0.6522	27	3226.629
φ(Top+Inj+t)	*P*(Top+Inj)	3283.234	2.38	0.13748	0.3038	29	3223.993
φ(Top×Inj+t)	*P*(Top×Inj+t)	3284.920	4.07	0.05916	0.1307	56	3168.277
φ(Top×Inj+t)	*P*(Top)	3287.154	6.30	0.01936	0.0428	30	3225.826
φ(Inj+t)	*P*(Top)	3288.264	7.41	0.01112	0.0246	24	3239.411
φ(Top+Inj+t)	*P*(Top+Inj+t)	3289.749	8.90	0.00529	0.0117	47	3192.489
φ(Inj+t)	*P*(Top+Inj+t)	3289.873	9.02	0.00497	0.0110	46	3194.751
φ(Top×Inj+t)	*P*(Top+Inj+t)	3289.977	9.13	0.00472	0.0104	52	3181.980
φ(Inj+t)	*P*(Inj)	3291.528	10.68	0.00217	0.0048	24	3242.675
φ(Top+Inj+t)	*P*(Top)	3291.729	10.88	0.00197	0.0044	27	3236.652
φ(Top×Inj+t)	*P*(Inj)	3293.689	12.84	0.00074	0.0016	30	3232.361
φ(Top+Inj+t)	*P*(Inj)	3294.717	13.87	0.00044	0.0010	26	3241.718
φ(Top+Inj+t)	*P*(Top+t)	3299.390	18.54	0.00004	0.0001	46	3204.268

The best supported model is in bold. Top, topical treatment (Met, Ac, Control); Inj, injected treatment (Bac, PBS, Control); t, time.

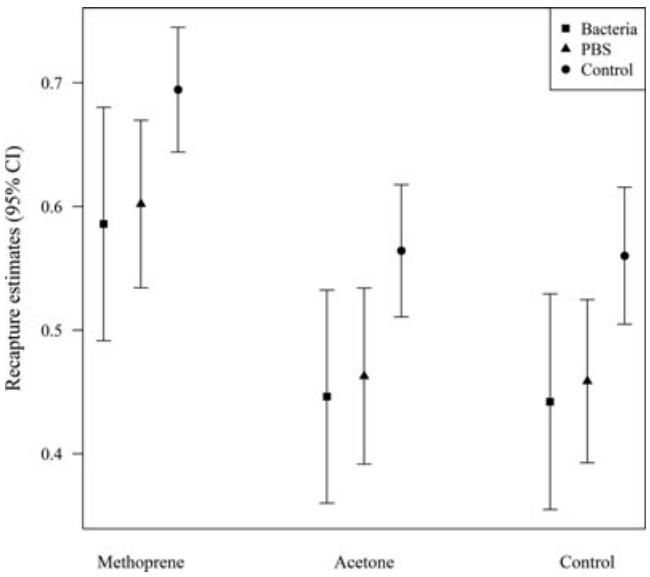

Figure 2. Estimates of mean daily recapture probabilities of wild American rubyspot males exposed to one Top and one Inj treatment. Estimates were calculated from a variance components approach of the best fitted model φ(Top × Inj + t) *P*(Top + Inj).

evaluated yet. If experimentally infected animals are not used, hormonal supplementation costs may arise but it may not be clear whether a hormone treatment in combination with an infection affects survival too. It seems likely that mortality was the highest in Met–Bac-treated animals because bacteria grew faster in Met males that were presumably immunosuppressed. Despite we did not measure any immune parameter, immunosuppression is a common consequence of high levels of JH (Rantala et al. 2003; Contreras-Garduño et al. 2009). Given that immunosuppressed animals can become more susceptible to predation (Rantala et al. 2011), we cannot discard predation as a consequent source of mortality associated to immunosuppression caused by Met in infected animals. This source remains to be tested.

Immunosuppression is not the only effect of JH in adult insects, so one has to keep in mind other sources of mortality associated to the combination of methoprene and bacteria. For example, resistance to environmental and physiological stress has been shown to decrease with JHa (e.g., Salmon et al. 2001; Tatar et al. 2001). Related to this, *Drosophila melanogaster* flies treated with high doses of methoprene are less resistant to starvation so that they die faster than control flies (Salmon et al. 2001). Such situation could be intensified in the presence of additional stressors such as pathogens (e.g., Bac treatment), leading infected animals to be more susceptible when JH levels are high (e.g., Met treatment). However, male survival was not affected by methoprene and/or bacteria in our experiment in captivity, where starvation was a main source of stress. Hence, we can argue that the main causes of male mortality in Met–Bac animals in the field experiment were more probably related to changes in behavior or immunosuppression, which are common effects of JH in damselflies (Contreras-Garduño et al. 2009, 2011).

Physical activity seems responsible of differences in survival, given that treatment did not affect survival in captivity

(where movement was prevented) whereas it did in the wild. In our study species, territorial defense can be an extremely costly activity (Contreras-Garduño et al. 2008). During territorial contests, male lipid resources are heavily used (Plaistow and Siva-Jothy 1996; Contreras-Garduño et al. 2008) and are not renewable during adulthood (Raihani et al. 2008). Moreover, nonterritorial animals that have had their lipidic reserves exhausted survived for a shorter period during an experimental infection compared to territorial animals when both animals were kept inactive (Contreras-Garduño et al. 2007). This implies a close relationship between lipid resources and immunocompetence (Hernández-Hernández et al. 2003). Costs of territoriality could have driven to fast mortality in highly territorial males (for a similar rationale see Munguía-Steyer et al. 2010), especially if territoriality was enhanced with Met and the immune system was simultaneously activated with Bac.

The recapture rates (detectability) we found give support to the idea that Met increased territorial activity in a long term: Met-treated males were easier to detect during the daily surveys, presumably because they became more territorial and therefore more faithful to their defended sites (Munguía-Steyer et al. 2010). The reduced effect of bacterial treatment on recapture probabilities seems unexpected according to previous findings in other calopterygids, in which immune response activation has been associated with enhanced dispersal (Suhonen et al. 2010) and reduced territorial behavior (Rantala et al. 2010), which would lead to lower recapture rates in infected animals. Unfortunately, our capture–recapture approach does not allow determining precise causes for the observed recapture rates. However, our results are consistent with long-term effects on site fidelity observed in other species of territorial odonates (e.g., Munguía-Steyer et al. 2010).

Tests of the ICHH have been primarily conducted in vertebrates. Support for the ICHH has been mixed in vertebrate studies. Apart from the methodological challenges we outlined in the introduction, one physiological reason is that the action of testosterone on sexual selection and immunocompetence is far from simple (Marsh 1996). Contrary to expectations from the ICHH, testosterone in some vertebrates has shown to affect immunocompence positively and sexual traits negatively (Roberts et al. 2004). Research in invertebrates has provided a clearer support for the ICHH despite most studies failing to manipulate hormonal levels (Marsh 1996). In those cases where hormonal levels have been experimentally augmented, sexual traits have been found to increase in expression while immune functions become negatively affected (e.g., Rantala et al. 2003; Fedorka and Mousseau 2007; Contreras-Garduño et al. 2009, 2011). One reason of why ICHH has found stronger support in invertebrates than in vertebrates is that the action of JH is more ubiquitous (controlling more functions), direct (i.e., acting directly on gene expression), and thus less complex than testosterone (e.g., Flatt et al. 2005; Riddiford 2008). Here, we have shown under natural conditions that JH is an important mediator of life-history trade-offs in insects (Flatt et al. 2005).

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Supporting Information

The following supporting information is available for this article:

Table S1. Model selection process to test the effect of one topically applied treatment and one injected treatment on daily survival and recapture probabilities of marked American rubyspot males in the wild.

Supporting Information may be found in the online version of this article.

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Capítulo 2.

Efecto de la hormona juvenil sobre la senescencia en machos con inversión terminal

Effect of juvenile hormone on senescence in males with terminal investment

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Abstract

Senescence is a universal property of living organisms and is controlled by hormones. Despite this, our knowledge of how hormones modulate senescence and survival in wild animals is limited. We have addressed this gap by increasing juvenile hormone (JH) levels in *Hetaerina americana* males, a species with terminal investment. More specifically, we assessed survival in young and old males that were treated with a JH analog (methoprene) and immune challenged to promote terminal investment. We replicated the same procedure in captivity where males were deprived of any activity or food. We expected old males to show the lowest survival when they have been treated with JH and immune challenged. This should be the case for wild animals but not for captive animals as the effects of JH and immune challenge should lead to an increase in high energetic-demanding activities only in the wild. Old animals died sooner compared to young animals in both the wild and captivity, confirming that males are subject to senescence. In wild, but not in captive animals, JH decreased survival in young males and increased it in old males, independently of immune challenge. Additionally, immune challenge increased recapture rates in young males, and decreased it in old males. Our results confirm a senescent pattern in the wild that is mediated by JH, but we found no effect of terminal investment on survival. One explanation is that animals undergoing senescence and terminal investment modify their feeding behavior to compensate their physiological state.

Introduction

Senescence, or aging, is an inevitable decline in survival and reproductive prospects with age, that is caused by a progressive reduction in physiological function (Partridge & Gems 2006; Flatt & Schmidt 2009). Every living organism is subject to senescence (Ackermann *et al.* 2007; Książek 2010). One of the main explanations for the origin of senescence, called 'antagonistic pleiotropy', suggests that those mutations that are deleterious at old ages had beneficial pleiotropic effects earlier in life (Williams 1957; Flatt & Promislow 2007), implying that senescence is an inevitable part of trade offs.

Trade offs are not only genetic, but can also be physiological, occurring within individuals when resources are limited and need to be used for different functions related, for example, to survival and reproduction (Zera & Harshman 2001). Given these physiological trade offs underlying senescence, animals should balance their resources among reproductive events (Stearns 1992; Roff 2002). This balance occurs even at the risk of increased mortality, an idea otherwise known as terminal investment. Although senescence and terminal investment processes should interact, such interaction has been examined mostly on effects on reproduction investment (Hoffman *et al.* 2010; Massot *et al.* 2011), but its effects on survival still need experimental evaluation (but see Part, Gustafsson, & Moreno 1992; Festa-Bianchet & King 2007).

Senescence is associated with changes in the immune system (DeVeale, Brummel, & Seroude 2004; Stanley 2012). Immune response involves the release of reactive oxygen species that leads to the accumulation of oxidative damage (Nappi & Christensen 2005;

González-Santoyo & Córdoba-Aguilar 2012), which is also associated with senescence (Finkel & Holbrook 2000). In general, immune response declines with age (Doums *et al.* 2002; Moret & Schmid-Hempel 2009), and high demand of the immune system in youth inevitably shortens individual lifespan, especially when resources are scarce (Moret & Schmid-Hempel 2000). Despite a decrease in the immune response during senescence has been documented in both vertebrates and invertebrates (Miller 1991; Doums *et al.* 2002), the effect of activation and usage of immune system on senescence has received much less attention (but see Moret & Schmid-Hempel 2009). Invertebrates are excellent models for studying the effect of immune response on senescence given that their immune system, unlike vertebrates, lack adaptive immune machinery that complicates the scenario where immunity and aging interact (DeVeale *et al.* 2004; Keller & Jemielity 2006).

In insects, juvenile hormone (JH) is a key regulator of aging (Tu, Flatt, & Tatar 2006) and terminal investment (i.e. Fronstin & Hatle 2008). Juvenile hormone is naturally synthesized in the *corpora allata*, an endocrine gland that is attached to the insect's brain and produces JH in response to stimulation from the central nervous system, via insulin signaling (reviewed by Flatt et al. 2005). JH regulates many developmental and life history traits such as resource allocation to reproductive activities or self physiological maintenance, including immunity (Flatt, Tu, & Tatar 2005; González-Tokman *et al.* 2012b). In general, old animals have higher titers of JH than younger individuals (Herman, Lessman, & Johnson 1981; Fluri *et al.* 1982; Jesudason, Venkatesh, & Roe 1990). Providing insects with JH or JH analogs like methoprene accelerates aging (i. e. probability of mortality, physiological deterioration), while JH downregulation slows aging (Tatar, Chien, & Priest 2001; Herman & Tatar 2001; Tatar & Yin 2001). Synthesis of JH is strongly sensitive to

89 the environment, and can be affected by nutrient intake and mating activity (Tobe &
90 Chapman 1979; Lessman & Herman 1983; Lee & Horodyski 2006; Nouzova *et al.* 2012).
91 However, studies on the effects of JH on aging have been done under captivity, where
92 feeding and mating activities are controlled (Keller & Jemielity 2006). This limitation is
93 especially serious if we consider that aging is also sensitive to resource availability, which
94 is highly variable in nature (Pletcher *et al.* 2002).
95
96 Recent evidence in rubyspot damselflies, *Hetaerina americana*, indicates that males show
97 terminal investment as reproduction changes with age, strongly depending on health status
98 (González-Tokman *et al.* 2013). Particularly, old males exhibit high territorial activity
99 when their risk of mortality is experimentally increased with an immune challenge. Young
100 males, on the other hand, reduce their territorial activity when immune-challenged
101 (González-Tokman *et al.* 2013), presumably as a cautious behavior to save resources for
102 future prospects of reproduction (McNamara *et al.* 2009). Given that terminal investment
103 has negative repercussions in animal energetic condition (González-Tokman *et al.*2013), it
104 must impact individual survival, and must be regulated by a mechanism involved in
105 allocation of resources to different life history traits. Juvenile hormone is an evident
106 candidate given that it is involved in trade offs between immunity and reproduction in
107 calopterygid damselflies and other insects (Rolff & Siva-Jothy 2002; Rantala, Vainikka, &
108 Kortet 2003; Contreras-Garduño *et al.* 2009, 2011; González-Tokman *et al.* 2012b).
109
110 Taking advantage that *H. americana* males show terminal investment, in the present study
111 we investigated the role of JH on male senescence and survival using an experimental
112 approach. The main goal of the present study is to understand the consequences of JH

113 treatment and male terminal investment on individual survival. We used a mark recapture
114 procedure which allows the decoupling of survival from recapture rates (Lebreton *et al.*
115 1992). First, we tested the effect of JH supplementation on survival probabilities of young
116 and old males under natural conditions. Second, we tested the effect of adding an immune
117 challenge to test for a possible effect of JH on potential terminal investment and its
118 consequence on senescence. These two tests were carried out in the wild, specifically on
119 males' mating sites. We produced a similar manipulation in captivity, to control for varying
120 environmental conditions and energetic expenditure that animals would incur in the wild
121 (Adamo *et al.* 2010; González-Tokman *et al.* 2011). We had four predictions: a) that old
122 animals will die sooner than young animals both in the wild and in captivity (i. e. show
123 senescence); b) that JH and immune challenge would interact to explain senescence in the
124 wild, but not in captivity, where feeding and sexual activities are prevented; c) that
125 mortality in the wild will be caused by the synergistic effect of JH on senescence and
126 terminal investment; and d) that terminal investment and senescence will determine fidelity
127 to reproductive sites and, consequently, recapture rates derived from our analyses in the
128 wild.
129
130 **Materials and methods**
131
132 *Study subject*
133
134 The damselfly *H. americana* shows intense male-male competition for riverine territories,
135 where females arrive with the only purpose of copulation (Grether 1996; Córdoba-Aguilar
136 *et al.* 2009). Males that can acquire and defend territories achieve much higher mating

success than nonterritorial males (Grether 1996; Serrano-Meneses *et al.* 2007). Territorial status is related to physiological condition: compared to nonterritorial males, territorial males have more energetic reserves and a better immune response, measured as encapsulation, phenoloxidase and lytic activity, and survival following bacterial infection (Contreras-Garduño, Canales-Lazcano, & Córdoba-Aguilar 2006; Contreras-Garduño *et al.* 2007).

Field work

The present study was carried out along the Apatlaco River, Morelos, México (18°45’55’’N, 99°14’45’’W), in October and November 2012. Between 1100 and 1500 hours, time at which animals show highest territorial activity, we captured males with a butterfly net on the river shore. To classify male age we used criteria described in Plaistow and Siva Jothy (1996), that separate adult damselflies into four age classes according to their appearance: age 1 comprises the youngest, teneral animals, that have just emerged and their bodies are still soft, the wings are flexible and undamaged, and typical adult pigmentation is still not fixed. Age 2 encompasses young and sexually mature animals that already fight for territories; their wings are flexible from the nodus to the tip and typical adult pigmentation is already fixed. Age 3 animals have fully matured and their bodies are harder and the wings less flexible, with some signs of pruinescence in the abdomen and thorax. Age 4 animals are the oldest; their wings are not flexible, papery-like and frequently damaged, and their bodies show abdominal and thoracic pruinescence. In the present study we only used males of ages 2 (young) and 4 (old).

Experimental protocol

Right after capture, each male was kept on ice for ten minutes prior to manipulation with a combination of two treatments: one of two hormonal treatments (JH) and one of two immune treatments (Imm). Individuals were randomly allocated to those treatments. Hormonal treatment (Met) consisted of a topical application of methoprene acid, an analog to juvenile hormone. From a dilution of 5 mg of methoprene acid in 1 mL of distilled water, we took 1 µL and diluted it in 1 mL of acetone. Using a micropipette, we took 3 µL of the solution (15 ng of methoprene acid) and placed on the male dorsal part of the head, so it rapidly penetrates the cuticle near the *corpora allata* (Flatt *et al.* 2005). This dose has been calculated for damselflies based on JH titers found in other insects (Contreras-Garduño *et al.* 2011). This treatment has been successfully used to increase reproductive behavior and affects immunity and survival in damselflies (including *H. americana*) and other insects (Teal, Gomez-Simuta, & Proveaux 2000; Rantala *et al.* 2003; Contreras-Garduño *et al.* 2009; González-Tokman *et al.* 2012b). As a control for the hormonal treatment we used 3 µL of acetone (Ac). Right after JH treatment application, all males received an immune treatment (Imm). The experimental group was implanted a 2 mm piece of rubbed nylon (0.18 mm thick) in the ventral part of the fourth abdominal segment (Rantala *et al.* 2000). When implanted, insects respond immunologically by depositing a melanin layer around the nylon piece (Rantala & Roff 2007; González-Tokman, Córdoba-Aguilar, & Forbes 2012a; Moreno-García, Lanz-Mendoza, & Córdoba-Aguilar 2013). As a control for the immune treatment (Control) a similar piece of nylon was inserted and immediately removed from the abdomen, so melanization was not allowed. To estimate body size, the

184 left anterior wing was measured from the insertion to the tip with a digital caliper (± 0.01
185 mm).
186
187 *Survival in the field*
188
189 In order to estimate survival in the field in response to age and treatments we used a
190 capture-mark-recapture approach (for similar methods see Munguía-Steyer, Córdoba-
191 Aguilar, & Romo-Beltrán 2010; Buzatto *et al.* 2010; González-Tokman *et al.* 2012b), and
192 analyzed data in program MARK 6.1 (White & Burnham 1999). This approach allows to
193 distinguish survival probabilities (φ) from recapture probabilities (p) by calculating
194 maximum likelihood estimates from encounter histories of marked animals (Lebreton *et al.*
195 1992). With this method, a group of models is constructed and the best model is selected
196 based on Akaike Information Criterion (AIC). In this study we tested a total of 73 models
197 (see supplementary Table S1) that included Age, JH treatment and Imm treatment as
198 predictors of survival and recapture.
199
200 Right after receiving the combination of JH and Imm treatments, males were marked with a
201 three-digit number made with a black permanent marker in the left anterior wing. From
202 October 25th to 29th we collected a total of 325 males and allocated them to the following
203 treatments: young (Ac-Control, N = 41; Ac-Imp, N = 41; Met-Control, N = 41; Met-Imp, N
204 = 41); old (Ac-Control, N = 41; Ac-Imp, N = 40; Met-Control, N = 40; Met-Imp, N = 40).
205 Each capture and marking day we collected the following number of young and old males:
206 day 1, 39 young and 43 old; day 2, 38 young and 38 old; day 3, 45 young and 31 old; day 4,
207 42 young and 49 old. Marked animals were released to the same site where they were

captured and the presence of marked animals was recorded daily for 22 days (from October 26th to November 16th). Detection of marked animals was based on daily surveys carried out from 1100 to 1400 hours by two observers walking in the same areas of the river.

Survival in captivity

An additional set of 140 old and young males were collected and manipulated with the same combinations of treatments mentioned above. Sample sizes remained as follows: young (Ac-Control, N = 17; Ac-Imp, N = 20; Met-Control, N = 20; Met-Imp, N = 20); old (Ac-Control, N = 14; Ac-Imp, N = 16; Met-Control, N = 16; Met-Imp, N = 17). After manipulation, animals were kept in captivity individually in 5 mL essay tubes in a shaded place, with a perch and a cap of humid cotton to keep humidity inside the containers. Water and food were not provided. The presence of dead animals was registered every four hours during the first day and every eight hours the following days. The experiment ended when the last animal died.

