What doesn't kill you makes you stronger: detoxification ability as mechanism of honesty in a sexually selected signal

Isaac G-Santoyo[1], Daniel González-Tokman[2], Miguel Tapia-Rodríguez[3], Alex Córdoba-Aguilar[4]

Affiliations:

[1]Neuroecology Lab. Facultad de Psicología, Universidad Nacional Autónoma de México, Ciudad Universitaria, 04510 Ciudad de México, México.

E-mail:isantoyo.unam@gmail.com

[2]CONACYT, Red de Ecoetología, Instituto de Ecología A. C., Xalapa, México.

E-mail: daniel.gt@inecol.mx

[3]Unidad de Microscopía, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Ciudad Universitaria, 04510, Ciudad de México, México.

E-mail: mtapia@biomedicas.unam.mx

[4]Instituto de Ecología, Universidad Nacional Autónoma de México, Ciudad Universitaria, 04510, Ciudad de México, México.

E-mail: acordoba@iecologia.unam.mx

Short running title: Detoxification ability by synthesis of ommochromes

Keywords: Detoxification ability, Ommochromes, Kynurenine, Confocal Microscopy, Odonata, Storage excretion, Sexually Selected Colouration.

Type of Article: Letters

Number of words in the abstract: 150

Number of words in the main text: 4943

Number of references: 49

Number of figures, tables, and text boxes: 4 figures

Corresponding authors:

Isaac G-Santoyo ; Neuroecology Lab. Facultad de Psicología, Universidad Nacional Autónoma de México, Ciudad Universitaria, 04510 Ciudad de México, México;Telephone nunmber: (552) 55- 56228222, Ext.41283.

E-mail address: isantoyo.unam@gmail.com.

Alex- Cordoba Aguilar; Instituto de Ecología, Universidad Nacional Autónoma de México, Ciudad Universitaria, 04510, Ciudad de México, México. Tel. (552) 55-56228222, Ext.9003.

E-mail: acordoba@iecologia.unam.mx

Statement of authorship:

I.G.-S conceived this study. All authors designed this study. I.G.-S., and D. G.-T. collected fieldwork data, M.T.-R. collected and analyzed microscopy data, I.G.-S. and D. G.-T. analyzed all data. All authors contributed in writing the first draft and approved the final version of the manuscript.

Data accessibility statement: In case the manuscript is accepted, the data supporting the results will be archived in an appropriate public repository, and the data DOI will be included.

Abstract

Sexual selection maintains colourful signals. The metabolic pathways to produce them often involve toxic byproducts that can reduce survival. However, rather than discarding these otherwise harmful byproducts, animals may use them by integrating them into sexually-selected traits. We tested this using the damselfly *Hetaerina americana*, where males bear a red wing spot evolved by intrasexual competition. We determined that red wing spots are generated by ommochrome pigments derived from the toxic metabolite, 3-hydroxy-kynurenine (3-Hk). We also found that males treated with 3-Hk had more ommochromes than controls but similar survival, suggesting that deposition of ommochromes counteracts the 3-HK toxicity. Thus, we report that sexually selected signals involve the treatment of excreted compounds that could otherwise have lethal effects, a hypothesis we call “detoxifying ability signalling”. Our results provide new insights about the evolution of sexual signals, elucidating a mechanism of honest indicators that could have arisen due to natural selection.

Introduction

Some animal colours result from the sequestration as pigments in the integument of harmful metabolic products that cannot be easily eliminated from the body during digestion (Cochran 1975). This mechanism is called storage excretion. What makes this mechanism particularly interesting is that rather than being harmful, the excreted pigments can generate external colouration that has adaptive functions such as camouflage or signalling information to conspecifics and heterospecifics. For example, excessive uric acid, the main nitrogen metabolic waste of terrestrial insects, sometimes cannot be completely eliminated during digestion but is excreted as a white or yellow pigment that is deposited in the larval cuticle of several species of pierid butterflies (Timmerman & Berenbaum 1999). This deposition creates the impression of bird droppings, which looks distasteful for predators, decreasing predation and thus increasing larval survival (Timmerman & Berenbaum 1999). Similar pigmentation patterns in adult butterflies, resulting from storage excretion, have been proposed to be involved in sexual selection processes, such as colourful traits used for attracting mates or dissuading conspecifics during male-male competition.