Statistics

Survival in the field was analyzed with Cormack-Jolly-Seber models, that estimate survival and recapture parameters from encounter histories (Lebreton *et al.* 1992). Model selection was based on AIC, and we considered that a difference in 2 AIC units was significant. Given the high number of factors in our study (Age, hormone treatment (JH), immune treatment (Imm), there is an extremely large number of possible models to test in our mark-recapture analysis. Therefore, we were not able to test all possible models, so that we used

232 the following criteria for selecting which models to test: we started fixing survival (φ)
233 parameters in the global model φ(Age * JH * Imm) and varying recapture (p) parameters,
234 starting with the global model p(Age * JH * Imm) and reducing it by removing terms one
235 by one until we got the simplest model, where recapture probabilities are constant (p(.)).
236 Until this point, we tested 19 different models. By doing this, we selected the best three
237 models (based on the ΔAIC < 2 units) explaining recapture probability: p(Age + JH + Imm
238 + Age:JH + Age:Imm), p(Age + Imm + Age:Imm + JH:Imm), and p(Age + JH + Imm +
239 Age:Imm). These three models were considered in the second step of model selection,
240 where we fixed recapture (p) and varied survival (φ) parameters, starting with the global
241 model φ(Age * JH * Imm) and reducing it by removing terms one by one until we got the
242 simplest model, where survival probabilities are constant (φ(.)). Model goodness of fit was
243 tested in the global model φ(Age * JH * Imm), p(Age * JH * Imm) by estimating
244 overdispersion with a median c-hat approach (White & Burnham 1999). Values higher than
245 3 units indicate high overdispersion. Our estimated overdispersion was relatively low
246 (median c-hat = 1.495) and it was corrected in all models, so we used AIC for
247 overdispersed data (*Q*AIC) in our model selection process (Burnham & Anderson 2002).
248 All models used a logit link function. Given that there was no single model with
249 considerably higher support than the others (i. e. $\Delta QAIC \geq 2$), we used model averaging of
250 models with $\Delta QAIC \leq 6$ (Richards 2008; Grueber *et al.* 2011). Averaged models had an
251 explanatory power of 92 % (*Q*AIC weight sum = 0.92; Burnham & Anderson, 2002).
252
253 Survival in captivity was analyzed with a proportional hazard Cox regression model that
254 included Age * JH * Imm + Body size. Again, the best model was selected based on AIC.
255 Differences in body size were analyzed with t-test and one-way ANOVA. Analyses were

carried out in R software 2.10.0 (R Development Core Team 2009) and MARK 6.1 (White & Burnham 1999).

Results

Survival in the field

Despite the fact that old males were significantly larger that young males (t-test t = 7.516, P < 0.001, N = 325), there were no differences in size between treatments in either young (ANOVA $F_{3,160} = 0.107$, P = 0.956) or old males (ANOVA $F_{3,157} = 0.518$, P = 0.671).

Most of the best supported models to explain differences in survival of marked animals in the field suggest that survival depended on the interaction of age and hormonal treatment (Age * JH) (Table 1), and that there was no effect of immune treatment (Imm) on male survival (Table 1, Figure 1). The general trend is that Met decreased survival in young males, and increased it in old males, independently of immune treatment (Figure 1). In young males, the negative effect of Met treatment on survival was not significant (Table 2). In old males, Met treatment increased survival significantly when males were immune challenged (Table 2; Figure 1). Old males that were only manipulated with control treatments (Ac-Control) survived less than young control males (Table 2; Figure 1), which confirms that senescence existed in control animals.

In recapture probabilities, there is a clear interaction effect of age and immune treatment (Age * Imm) (Table 1; Figure 2): in young males, recapture probability is increased by

immune activation (Imp), while in old males it decreases with Imp. In both young and old males, Met treatment has a negative effect on recapture probabilities, which is significant in old males (Table 2; Figure 2).

Survival in captivity

Survival of males in captivity was only explained by age, with young males surviving significantly more than old males ($z = 6.17$, $P < 0.001$, $N = 140$; Figure 3). Neither hormonal (JH), immune (Imm) treatments nor body size explained differences in male survival in captivity.

Discussion

In the present study we have shown that males of the territorial damselfly *H. americana* are subject to senescence, consistent with our first prediction: survival probabilities are lower for old than for young control animals both in the wild and in captivity. This occurred in spite of the inevitable sampling bias resulting from our cross-sectional experimental design: when sampling old males we are biasing our sample towards long-lived individuals (only individuals that were able to survive until old age), while when sampling young males we are including both short- and long-lived individuals. Partly coherent with our second prediction, JH analog ameliorated senescence in wild animals and had no effect in captive animals, that were prevented from feeding and mating. This suggests that either foraging or territorial activity could have been altered by JH analog treatment, leading to the observed effects in the field.

Our finding that Met increased survival in old, immune-challenged males, was unexpected as most evidence associates senescence with high levels of JH (Tatar *et al.* 2001; Herman & Tatar 2001; Flatt & Kawecki 2007). However, this previous evidence comes from laboratory studies. In natural conditions, on the other hand, there are many uncontrolled factors that can be affected by JH and favor senescence. One such factor is how animals can compensate experimentally-induced high JH levels in the field. It is known that JH has different physiological effects when resource availability varies (Trumbo & Robinson 2004), which occurs in natural conditions. Nutritrional state is a key regulator of the neuroendocrine system, because it is implied in the insulin signaling pathway leading to JH production. In particular, when food is scarce, insulin-like peptide production decreases, and expression of insulin-like genes is repressed (Ikeya *et al.* 2002), leading to low JH synthesis and increased lifespan (Flatt et al. 2005). Animals can vary their feeding activity in response to environmental pressures, either by increasing it (Lee, Raubenheimer, & Simpson 2004; González-Tokman *et al.* 2011), decreasing it (Adamo *et al.* 2010), or selecting their diets adaptively (Mayntz *et al.* 2005; Pekár *et al.* 2010; Ponton *et al.* 2011). Given that dietary restriction is associated with prolonged lifespan in insects (Grandison, Piper, & Partridge 2009), JH in old males may have induced a reduction in their foraging activity, thereby explaining their observed increased lifespan. Unfortunately, we did not conduct any behavioral study along with our experiment. Future studies should evaluate the effect of JH on feeding behavior.

There was no clear support for our third prediction in relation to a synergistic effect of JH and immune challenge to affect survival of old males more intensively than the other males.

328 According to this, Met would increase reproductive activity, which would lead to faster
329 mortality, especially in immune challenged males (González-Tokman *et al.* 2012b;
330 González-Tokman *et al.* 2013). This expectation is based on previous evidence in terms of
331 resource allocation to sexual traits in damselflies (Rantala *et al.* 2003; Contreras-Garduño
332 *et al.* 2009, 2011). This was possibly the case for young males in our study, who showed
333 increased mortality when treated with Met. Nevertheless, old males supplemented with Met
334 showed reduced mortality, especially when they were immune challenged, suggesting that
335 JH largely determines how organisms schedule their investment in reproduction during
336 their lifetime (McNamara *et al.* 2009). It seems like JH mediates a switch towards an
337 alternative reproductive trajectory by which animals would save resources for better
338 chances of reproduction in the future (McNamara *et al.* 2009). Besides modifying nutrient
339 intake, JH could have modified reproductive behavior in males treated with Met, altering
340 survival probabilities in our study in the field.
341
342 In regards to our fourth prediction, a high recapture rate should be expected for animals
343 whose mortality risk is also high, as a consequence of terminal investment. This prediction
344 was not satisfied in the present study. However, this prediction would imply that high
345 recapture rates imply high territorial activity, which is not necessarily the case. Indeed,
346 there are factors different from territorial status that can determine recapture rates and, as a
347 consequence, territorial animals not always show higher recaptures than non territorials
348 (Munguía-Steyer et al. 2010). It seems that immune challenge, rather than hormone
349 treatment, influenced faithfulness to mating sites. In particular, immune challenge increased
350 recapture probability in young males but decreased it in old males. It is unclear why young
351 males had their recapture rate increased. There are at least three factors associated to

faithfulness to a mating site in damselflies: mating experience (i.e. acquisition of a mating in the past leads to further faithfulness; Switzer 1997; Nagy, Szallassy, & Devai 2008), infection (i.e. the higher the re-infection probability, the more likely to abandon a mating area; Suhonen, Honkavaara, & Rantala 2010; Rantala, Honkavaara, & Suhonen 2010) and male aggression (the higher the male aggression, the less likely to stay in a mating place; Córdoba-Aguilar 1994). We do not have data to assess the effect of mating experience. As for infection, the fact that we used the immune challenge approach suggests that avoiding re-infection may explain our results but only for old males. In terms of avoiding aggression, possibly old males searched for mates outside the mating riverine territories as they were unable to face the intense male-male competition in these areas. That relatively old males are chased away from mating territories is common in *Hetaerina* (Córdoba-Aguilar 1994; Guillermo-Ferreira & Del-Claro 2011). These old males look for mating opportunities elsewhere perhaps to save their already reduced energetic stores or to replenish energy reserves. Related to energy, it is known that *H. americana* males may engage in a territorial/nonterritorial tactic: males leave their defended territories for a few days to then re-assume their positions (Raihani, Serrano-Meneses, & Córdoba-Aguilar 2008). One explanation for this "switcher" tactic is that these males leave their defended sites to replenish their energetic stores by feeding outside territories (Raihani *et al.* 2008). One would expect that this should occur more to old males as they have lower energetic reserves compared to young males (Contreras-Garduño et al. 2006). Despite this, switcher males do not differ in age from territorial and nonterritorial males (Raihani et al. 2008). Given this, it is unlikely that our JH-treated old males were switchers.

Environmental conditions are key determinants of animal senescence; consequently, evaluating senescence under controlled laboratory conditions can provide an incomplete picture of natural selection acting on senescent animals (Williams *et al.* 2006). On the other hand, detecting aging rates under natural conditions has been challenging because many factors can mask senescence and because it requires periodical monitoring of animals across their lifetimes, which can be logistically demanding (Nussey *et al.* 2008). By using a capture-mark-recapture model we have detected senescence in a wild population of a short-lived territorial insect. Our unexpected findings concerning the effect of JH on senescence in the wild highlight the importance of using longitudinal studies in natural populations in order to understand the evolution of senescence (Williams *et al.* 2006; Nussey *et al.* 2008).

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601 Table 1. Summary of model selection process to test the effect of age and one hormonal
602 (JH) and one immune treatment (Imp) on daily survival and recapture probabilities of
603 marked *Hetaerina americana* males in the wild.

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Model description							
Survival components	**Recapture components**	***Q*AICc**	**Δ *Q*AICc**	**AICc weight**	**Model likelihood**	**No. of parameters**	***Q* Deviance**
Age+JH+Age:JH	Age+JH+Imm+Age:Imm	2589.297	0.000	0.174	1.000	9	2571.154
Age+JH+Imm+Age:JH+Age:Imm	Age+JH+Imm+Age:Imm	2590.513	1.216	0.095	0.544	11	2568.303
Age+JH+Age:JH	Age+JH+Imm+Age:Imm+JH:Imm	2590.515	1.218	0.095	0.544	10	2570.340
Age+JH+Age:JH	Age+JH+Imm+Age:JH+Age:Imm	2591.073	1.777	0.072	0.411	10	2570.898
Age+JH+Imm+Age:JH	Age+JH+Imm+Age:Imm	2591.242	1.945	0.066	0.378	10	2571.067
Age+JH+Imm+Age:JH+Age:Imm+JH:Imm	Age+JH+Imm+Age:Imm	2591.328	2.032	0.063	0.362	12	2567.080
Age+JH+Imm+Age:JH+Age:Imm	Age+JH+Imm+Age:Imm+JH:Imm	2591.821	2.524	0.049	0.283	12	2567.572
Age+JH+Imm+Age:JH+JH:Imm	Age+JH+Imm+Age:Imm	2591.857	2.560	0.048	0.278	11	2569.647
Age+JH+Imm+Age:JH+Age:Imm	Age+JH+Imm+Age:JH+Age:Imm	2592.257	2.960	0.040	0.228	12	2568.008
Age+JH+Imm+Age:JH	Age+JH+Imm+Age:Imm+JH:Imm	2592.458	3.161	0.036	0.206	11	2570.247
Age+JH+Imm+Age:JH+Age:Imm+JH:Imm	Age+JH+Imm+Age:Imm+JH:Imm	2592.854	3.557	0.029	0.169	13	2566.564
Age+JH+Imm+Age:JH	Age+JH+Imm+Age:JH+Age:Imm	2593.020	3.723	0.027	0.155	11	2570.810
Age+JH+Imm+Age:JH+Age:Imm+JH:Imm	Age+JH+Imm+Age:JH+Age:Imm	2593.103	3.806	0.026	0.149	13	2566.813
Age+JH+Imm+Age:JH+JH:Imm	Age+JH+Imm+Age:Imm+JH:Imm	2593.319	4.023	0.023	0.134	12	2569.071
Age*JH*Imm	Age+JH+Imm+Age:Imm	2593.320	4.023	0.023	0.134	13	2567.030
Age+JH+Imm+Age:JH+JH:Imm	Age+JH+Imm+Age:JH+Age:Imm	2593.653	4.356	0.020	0.113	12	2569.404
Constant (.)	Age+JH+Imm+Age:Imm	2594.350	5.053	0.014	0.080	6	2582.283
Age*JH*Imm	Age+JH+Imm+Age:Imm+JH:Imm	2594.851	5.554	0.011	0.062	14	2566.515
Age*JH*Imm	Age+JH+Imm+Age:JH+Age:Imm	2595.099	5.802	0.010	0.055	14	2566.763

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Table 2. Daily survival (φ) and recapture (p) probabilities of *Hetaerina americana* males of different ages exposed to one hormonal (Ac or Met) and one immune (Imp or Control) treatment. Estimates and standard errors (SE) were calculated from model averaging of the best supported models (top 6 AIC, Table 1).

			Treatment				95% C. I.	
Parameter		**Age**	**JH**	**Imm**	**Estimate**	**SE**	**Lower**	**Upper**
1	φ	Young	Ac	Control	0.913	0.015	0.882	0.943
2	φ	Young	Ac	Imp	0.911	0.016	0.880	0.941
3	φ	Young	Met	Control	0.874	0.020	0.834	0.914
4	φ	Young	Met	Imp	0.860	0.022	0.816	0.904
5	φ	Old	Ac	Control	0.831	0.026	0.779	0.882
6	φ	Old	Ac	Imp	0.856	0.023	0.811	0.900
7	φ	Old	Met	Control	0.906	0.017	0.873	0.938
8	φ	Old	Met	Imp	0.912	0.016	0.882	0.943
9	p	Young	Ac	Control	0.444	0.032	0.382	0.507
10	p	Young	Ac	Imp	0.564	0.033	0.500	0.628
11	p	Young	Met	Control	0.372	0.034	0.306	0.438
12	p	Young	Met	Imp	0.476	0.038	0.401	0.551
13	p	Old	Ac	Control	0.540	0.041	0.459	0.621
14	p	Old	Ac	Imp	0.443	0.036	0.372	0.515
15	p	Old	Met	Control	0.458	0.035	0.390	0.526
16	p	Old	Met	Imp	0.352	0.031	0.291	0.412

Figure 1. Daily survival probabilities (estimates ± 95% C. I.) of *Hetaerina americana* males of different ages exposed to one hormonal (Met or Ac) and one immune (Imp or Control) treatment. Estimates and C. I. were calculated from model averaging of the best supported models. Sample sizes are showed above the bars. Confidence intervals can be seen in Table 2.

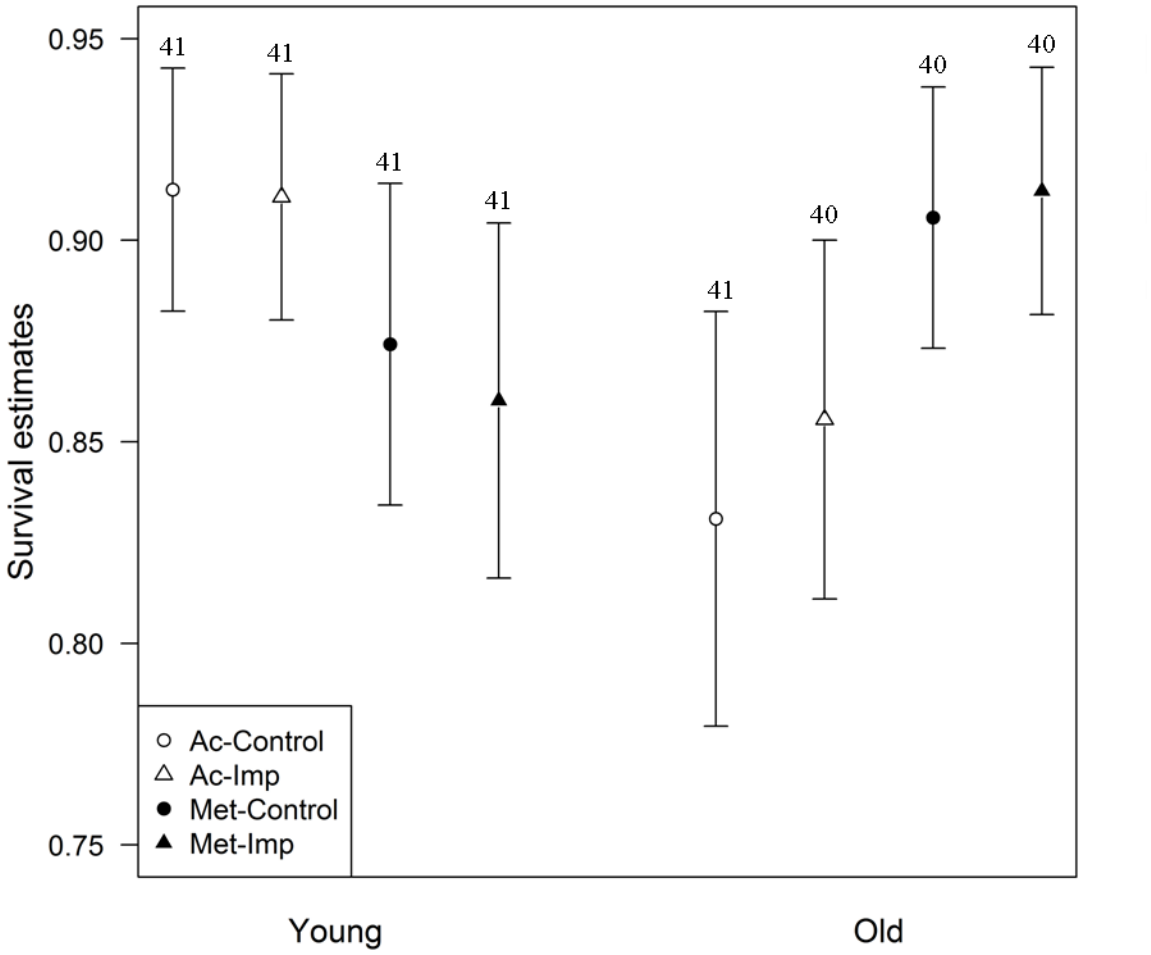
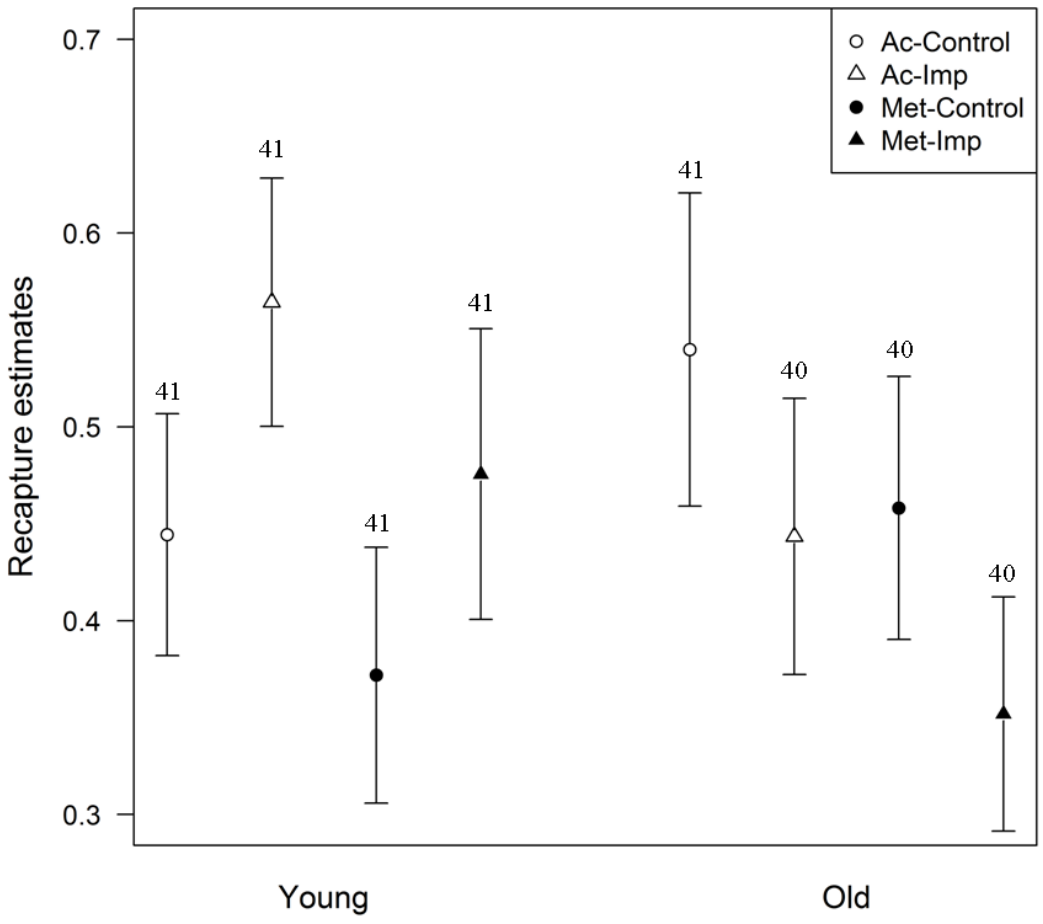
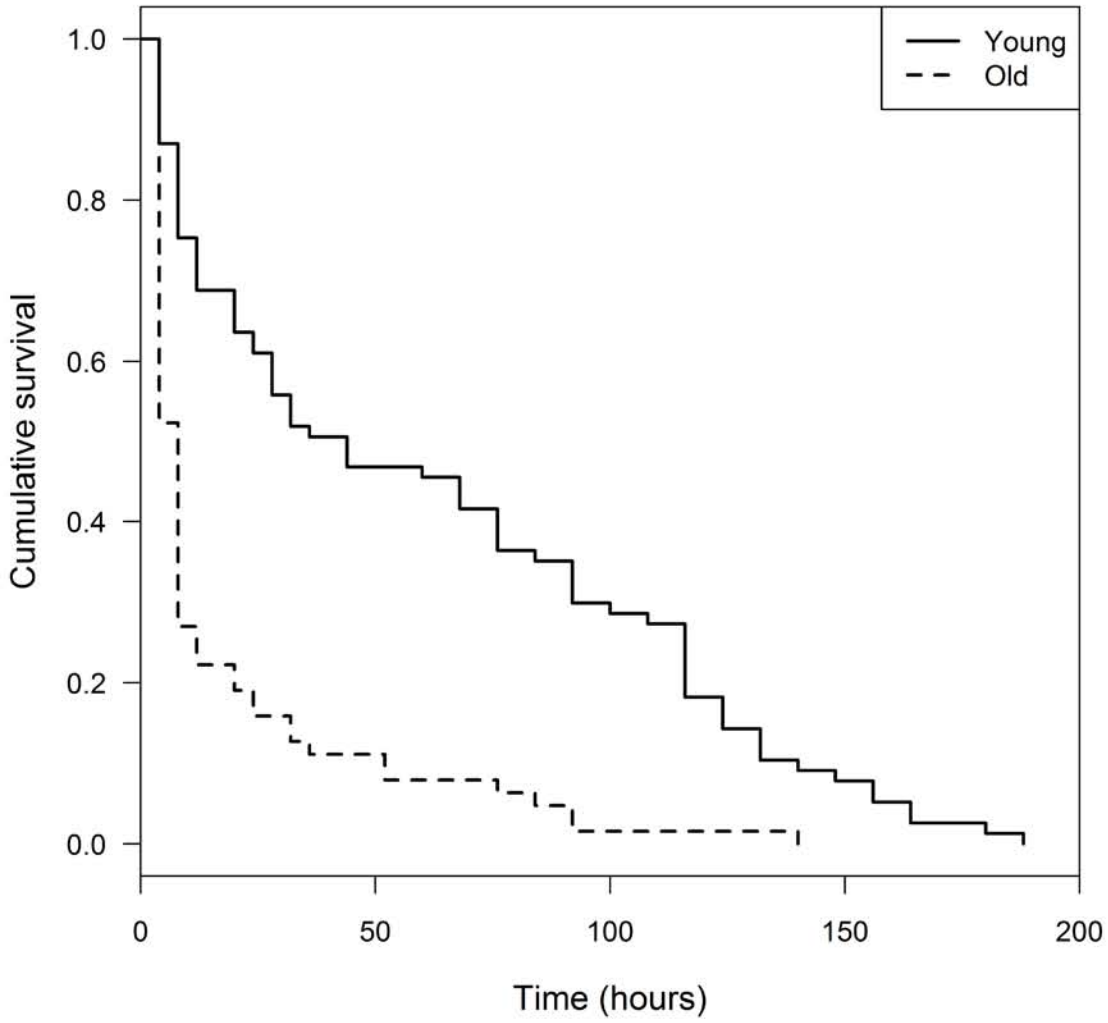


Figure 2. Daily recapture probabilities (estimates ± 95% C. I.) of *Hetaerina americana* males of different ages exposed to one hormonal (Met or Ac) and one immune (Imp or Control) treatment. Estimates and C. I. were calculated from model averaging of the best supported models. Sample sizes are showed above the bars. Confidence intervals can be seen in Table 2.



634 Figure 3. Survival in captivity of young (N = 164) and old (N = 161) *Hetaerina americana*

635 males.



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Capítulo 3.

Éxito de apareamiento y su efecto sobre la condición energética mediados por inversión terminal en machos territoriales de un invertebrado de vida corta

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Mating success and energetic condition effects driven by terminal investment in territorial males of a short-lived invertebrate

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Summary

1. The terminal investment hypothesis has two predictions: in the face of an infection (i) mature males will increase investment to traits that increase mating success, while such investments will occur to a less extent in young males; and (ii) physiological costs of resource reallocation will be more severe for infected mature males than for infected young males.

2. Although these predictions have been tested in long-lived vertebrates, prior studies have not examined actual resource allocation conflicts. Here, we have tested the above predictions and have investigated the energetic costs of increased mating by old males, using a short-lived invertebrate, the damselfly *Hetaerina americana*. Males of this species defend territories as the main way to obtain access to females.

3. Using groups of infected vs. noninfected males of two different ages, we found that compared to young infected males, mature infected males defended territories for longer, had higher mating success and directed agonistic behaviour to conspecific males more frequently. Despite similar immune responses by mature and young males, infected mature males ended up with less fat reserves compared to infected young males. This suggests that resource allocation conflicts are more severe for mature than for young males.

4. In general, these results suggest that the terminal investment hypothesis applies in males of short-lived invertebrates and that a cause of increased mating success for males of advanced ages is reduced energetic stores.

Key-words: immunity, invertebrate, males, mating success, terminal investment

Introduction

When animals reproduce several times in their lifetime, allocation of resources to current reproduction will constrain resources devoted to future reproduction (Clutton-Brock 1984; Reznick 1985; Creighton, Heflin & Belk 2009). To deal with this trade-off and thus maximize lifetime reproductive success, animals should balance resources among their expected reproductive events (Stearns 1992; Roff 2002). So, when animals perceive that they are likely to reproduce in the future or that it is not affordable to reproduce at present, they can adopt a strategy of restricting their investment to current reproduction and skip some reproductive events (e.g. Velando, Drummond & Torres 2010; Goutte *et al.* 2011). On the other hand, when animals perceive that the probability of mortality is high and there are low chances of reproducing in the future, they should expend higher amount of resources in current reproduction even if it ends with animal's death (Williams 1966; Javois & Tammaru 2004; Kivleniece *et al.* 2010). This idea is known as the 'terminal investment hypothesis'. The predictions of the terminal investment hypothesis have been supported by studies of long-lived vertebrates (e.g. Velando, Drummond & Torres 2006; Hoffman *et al.* 2010) and studies of invertebrates (e.g. Adamo 1999; Javois & Tammaru 2004; Sadd *et al.* 2006).

The terminal investment hypothesis has two main assumptions: (i) that an investment in current reproduction is traded off against investments in future reproduction and (ii) that animals perceive their life span and thus modulate their investment in a reproductive event accordingly (Nielsen & Holman 2011). Given that prospects for future reproduction depend on individual age, the terminal investment hypothesis needs to be evaluated in individuals of different age classes, whose residual reproductive values differ (Adamo 1999; Velando, Drummond & Torres 2006).

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Under adverse conditions, young animals, with high residual reproductive values, should prioritize investment in self –maintenance, so that survival will not decrease drastically. On the other hand, old senescing animals, whose residual reproductive values are low, should prioritize investment in current reproduction (Forbes 1993).

A successful experimental method to probe terminal investment predictions is to reduce an animal's life expectancy via using an immune challenge in young and old animals. The immune response is a costly function (Sheldon & Verhulst 1996; Rolff & Siva-Jothy 2003) that is sensitive to both individual condition (Rantala *et al.* 2003; Contreras-Garduño *et al.* 2008) and senescence (e.g. Saino *et al.* 2003; Moret & Schmid-Hempel 2009). Consequently, young animals are more likely to recover from an immune insult than old animals. Terminal investment occurs when insulted animals increase their investment in reproduction when they are old but not when they are young.

Terminal investment has been mainly studied in females of both vertebrates (e.g. Hoffman *et al.* 2010; Weladji *et al.* 2010) and invertebrates (e.g. Adamo 1999; Javois & Tammaru 2004; Creighton, Heflin & Belk 2009; Cotter, Ward & Kilner 2010). In general, it has been documented that old and/or immune challenged females end up increasing investment in progeny compared to young or control females (Adamo 1999; Javois & Tammaru 2004; Creighton, Heflin & Belk 2009; Cotter, Ward & Kilner 2010; Hoffman *et al.* 2010 but see Weladji *et al.* 2010). In males that do not contribute to parental care, terminal investment can be detected as increases in the expression of sexually selected traits. Sexually selected traits are costly signals that reflect male quality and are key for male fitness (Hamilton & Zuk 1982; Anderson 1994). Several laboratory studies have investigated terminal male investment in sexually selected traits (e.g. Sadd *et al.* 2006; Kivleniece *et al.* 2010; Krams *et al.* 2011) and have often failed to detect shifts in male allocations (Vainikka *et al.* 2007). One explanation for the failure to detect this pattern is that selective pressures, such as limited food availability, that act in the wild but not under laboratory conditions are necessary to detect this pattern. A single study has been conducted in the wild, with the blue-footed booby *Sula nebouxii* (Velando, Drummond & Torres 2006), a long-lived bird. When old, senescing males were immune insulted, they responded by increasing their reproductive success, while younger males showed a decline in reproductive success. This study is the only experimental evidence supporting male terminal investment in the wild. Further evidence from similar wild conditions is therefore sorely needed.

An underlying assumption in studies of terminal investment is that there exists a conflict in the allocation of resources to different time-related reproductive functions (Clutton-Brock 1984; Reznick 1985; Creighton, Heflin & Belk 2009). Few studies, however, have actually assessed the energetics of resource allocation conflict. This is especially important for experimental studies as it is not at all clear, for example, whether energy devoted to immune response is subsequently not available for reproductive investment. Ignoring this energy allocation has at least two confounding effects: (i) it may be that it is not really the immune response that is costly but another noncontrolled function and (ii) the imposed challenge may not be costly at all. This may explain a few cases where the evidence of whether an energy conflict is occurring for the targeted, experimentally affected function is unclear (e.g. Langley & Clutton-Brock, 1998, Vainikka *et al.* 2007). An example that clearly illustrates the importance of an approach that measures energy allocation is that of the beetle *Tenebrio molitor*: males face an energy-based trade-off only above a threshold of immune challenge (Krams *et al.* 2011). Below such threshold, no energy spent in immune response was detected yet experimentally challenged males were less attractive (Kivleniece *et al.* 2010; Krams *et al.* 2011).

In the present study, we manipulated life expectancy in both young and mature males of the territorial damselfly *Hetaerina americana* by activating a costly immune response with a nonpathogenic elicitor (a nylon implant). We measured both aggressive territorial behaviour (used here as a sexually selected trait, cf. Contreras-Garduño *et al.* 2009) and mating success of implanted vs. control insects. We also measured immune response (i.e. nylon encapsulation) and energetic condition (i.e. fat reserves) after treatment. According to the terminal investment hypothesis, we predicted that mature males would show higher territorial aggression when implanted, while the opposite trend would occur in young males. We also predicted that the physiological costs of terminal investment would be higher in mature, senescent males than in young males. Specifically, we predicted that old males would lose energetic stores more drastically than young males. To our knowledge, this is the first study that experimentally evaluates the terminal investment hypothesis in males of a short-lived animal in the wild by measuring energetic costs.

Materials and methods

STUDY SUBJECT

We used adult males of the damselfly *Hetaerina americana* (Odonata: Calopterygidae), which contend for riverine territories where females arrive to mate (Córdoba-Aguilar, Jiménez-Cortés & Lanz-Mendoza 2009a; Córdoba-Aguilar *et al.* 2009b). Males able to acquire and successfully defend territories usually have larger red wing spots and body sizes, which allow them to accrue a higher mating success compared to nonterritorial males (Grether 1996; Serrano-Meneses *et al.* 2007; Contreras-Garduño *et al.* 2008). Territorial ability, expressed in the form of aggressive flights against conspecifics, is one honest signal of male immune and energetic condition (Contreras-Garduño, Canales-Lazcano & Córdoba-Aguilar 2006; Contreras-Garduño, Lanz-Mendoza & Córdoba-Aguilar 2007; Contreras-Garduño *et al.* 2008). Energetic stores are present in the form of lipidic fat reserves stored in the thoracic muscle (Contreras-Garduño, Canales-Lazcano & Córdoba-Aguilar 2006). Such reserves serve as fuel during aggressive flights, and

experimental work with larvae has shown that fat reserves are condition dependent (Jiménez-Cortés, Serrano-Meneses & Córdoba-Aguilar 2012). *H. americana* males typically have no more than three matings in their lifetime (Serrano-Meneses *et al.* 2007), which makes them suitable subjects for studying senescence in reproductive activity and terminal investment.

FIELD WORK

Field work was carried out in the Apatlaco river, Morelos, Mexico (18°45′55″N, 99°14′45″W), from November to December 2011, and in Metztitlán river, Hidalgo, Mexico (20°32′30″N, 98°43′45″W), from December 2003 to February 2004. Animals were captured with a butterfly net between 1100 and 1400 hours, the time at which territorial activity is highest at our study site (all authors' personal observations). Behavioural observations were also carried out in this time interval. Given that our main aim is to study senescence, we took special care to assess male age. We classified ages in categories according to visible features of the wings, thorax and abdomen (Plaistow & Siva-Jothy 1996): age 1 males have soft, undamaged and dorsoventrally flexible wings; age 2 males are young and sexually mature individuals that fight for territories, have harder wings that are flexible from the nodus to the tip and are already pigmented; age 3 males are fully sexually mature but have less flexible wings and show some signs of pruinescence in the thorax; age 4 males, the oldest, show abundant thoracic pruinescence and inflexible wings that are occasionally damaged (broken at their tips). We were also careful about territorial status and considered that a male was territorial when it was observed involved in contests with conspecific males and actively defending a site. Territorial males also reacted aggressively to the presentation of an experimental conspecific intruder. Otherwise, males were considered nonterritorial. We only collected territorial, sexually mature males of ages 2 and 3. Based on the age categories mentioned above, we created two experimental categories: 'young' and 'mature'. Males were considered 'young' when their wings, thorax and abdomen were still brilliant and the pterostigmata were still clear. All young animals were of age class 2. Some males of age 3 were marked with a small dot in the right anterior wing, released and recaptured 8–10 days later only if they were territorial at that time. These are 'mature' males that we estimate to be at least 10–15 days older than young males. Marked males that were already from age class 4 were excluded from the study. We considered that an age difference of 10–15 days was appropriate for our experiment given that the longevity of these animals is around 30 days (all authors' unpublished data) and at the age of 15 days since emergence, damselflies of similar life span can start showing senescence (Sherratt *et al.* 2010). While our ageing method is based on subjective traits (appearance of the animals), the fact that young and mature males differed in body size in our experiments suggests that they emerged at different times and thus come from different but overlapping cohorts (see also Córdoba-Aguilar 2009a).

EXPERIMENTAL PROTOCOL

Both young and mature males were manipulated with either an experimental or a sham treatment. In the experimental group, an immune response was triggered by inserting a nylon monofilament (previously rubbed with fine sandpaper, 2 mm long, 0·18 mm width) in the ventral part of the fourth abdominal segment (Rantala *et al.* 2000). When implanted, insects respond by melanizing the implant (for similar methods, see Rantala & Roff 2007; González-Tokman, Córdoba-Aguilar & Forbes 2012). Males in the sham group received the same manipulation, but the implant was immediately removed. Males were anaesthetized in ice for 10 min, marked with a unique number made with permanent ink on the right anterior wing (numbers can be seen from 3 to 5 m away) and released to the same place where they were collected.

EXPERIMENT 1: LONG-TERM TERRITORIAL DEFENCE AND MATING SUCCESS

In the Metztitlán river, we collected and manipulated 51 experimental young males, 59 sham young males, 56 experimental mature males and 73 sham mature males. Wing length, measured from the site of wing insertion in the thorax to the distal end of the wing, was considered as the measure of body size (Serrano-Meneses *et al.* 2007). Starting 24 h after manipulation (experimental and sham), animals were tracked daily to record territory tenancy and mating success for 19–20 days. For territory tenancy, we walked three times daily along the river stretch (*c.* 200 m length) where marked males were released and recorded which males were present on each site. Sites were depicted on a map of the river stretch and were updated daily (as defended areas may move depending on sun conditions; Córdoba-Aguilar *et al.* 2009b; Córdoba-Aguilar, Jiménez-Cortés & Lanz-Mendoza 2009a; see also Córdoba-Aguilar 1995). Unlike males of the experiment 2 described below, territory tenancy was estimated by seeing whether males remained at the same site in at least on two of the three surveys. We did not directly experimentally assay territoriality as there was too much vegetation in the Metztitlán river to simulate intrusions as in experiment 2. Despite this, we are confident that this approach was useful to record territory tenancy as males that failed to appear on a particular day at their previously recorded site did not subsequently reappear on the same spot and actually wandered to different sites. This indicates that males became nonterritorial after losing a territory (see also Raihani, Serrano-Meneses & Córdoba-Aguilar 2008). Mating success was also recorded via these daily censuses. Although matings are relatively short (2–3 min), the fact that a copulating pair usually flies continuously for up to 40 min to reduce male harassment makes visually recording each mating relatively tractable (Córdoba-Aguilar 2009b).

EXPERIMENT 2: SHORT-TERM MALE AGGRESSIVENESS AND PHYSIOLOGICAL CONDITION

In the Tetlama river, we collected and manipulated 52 experimental young males, 48 sham young males, 52 experimental mature males and 53 sham mature males. Twenty-four hours after manipulation (nylon insertion or sham treatment), we made behavioural observations and recaptured the animals to recover the implants and measure the extent of melanization. Animals were then sacrificed and stored in 70% ethanol for subsequent measurement of fat content. We took care to observe young and mature animals simultaneously, so differences in behaviour or physiology between age classes were not due to climatic conditions. Given that within odonates it is common that time of emergence affects morphological features (Stoks & Córdoba-Aguilar 2012), there were unavoidable differences in the body sizes of young and mature males in our study (see Results). We addressed this issue by including body size as covariate in statistical analyses. Some animals could not be recaptured after observation and for some of them implants could not be recovered, so sample sizes may differ among different analyses.

EXPERIMENT 2: BEHAVIOUR

Each focal male's territorial behaviour was recorded in response to simulated intrusions of conspecific males. Intruding conspecifics were fully mature males of age class 3 that were captured at the moment of observation and tethered to a 50-cm nylon thread attached to a stick (Anderson & Grether 2010). Tethered intruders

were presented ten times to each focal male, and we waited 2 min between intrusions. A presentation was considered complete when the tethered intruder flew for at least 5 s not further than 20 cm from the front or side of the focal male. Tethered intruders were replaced when they stopped flying, so that in some cases, more than one was used per focal male or sometimes one intruder was used for more than one focal male. For each presentation, we classified focal behaviour as one of three possible behavioural responses: (i) a focal male was considered to 'attack' the model when he responded to the presentation by flying towards and chasing the intruder. This is the clearest territorial response (Anderson & Grether 2010); (ii) a male was considered to do a 'wing display' when it opened its four wings without moving from its site. While this behaviour has not been considered as a territorial display in *Hetaerina* damselflies (Anderson & Grether 2010), it is used to intimidate males in odonates (Utzeri 1988). Finally, (iii) a male was considered to 'getaway' if it flew at least 2 m apart of its site when the intruder was presented (see also Córdoba-Aguilar 1995). We only considered individuals with at least eight recordings. Sample sizes were as follows: young implanted $N = 18$, young sham $N = 19$, mature implanted $N = 22$ and mature sham $N = 26$.

EXPERIMENT 2: IMMUNE RESPONSE, FAT CONTENT AND BODY SIZE

Melanization of nylon implants was measured as the darkness of an implant relative to a control piece of nylon that was not implanted. Each implant was photographed from three different angles at one side of the control, and darkness was obtained with ADOBE PHOTOSHOP 7.0 (Adobe Systems Inc., San Francisco, CA, USA), using a scale where 0 is the minimum possible value and means black, and 255 is the maximum and means white. The average from the three pictures of the experimental nylon divided by the average of three pictures of an uninserted nylon was considered implant darkness. Sample sizes for melanization of nylon implants remained as follows: young $N = 14$ and mature $N = 21$.