A classical tenet in sexual selection theory is that the expression of pigmented traits has evolved because these colours grant individuals an advantage during mate choice or intrasexual competition for mates (Andersson 2019). For instance, it has been observed that bright male colouration is maintained by sexual selection because it can only be produced by high quality (e.g. well-fed) individuals that can afford the production of pigments without compromising other traits that require the same limiting resource (Zera & Harshman 2001). Interestingly, genes responsible for colouration interact

pleiotropically with other genes that can be involved in important physiological functions such as thermoregulation, photoprotection, desiccation resistance, immunoregulation, antioxidation and excretion of toxic compounds that may result from metabolism and immune response (von Schantz *et al.* 1999; McGraw 2005; Vukusic *et al.* 2013). Therefore, pigmentation that reflects efficient metabolism may be subject to strong sexual selection if it honestly reflects individual genetic and physiological condition (von Schantz *et al.* 1999; Monaghan *et al.* 2009).

Storage excretion as a mechanism of sexual pigmentation has not been discussed and examined in detail despite providing a clear physiological and evolutionary explanation of the origin and maintenance of many sexual signals. Storage excretion can be particularly important when the synthesis of such pigments is involved in physiological pathways that generate waste products that are difficult to eliminate or inactivate. Moreover, the assumption that the trait provides honest information about the quality of the bearer is upheld, since the use of toxic byproducts to generate visible colouration could be doubly informative: pigments signal the bearer's ability to deal with toxic metabolites that could otherwise impair survival, as well as the genetic and/or physiological condition since for example, because only individuals with better diets or protein metabolism will produce a large enough amount of the byproducts to require storage excretion of the pigments (as suggested by (von Schantz *et al.* 1999)).

Red pigments are common in several invertebrates, and most of their underlying components are ommochromes (Linzen 1974). In insects, ommochromes give colour to structures such as the eyes and the wings of flies, butterflies and damselflies (Chapman 1998; Riou & Christidès 2010; Futahashi *et al.* 2012). These red pigments result from the

metabolism of tryptophan, an essential amino acid that is consumed from food (Kayser 1979; Chapman 1998). Ommochrome pigments are synthetized from the tryptophan metabolite 3-hydroxykynurenine (3-Hk), which together with tryptophan, are considered neurotoxic compounds that cause paralysis, altered mating behaviour, aging and high mortality in adult insects (Connolly *et al.* 1969; Cerstiaens *et al.* 2003; Oxenkrug 2010; Oxenkrug *et al.* 2011). In fact, wild type flies fed with inhibitors of tryptophan-kynurenine metabolism show increased survival compared to control flies (Oxenkrug *et al.* 2011). 3-Hk is present at high concentrations during metamorphosis when protein breakdown releases high quantities of this tryptophan product (Chapman 1998). Given the noxious effects of 3-Hk, an important way in which insects can deal with excessive neurotoxic byproducts is via the synthesis of ommochromes, which can be eliminated in the meconium during pupation in holometabolous insects, in the excreta or by storage excretion in the form of cuticular pigmentations, a hypothesis proposed by Linzen in 1974 and called "the tryptophan-detoxification hypothesis" (Linzen 1974; Vukusic *et al.* 2013; Figon & Casas 2019). In fact, both 3-Hk and ommochrome pigments are found at high concentrations in the meconium of lepidopterans, the waste product from the pupal stage (Ogawa & Hasegawa 1980).

Ommochromes can function as sexually selected signals and may be considered honest indicators of individual quality, since only well-fed individuals will accumulated high enough amounts of these metabolites to develop conspicuous signals (Hooper *et al.* 1999).

Here, we propose that ommochromes are components of male sexual traits that can be used for detoxification, providing a new mechanism of sexual signal production and

fulfilling the honesty principle. We tested these ideas using males of the rubyspot damselfly *Hetaerina americana*. This species is a classic model in sexual selection studies: males bear a red wing spot (RWS; Fig. 1a) whose size is an honest indicator of individual condition, and which provides an advantage during male-male competition (Contreras-Garduno *et al.* 2008; Córdoba-Aguilar & González-Tokman 2014). We (1) determined the presence of ommochromes in the wing spot, (2) tested the toxic effect of 3-hydroxykynurerine (3-Hk), the most toxic tryptophan metabolite and precursor of ommochromes, and (3) measured deposition of ommochromes in wing spots of young adult males injected with 3-Hk.