For measurements of fat content, animals were dried in a desiccator, weighed ($\pm 0{\cdot}1$ mg), submerged in chloroform for 24 h for fat extraction, redesiccated and reweighed (for similar procedures, see Marden 1989; Pekár *et al.* 2010; González-Tokman, Córdoba-Aguilar & Forbes 2012). The difference between the initial and the final weights was considered fat content. Sample sizes for fat content were as follows: young implanted $N = 19$, young sham $N = 19$, mature implanted $N = 21$ and mature sham $N = 22$.

As a measure of body size, we used the area of the right anterior wing (in mm^2). We used wing area instead of wing length because in experiment 2, we photographed males with a scale of known area and did not measure wing length, as it has been done in experiment 1. To measure wing area, we analysed pictures of the animals with ADOBE PHOTOSHOP 7.0.

STATISTICAL ANALYSES

Initial differences in body size between males of different age classes and treatments were tested with two-way analysis of variance (ANOVA), with age and treatment as factors. Given that body size was different between young and mature males in both the long- and the short-term experiments (see Results section), body size was always included as a covariate in subsequent analyses.

In experiment 1 (long term), the number of days that males defended a territory and the probability of copulating were analysed with Generalized Linear Models (GLM). The initial models tested included age, treatment, body size and all interactions (age × treatment × body size). Model selection was carried out both backwards and forward based on AIC values of all competing models (Johnson & Omland 2004). Overdispersion (residual deviance/residual d.f.) was low (< 2) in all global models. When the response variable was the proportion of days where focal males defended their territory, we used a GLM with binomial distribution of errors and logit link function. When the response variable was the number of copulations, we used a GLM with Poisson distribution of errors and log link function.

In experiment 2 (short term), male behaviour in response to simulated intrusions of conspecific males was also analysed with GLM. The initial models tested included age, treatment, body size and all interactions (age * treatment * body size). As above, model selection was based on AICs. Overdispersion was tested in the global models and was low (< 2) unless otherwise specified. When the response variable was the proportion of events where the focal male attacked the intruder, overdispersion of the global model was high (residual deviance/residual d.f. = 6·55), and then, we used quasi-binomial errors and logit link function. Starting with nonsignificant interactions, less significant covariates were eliminated one by one from the initial model based on the significance of a likelihood ratio tests (LRT) between the initial model and the model without the covariate. A significant LRT ($P < 0{\cdot}05$) means that the exclusion of the covariate does not affect model fit, and then, the covariate can be removed from the analysis (Johnson & Omland 2004). When the response variable was the proportion of intrusions where the focal male showed his wings without flying, we used a GLM with binomial errors and logit link function. Including the effect of heterogeneous variances in males of different age and treatment was not significant (beta-binomial model LRT age: $P = 0{\cdot}48$, treatment: $P = 0{\cdot}37$). When the response variable was the proportion of intrusions where the focal male left his perch, we used a GLM with binomial errors and logit link function. Including the effect of heterogeneous variances in males of different age was not significant (LRT between beta-binomial models $P = 0{\cdot}66$).

Melanization of nylon implants and fat content were analysed with general linear models (LM). Model selection was performed backwards and forward based on AIC values and LRT. When implant darkness was the response variable, the initial LM included age, body size and the interaction (age * body size). When fat content was the response variable, the initial LM included age, treatment, body size and all interactions (age × treatment × body size). Homogeneity of variances was tested with Fligner–Killeen tests.

In the Results section, we only show the best supported models. The presence of outliers was tested with Cook's distance, but no outliers or influential points were detected in any analysis (all Cook's distances < 0·5; Crawley 2007). Analyses were carried out in R version 2.10.0 (R Development Core Team 2009).

Results

EXPERIMENT 1: LONG-TERM TERRITORIAL DEFENCE AND MATING SUCCESS

Young males were significantly larger than mature males ($F_{1,236} = 12{\cdot}42$, $P < 0{\cdot}01$, $N = 239$; mature: $25{\cdot}40 \pm 0{\cdot}05$ mm, $N = 129$; young: $25{\cdot}70 \pm 0{\cdot}07$ mm, $N = 110$). There were no differences in male body size between treatments ($F_{1,236} = 0{\cdot}32$, $P = 0{\cdot}57$).

The probability of holding a territory depended on the interaction of age and treatment and on the interaction of body size and treatment (Figs 1 and 2; binomial GLM d.f. = 233, age $z = 1{\cdot}17$, $P = 0{\cdot}24$; treatment $z = 1{\cdot}55$, $P = 0{\cdot}12$; body size $z = 3{\cdot}37$, $P < 0{\cdot}01$; age: treatment $z = 7{\cdot}32$, P 0·01; treatment: body size $z = -1{\cdot}95$,

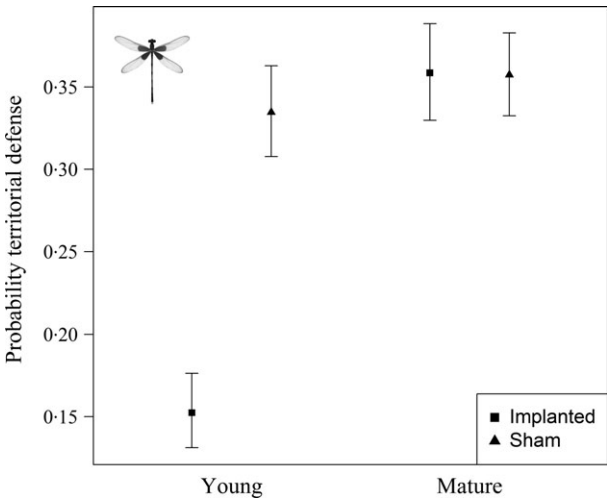

Fig. 1. Effect of the interaction age: treatment to explain differences on the proportion of days that *Hetaerina americana* males defended their territories. Bars represent estimates ±95% confidence intervals.

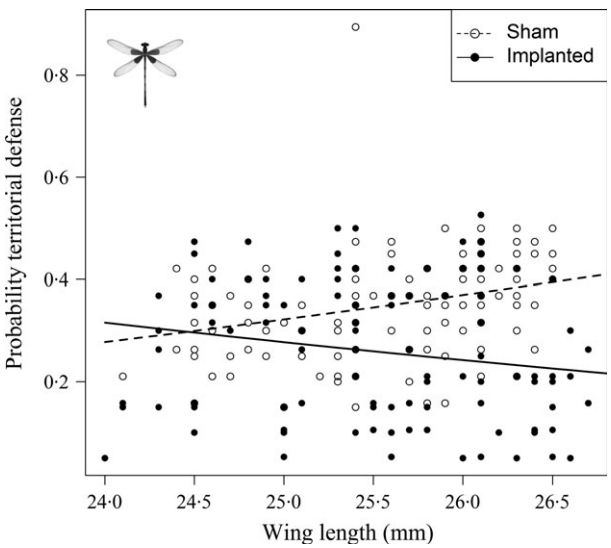

Fig. 2. Effect of the interaction treatment: body size to explain differences on the proportion of days that *Hetaerina americana* males defended their territories. Lines were predicted by the best supported model.

$P = 0{\cdot}05$). Young males lost their ability to defend a territory when implanted, while no effect of implant was found in mature males (Fig. 1). Also, larger males remained territorial for more days in the sham treatment but not in the implanted treatment (Fig. 2). The number of copulations was higher in mature and large males (Poisson GLM d.f. = 236, age $z = 3{\cdot}64$, $P < 0{\cdot}01$; body size $z = 3{\cdot}88$, $P < 0{\cdot}01$), independently of treatment (Fig. 3).

EXPERIMENT 2: SHORT-TERM MALE AGGRESSIVENESS AND PHYSIOLOGICAL CONDITION

Contrary to the long-term experiment, mature males were significantly larger than young males (ANCOVA

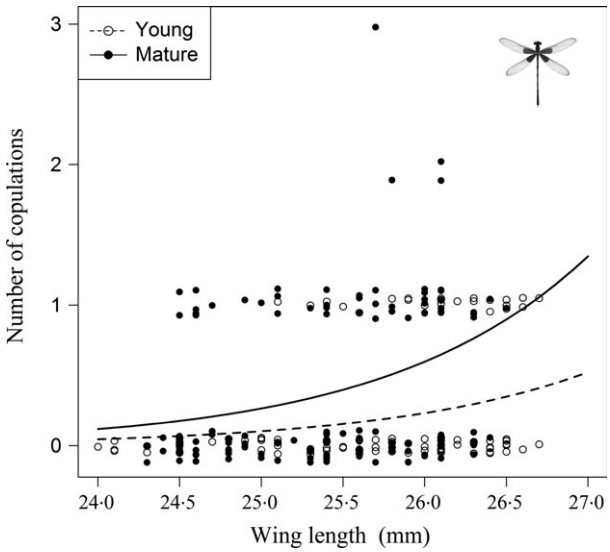

Fig. 3. Effect of the interaction age: body size to explain differences on the number of copulations observed in *Hetaerina americana* males. Points are displaced in the Y axis only for visual purpose. Curves were predicted by the best supported model.

$F_{1,199} = 12{\cdot}33$, $P < 0{\cdot}001$; mature: $115{\cdot}75 \pm 8{\cdot}97$ mm^2, $N = 104$; young: $110{\cdot}95 \pm 10{\cdot}41$ mm^2, $N = 98$). There were no differences in male body size between treatments ($F_{1,199} = 0{\cdot}08$, $P = 0{\cdot}78$).

Male aggressiveness, measured as the proportion of encounters where a focal male attacked the conspecific intruder, was not dependent on any of the tested covariates (and interactions): age (young/old), treatment (implanted/control) and body size (quasi-binomial GLM, Table S1).

The proportion of encounters where the focal male opened his wings (wing display) in response to the intruder was marginally dependent on the interaction of age and treatment (binomial GLM d.f. = 80, age: $z = 2{\cdot}89$, $P < 0{\cdot}01$; treatment: $z = 1{\cdot}08$, $P = 0{\cdot}28$; age: treatment: $z = -1{\cdot}92$, $P = 0{\cdot}06$), with mature males displaying more intensively when infected and young males showing the opposite trend (Fig. 4).

The proportion of encounters in which the focal male left his place in the presence of an intruder was marginally dependent on the interaction of age and body size (binomial GLM d.f. = 80, age $z = -1{\cdot}85$, $P = 0{\cdot}07$; body size $z = -0{\cdot}58$, $P = 0{\cdot}56$; age: body size $z = 1{\cdot}71$, $P = 0{\cdot}09$). The trend was that young males left the sites more often when they were smaller, while mature males left their sites more often when they were larger (Fig. 5). In general, young males left their site more often than mature males, and this effect was independent of treatment.

Melanization of nylon implants was not dependent on age or body size (LM; Table S2). The amount of fat reserves in male's thoraces was dependent on body size and on the interaction age: treatment (LM d.f. = 76; age $t = 1{\cdot}87$, $P = 0{\cdot}07$; treatment $t = 1{\cdot}80$, $P = 0{\cdot}08$, body size $t = 2{\cdot}24$, $P = 0{\cdot}03$; age: treatment $t = 2{\cdot}22$, $P = 0{\cdot}03$).

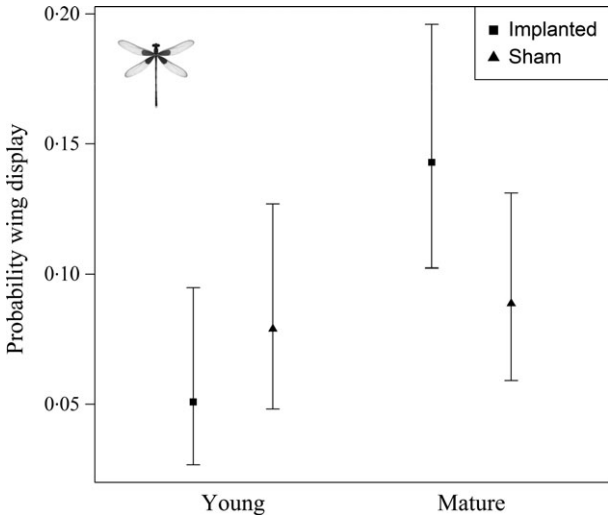

Fig. 4. Effect of the interaction age: treatment to explain differences on the probability that *Hetaerina americana* males respond to the presence of a conspecific intruder by showing their pigmented wings. Bars represent estimates ±95% confidence intervals.

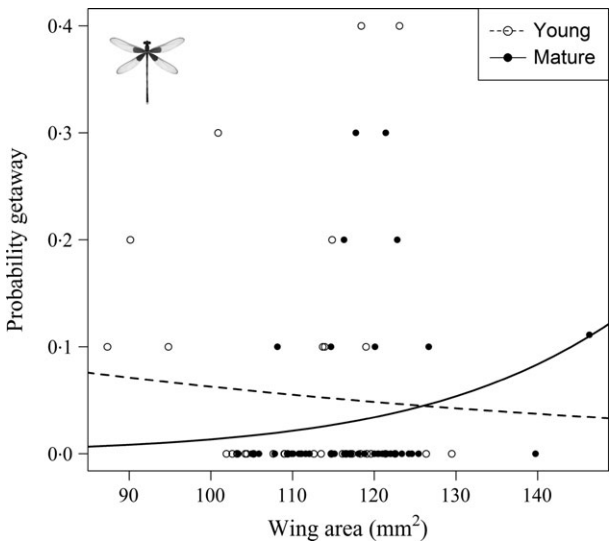

Fig. 5. Effect of the interaction age: body size to explain differences on the probability that *Hetaerina americana* males leave their site in the presence of a conspecific intruder. Curves were predicted by the best supported model.

Larger males had more fat reserves. Young animals had more fat reserves when implanted, and old animals had less fat reserves when implanted (Fig. 6).

Discussion

When animals get old, they adjust their investment in reproduction in two possible ways: first, old animals can increase their investment in current reproduction as a strategy of terminal investment if they perceive that their chances of future reproduction (residual reproductive value) are reduced (Clutton-Brock 1984; Velando,

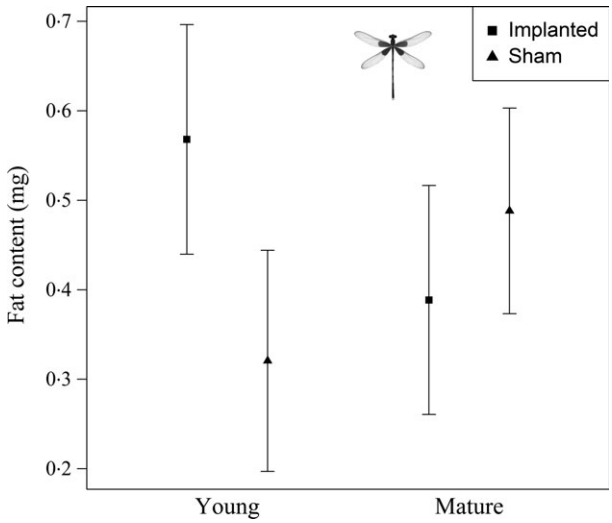

Fig. 6. Effect of the interaction age: treatment on energetic reserves of *Hetaerina americana* males. Bars represent estimates ±95% confidence intervals.

Drummond & Torres 2006). Alternatively, old animals can reduce their investment in current reproduction either because they end up deteriorated as a result of senescence or because of an adaptive reproductive restraint (McNamara *et al.* 2009). Here, we have used male *H. americana* damselflies to demonstrate that when chances for future reproduction are experimentally reduced by an immune challenge, old animals show terminal investment because they maintain elevated territorial activity despite a deteriorated physical condition. Our results imply that damselflies somehow perceive their future chances for reproduction and allocate their resources accordingly. Unlike other systems (Velando, Drummond & Torres 2006; Cotter, Ward & Kilner 2010), old *H. americana* males do not seem to be cautious about their investment in reproduction, and as a consequence, their energetic reserves become depleted.

To evaluate our first prediction – that mature implanted males would have higher mating success than young implanted males – we found mixed support. Mature males indeed did accrue higher mating success than young males; however, this effect was independent of treatment. In our study species, territorial males rarely get more than three matings in their life, while most nonterritorial males obtain no matings at all (Serrano-Meneses *et al.* 2007). Furthermore, only 10–15% territorial males obtain some matings (Serrano-Meneses *et al.* 2007). Such strict characteristics make the *Hetaerina* mating system an extremely competitive biological system. Thus, that implanted mature males invest their energy and time to still hold a territory (and so accrue some matings) can be interpreted as a strategy of terminal investment.

We predicted that higher mating success in mature implanted males would be linked to enhanced territorial aggression. However, we did not find an effect of age or treatment in aggressiveness towards intruder males. Instead, our evidence, although marginally significant

($P < 0{\cdot}10$), suggests that there is an increased frequency of wing displays in mature implanted males and that mature males are less likely to abandon their territories. Why would wing displays be functional in the context of territorial defence? In odonates, these displays are considered as threat signals that males and females use against any sex of the same or different species (Corbet 1999). These type of displays in territorial animals can be used as alerting signals that prevent direct confrontation between males (Maynard-Smith & Harper 2003; Searcy & Nowicki 2005; Grether 2011). We suggest that *H. americana* wing display by males has the same function and that, when used against conspecific males, is to show the red spot males have on at the base of their wings. Previous studies have indicated that the size of such spot is a condition-dependent trait (Contreras-Garduño *et al.* 2008; Jiménez-Cortés, Serrano-Meneses & Córdoba-Aguilar 2012). Displaying this trait in the presence of intruder males could be a way to avoid confrontations especially when the territory holder is sick, as it was the case with implanted animals. Interestingly, even when mature implanted males had less fat reserves (the prime fuel used during odonate flying contests; Marden 2008) than young implanted males, mature males still pursued a more aggressive strategy to hold their territories. This can be explained in terms of the fitness pay-offs for mature males as these had more to lose than young males.

Our study is not the first to examine the effect of activation of the immune response on territoriality in the same and closely related species. In *H. americana*, prior studies have found that mature males infected with bacteria defended their territories with the same intensity as healthy mature males (González-Tokman *et al.* 2011). In young males, on the other hand, the probability of becoming territorial is lower for animals that were immune challenged in closely related species of the genus *Calopteryx* (Rantala, Honkavaara & Suhonen 2010). This probably occurs because young infected individuals disperse further to get new territories, to feed better or to avoid reinfection (Suhonen, Honkavaara & Rantala 2010).

We believe that losing a territory rather than defending it can be an adaptive strategy used by young males to increase their fitness, especially when infected. Previous research in *H. americana* has found that despite the fact that being nonterritorial means nearly zero matings, there are two options to gain at least one mating. First, if a nonterritorial male is large enough, he can regain a territory (Raihani, Serrano-Meneses & Córdoba-Aguilar 2008). Of course, this option depends on whether the male has enough energetic resources in the form of fat reserves (Raihani, Serrano-Meneses & Córdoba-Aguilar 2008), which seems the case of our infected young males. A second option, which produces a reduced fitness outcome, is to wander over several territories as a nonterritorial male and opportunistically take over flying mating couples to displace the mating male (Córdoba-Aguilar *et al.* 2009b). This strategy can be effective only if the nonterritorial male is larger than territorial males, which is not that common in odonates (Suhonen, Rantala & Honkavaara 2008).

Prior studies using immune challenges to modify animal condition and thus test predictions from the terminal investment hypothesis have not looked at potential resource allocation costs in energetic terms. In our work, we examined allocation costs in energetic terms by measuring thoracic fat reserves. We found that melanization of the nylon implants did not differ according to age. This means that males of different ages invest the same in immune response, but despite this, infected mature males suffered increased losses to fat reserves. A reduction in fat reserves at any age can have varied negative effects. One negative effect is reduced survival (Contreras-Garduño, Lanz-Mendoza & Córdoba-Aguilar 2007). In functional terms, the reduction in fat stores in mature males may be partially explained by a resource reallocation from immunity to fat reserves, which affected mature males more than young males. Such resource reallocation cannot be confused with the cost of territorial defence as the rate of territorial attacks to intruders was the same for all experimental groups.

Young infected males may have engaged in compensatory resource intake. This would account for their ability to maintain energetic reserves following the immune challenge. Compensatory resource intake has been observed in the same species (González-Tokman *et al.* 2011) and other insects (Lee *et al.* 2006; Povey *et al.* 2009) and is used to compensate an energetic imbalance when faced with energetic problems such as an infection. Old infected males did not compensate probably because either they invested their remaining time to get more matings or they were physiologically incapable of transforming more food into energetic reserves (e.g. Siva-Jothy & Plaistow 1999). However, previous results in this species indicate that even relatively old animals are able to restore fat reserves, which suggest that feeding can occur at different ages and not only young ones (Raihani, Serrano-Meneses & Córdoba-Aguilar 2008).

Body size showed different trends in both experiments, which can be partially understood on the basis of effects of seasonality that have been detected in this (Córdoba-Aguilar 2009a) and other insect and odonate species (Forrest 1987; Kause *et al.* 2001; Stoks & Córdoba-Aguilar 2012). According to such effects, body size tends to change along the season in *H. americana*. The fact that both experiments were carried out in relatively different times and sites may explain such differences in body size.

Given that our study was carried out in wild animals under natural conditions, there are some factors that escaped our control. Unlike studies in laboratory reared animals, we could not have absolute control of initial male age, feeding intensity or mating experience. Our ageing method guaranteed that mature males were at least 15 days older than young males. However, young and mature males emerged at different times (and therefore

they differed in body size), so knowing the precise age would have been desirable. Moreover, we have argued that compensatory feeding could have occurred in our wild population, but we did not observe nor have control of foraging behaviour. Also, mating experience could have caused differences in behaviour and condition that we could not control. Although studies in captivity can control all these factors, they not always represent real situations faced by wild animals and that is why studies in wild animals need to be complemented with studies in captivity. Despite the heterogeneity in the initial conditions of the animals we tested, we still found clear trends supporting terminal investment hypothesis in males of a territorial insect.

The terminal investment hypothesis in short-lived animals has been poorly studied, especially in males. Our results show that this hypothesis applies to males of a short-lived invertebrate. In *H. americana*, young infected males seem to take the option of leaving a territory and possibly wait for more favourable conditions, while old infected males prefer to stay in a territory and consume their reduced energetic stores to get as many matings as noninfected males.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Model selection to explain differences in the probability that focal *Hetaerina americana* males attack a conspecific intruder.
Table S2. Selection of linear models to explain melanization of a nylon implant in *Hetaerina americana* males of different age classes.

Discusión

En la presente tesis encontré que la hormona juvenil (HJ) es fundamental durante la etapa adulta de los insectos porque regula diferentes funciones directamente asociadas con su adecuación. La táctica reproductiva, la longevidad y el estado fisiológico de los machos de la especie estudiada son regulados por la HJ, pero no necesariamente en el sentido esperado. Hasta este trabajo se conocía el papel de la HJ principalmente por estudios en moscas *Drosophila*, y su papel durante la etapa adulta había sido estudiado principalmente en las hembras (aunque ver el caso para otros insectos por Rolff & Siva-Jothy 2002; Rantala *et al.* 2003a; Contreras-Garduño *et al.* 2009). Poco se sabía del efecto de esta hormona en machos y menos en condiciones naturales, donde las presiones son distintas a las del cautiverio (escasez de alimento, parásitos, dispersión).

El primer resultado de esta tesis apoya la hipótesis de la desventaja en la inmunocompetencia (HDIC; Folstad & Karter 1992), que predice un compromiso mediado por hormonas entre la inversión a caracteres sexuales y a respuesta inmune. Este trabajo aporta importante evidencia sobre esta hipótesis en machos de libélulas en condiciones naturales, mediado por la HJ (Cap. 1; González-Tokman *et al.* 2012). Complementé además este estudio con otro utilizando parásitos naturales en otra especie de libélula, donde encontré que una de las ventajas de la HJ desde el punto de vista del parásito, es que éste puede aprovechar tomando más recursos del hospedero (anexo 1; González-Tokman et al. 2012). Hasta el desarrollo de esta investigación, la HDIC había sido estudiada en vertebrados, siendo la testosterona la hormona causante del

compromiso entre las funciones sexual e inmune. Sin embargo, dichos estudios aportaban evidencia mezclada, no siempre acorde a la HDIC (Roberts, Buchanan, & Evans 2004). El hecho de que la HJ regula tantas funciones resalta la importancia de incluir a los insectos y a diseños experimentales bajo condiciones naturales en los estudios de biología evolutiva. No obstante, la evidencia en insectos sigue siendo escasa y se desconocen en gran medida los efectos de la HJ en muchas funciones, principalmente durante la etapa adulta.

Otro de los efectos de la HJ que puse a prueba en insectos adultos es sobre la senescencia (envejecimiento). Contrario a lo esperado, encontré que la HJ detiene la senescencia de los machos, tal vez debido a que provoca cambios en la conducta sexual o de alimentación, lo cual aun no ha sido evaluado. La evidencia previa indicaba que la HJ era promotora del envejecimiento (Tatar *et al.* 2001; Herman & Tatar 2001), sin embargo nunca se había visto su efecto en condiciones naturales a pesar de que éstas determinan en gran medida los patrones de senescencia (Williams *et al.* 2006). Una de las presiones de la naturaleza que los estudios en condiciones de laboratorio no han controlado es la presencia de patógenos, que activan el sistema inmune y causan daño oxidativo (Nappi & Christensen 2005; González-Santoyo & Córdoba-Aguilar 2012), lo cual debería favorecer la senescencia (Moret & Schmid-Hempel 2009). El presente estudio aporta evidencia experimental de que el uso del sistema inmune, en interacción con las hormonas, moldea los patrones de senescencia en la naturaleza. El presente estudio confirma que la HJ tiene distintas funciones a lo largo de la etapa adulta de los insectos y que las presiones que existen bajo condiciones naturales deben ser consideradas en estudios futuros. Resta

evaluar el efecto de las hormonas y los patógenos sobre la conducta sexual y la de alimentación, ya que estos cambios conductuales definirían la disponibilidad y el requerimiento de recursos de un individuo en su ambiente, especialmente considerando que los insectos estudiados son depredadores que podrían alterar su conducta alimenticia de manera adaptativa (Mayntz *et al.* 2005). Relacionado con esto, en el anexo 2 de mi tesis puse a prueba la idea de si los machos de *H. americana* podrían modificar su conducta alimenticia ante situaciones de riesgo, como es un reto inmune. En esta investigación documento que efectivamente, un depredador en condiciones naturales y ante la amenaza de un reto, puede modificar sus patrones alimenticios (Anexo 2; González-Tokman *et al.* 2011).

La necesidad y disponibilidad de recursos varían a lo largo de la vida de un individuo. De acuerdo con los resultados de la presente tesis, los machos reaccionan conductual y fisiológicamente de forma plástica al deterioro que sufren con la edad. En particular, cuando la esperanza de vida de un individuo viejo es reducida con un reto inmunológico, hay inversión terminal en la reproducción, la cual ocurre a costa de un deterioro fisiológico (reservas energéticas) pero a la larga representa un incremento en el éxito de apareamiento (Cap. 3). Por el contrario, cuando los machos jóvenes son retados de igual forma se vuelven cautelosos, evitando el desgaste de encuentros sexuales agonísticos, probablemente con la esperanza de recuperarse y reproducirse en el futuro (McNamara *et al.* 2009). Las diversas estrategias reproductivas de individuos jóvenes y viejos repercuten en su proceso de senescencia y parecen estar mediadas por la hormona juvenil (Cap. 2).

El presente trabajo resalta la importancia de combinar estudios en laboratorio con estudios en condiciones naturales para responder a preguntas evolutivas. Los insectos han sido menos estudiados que los vertebrados pero resultan fundamentales en la comprensión de las razones conductuales y fisiológicas que repercuten en la evolución de los seres vivos.

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Anexo 1.

Efecto de un análogo a la hormona juvenil sobre un sistema natural parásito-hospedero

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ORIGINAL PAPER

Effect of juvenile hormone analog in a natural host-parasite system

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Abstract Hormones mediate the physiological responses of animals to environmental changes. Consequently, hormones can be responsible of trade offs between different life history traits. Juvenile hormone (JH) is thought to mediate resource allocation in insects: specifically, it is thought to enhance the expression of condition-related traits like sexual signals, whilst reducing immune responsiveness. Here, we experimentally test whether a JH analog (JHa) had an effect on immunity of male dragonflies *Celithemis eponina*, and if such effects are translated into faster growth or development of a natural parasite (water mite). We also tested the effects of JHa on host condition (muscular mass and fat reserves) of mature male dragonflies. Mites from JHa treated dragonflies grew faster than mites from control dragonflies receiving just an acetone carrier. However, there was no effect of JHa on measures of host immune response (melanization of a nylon implant) or condition of mature males. We suggest that better parasite growth in JHa treated males does not result from the JH immunosuppressive function, but instead it appears that parasites receive hormone signals from the host and alter their development without affecting host condition measurably. Our work highlights the importance of measuring both immune parameters and response to real parasites when studying evolutionary trade offs.

Keywords Juvenile hormone · Trade off · Dragonflies · Parasites · Mites · Immunocompetence

Introduction

By definition, parasites reduce their host's fitness, so resisting parasites via immune response can be beneficial for the host (Lochmiller and Deerenberg 2000; Schmid-Hempel

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2005; Schulenburg et al. 2009). However, immune response uses resources at the expense of other functions (Sheldon and Verhulst 1996; Kotiaho 2001) and it is not well understood how an organism allocates its resources to immunity versus to other functions. One functional candidate that may regulate resource allocation is the endocrine system, whose main products are hormones. Hormones mediate the animal's physiological response to environmental changes (Finch and Rose 1995; Zera and Harshman 2001); as such, hormones could have an important role in mediating resource allocation to different life history traits, including resistance to parasites (Zera and Harshman 2001).

In fact, hormones have long been suspected to play a key role in allocation of resources to immunity in both vertebrates and invertebrates. One hormone that has been of special interest to evolutionary ecologists given its versatility is juvenile hormone (JH), that is absent in vertebrates but mediates a number of life history trade offs and functions in insects, including immunity (Flatt et al. 2005). JH promotes metabolism and reproduction, but reduces stress resistance, lifespan and different immunological parameters (reviewed by Flatt et al. 2005). Although its role on parasite resistance is not completely clear, JH is known to have important immunosuppressor effects caused by trade offs with other traits related to fitness, some of which are known to be dependent on nutritional condition (Rolff and Siva-Jothy 2002; Rantala et al. 2003; Contreras-Garduño et al. 2009, 2011).

Four studies have explored the possible immunity-condition trade offs in insects mediated by JH. In *Tenebrio molitor* beetles, an experimental increase of JH led to a reduction in phenoloxidase (a key enzyme in invertebrate immune response) activity and melanization of a nylon implant (an artificial, non pathogenic immune elicitor; Rolff and Siva-Jothy 2002; Rantala et al. 2003), which indicates a poor immune response. However, there was no effect of JH on lytic activity and survival (Rantala et al. 2003). Two other studies with calopterygid damselflies showed that males treated with methoprene (an analogue of JH) had reduced levels of phenoloxidase activity after a bacterial challenge (Contreras-Garduño et al. 2009), but had higher condition and fared better in territorial competition (Contreras-Garduño et al. 2011).

Although these studies appear to provide compelling support for the immunomodulatory role that JH has in insects, some researchers have suggested that functional, rather than immunological responses, should be measured (Adamo 2004; Viney et al. 2005). There are important reasons for this: first, using a single immune parameter is a poor characterization of immune function due to potential trade offs with other immune parameters (Rantala and Roff 2005). Second, the immune challenges that have been used have either been artificial (i.e. nylon implants) or pathogens that are not known to occur naturally with the host population. The biological relevance of these studies is therefore questionable (for a similar claim see Keil et al. 2001). Use of real parasites and pathogens in tests are not without their problems, which include logistics but also whether or not the parasite or pathogen has evolved to be not recognized by its hosts. Notwithstanding, such *real* tests are needed.

For these reasons, here we tested the effect of methoprene, an analog of JH (JHa), in a real, well studied host-parasite system (Corbet 1999). Specifically, we studied the effect of JHa on the development and success of parasitic water mites on males of a libelullid dragonfly host, *Celithemis eponina* (Drury). A number of studies have shown that water mites are agents of selection that impinge upon odonate adults' fitness (e.g. longevity, fecundity, mating success; reviewed by Forbes and Robb 2008). Dragonflies often show a melanization response against the mite's feeding tube (stylostome) which, when successful, kills the ectoparasitic mite (Yourth et al. 2002). Here we have measured the effect of JHa on melanization response. We also explored the effect of JHa on host condition,

measured as muscle mass and fat reserves. Both muscle and fat are condition-dependent traits (i.e. whose expression is dependent on resource availability) that develop during sexual maturation (Contreras-Garduño et al. 2011; González-Tokman et al. 2011), and are important for intra male competition (which takes place in the air) and egg production (Corbet 1999).

We had two main predictions. First, mites should engorge faster and larger on hosts treated with JHa. Second, non-immunologically based functions (i.e. muscle mass and fat reserves) should improve in JHa-treated animals because of the trade off to a decreased immune response. If met, these predictions would support the idea that JH depresses the host immune system at the expense of an increase in host condition.

Materials and methods

Study system and general procedure

We collected male *C. eponina* (Odonata: Libellulidae) haphazardly with an insect net on July 2010 in field around Queens University Biological Station, Ontario, Canada (44°34′25″N, 76°20′5″W). Libellulid dragonflies from this area are parasitized by mites from the subgenus *Arrenurus* (B. P. Smith, personal observations). Mite taxonomy based on morphology for this group is unknown, so we were unable to identify the species of mite(s). In their larval stage, arrenurid water mites are ectoparasites of adult odonates (and other insects) when the latter emerge from water. The mite larvae produce a feeding tube or stylostome in the host and begin absorbing the haemolymph within the first 24 h (Corbet 1999; Forbes and Robb 2008). Once the larval mite has fully engorged, it detaches when the host returns to water to breed (and the mite completes its life cycle as a predatory nymph and then adult). Mites cannot move to other hosts or form another feeding tube once feeding begins, so enumerating them upon host emergence provides accurate data on parasitism (see Hassall et al. 2010 for a notable exception).

We only used juvenile and recently mature *C. eponina*, dragonflies. Juveniles have soft and fragile wings that are pale yellow (Dunkle 2000). Mature and old males have brownish orange wings and are pigmented with reddish coloration on the face, wing veins and pterostigma. Old males show thoracic and abdominal pruinescence, and their wings are frequently broken (see Plaistow and Siva-Jothy 1996 for a similar aging method). In addition, when dragonflies are mature or old, mites have usually detached, so the presence of mites indicates a young insect. As a proxy of body size we measured each male's forewing (±0.1 mm). At the end of each experiment, we measured fat content and muscular thoracic mass by drying (72 h in a desiccator at room temperature) and weighing thoraces on a digital scale (±0.1 mg). We estimated fat content by submerging thoraces in chloroform for 24 h, re-drying and re-weighing each sample. The difference between the first and the second weights was considered fat content (Plaistow and Siva-Jothy 1996). After fat extraction we obtained a measure of muscle mass by submerging samples in potassium hydroxide (KOH, 0.8 M) for 48 h. We re-dried and re-weighed samples to obtain the weight difference before and after KOH treatment (Plaistow and Siva-Jothy 1996).

Experimental protocol

A precursor of juvenile hormone, methoprene acid, was used as a juvenile hormone analog (JHa). We chose the JHa dose based on the average mass of the insects (see Contreras-

Garduño et al. 2009). Although it is always desirable to measure directly the JH titers in haemolymph (see Zera and Harshman 2001), the body mass-corrected JHa dose still produces coherent results (Contreras-Garduño et al. 2009). The dragonflies we studied weighed 125 ± 20 mg (N = 80), which is about 30% more than that of the only odonate used in a JHa-induced study (95 mg for *Calopteryx virgo*; Contreras-Garduño et al. 2009). We therefore initially used two different JHa doses: 15 ng and 30 ng. The higher dose caused high mortality (N = 19; see "Results" section), so we used the lower dose for our experiment.

Five mg of methoprene acid (Sigma) were diluted in 1 mL of distilled water. The mixture, re-diluted 1:1,000 in acetone, was used as JHa. Using a micropipette, 3 μL of the JHa solution (5 ng μL^{-1}) were applied dorsally and topically between the head and the thorax of each experimental male (for a similar procedure see Contreras-Garduño et al. 2009). This application method allowed the JHa to penetrate near the *corpora allata*, where JH is naturally synthesized (reviewed by Flatt et al. 2005). As a control treatment, 3 μL of acetone were applied the same way. Unmanipulated controls were also included in the mite engorgement experiment.

Effect of JHa on mite engorgement

Since the process of mite engorgement occurs while the odonate hosts are not still sexually mature, the present study was carried out with pre-reproductive, juvenile males. Only juveniles with attached mites were used for testing the effect of the JHa on mite engorgement and detachment. Males were allocated to the different treatments (JHa, N = 26; acetone control, N = 27; unmanipulated control, N = 27) matched by their number of attached mites, so that at the end there were no differences in initial mite number between treatments (see "Results"). The number of attached mites on each male was counted twice under a stereoscopic microscope, first before the experimental manipulations and then 2 days later. During the 2 days following manipulation, all males were fed manually once a day with one alive, sexually mature (as assessed from fully bright coloration), small damselfly (*Nehalennia irene* male or female) collected in the nearby marsh. Prey were randomly given to each dragonfly and feeding periods never lasted longer than 10 min. A dragonfly was considered to reject a prey when it did not bite it in a 3 min period.

To stimulate mite detachment 48 h after experimental manipulation, each dragonfly was gently brushed with a piece of soft wet cotton for 1 min. This process has been shown to promote mite detachment (Smith 1988, but see Smith and Laughland 1990). The brushing process was blind (the identity of dragonflies was unknown) and consistent (dragonflies were first submerged in water for 10 s, brushed against the cotton 50 times and submerged again for 10 s, all animals manipulated by the same person). The number of remaining mites after brushing was counted to determine how many mites had detached. After the second count, dragonflies were frozen. All detached mites were photographed, and their area was measured by duplicate (both measurements were closely related, $R^2 = 0.999$) using Image J software 1.42q (http://rsb.info.nih.gov/ij), and the average between both measurements was used.

Effect of JHa on immune function and condition

A subset of mature *C. eponina* males (N = 52) was captured to test the effect of JHa on melanization immune response. Twenty-nine males were allocated to the JHa treatment

and the remaining 23 to the acetone control treatment. Unmanipulated controls could not be included in this section because of low availability of males in the studied population, as the season advanced. To be clear, males used to determine effects of JHa on condition and immune response to a nylon filament were not the same males used to assess effects of JHa on mite growth and development (or detachment readiness). Mature males were fed as above. Treatments were applied 12 h before the immune challenge, and animals were not fed during the experiment, so that all of them were in poor nutritional condition when challenged. The immune challenge consisted of inserting a 2 mm long nylon piece (diameter 0.18 mm, rubbed with fine sandpaper) into the lateral thoracic region. The nylon implant was removed 24 h later and kept in ethanol 70% for subsequent measurements of melanization. This method of immune challenge has been successfully used in previous immunoecological studies and is thought to be a good measure of a host's response to a natural pathogen (Rantala and Roff 2007; Smilanich et al. 2009; but see Rantala et al. 2011). Three digital photographs were taken of each implant and the average proportion melanized (Bascuñán-García et al. 2010; González-Santoyo et al. 2010) and the darkness of the implants relative to a grey reference (lower values are from darker implants; Rantala and Roff 2005) were calculated using Adobe Photoshop ver. 7.0. Proportion melanized and implant darkness were measured twice (both measurements were closely related: proportion, $R^2 = 0.955$; darkness, $R^2 = 0.980$) and the average values were taken as the measures of melanization. The quotient between proportion melanized and implant darkness was used as an index of melanization, so that larger values are from dark implants that have higher proportions melanized. We excluded 24 males (11 JHa and 13 acetone controls) that died before implant removal. It was impossible to recover the nylon implant in some cases, so the sample size was further reduced. For some other cases, the thorax had to be destroyed for obtaining the implant and sample size was also reduced when analyzing muscle mass.

Statistics

To test for differences in dragonfly mortality and willingness to feed, generalized linear models (GLMs) with binomial distribution and logit link function were used. Differences in wing length were tested with one way analyses of variance or t-tests. Differences in the initial number of mites between dragonflies from the different treatments were tested using a GLM with negative binomial distribution and log link function. Mite detaching in response to JHa was also analyzed with a GLM. We assumed that the number of mites detaching from a host was directly related to the number of mites that the host originally had, so the model included the initial number of mites as an explanatory covariate; treatment was included as a factor. The interaction between treatment and initial mite load was also included in the model. If JHa affects mite detachment, the interaction should be significant. Willingness to feed was controlled in the model by including it as a factor of two levels (males that ate and males that did not eat). The final model used a negative binomial distribution and a log link function. Two outliers were removed from the analyses (Cook's distance > 1), leaving a final sample size of 64 individuals. Values with Cook's distance < 1 were retained in the analyses (Quinn and Keough 2002). One way analysis of variance with Tukey post hoc tests was used for comparing the size of mites that detached. Differences in fat content and muscle mass of juvenile males of the mite engorgement experiment were tested with two way analyses of variance (with treatment and willingness to feed as factors; the interaction between both factors was excluded because it did not account for significant variation in the dependent variable).

For testing the effect of treatment on melanization, muscle mass and fat content data were bootstrapped 10,000 times and 95% confidence intervals were calculated. Overlapping intervals mean non significant differences (Crawley 2007). Associated Kolmogorov–Smirnov *P*-values were calculated for these intervals (Sekhon 2011). Prior to parametric analyses, homogeneity of variances was tested with Levene or Fligner-Killeen tests (Crawley 2007). Data are shown as mean ± SD. Analyses were conducted with SPSS 15.0 and R software (R Core Development Team 2009, version 2.10.0).

Results

Effect of JHa dose on survival

The different JHa doses resulted in large differences in dragonfly mortality 48 h after its application. Three times as many treated with the larger dose (30 ng) died compared with the smaller dose (15 ng; from 19 to 58%; GLM $z = 2.897$, $P = 0.004$, $N = 46$). The 30 ng dose was therefore not used in experiments. The mortality rates of the acetone-treated controls (19%) and the unmanipulated controls (15%) were not significantly different from each other or from the JHa dose of 15 ng (19%; GLM $z = -0.424$, $P = 0.671$, $N = 80$).