Materials and Methods

1. Animals

Adult males of *H. americana* were captured in the riverine areas of the Tetlama River, Mexico (18º 45' 55''N, 99º 14' 45'' W) with a butterfly net between 10.00 and 16.00h, the time at which males are most active (Contreras-Garduño *et al.* 2006). Males were aged according to three visual categories (Plaistow & Siva-Jothy 1996): 1) juvenile males, bearing soft, dorso-ventrally flexible, undamaged wings with a small and incompletely formed RWS; 2) sexually mature males, with less flexible wine same RWS size as sexually mature males. Given that animals from the second category have a better physiological condition and will remain alive for a longer period from the time of capture, we only used sexually mature males.

2. Presence of ommochromes in the RWS of *H. americana* males

Pigment extraction

To determine whether ommochrome pigments are present in the RWS, we sacrificed 30 adult males by freezing for 20min at -20°C soon after collection. Animals were then dried in an oven (Heratherm-ThermoFisher) for 24h at 30°C. To extract the red pigment from the wings we used the methodology proposed by Nijhout (Nijhout 1997), with some modifications (Rioy & Christides 2010)f. Red spots exclusively obtained from the forewings were pooled and homogenized in methanol at 4°C and centrifuged for 5min at 14,000g, and the precipitate was washed twice with 99% methanol and three times with ethyl ether by repeated suspension and centrifugation (5min at 14,000g). After ether was evaporated from the precipitate, 4mL of acidic methanol (100% methanol plus 0.5%

hydrochloric acid) were added to the residue and the suspension was centrifuged for 10min at 14,000g. The supernatant was concentrated to one-quarter of its volume using a vacuum concentrator (SpeedVac-Thermo®). Later, we added 2mL of distilled water and SO gas to this solution and we incubated it overnight for 8h at 2°C. The red pigment obtained was precipitated by centrifugation for 10min at 14,000g and washed with a 2mL of cold water. The sample was then dried in a drying oven (Heratherm-ThermoFisher) at 28°C until water evaporated. The powder obtained was used to determine the nature of the red wing pigment.

Nature of the red wing pigment

To evaluate presence of ommochromes in the red wing pigment, we performed four complementary methods based on biochemical properties: Redox behaviour, spectral absorbance, thin layer chromatography and autofluorescence by confocal microscopy.

1. *Redox behaviour*

The most conspicuous property of ommochromes is their reduction-oxidation behaviour (i.e. Redox), which is observed by a colour change from red when reduced to yellow when oxidized (Linzen 1974). Hence, to evaluate this the red spot powder was dissolved in phosphate buffer (0.067M, pH=8.5) containing 10µL of 1% ascorbic acid as a reductant to observe the resulting colour. Next, we added 10µL of 1% $NaNO_2$ as an oxidant component and the colour changes were observed. Finally, we again added 10µL of 1% ascorbic acid to test whether the colour reverted to its previous state.

2. *Spectral absorbance*

Ommochromes have a particular spectral behaviour characterized by 2 to 4 spectral peaks distributed throughout the UV and visible ranges of the spectrum (Linzen 1974; Bolognese *et al.* 1988). These peaks have been characterized when ommochromes are dissolved in both phosphate buffer (pH=7.0) and 5N HCl (Umebachi & Uchida 1970; Nijhout 1997). Therefore, to explore the presence of ommochromes in the RWS, we prepared two different solutions using 20µg of the red powder extracted and 100µL of phosphate buffer or 5N HCl. Spectral peaks of the ommochrome xanthommatin are the best characterized by previous studies (Linzen 1974; Figon & Casas 2019). Hence, we convert any ommochromes present in the RWS powder (e.g. ommatin D or dihydroxanthommatin) into xanthommatin. This conversion allows us to ensure the specificity in the resulting peaks. For the case of the phosphate buffer, the solution was refrigerated at 4°C for 12h, while the 5N HCl solution was kept at 25°C in a drying oven (Heratherm-ThermoFisher) for 5 days (Umebachi & Uchida 1970). After this period, both solutions were centrifuged (14,000g for 5min) and the absorbance of the supernatant was measured from 220-600nm wavelength using a spectrophotometer (Hitachi, Mod. U-3900). We compared the peaks obtained in both solvents with those described by Nijhout (Nijhout 1997), who used the same solvents to evaluate spectral peaks of ommochromes extracted from the ventral hind wings of the lepidopteran *Precis coenia* (Nijhout 1997).