Effect of JHa on mite engorgement

Males from the different treatments did not differ in wing length ($F_{2,77} = 0.715$, $P = 0.492$, $N = 80$) or initial mite load (GLM $df = 77$, $P = 0.894$). Of the sample of 80 males, 14 appeared moribund or listless and died before the end of the experiment (5 JHa, 5 Acetone and 4 Control). For the 66 males that were not listless and remained alive, and for which mite engorgement and detachment were recorded, there were no differences in wing length between treatments (ANOVA $F_{2,63} = 0.213$, $P = 0.808$, $N = 66$).

Treatment did not affect male willingness to feed when *N. irene* prey were offered. There were 9 males who did not eat (5 in JHa treatment [24%], 3 in acetone control treatment 237 [14%] and 1 in unmanipulated control treatment [4%]); the difference between treatments in the proportion of males that did not eat was not significant (GLM $z = -1.789$, $P = 0.074$, $N = 66$). Also, males that did not eat were not different in wing length from males that did ($t = 0.904$, $P = 0.369$, $df = 64$).

Treatment had an effect on mite detachment when initial mite number was controlled (Table 1; Fig. 1): dragonfly hosts exposed to JHa lost more mites than acetone controls ($z = 2.637$, $P < 0.001$) but not more than unmanipulated controls ($z = -0.026$,

Table 1 Analysis of deviance from the model: lost mites = treatment × initial mites + willingness to feed

	df	Deviance	Residual *df*	Residual deviance	*P*
Treatment	2	0.487	61	205.333	0.784
Initial mites	1	102.833	60	102.500	**<0.001**
Treatment × initial mites	2	29.021	57	73.362	**<0.001**
Willingness to feed	1	0.117	59	102.382	0.732

Theta = 2.508, SE = 0.724, N = 64

Significant values are provided in bold

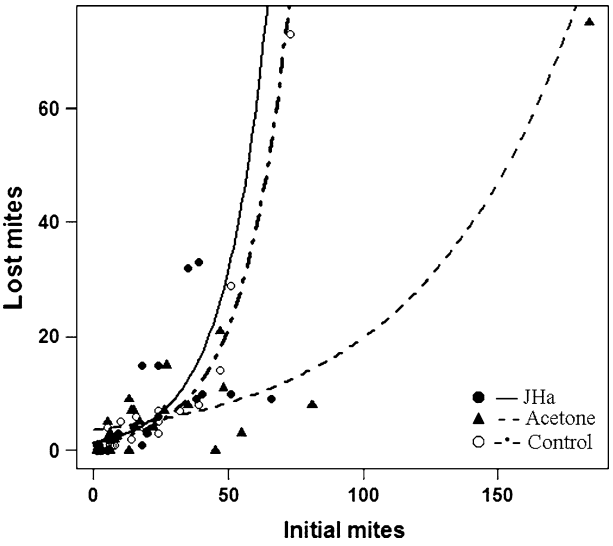

Fig. 1 Initial mite load on *C. eponina* males plotted against mite detachment for each host treatment. *Lines* were calculated from the statistical model used

$P = 0.979$). Acetone treated controls lost fewer mites than unmanipulated controls ($z = 4.877$, $P < 0.001$). Host willingness to feed was controlled in the model but was not significant in explaining mite detachment (Table 1).

The size of mites that detached from hosts after brushing differed between treatments (ANOVA $F_{2,248} = 3.582$, $P = 0.029$, $N = 251$; Fig. 2): mites detached from JHa treated hosts were significantly larger (0.228 ± 0.059 mm^2) than mites detached from acetone control treated hosts (0.204 ± 0.065 mm^2; Tukey's $P = 0.027$). Mites detached from unmanipulated control hosts (0.219 ± 0.059 mm^2) did not differ in size from JHa (Tukey's $P = 0.700$) or acetone controls (Tukey's $P = 0.263$). There was no effect of treatment or willingness to feed on muscle mass (two way ANOVA, treatment:

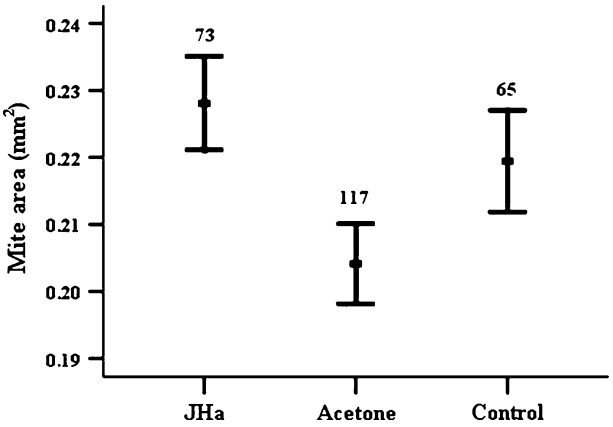

Fig. 2 Size of mites that detached from *C. eponina* males for each host treatment. *Bars* are standard errors and numbers about bars are sample sizes

$F_{2,62} = 0.865$, $P = 0.426$, N = 66 [JHa: 7.2 ± 2.9 mg, N = 21; acetone: 8.1 ± 3.4 mg, N = 22; unmanipulated 7.1 ± 3.1 mg, N = 23]; willingness to feed: $F_{1,62} = 2.684$, $P = 0.106$ [ate: 7.7 ± 3.2 mg, N = 57; fasted: 5.9 ± 2.4 mg, N = 9]).

There was no effect of treatment or willingness to feed on fat content (two way ANOVA, treatment: $F_{2,62} = 2.241$, $P = 0.115$, N = 66 [JHa: 0.17 ± 0.20 mg, N = 21; acetone: 0.10 ± 0.16 mg, N = 22; unmanipulated 0.08 ± 0.14 mg, N = 23]; willingness to feed: $F_{1,62} = 3.283$, $P = 0.075$ [ate: 0.13 ± 0.18 mg, N = 57; fasted: 0.04 ± 0.10 mg, N = 9]).

Effect of JHa on immune function and condition

Males initially allocated to JHa and acetone treatments did not differ in wing length ($t = 0.982$, $P = 0.331$, N = 52). Males from both treatments that survived until the end of the experiment also did not differ in wing length ($t = 0.889$, $P = 0.383$, N = 27).

Males treated with JHa and acetone did not differ in the melanization of the nylon implant ($P = 0.49$), in muscle mass ($P = 0.88$) or in fat content ($P = 0.23$) (Table 2).

Discussion

Applying JHa to dragonfly hosts enhanced growth of their natural parasitic mites in a short period of time (48 h), as we had predicted. This occurred despite a marginally significant trend ($P = 0.07$) of JHa treated males to decrease feeding and consequently reducing available resources for parasites. However, contrary to our predictions, JHa did not appear to decrease melanization immune response to nylon implants as a consequence of increasing condition. Thus, these results do not echo previous evidence in calopterygid damselflies where JHa drives resource allocation after adult emergence (Contreras-Garduño et al. 2011). We suggest that, rather than an immunosuppressive effect, additional JHa treatment was absorbed by mites, and this enhanced their development (see Lawrence 1986). In fact, there is evidence that parasitic mites depend on the JH levels of their hosts in order to complete their life cycle: for example, *Varroa* mites that are parasitic of honey bees are prevented from reproducing if JH titres in their host's haemolymph do not reach a threshold level (Hänel and Koeniger 1986). A similar rationale makes sense in our study system, where host and parasite life cycles are also finely synchronized (Forbes and Robb

Table 2 Descriptive statistics of different measures of individual condition after experimental manipulation

	JHa Mean ± SD (95% CI)	Acetone Mean ± SD (95% CI)
Melanization index (proportion encapsulated/implant darkness)	0.80 ± 0.45, n = 15 (0.58–1.02)	0.65 ± 0.31, n = 11 (0.48–0.83)
Muscle mass (mg)	15.0 ± 1.9, n = 15 (13.92–15.76)	15.1 ± 1.9, n = 10 (13.98–16.27)
Fat content (mg)	0.20 ± 0.36, n = 15 (0.1–0.5)	0.12 ± 0.28, n = 10 (0–0.4)

95% confidence intervals were estimated from 10,000 bootstraps. If CIs are overlapped, there is no significant difference between treatments

2008). In such a system, natural titres of JH may increase in the dragonfly when it reaches maturity (as occurs with other insects; Fluri et al. 1982; Robinson et al. 1989; Cusson et al. 1990; Teal and Gómez-Simuta 2002) and returns to water to mate. This hormonal signal could be detected by the mites, causing them to detach from the host.

Our results do not fully discard the possibility that JHa suppresses immunity. Although melanization has been suggested to be the main mechanism of immune response of odonates against water mites (Åbro 1982; Forbes and Robb 2008); other unmeasured components of the immune response could also be used against mites. Furthermore, we did not actually replicate the timing of natural parasitism by the water mites. Most odonates mount their immune response to arrenurid mites just after they emerge as tenerals, when the mites first form their feeding tubes into the host. The hosts then mount their resistance response by encapsulating the mite feeding tubes. The melanization response of these dragonflies had therefore occurred a week or more before we administrated JHa, so the dragonflies may have already made their main immune investment prior to this immune challenge.

The lacking effect of JHa on host muscle mass and fat reserves is contrary to what we had predicted based on previous studies in which JHa administration had a positive effect (at least on fat reserves in territorial damselflies; Contreras-Garduño et al. 2011). One possible reason is that both traits may have been formed by the time JHa was applied. This, however, would be surprising since odonates emerge with very low values of muscle mass and fat reserves and, in fact, both traits are constructed in the very first days after emergence (reviewed by Stoks and Córdoba-Aguilar in press). One exception to this rule in odonates is that of *Hetaerina americana*, a territorial damselfly in which a subset of adult males has been found to re-store levels of muscle mass and fat reserves after a period of energetically demanding activities (Raihani et al. 2008). This situation may well apply to *C. eponina*. A second possibility is that both traits are not condition-dependent. Partially supportive of this is that *C. eponina* is not a territorial species (Bried and Ervin 2006), which means that directional selection is not expected to operate on muscle mass and fat reserves with as much strength as it would in territorial odonates (Suhonen et al. 2008). In addition, although we controlled for diet during the experiment, diet prior to this may have had a strong effect on these measures, as well as on immune response.

We acknowledge the fact that males treated with acetone produced unexpected results. For example, we did not expect acetone treated males to have lost more mites than unmanipulated controls, but fewer than JHa treated animals. Although this seems that acetone is not the right control (despite the *status quo* of other studies that have used acetone to dissolve JH to penetrate into the corpora allata; e.g. Contreras-Garduño et al. 2009, 2011), acetone treated males did not differ in the physiological traits we measured (melanization, muscle mass and fat reserves) which indicates that whatever the reason by which acetone affected mite detachment, this does not affect host's condition (at least no more than the other treatments). In a way, this means that acetone is not the worst control at all. One potential reason of the mite detachment effect of acetone is that it may have negatively affected JH natural levels so that JH does not reach its target tissues as effectively as JH in the unmanipulated males. Nevertheless, the potential effects of acetone need to be addressed in the future.

Our results also have implications to sexual selection theory, given their relation with the immunocompetence handicap hypothesis (ICHH; Folstad and Karter 1992). According to this idea, hormones (explicitly testosterone, but the same rationale works for JH) mediate resource allocation to immunity and male sexual traits. One main assumption of ICHH is that testosterone reduces immunocompetence and increases parasitism because it privileges investment to sexual traits. Although our results do not provide clear support for

the hypothesis (i.e. JHa did not reduce immune response), we do not discard the possibility that it was the reduction in an unmeasured immune parameter which caused faster growth in parasitic mites of JHa treated males. We highlight the importance of measuring both immunological parameters and functional responses when studying evolution of sexual and life history traits.

Although we cannot conclude with certainty why there was faster engorgement of mites when host levels of JH were augmented, here we show that higher host's hormonal levels enhanced parasite growth. Unlike previous studies, we used a natural host-parasite system in which the parasite is known to have detrimental effects on host's fitness. Faster engorgement to larger sizes probably translates into higher mite success because mites should experience selection to time their engorgement in order to increase their chances of returning to water to complete their life cycle as adults. If a mite has attained its full size and is ready to detach from its host, it can leave as soon as the host is near water. Otherwise, staying on a host could increase the possibility of dying on the host (e.g. being consumed by a host's predator, Nagel et al. 2011).

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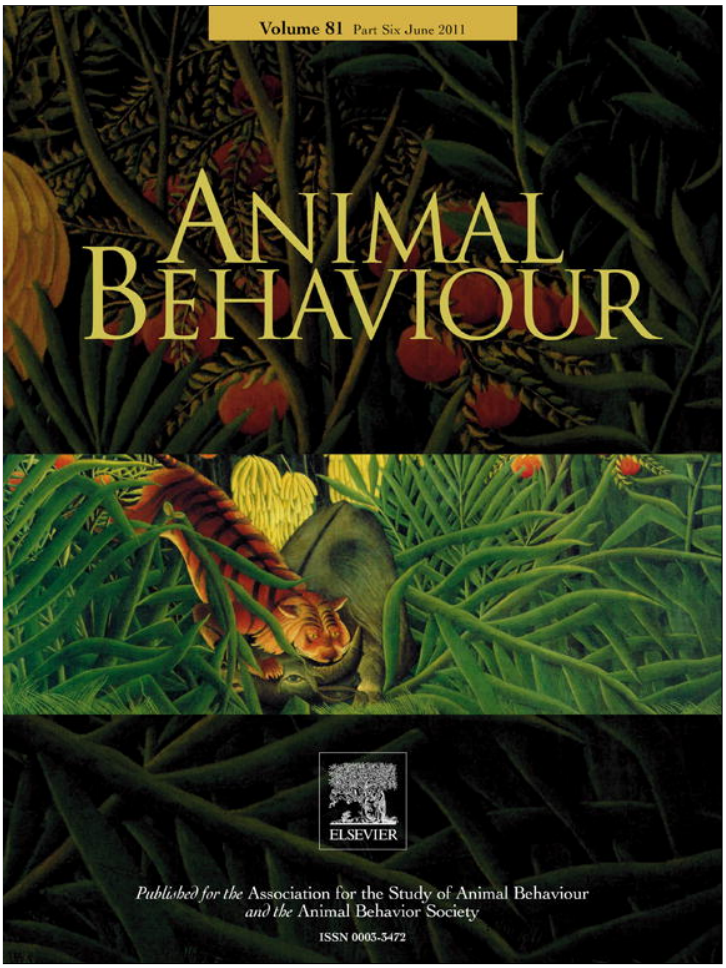
Anexo 2.

Efectos de una infección sobre las conductas de alimentación y reproducción en un insecto depredador en vida libre



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Infection effects on feeding and territorial behaviour in a predatory insect in the wild

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Sick animals may change their feeding behaviour to compensate for infections. However, there is little information regarding whether infection affects (1) feeding behaviour of predators, (2) feeding behaviour using an experimental approach in the wild, (3) other costly behaviours and/or (4) physiological components of condition. We experimentally infected males of the predatory damselfly *Hetaerina americana* in a field experiment. We hypothesized that infection would reduce feeding behaviour. We further predicted a reduction in territorial activity, an increase in immune response (measured by the activity of phenoloxidase, PO) and a reduction of fat reserves and flight-associated muscle mass (two traits usually traded off with immune ability and territorial behaviour). We also infected males in a laboratory experiment that controlled for food supply and territorial activity, and measured the same physiological characters. Immune challenges in the field experiment unexpectedly increased feeding rate but did not change territorial activities. Muscle mass was reduced in the field but not in the laboratory, probably because of differences in the presence of energetically expensive territorial activities. In the laboratory, starvation and infection reduced PO activity and fat stores but did not affect muscle mass. Thus, our field and laboratory results support the idea that increased feeding compensates for infections in predators.

Changes in feeding behaviour are expected after infections. Infected individuals may change feeding to balance nutrients (i.e. reviewed by Huffman 2003), which may be used as a form of self-medication (Raubenheimer & Simpson 2009). The field of self-medication strategies and adaptive behavioural changes in feeding behaviour is recent (Raubenheimer & Simpson 2009), although the foundations of the field have long been known, especially in vertebrates (reviewed by Villalba & Provenza 2007). One putative compensatory change after infection is to increase food intake to compensate for the energetic costs demanded by immune action (Moret & Schmid-Hempel 2000). Supporting evidence for this pattern was found in armyworm caterpillars, in which infected individuals increased their protein intake to elevate immune ability (Povey et al. 2009; for similar experiments and rationale see also Lee et al. 2006; Kolluru et al. 2009). Furthermore, a number of studies have found that animals may be highly selective in their diets to balance and/or to obtain those nutrients that can be of better use against pathogen attacks (e.g. Povey et al. 2009; Adamo et al. 2010).

Given that the self-medication field is recent, there are a number of gaps that still remain to be clarified. First, our knowledge of adaptive changes in feeding regimes comes from primary consumers (e.g. bumblebees; König & Schmid-Hempel 1995; butterflies; Povey et al. 2009; crickets; Adamo et al. 2010). Many predators can be highly specific in nutrient acquisition to redress nutritional imbalances (Mayntz et al. 2005). For example, a predator can choose a particular prey of specific nutritional composition, consume more of prey that contains nutrients demanded by the predator, or use only some particular nutrients of a caught prey (Mayntz et al. 2005). Second, most studies of feeding behaviour after infection have been conducted in the laboratory. Whether an increase in feeding rate occurs in natural conditions (where natural selection may constrain the feeding rate) is unclear (Cézilly & Benhamou 1996). In contrast to the armyworm results described above, a seminatural study found reduced foraging activity after infection (König & Schmid-Hempel 1995). Hence, illness-induced anorexia, the opposite response, has been proposed as a compensatory response to deal with an infection (Ayres &

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Schneider 2009; Adamo et al. 2010). Illness-induced anorexia occurs both in vertebrates and invertebrates (reviewed by Exton 1997; Langhans 2007). On a proximate level, increased octopamine levels (expected as part of an insect immune response) affect the central nervous system region that determines feeding in the caterpillar *Manduca sexta* (Adamo 2005). Increased feeding after infection may be maladaptive as lipid intake (via food) may negatively affect immune function. In insects, both ingestion and immunity share the same protein, apolipophorin III. Apolipophorin III functions in lipid transport and in pathogen binding to activate the immune response (Weers & Ryan 2006). Thus, the explanation for the lipid ingestion–immune function trade-off is that by reducing food intake (especially for animals that contain more lipid than plants), apolipophorin III is not used to transport lipids but may be alternatively utilized for an immune function (Adamo et al. 2007, 2008, 2010).

An investigation of changes in feeding behaviour by experimentally infected predators in the wild will provide a realistic assessment of the costs and benefits of different behavioural strategies. Laboratory studies may offer animals food choices with no apparent natural selection costs, a situation that has no parallel to what occurs in nature. Furthermore, we know little about the effects of feeding changes on other activities of infected animals, which in many cases are energetically costly behaviours. For example, migrating Mormon crickets, *Anabrus simplex*, are protein deficient and have impaired immunocompetence (Srygley et al. 2009). Given that immunity is traded off against a number of condition-related components, dealing with an infection may still induce an animal to end up in poor condition even when it increases or decreases its feeding rate. Usually only changes in immunity following changes in feeding behaviour have been measured, but the effect on other physiological components has been ignored. Such an investigation should provide insights on the fitness effects of changes in feeding activities for sick animals.

Here we experimentally induced an immune response via bacterial infections in males of a territorial damselfly. These animals are predators in all life stages. Feeding behaviour has been well described in odonates; they are commonly regarded as food generalists and opportunistic predators (reviewed by Corbet 1999). However, to our knowledge, no study has documented the effect of illness on odonate feeding behaviour. We expected adaptive feeding changes, as other invertebrate predators are capable of highly nutrient specific prey use (Mayntz et al. 2005). In particular, we expected that after infection animals would show an anorexic period or at least reduce their feeding rate. We studied these animals in the wild to examine the effects of infection on territorial behaviour. Odonate territorial behaviour has been widely investigated given its high energetic costs (i.e. Marden & Waage 1990; Plaistow & Siva-Jothy 1996): winners of territorial fights typically have higher lipid reserves and flight-associated muscle mass (reviewed by Suhonen et al. 2008). We predicted that infected animals would reduce their territorial activity compared with control animals. Finally, we assessed physiological effects of altered feeding behaviour following infection. We measured a number of male indicators of physiological condition: activity of phenoloxidase (PO, an enzyme that plays a role in the insect immune defence against pathogens; Cerenius & Söderhäll 2004), fat reserves and muscle mass. These three physiological variables are good indicators of condition (reviewed by Schmid-Hempel 2005). PO activity is directly dependent on food access (Pascual et al. 2006; Alaux et al. 2010) and food quality (Babin et al. 2010; Chiu et al. 2010). In relation to fat reserves and muscle mass, both traits are positively correlated with condition, given their use in energetically exhaustive activities in insects (e.g. Gray & Eckhardt 2001; Kemp & Alcock 2008) and in odonates (Marden 1989; Plaistow & Siva-Jothy 1996). We predicted that PO would increase as a natural response to deal with pathogen infection. Previous studies in territorial damselflies showed that, regardless of condition (as assessed by fat reserves and muscle mass), males augmented PO levels after infection (Contreras-Garduño et al. 2007). Since trade-offs should exist between the use of immune resources and other energetically costly functions, we expected that both fat reserves and muscle mass would decrease. Support for this expectation comes from several studies in territorial odonates in which fat reserves are affected by fighting behaviour and secondary sexual traits (Contreras-Garduño et al., in press), and muscle mass is affected by parasite defence (Marden & Cobb 2004) or territorial activities (Marden et al. 1998).