3. Thin layer chromatography (TLC)

TLC allows the separation and characterization of ommochromes if these pigments are present in our red powder (Nijhout 1997). Hence, we implemented a TLC using a

10x20cm silica gel plate (Merck, Darmstadt, Germany, Silica Gel 60 F254). This plate was divided into 4 channels (2cm between channels). The division was made from the narrower side of the plate (10cm) and a thin base line was drawn with a pencil at 1cm from the bottom of the plate in each channel. At this base line, we added 10µL from the red powder previously diluted in acidic methanol (1mL (Nijhout 1997)). The plate was left in a vertical position inside a chromatographic chamber (Aldrich) for 2h to allow the complete separation of pigments. This chamber was previously saturated with a developing solvent of phenol:water (3:1 by volume) using a small piece of filter paper (JM 3639, JoyLab) around the chamber. After this time, we identified the bands formed along the plate using an UV/White transilluminator (Safe Imager 2.0, Invitrogen). Then, we measured the distance (mm) at which these bands were located with respect to the base line as well as the distance travelled by the developing solvent in each channel. These distances were used to obtain the retention factor (R_f) for each pigment found. To determine whether any R_f obtained in our sample corresponded to ommochromes, these values were compared with the R_f of authentic synthetic ommochromes (Nijhout 1997), since there are no commercially ommochrome standards available to date (Figon & Casas 2019).

4. *Autofluorescence analysis*

The presence of indole groups in all ommochromes gives them the property of autofluorescence at certain wavelengths (Linzen 1974). Therefore, one last test to determine the presence of ommochromes in *H. americana* wings was observing autofluorescence induced by excitation with specific laser lines and evaluating with a

spectral detector using confocal microscopy. To accomplish this, five forewings were mounted onto pre-cleaned microscope slides (Lauka, México) and covered with 0.17mm thick 180x180mm coverslips. Bright field tile imaging was carried out with an upright microscope (Olympus BX51-WI, Tokyo, Japan) equipped with an XYZ motorized stage (MAC6000, Ludl Electronic Products, Hawthorne, USA), and a RGB CCD camera (MBF CX9000, MBF Bioscience, Williston, USA). Imaging was done using a UPLAN FL N 10X N.A. objective.

To visualize the autofluorescence produced by the RWS, we acquired XYZλ images from both pigmented and non-pigmented regions of the same wing with a Nikon A1R^{+} laser confocal scanning head coupled with an Eclipse Ti-E inverted microscope (Nikon Corporation, Japan). We thus excited the samples using four lasers of different wavelengths: 405 (2mW), 488 (70mW), 561 (1.4mW) and 647nm (1.25mW). We evaluated the resulting signals using a 32-channel spectral detector (10nm resolution, from 425 to 735nm wavelength) plus a transmitted light detector. The pinhole value was set at 29.4µm for all lasers. Images were captured with NIS Elements C software v. 4.50 (Nikon); and XYZ□ resulting images were converted to single-plane images by applying maximum intensity projections (MIP) in order to show the brightest fluorescence information for all Z planes for all wavelengths at a glance with the same software. Fluorescence single plane tile imaging of the wings was done with a CFI Plan Fluor 10X N.A. 0.3 objective, using 7mW of 561nm laser power, pinhole aperture of 195.4µm, and a GaAsP detector, all controlled through NIS Elements C software v4.50 (Nikon).

3. Toxic effects of 3-Hk in *H. americana* males

Given that 3-Hk has been reported to be fatal to insects (Cerstiaens *et al.* 2003), we evaluated whether this substance kills *H. americana* males. We performed an experiment in captivity to calculate the median lethal concentration (LC_{50}) of 3-Hk. 3-Hk (Sigma-Andrich) was dissolved in distilled water. Five 3-Hk concentrations were injected into 75 adult males (15 per group): 0 (only distilled water), 1, 100, 1000 and 10,000µg mL^{-1}. The injection took place in the dorsal thoracic region, where the wings are inserted. Animals received 4µl of each 3-Hk concentration using a microsyringe (10µL, Hamilton 80330). Manipulation lasted less than 1 min. Males were then placed individually into transparent plastic 5-mL assay tubes (Simport,) with a piece of wood as a perch, moist pieces of cotton to provide humidity and a temperature of 26°C inside the tubes. Males were not fed during the experiment and conditions of captivity were always the same. Twenty-four hours after injection, male mortality was recorded for all the five groups. This survival experiment concluded that 3-Hk is toxic for adult males of *H. americana* (see results for further details).

4. Mitigation of 3-Hk toxic effects by deposition of ommochromes in RWS

To evaluate whether males are able to counteract the toxic effect of 3-Hk by depositing ommochromes into their wings, forming the red wing spots, we performed another captivity experiment administrating 3-Hk in males, then determining whether the ommochromes were subsequently deposited into their RWS. To accomplish this, 54 adult males were captured and randomly allocated to three different treatments: 1) 3-Hk treatment (N=20 males injected with 363µg of 3-Hk diluted in 4µL of PBS 1x—the

previously estimated LC_{50}), 2) Sham treatment (N=17 males injected with 4µL of PBS 1X), and 3) Control treatment (N=17 males with no manipulation). After manipulation, males were monitored in captivity every 4h to record the time to death. The experiment ended when the last male died. Captivity conditions were the same as those used to calculate the LC_{50}. After the last male died, forewings from all individuals were removed from the body. The effect of 3-Hk on red wing pigmentation was evaluated by two complementary techniques: 1) quantification of red chroma (*Rc*) by spectrophotometry (Contreras-Garduno *et al.* 2008), and 2) the relative fluorescence *(Rf)* by confocal microscopy.

The red chroma of RWS from the different treatments was calculated as the proportion of total reflectance (from 360 to 740nm) occurring in red wavelengths (600-700nm) using a spectrophotometer (MINOLTA CR-200, Konica Sensing Inc., Osaka, Japan; for a similar procedure see (González-Santoyo *et al.* 2014)).

Rf was determined from confocal XYZ images obtained from a random sample of 10 forewings from males of each treatment. Laser scanning confocal microscope Z-stack images (512x512 pixels, 12-bits, 3µm interval) were acquired with a CFI Plan Fluor, 10X, N.A. 0.3 objective, using 1.4mW of 561nm laser power for excitation, pinhole aperture of 20.43µm, emission filter 595/50, and a GaAsP detector, all controlled through NIS Elements C software v4.50 (Nikon). Z-images were then processed using Image J software (Abramoff *et al.* 2004), performing a Z-projection with the pixels obtained from maximum intensity projection (MIP) as the reference. The MIP image was converted from 12 to 8-bits and a histogram of the pixel intensity value was extracted. *Rf* value from each Z-projection was calculated by multiplying the intensity value of the histogram with

its corresponding number of pixels then dividing by the total number of pixels present in each image (262144 pixels (Abramoff *et al.* 2004)). Finally, *Rf* values of all Z-projections were averaged to obtained a unique *Rf* value per individual.

5. Statistical analyses

To determine whether ommochromes are responsible for the male RWS we qualitatively analysed redox behaviour, spectral absorbance, *Rf* of purified ommochromes, and autoflourescence. To evaluate whether 3-Hk, the precursor of ommochrome pigments, is toxic for adult males, we calculated the LC_{50} through the Trimmed Spearman-Karber method (Hamilton *et al.* 1977). And performed a survival analysis with the following predictors: treatment (3-Hk, Sham, and Control), wing length, RWS area, and their interactions. Analysis was done in R (Team 2019) using the tsk package (Stone 2012).