METHODS

Study Subject

We conducted this study on adult *Hetaerina americana* damselflies (Insecta: Odonata). After becoming sexually mature, males search for available territories in river banks that females visit for mating (e.g. Grether 1996; Córdoba-Aguilar et al. 2009a). Territorial competition is intense so that only males in good physiological condition are able to acquire and defend a territory against conspecifics. Success in territorial acquisition and defence is correlated with fat reserves and muscular mass (Contreras-Garduño et al. 2006, 2008; Serrano-Meneses et al. 2007). These traits are functionally linked with the ability to sustain flight for long periods (Marden 1989; Marden et al. 1998). Perhaps because of the overall good condition that males holding a territory have, other physiological traits not related to territorial defence appear indirectly correlated. This is the case with immune ability in the form of PO and hydrolytic enzyme activity, nitric oxide levels and survival after pathogenic (e.g. insect-specific bacteria) and nonpathogenic challenges (a nylon filament) (Contreras-Garduño et al. 2006, 2007, 2008, 2009; Córdoba-Aguilar et al. 2009b; however, see González-Santoyo et al. 2010). Several studies have focused on prey capture behaviour in this animal (e.g. Grether & Grey 1996), but it is unknown whether infection may affect feeding behaviour.

Field Work

Field work was conducted along the Tetlama River, 1 km from Palo Bolero, Morelos, Mexico (18°45′55F0B2N, 99°14′45F0B2W). At the study site, Tetlama River is about 8 m wide, and the current of the river varies according to precipitation. During the day, *H. americana* males perch along the shore either on vegetation (mainly consisting of shrubs and grass $\leq$ 1.5 m height) or on stones. The selected study site was approximately 500 m long. We captured adult males with an aerial insect net between 1000 and 1600 hours, the time at which males are active and territorial (Contreras-Garduño et al. 2006). To minimize impact on animal welfare, all further manipulations (wing marking, injections and haemolymph extraction) were done by grabbing the wings of the males while they were still alive. We estimated the age of males using categories proposed by Plaistow & Siva-Jothy (1996): age 1, teneral males, have soft, dorsoventrally flexible undamaged wings; age 2 males have harder wings, flexible from the nodus to the tip; age 3 males have less flexible wings and show some signs of pruinescence in the thorax; age 4 males, the oldest, show abundant thoracic pruinescence and unflexible, damaged wings. Since immune ability and muscle mass may vary with age (unpublished data), we used only sexually mature males of age 3. Indeed, a better estimate of age could have been accomplished by marking

individuals of age 1, but manipulation at this age causes high mortality as tenerals are extremely fragile (A.C.-A., unpublished data). Still, using males of class 3 is an appropriate approach because at this age males show the highest levels in immunity and fighting ability (Contreras-Garduño et al. 2008). We measured each male's right anterior wing with a digital calliper (±0.01 mm) as an indicator of body size.

Experimental Infection and Behavioural Recordings

In December 2009, we captured 119 males (52 males on 11 December, 67 males on 14 December) and numbered them with a permanent marker in the left posterior wing for subsequent behavioural recordings (hereafter termed the field experiment; see below). As an experimental pathogen we used living *Serratia marcescens*, which is a Gram-negative bacterium that is pathogenic for insects and exists in the gut of *H. americana* males in natural populations of central Mexico (identified by API 20E system, BioMerieux; unpublished data). Furthermore, this bacterium can trigger the PO cascade (Contreras-Garduño et al. 2007), and, in high dosages, regularly induces mortality (González-Tokman & Córdoba-Aguilar 2010). Bacteria used were obtained from *Anopheles albimanus* mosquitoes grown at the Instituto Nacional de Salud Pública (Cuernavaca, Morelos, Mexico) in LB agar at 37 °C ('bacterial' or 'infected' treatment). Bacteria were resuspended in phosphate buffer saline (PBS 1×, pH = 7). We injected bacteria in a concentration of approximately 700 colony formation units (cfu) per μl of solution in the male's thoracic muscle, at the point of wing insertion, using a 10 μl Hamilton microsyringe. This bacterial dosage was derived from the LD_{50} previously estimated in males kept in captivity and injected with varying doses of *S. marcescens* (González-Tokman et al. 2010). The calculated LD_{50} was 14×10^3 cfu per μl of solution, which was diluted (1:20) to assure a low toxic effect of the experimental infections. The volume injected was always 1 μl. Chosen randomly, 47 males were injected with living bacteria (15 and 32 males on day 1 and 2 of marking, respectively), 42 males were injected with PBS (sham: 16 and 26 males on day 1 and 2 of marking, respectively) and 30 males received the same manipulation as the other treatments except for the injection (unhandled control: 15 males/marking day). Marking and manipulation occurred on the river bank. Time between capture and release took no more than 30 min, and released males often returned to their original defended sites and reassumed their activities. We conducted behavioural observations 1, 2 and 3 days postinoculation. In some cases a male was subject to a single observation, but other males were observed up to two and three times. To avoid pseudoreplication, we compared only treatments from a given day. It would have been desirable to have observations made later than 3 days postinfection, but the number of experimental animals at the study site was drastically reduced after the third day. This result is not anomalous. Rantala et al. (2010), for example, found that immune activation in damselfly adults in the wild led to higher mortality via predation.

In our study site, damselflies were active from 1000 to 1500 hours, during which males engaged in both opportunistic feeding and territorial behaviour (i.e. while defending a territory, they may take opportunities to feed when prey pass nearby). We made observations during this interval. We conducted focal observations on marked animals for 30 min and recorded feeding and territorial activities. Repeatability measures showed that our observers coincided in more than 95% of recordings (Martin & Bateson 1993). Foraging takes place when a perching damselfly flies towards small insect items (usually dipterans) that pass through its territory. After each attack, the damselfly usually returns to the same perching site, where mandible movements can be clearly observed if a capture attempt was successful. Most captured prey are not visible by the naked eye, and so the effectiveness of an attempt was assessed by the mandible movements (see also Grether & Grey 1996). Damselflies may capture prey of different sizes, which represent different energetic supplies (e.g. Fried & May 1983). To deal with this, Grether & Grey (1996) divided prey size in two groups: (1) minute, nonvisible prey weighing about 0.33 mg that are detected indirectly via the damselfly's mouth movements; and (2) visible prey weighing 1–9 mg that protrude from the damselfly's mouthparts. We recorded the number of prey captured (minute and visible) and the total number of capture attempts, and calculated rates of prey capture or capture attempts/h. In this study, visible prey represented less than 5% of total intake, so we used only recordings equivalent to minute prey for analysing differences in foraging behaviour caused by experimental treatment (see Results).

We recorded the total time spent in territorial activity from the duration of all flights that the focal male engaged in during chases against other conspecific males that entered the focal male's territory or flew nearby. We also registered the total number of contests, as well as the winner of each contest. A male was considered to have won a contest if he returned to his original perching site, and he was considered to have lost if he was displaced to a different site and the opponent male took over and defended the focal male's territory. We also recorded copulation attempts, but only three males attempted to copulate so we did not analyse these data.

Assessing Condition Components following Infection in the Field Experiment

Infected and sham males that were marked and manipulated on 14 December were recaptured from the field on 17 December for laboratory analyses of three indicators of condition (regardless of whether their behaviour had been previously recorded): PO activity in the haemolymph, fat load and muscle mass. As controls, we included males that had not been captured previously for having a baseline measure of condition. We included two more groups of males that were captured on 17 December and injected with the bacterial or the sham treatment 12 h before haemolymph extraction. During this 12 h interval (from 1900 hours, when injections were made, to 0700 hours, when haemolymph was extracted) males remained captive in transparent, plastic assay tubes (5 ml), with a perch and a cap of humid cotton to maintain constant room temperature (around 26 °C). It is unlikely that these newly captured males would have engaged in flying or feeding activities if left under natural conditions in this time period, since these animals remain inactive at night (Corbet 1999; personal observations). For this reason, further measurements of condition in the newly captured males can be considered as real responses 12 h after an infection in the field, despite having been kept in captivity during that time. To control for possible stressing effects due to captivity, males in the unhandled and the recaptured (3 days after injection) treatments were also kept 12 h in the same conditions as above. Regardless of treatment, all males spent between 12–14 h starved and captive prior to haemolymph extraction and fat and muscle measurements. Final treatments were as follows: bacteria-injected 3 days ($N = 4$) or 12 h ($N = 7$) before the assay; sham-injected 3 days ($N = 7$) or 12 h ($N = 8$) before the assay; unhandled control ($N = 11$). In some cases the immune assay could not be performed because of insufficient haemolymph sample, resulting in unequal sample sizes among groups.

Assessing Effects of Food Availability on PO, Fat Reserves and Muscle Mass in Captivity

All field experiments share the problem of uncontrolled environmental variables; the most relevant for this study is that the field experiment did not control for consumed food or the level of aggression during territorial defence. To control for these variables, we conducted a laboratory experiment in which we manipulated food level and reduced male activity (hereafter, the laboratory experiment). In March 2010, we captured and kept 110 males in captivity under the same conditions described above. We randomly assigned these animals to three experimental treatments, one injected with 1 μl of *S. marcescens* inoculated PBS solution (700 cfu/μl; $N = 38$), another injected with 1 μl of PBS solution (sham-injected; $N = 36$) and a third remained nonmanipulated (unhandled control; $N = 36$). A proportion of males in each treatment was kept under starvation (24, 21 and 20 males, respectively), while the remaining (14, 15 and 16 males, respectively) was fed ad libitum with *Drosophila melanogaster* (Nubbin unwinged strain). Males from the ad libitum fed group were provided prey until they took no more (range of eaten flies: 1–8). Duration of the feeding session was 24 h, during which each male was offered prey four times. After that time we sacrificed all males for the measurement of PO activity, fat and muscle mass. Males that were originally allocated to the ad libitum fed group but did not accept at least one prey were excluded from the analyses (6 infected, 3 sham, 2 unhandled). Males that died before 24 h elapsed were also excluded from the analyses (starved: 8 unhandled, 3 sham, 2 infected; fed: 3 unhandled, 3 sham, 6 infected).

PO activity

We measured PO activity from the haemolymph, which was extracted by perfusion with approximately 200 μl of PBS (see details in González-Santoyo et al. 2010). PO activity was measured as the formation of dopachrome from L-dihydrophenylalanine (L-DOPA, Sigma; Söderhäll & Hall 1984; Contreras-Garduño et al. 2007), in propylene 96-Microwell plates (Eppendorf). We mixed haemolymph sample volumes corresponding to 20 μg of protein (see below) with PBS to reach 100 μl in each well, and then added 50 μl of L-DOPA (4 mg/ml of PBS), the substrate for PO. We incubated the plate for 30 min at room temperature. PO activity was then recorded every 10 min for 1 h using a spectrophotometer (Biorad 350) at 490 nm. PO activity is expressed as optical density/μg of protein. As a blank we used a mixture of 100 μl of PBS and 50 μl of L-DOPA, and its absorbance value was subtracted from the reading of each sample. Haemolymph extraction took no more than 10 min, after which the heads of all animals were manually squashed so that animals died in a relatively short time. Haemolymph samples were stored in ice from the time of extraction until the end of the assay.

Protein determination

Since haemolymph samples obtained by perfusion are a mixture of haemolymph and PBS in unknown proportions (i.e. some haemolymph samples can be more diluted than others), PO activity was not measured per haemolymph volume, but instead was standardized for the total protein mass in the sample (Contreras-Garduño et al. 2007; González-Santoyo et al. 2010). Protein mass was determined using the method of the bicinchoninic acid assay (BCA) with the PIERCE protein assay kit. Each well of a propylene 96-Microwell plate (Eppendorf) was filled with 10 μl of the haemolymph sample, plus 40 μl of PBS and 150 μl of BCA reagent. We incubated the plate at 37 °C for 20 min and recorded the absorbance at 560 nm. We calculated the mass of protein in each sample from a reference standard curve that contained different concentrations of albumin (0, 2.5, 5, 7.5, 10, 12.5, 20 and 30 μg of albumin/ml).

Fat and muscle mass quantification

We measured fat and muscle mass from the thorax and abdomen. After removing the head and the legs, we dried males' thoraces and abdomens by placing them in a desiccator (for 24 h) and obtained their dry weight to the nearest 0.1 mg. Initial dry weight included the exoskeleton, fat and muscle. We then placed samples in chloroform for 24 h to extract fat. Samples were then redesiccated and reweighed. We quantified fat content as the difference between the initial weight and the second weight. Other methods of lipid quantification have been considered more accurate for extracting lipids from other animal tissues, since nonlipidic substances such as urea, glucose, aminoacids and salts could be also extracted with the chloroform (Barnes & Blackstock 1973). However, chloroform extraction has been widely used in insects (e.g. Zanotto et al. 1993; Lee et al. 2004; Contreras-Garduño et al. 2008). After lipid extraction, we submerged samples in 0.8 M potassium hydroxide for 48 h to extract muscle. Again, we dried bodies and reweighed them. We assumed the difference between the initial weight and the second weight to be muscle mass (for a similar procedure see Contreras-Garduño et al. 2008).

Statistics

To assess the effect of the bacterial treatment on foraging and territorial behaviour, all statistical tests included treatment and wing length as predictor variables. We constructed GLM models with and without the interaction (treatment*wing length), and selected the simplest model with the lowest Akaike Information Criterion (AIC; Hegyi & Zsolt 2011). We analysed separately each response variable for each postmanipulation day. We used a priori contrasts to test for differences between treatments (Crawley 2007). Behaviour of infected and sham males was always compared to that of the same eight unhandled males, four of which were observed between the first and the second marking events (11–14 December) and four after the second marking event (14–17 December). We used Cook's distance to test for the presence of outliers and removed values greater than 1 from the analyses. In models with fighting time as the response variable, we excluded from the analyses one unhandled male that was always detected as an outlier and one sham male that was an outlier only on the first day after manipulation. We used Fligner–Killeen tests (Crawley 2007) to assess homogeneity of variance in models where the response variable was continuous. When the assumption of homogeneity of variance was not satisfied, we used generalized least squares (GLS; Crawley 2007; Zuur et al. 2010). To compare the proportion of males that ate at least one visible prey across the different treatments, or the proportion of contests won by males across the different treatments, we made generalized linear models (GLM) with binomial distribution and logit link; as the response variables were binary (ate or did not eat, won or lost, respectively). When the response variables were counts (number of contests, number of prey and number of foraging attempts), we used GLMs with negative binomial distribution (Crawley 2007).

In the field experiment, where we recaptured males 12 h or 3 days postmanipulation to measure PO activity, fat load and muscle mass, the predictor variables in each model were treatment, time pre-assay and wing length. We also tested the interaction (treatment*time pre-assay) and removed it when its inclusion increased the model's AIC. In this case we did not test interactions of wing length with treatment or time pre-assay because of insufficient sample sizes.

For the laboratory experiment, in which males from the different treatments were fed or starved to measure PO activity, fat load and muscle mass, the predictor variables in each model were treatment (infected, sham, unhandled), nutrition (fed or starved) and wing length. We also tested interactions and, again, the final model selected was the simplest with the lowest AIC (Hegyi & Zsolt 2011). For analyses we used SPSS 15.0 and R software (R Development Core Team 2009, v.2.10.0).

RESULTS

Effect of Bacterial Infection on Foraging Behaviour in the Field Experiment

Neither treatment nor wing length affected total prey capture rate on the first or the second day following application (first day GLM: treatment: $df = 44$, $P = 0.112$, $\theta = 0.390$; wing length: $df = 43$, $P = 0.879$; second day GLM: treatment: $df = 18$, $P = 0.248$, $\theta = 0.885$; wing length: $df = 17$, $P = 0.171$; Table 1). Treatment but not wing length significantly affected total prey capture rate on the third day after manipulation (GLM: treatment: $df = 26$, $P = 0.003$, $\theta = 0.445$; wing length: $df = 25$, $P = 0.395$; Fig. 1): sham males captured significantly fewer prey per hour than infected (GLM: $Z = -2.948$, $P = 0.003$) and unhandled ($Z = -2.974$, $P = 0.003$) males, and the difference in prey capture rate between unhandled and infected males was not significant ($Z = 0.325$, $P = 0.745$).

Treatment had no effect on the proportion of males that ate at least one visible prey on 1, 2 or 3 days following manipulation (1 day: $df = 1$, $N = 47$, $P = 0.357$; 2 days: $df = 1$, $N = 21$, $P = 0.471$; 3 days: $df = 1$, $N = 30$, $P = 0.412$; Table 1). Only 2 of 8 unhandled males consumed at least one visible prey. One day after manipulation, 4 of 21 sham and 2 of 18 infected ate at least one visible prey. Two days after manipulation 4 of 8 sham and 0 of 5 infected males ate visible prey. Three days after manipulation, 0 of 11 sham and 4 of 11 infected males consumed at least one visible prey.

Treatment but not wing length had an effect on foraging attempts 1 day after its application (GLM: treatment: $df = 44$, $P = 0.016$, $\theta = 0.628$; wing length: $df = 43$, $P = 0.733$; Fig. 2, Table 1): unhandled males made more attempts than sham ($Z = -2.055$, $P = 0.040$) and infected males ($Z = -2.279$, $P = 0.023$); sham and infected males did not differ in their foraging attempts ($Z = 0.329$, $P = 0.742$). Two days after application, treatment and wing length had no effect on foraging attempts (GLM: treatment: $df = 18$, $P = 0.248$, $\theta = 0.885$; wing length: $df = 17$, $P = 0.171$; Table 1). On the third day there was an effect of the interaction treatment*wing length on the number of foraging attempts (GLM: $df = 23$, $P = 0.001$, $\theta = 2.055$; treatment: $df = 26$, $P < 0.001$; wing length: $df = 25$, $P = 0.719$; Fig. 3, Tables 1, 2): while larger males tended to make more foraging attempts in unhandled and sham treatments, the opposite occurred with infected males (Tables 1, 2).

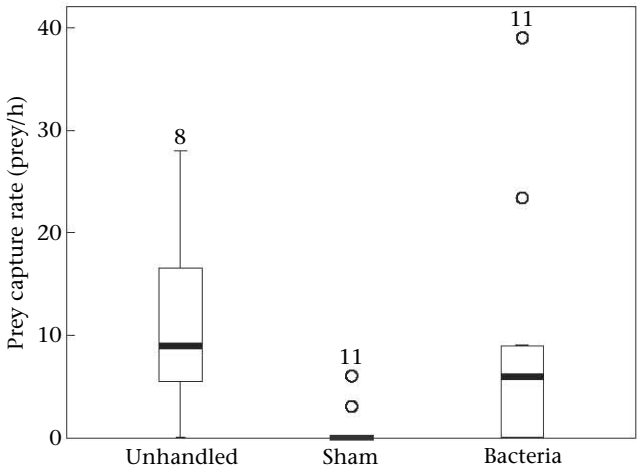

Figure 1. Effect of treatment over the number of prey captured by *H. americana* males from the field experiment 3 days after experimental manipulation. Circles represent extreme values; boxes represent first, second and third quartiles; whiskers are the sample maximum and minimum observations; sample sizes are shown above each plot.

Effect of Bacterial Infection on Territorial Behaviour in the Field Experiment

Neither treatment nor wing length had an effect on the total time that males spent contending for territories in any day following manipulation (first day: generalized least squares, GLS: treatment: $F_{1,42} = 2.355$, $N = 45$, $P = 0.132$; wing length: $F_{1,42} = 1.342$, $P = 0.253$; second day: ANCOVA: treatment: $F_{1,17} = 0.076$, $N = 20$, $P = 0.786$; wing length: $F_{1,17} = 0.007$, $P = 0.935$; third day: GLS: treatment: $F_{1,25} = 1.380$, $N = 28$, $P = 0.251$; wing length: $F_{1,25} = 0.413$, $P = 0.526$; Table 1). Neither treatment nor wing length had an effect on territorial contest rate on any of the 3 days (first day: GLM: treatment: $df = 44$, $P = 0.498$, $\theta = 0.532$; wing length: $df = 43$, $P = 0.100$; second day: GLM: treatment: $df = 18$, $P = 0.385$, $\theta = 0.948$; wing length: $df = 17$, $P = 0.906$; third day: GLM: treatment: $df = 26$, $P = 0.809$, $\theta = 1.429$; wing length: $df = 25$, $P = 0.204$; Table 1). Neither treatment nor wing length had an effect on the probability that a male would win a territorial contest 1 or 2 days after manipulation (1 day: GLM: treatment: $F_{2,43} = 1.175$, $N = 47$, $P = 0.319$; wing length: $F_{1,43} = 2.711$, $P = 0.107$; 2 days: GLM: treatment: $F_{2,17} = 0.787$, $N = 21$, $P = 0.471$; wing length: $F_{1,17} = 0.005$, $P = 0.943$; Table 1), but 3 days after manipulation the probability of winning a territorial contest depended on the interaction treatment*wing length (GLM: $F_{2,17} = 3.686$, $N = 24$, $P = 0.025$; treatment: $F_{2,20} = 1.507$, $P = 0.222$; wing length: $F_{1,19} = 7.397$, $P = 0.007$; Fig. 4, Tables 1, 3), even though larger males won more contests in all treatments (Fig. 4, Table 3).