To evaluate whether males of *H. americana* counteract the toxic effect of 3-Hk by depositing ommochrome in their RWS, we evaluated both the survival differences between treatments (i.e. 3-Hk, sham, and control) and the differences in colour properties of *Rc* and *Rf*. Survival after treatment was evaluated with a Cox proportional hazard regression, whose predictors were the additive effects of treatment and wing length (a proxy of body size (González-Santoyo *et al.* 2014). This model was simplified based on AIC values to obtain the best supported model (i.e. the model with the lowest AIC).

To determine differences in *Rc* and *Rf* we used linear mixed and linear models respectively. The predictor variables were treatment, survival time, wing length, RWS area and the interactions treatment*survival time, treatment*wing length and treatment*RWS. Given that *Rc* was measured for both forewings of each damselfly,

individual identity was included as a random effect in this analysis. The initial models were reduced based on AIC and the best supported model is reported. *P*-values of the predictor variables and interactions were obtained using likelihood ratio tests for *Rc* and with F tests for *Rf*. Variance homogeneity was tested using the Fligner-Killeen test, normality of residuals was inspected visually from normal q-q plots and the presence of outliers was evaluated with Cook's distances (none were found—all Cook's distances<1). All analyses were done in R software (Team 2019) according to (Crawley 2012) (Zuur *et al.* 2009).

Results

Biochemical tests confirmed the presence of ommochromes as main pigments in the RWS (Fig. 1a). Besides Redox behaviour, we observed that the absorption spectra from the red powder diluted in 5N HCl displayed three absorbance wavelength peaks; 249, 357 and 460nm with relative absorbances of 2.49, 0.52 and 0.74 respectively. The red pigment dissolved in phosphate buffer displayed peaks at two different wavelengths: 235 and 429nm, with relative absorbances of 2.68 and 0.68 respectively (Fig. 1b).

Moreover, our TLC analysis indicates that xanthommatin is an ommochome present in *H. americana* male RWS. When the silica gel plate was exposed to the UV spectrum, we observed a slightly defined band with a *Rf*=0.15$\pm$0.013 and a well-defined band with *Rf*=0.36$\pm$0.012 (Fig. 1b), which coincides with the standard synthetic ommochrome xanthommatin (*Rf*=0.36) when the same silica gel/phenol TLC system is implemented (Nijhout 1997). The other slightly defined band observed showed a similar *Rf* value to the same xanthommatin, but in its reduced form, dihydro-xanthommatin (Rf=0.13 (Nijhout 1997)). We also found a slight third band (Fig. 1b), which may correspond to 3-Hk (Rf=0.52 (Nijhout 1997)), although it was very faint.

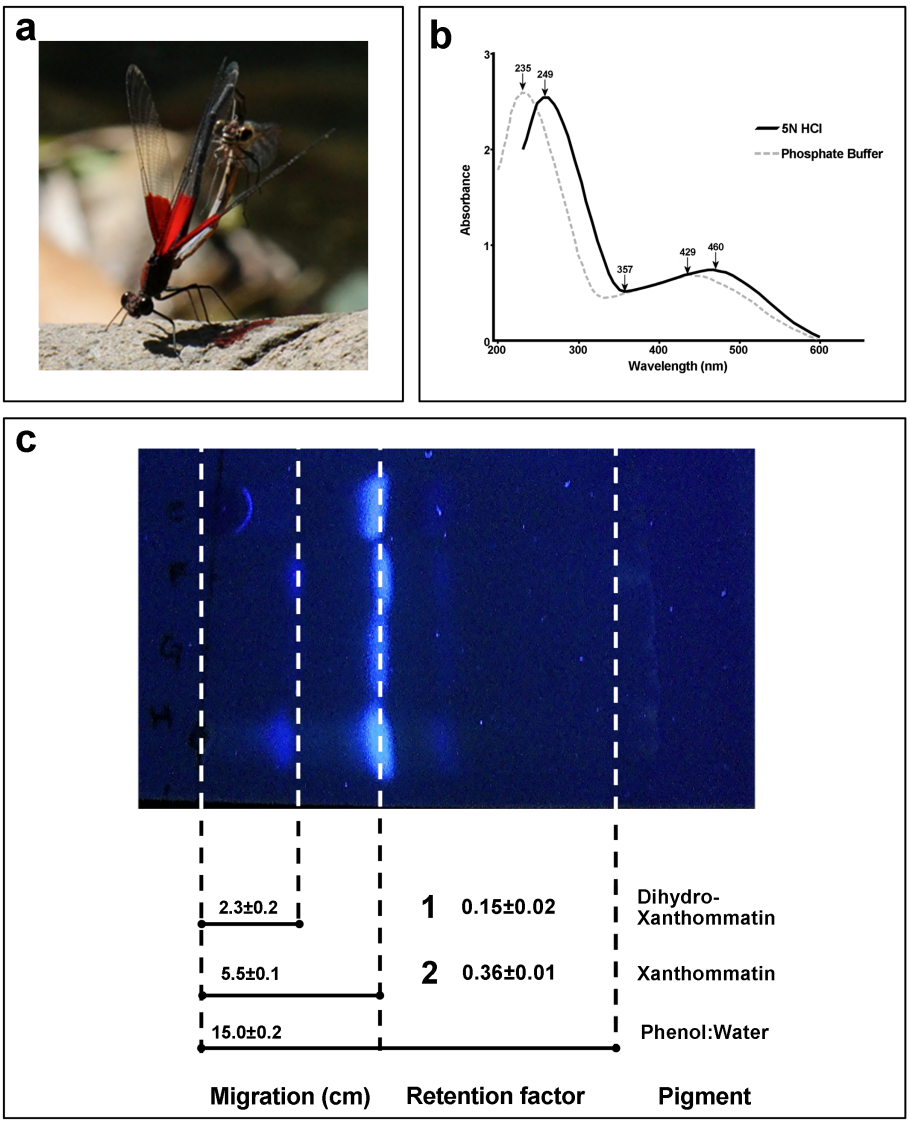


Figure 1. (a) *Hetaerina americana* male showing his red wing spot (RWS) during mating. (b) Spectral absorbance analysis showing the presence of ommochrome pigments in the RWS. The red powder dissolved in 5N HCl (solid black line) or phosphate buffer (dashed grey line) show a typical spectral behaviour of the pigment xanthommatin. (c) UV spectrum photography from the thin layer chromatography (TLC) confirmed the presence of xanthommatin. The column migration and dashed lines show the distance travelled (cm) by the two bands observed in the TLC and by the solvent used (Phenol:Water). The retention factor (Rf) for these bands (1 and 2) were consistent with the authentic standard of xanthommatin (number 2) and its reduced form dihydro-xanthommatin (number 1). The Rf of the authentic standard of xanthommatin was reported by Nijouth (1997) using the same TLC method.

In addition, patterns of autofluorescence in the RWS were observed in the spectral confocal microscope. Figure 2 shows MIP images obtained from XYZλ scans of both red (Fig. 2a) and transparent (Fig. 2b) regions of a representative control wing (Fig. 2c) excited with 561nm wavelength light. We excited separately with 405, 488, 561 and 647nm wavelengths and found two main types of fluorescence: the most conspicuous corresponds to ommochrome pigments, which are distributed along the wing veins (Fig. 2a, 2d) and spread over the wing tissue in the red pigmented area (Fig. 2d, 2e), but not in the transparent region (Fig. 2b). This signal was strongest when excited at 561 nm, but it was also visible when excited at 405 or 488 but not at 647 nm wavelengths (see supplementary figures S1, S2, S3). The other main type of fluorescence observed was autofluorescence of both the red and the transparent wing regions, which can be attributed to the reflection of light by the wax that covers the wings. This type of fluorescence occurred at all wavelengths (see supplementary figures S1, S2, S3) and shows a characteristic periodicity on its distribution pattern.