Table 1
Descriptive statistics of *H. americana* male foraging and territorial behaviour after experimental manipulation in the field

Variable	Unhandled (N=8)	Day 1		Day 2		Day 3	
		Sham (N=21)	Bacteria (N=18)	Sham (N=8)	Bacteria (N=5)	Sham (N=11)	Bacteria (N=11)
Total prey capture rate (prey/h)	11.2±9.2	3.9±4.8	3.0±7.2	15.8±16.3	5.1±6.3	0.8±1.9	8.9±12.1
Visible prey foragers (%)	25.0	19.0	11.1	50.0	0	0	36.4
Prey capture attempts (attempts/h)	32.6±24.3	9.8±8.8	8.6±16.4	47.6±46.9	15.7±9.7	8.4±7.6	31.5±31.2
Time fighting (s)	22.9±27.4	9.6±13.1	9.4±14.2	29.6±33.9	16.7±24.5	25.6±31.5	59.1±164
Contest rate (contests/h)	6.4±5.3	4.0±5.4	3.2±4.6	7.9±8.8	3.1±2.4	5.6±4.5	7.4±9.7
Won contests (%)*	79.1±33.2 (N=6)	54.2±48.8 (N=12)	29.2±45.2 (N=8)	27.8±40.4 (N=6)	12.5±25.0 (N=4)	26.7±43.6 (N=9)	59.3±40.2 (N=9)

Values are means ± SD.
* Excluding animals that did not fight.

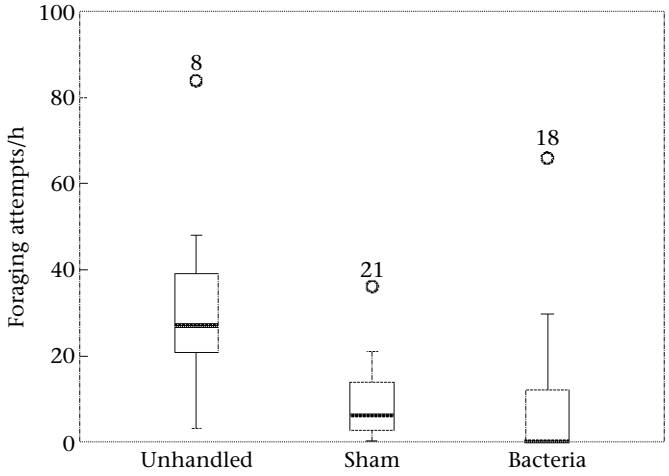

Figure 2. Effect of treatment over the number of prey capture attempts in *H. americana* males from the field experiment 1 day after experimental manipulation. Circles represent extreme values; boxes represent first, second and third quartiles; whiskers are the sample maximum and minimum observations; sample sizes are shown above each plot.

Effect of Bacterial Infection on Male Condition in the Field Experiment

PO activity of males did not differ significantly between treatments or injection times (12 h or 3 days before haemolymph extraction) (ANCOVA: treatment: $F_{2,27} = 0.802$, $N = 32$, $P = 0.459$; time pre-assay: $F_{1,27} = 2.016$, $P = 0.167$; wing length: $F_{1,27} = 0.228$, $P = 0.637$; Table 4). Fat load of males also did not differ significantly between treatments or injection times (ANCOVA: treatment: $F_{2,32} = 1.388$, $N = 37$, $P = 0.264$; time pre-assay: $F_{1,32} = 0.560$, $P = 0.460$; wing length: $F_{1,32} = 2.247$, $P = 0.144$; Table 4). Muscle mass was dependent on the interaction treatment*time pre-assay (ANCOVA: $F_{1,31} = 6.389$, $N = 37$, $P = 0.017$; treatment: $F_{2,31} = 4.141$, $P = 0.025$; time pre-assay: $F_{1,31} = 2.139$, $P = 0.154$; wing length: $F_{1,31} = 0.804$, $P = 0.377$; Fig. 5, Table 4): in the group of infected males, muscle mass was reduced 12 h after injection but recovered 3 days later, and such change did not occur in sham-injected males (notice that there was no effect of time pre-assay in unhandled males; Fig. 5).

Table 2
Generalized linear model for prey capture attempts by *H. americana* males 3 days after manipulation with three treatments. The model is prey capture attempts as a function of treatment*wing length

	Estimate	SE	*z*	*P*
Treatment				**<0.001***
Infected vs sham	−36.728	7.000	3.355	**<0.001**
Infected vs unhandled	−28.798	9.802	−3.747	**<0.001**
Sham vs unhandled	−7.930	11.405	−0.695	0.487
Wing length				0.719*
Treatment*wing length				**0.001***
Infected vs sham	1.408	0.389	3.624	**<0.001**
Infected vs unhandled	1.162	0.466	2.493	**0.013**
Unhandled vs sham	0.246	0.462	0.533	0.594

Parameters with $P < 0.05$ are in bold.
* Significance of the main effects.

Assessing Effects of Food Availability on Male Condition in the Laboratory Experiment

PO activity depended on the interaction treatment*nutrition (ANCOVA: $F_{2,64} = 3.318$, $N = 71$, $P = 0.043$; treatment: $F_{2,64} = 1.479$, $P = 0.235$; nutrition: $F_{1,64} = 2.130$, $P = 0.149$; wing length: $F_{1,64} = 0.004$, $P = 0.951$; Fig. 6, Table 5): while starvation had the same effect on PO activity when comparing sham with unhandled males ($t_{44} = 1.430$, $P = 0.158$) and sham with infected males ($t_{50} = -1.353$, $P = 0.181$), it had different effects when comparing unhandled and infected males ($t_{42} = -2.575$, $P = 0.012$), with the latter showing reduced PO activity under starvation. Fat load was significantly affected by the different injection treatments (ANCOVA: $F_{2,75} = 11.110$, $N = 79$, $P < 0.001$; Fig. 7, Table 5): while fat load did not differ between unhandled and sham males ($t_{51} = -1.304$, $P = 0.196$), infected males showed reduced levels of fat compared with unhandled ($t_{47} = -4.396$, $P < 0.001$) and sham ($t_{54} = 3.372$, $P = 0.001$) males. Fat load was independent of nutritional treatment ($F_{1,75} = 0.156$, $P = 0.698$) and wing length ($F_{1,75} = 0.280$, $P = 0.599$). Muscle mass did not differ between

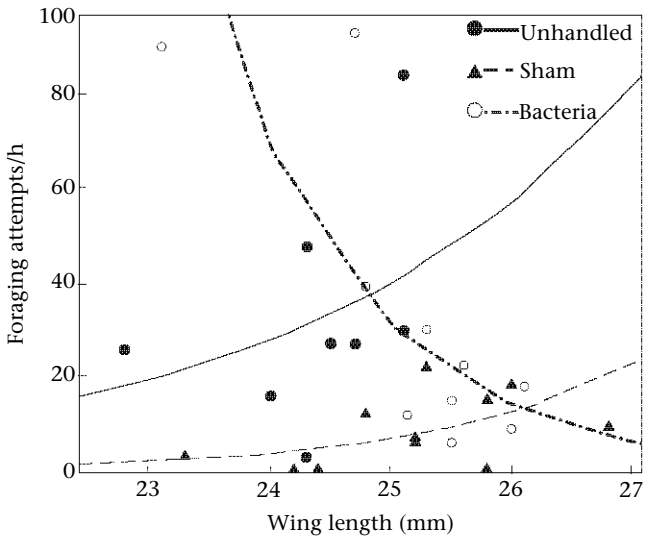

Figure 3. Effect of the interaction between treatment and wing length over foraging attempts in *H. americana* males from the field experiment 3 days after experimental manipulation.

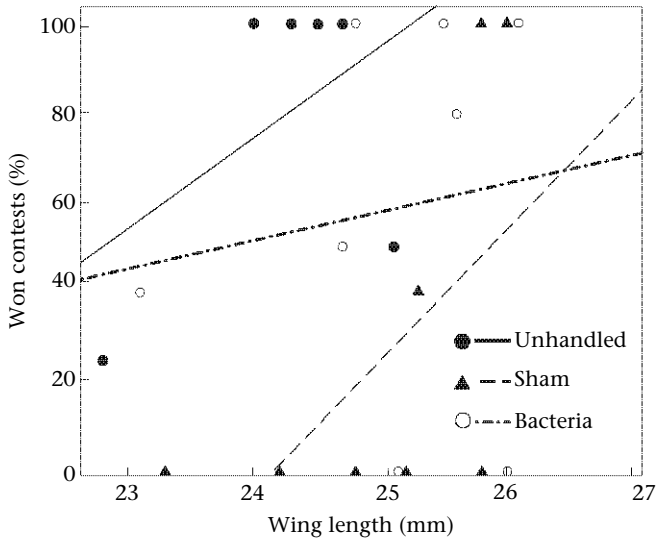

Figure 4. Effect of the interaction between wing length and treatment over the proportion of won territorial contests in *H. americana* males from the field experiment 3 days after experimental manipulation.

Table 3
Generalized linear model for the proportion of territorial contests won by *H. americana* males 3 days after manipulation with three treatments. The model is proportion of contests won as a function of treatment*wing length

	Estimate	SE	*z*	*P*
Treatment				0.222*
Wing length				**0.007***
Treatment*wing length				**0.025***
Infected vs sham	4.155	1.982	2.097	**0.036**
Infected vs unhandled	0.578	0.799	0.724	0.469
Unhandled vs sham	3.577	2.043	1.751	0.080

Parameters with $P < 0.05$ are in bold.
* Significance of the main effects.

males of the different injected (ANCOVA: $F_{2,75} = 0.390$, $P = 0.678$, $N = 79$) or nutritional ($F_{1,75} = 0.839$, $P = 0.363$) treatments, and wing length was not significant ($F_{1,75} = 2.569$, $P = 0.113$; Table 5).

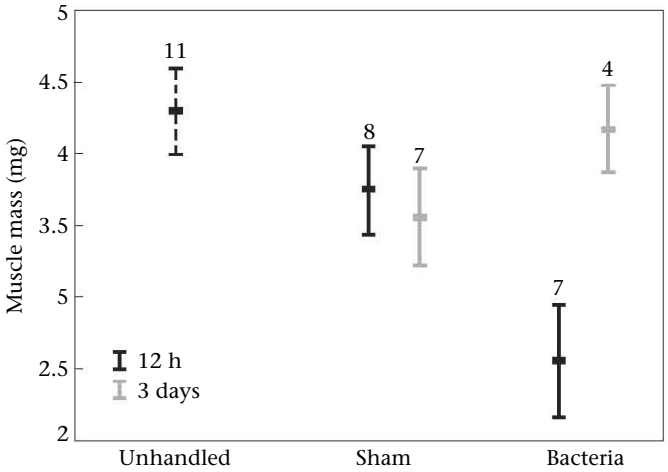

Figure 5. Effect of the interaction between treatment time pre-assay on muscle mass in *H. americana* males from the field experiment. Dotted line represents unhandled treatment, for which time was irrelevant. Bars represent standard errors. Samples sizes are shown above each plot.

DISCUSSION

In relation to adaptive changes in feeding behaviour in infected animals, our prediction was to see an anorexic period or at least a reduction in feeding activities, which would be linked to a reduction in lipid-based transportation costs. However, our results support the opposite trend, as infected males increased their prey consumption in relation to sham males. We did not find enough evidence to suggest that such change was related to whether prey were large or small. Besides foraging less than infected males, sham males also foraged less than unhandled males. These results can be interpreted as a side-effect of injection. The injection itself may induce an immune challenge due to the physical damage caused to the exoskeleton. That insects respond to exoskeleton injuries has been previously shown in the house cricket (Bascuñán-García et al. 2010). Nylon insertions that damaged the exoskeleton during the immature stage led to a reduction in adult size and an increase in exoskeleton thickness (Bascuñán-García et al. 2010). Our results and those of Bascuñán-García et al. (2010) should thus alert other researchers about the potential effects of injections in sham manipulations. Assuming that injection itself may induce an immune response, then the result that both infected and sham males decreased their feeding attempts but only sham males decreased their feeding rate partially supports the idea that reduced feeding behaviour could compensate for injury or illness at some point of a pathogenic infection. However, our results are more suggestive of a compensatory feeding. Why damselflies did not show anorexia as was described in herbivores is not clear. Perhaps the fact that predators depend mainly on lipid-based resources requires them to get at least some level of food to attend to costly behaviours (e.g. catching prey and/or defending a territory). However, some specific details of the reduction in feeding behaviour do not make much sense: (1) only sham males, but not infected males, showed the reduction in feeding rate; (2) sham and infected males reduced their feeding attempts 1 day after manipulation, whereas sham males consumed fewer prey 3 days after manipulation; and (3) on the third day larger unhandled and sham males foraged more intensively, whereas larger infected males foraged less intensively. One explanation for these anomalous results is that there are different effects for injections and injections plus bacteria treatment that reduce feeding rates or feeding attempts and that such effects occur at different times. These different effects may affect males according to their size, with small infected males doing worse at prey capturing. However, it is not clear exactly how such differential effects would work.

We expected that infections would not only cause a negative effect on feeding behaviour but also affect the ability of males to engage in costly territorial behaviours. Contrary to our prediction, infected animals did not change their territorial activities. This result may be due to the prioritization of sexual behaviour. Immunity and reproduction may be traded off with each other (Sheldon & Verhulst 1996; Lawniczak et al. 2007). However, abandoning territorial activities as a consequence of infection could be very costly if mating opportunities only come when a territory has been secured. In our study subject, males rarely get more than two matings in a 7–25 day period and only when they defend a territory (Serrano-Meneses et al. 2007; Córdoba-Aguilar 2009). Interestingly, although size has been previously found to correlate with contest success in this species (Serrano-Meneses et al. 2007), we found that this was the case but on the third day for all treatments. One interpretation is that independently of their size (which positively correlates with fat reserves; Serrano-Meneses et al. 2007), males in the present study invested all their energy to hold their territories on the first and second days, which was not further accomplished on the third day. Possibly on this last day, resources could no longer be allocated to resource holding and only

Table 4
Descriptive statistics of *H. americana* male condition measurements at different times after experimental manipulation in the field

Variable	Unhandled (*N*=9)	12 h		3 days	
		Sham (*N*=8)	Bacteria (*N*=7)	Sham (*N*=8)	Bacteria (*N*=4)
PO activity* (OD protein/μg)	0.05±0.03	0.14±0.27 (*N*=7)	0.23±0.37 (*N*=6)	0.05±0.02 (*N*=7)	0.05±0.02 (*N*=3)
Fat load (mg)	0.89±0.44	0.66±0.68	0.50±0.40	0.83±0.46	0.60±0.34
Muscle mass (mg)	4.39±1.08	3.75±0.86	2.56±1.02	3.56±0.90	4.18±0.61

Values are means ± SD.
* Excluding animals with insufficient haemolymph sample.

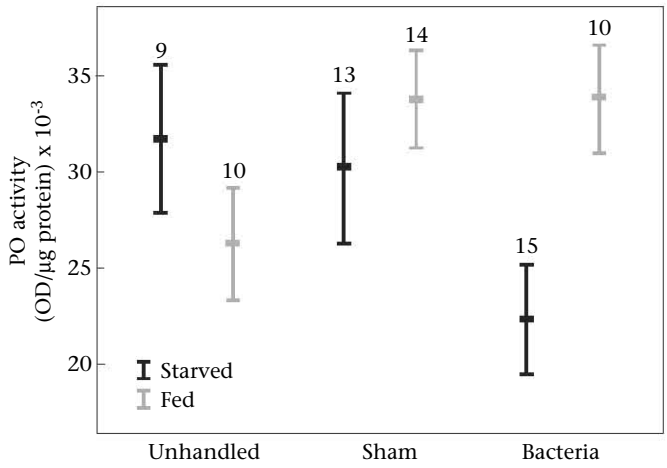

Figure 6. Interaction between injection and nutritional treatments on PO activity in *H. americana* males from the laboratory experiment. Bars represent standard errors. Sample sizes are shown above each plot.

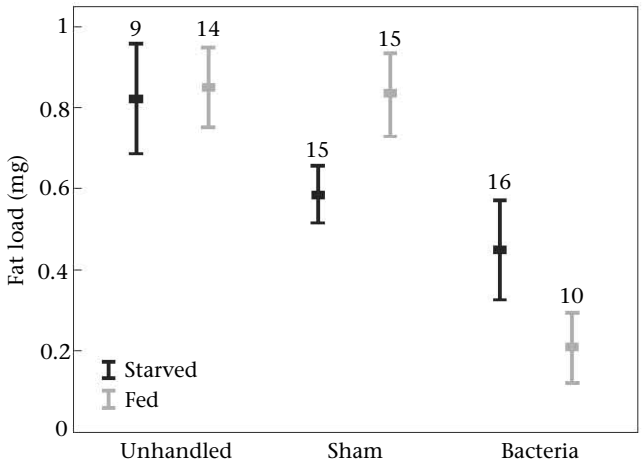

Figure 7. Effect of injection and nutritional treatment on fat load in *H. americana* males from the laboratory experiment (the interaction was not significant). Bars represent standard errors. Sample sizes are shown above each plot.

large males could keep such capacity. However, it is unclear why size was not important for the first 2 days in the unhandled males.

The prioritization of territorial activities does not mean that the activity is cost free. At the proximate level, we detected a physiological cost for infected males in the field experiment. Infected males showed reduced muscle mass after 12 h. In damselflies, muscle mass also becomes impaired after energetically costly activities such as territorial defence (Marden et al. 1998). Although our field results indicated no muscle mass differences between treatments after 3 days, the lack of significance may be explained by the small sample size. In the laboratory experiment, muscle mass was not affected by treatment or food access, while fat load levels were impaired in infected males independently of food access, and PO activity was reduced in infected males only if food was not provided. Given that the fundamental difference between both approaches is that the laboratory males were precluded from territorial activities, this corroborates the idea that the muscle mass reduction in the field experiment males was due to territorial defence. One explanation for the absence of an effect on fat in the field experiment was that these males always had access to food. Food has an effect on PO activity (Moret & Schmid-Hempel 2000; Siva-Jothy & Thompson 2002). In fact, our results in this respect are in agreement with those found by Moret & Schmid-Hempel (2000), who detected that *Bombus terrestris* bumblebees exposed to bacterial lipopolysaccharides and microlatex beads showed reduced survival only when access to food was not allowed. The negative effects on PO activity when no food was provided may explain why males in our field experiment did not cease food intake but rather just reduced food ingestion rate. Infected males may acquire sufficient food to maintain both PO levels and fat load but are unable to maintain muscle mass. Two recent studies have shown that fat load, PO activity and territorial behaviour in calopterygids may compete for identical resources and thus trade-off (Contreras-Garduño et al. 2009, in press). Although slightly different to our results here, these other studies have found that resource investment in territorial activity and fat load affects PO activity (Contreras-Garduño et al. 2009).

Finally, there are important critical issues of our work that may be dealt with in future studies. First, males of different bacterial treatments may have competed with each other for territories, which may have differentially affected their resource use. For example, a territorial contest between a focal infected male and a focal noninfected male is likely to be more costly for the former male. Given that we did not observe all fights, whether such influence exists cannot be assessed. Second, as outlined above, sample sizes for some comparisons were low, thus preventing robust analysis. For example, field experiment males whose mass was measured on the third day showed no differences between treatments while a similar analysis conducted after 12 h showed significance. Although we prefer the hypothesis that muscle mass was rebuilt, we cannot exclude alternative explanations such as that those animals observed on the third day were those capable of retaining their muscle mass values. Third, the quality and type of food provisioning that males obtained in the laboratory experiment is likely to be different from that obtained in the field. For example, males in the laboratory were provided one type of food, whereas field animals had the opportunity to choose prey of different quality (see Povey et al. 2009). Note that we found no differences in prey size according to treatment in the field experiment. However, if infected males begin to select specific prey to deal with specific immunological-digestive demands, our food provisioning in the laboratory may have prevented this adaptive feeding change. This

Table 5
Descriptive statistics of *H. americana* male condition under different injected and nutritional treatments in the laboratory experiment

Variable	Unhandled (N=14)	Fed		Unhandled (N=9)	Starved	
		Sham (N=15)	Bacteria (N=10)		Sham (N=15)	Bacteria (N=16)
PO activity* (OD protein/µg)	0.53±0.19 (N=10)	0.68±0.19 (N=14)	0.68±0.18 (N=10)	0.63±0.23 (N=9)	0.60±0.28 (N=13)	0.45±0.22 (N=15)
Fat load (mg)	0.94±0.45	0.83±0.39	0.22±0.27	0.84±0.45	0.62±0.34	0.43±0.46
Muscle mass (mg)	2.35±1.67	2.28±1.50	2.36±1.95	2.52±1.53	3.30±1.31	2.37±1.41

Values are means ± SD.
* Excluding animals with insufficient haemolymph sample.

problem can be circumvented by sampling prey caught by infected and noninfected males to see whether there are differences in food quality.

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