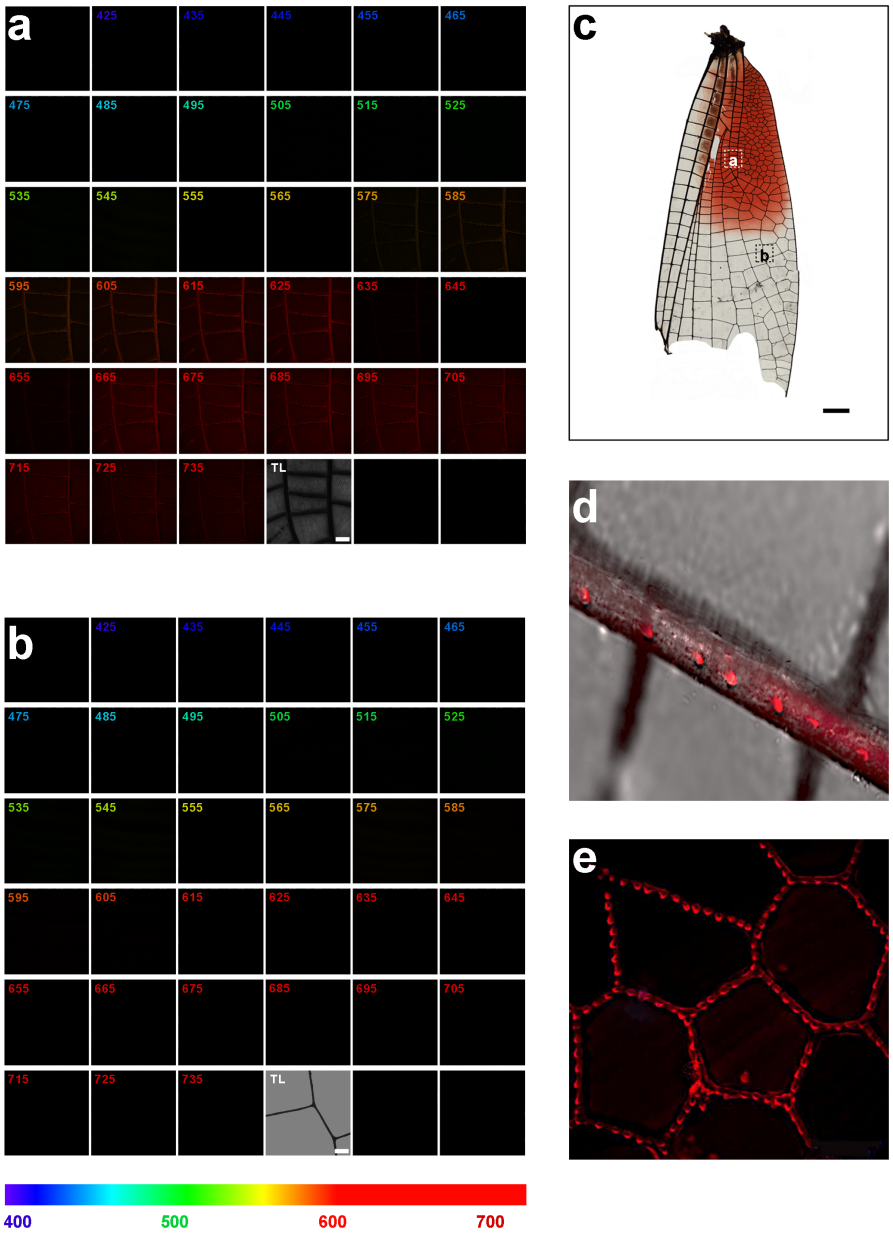


Figure 2. Fluorescence XYZλ scans (425-735 nm) of *H. americana* wings excited at 561 nm. Maximum intensity projection images of (a) a sub-region of the red-pigmented area, and (b) a sub-region of the transparent area, depicted in (c). Each frame in (a) and (b) represent a specific wavelength image, as indicated. Fluorescence intensity was highest between 615-725 nm in the red pigmented area but not in the transparent region. Higher magnifications show the presence of red fluorescence spots distributed along the wing veins ç(d) and diffusely spread over the wing tissue (e). Scale bars in a and b indicate 100μm and in c, 1mm.

3-Hk had a range of toxic effects on *H. americana* males, from partial immobility at the lowest concentrations tested (1 and 100µg mL^{-1}) to death for the highest concentrations (1000 and 10000µg mL^{-1}). This toxicity led us to determine the LC_{50} for sexually mature adult males. We found that the group with the lowest 3-Hk concentration (1µg mL^{-1}) showed a mortality rate of 13.13%, while subsequent doses (100, 1000 and 10000 µg mL^{-1}) increased mortality to 40%, 46% and 53% respectively. Consequently, LC_{50} of 3-Hk for males of this species was estimated at 368.69 µg mL^{-1} (C.I 95%: 78.5- 1729.9µg mL^{-1}).

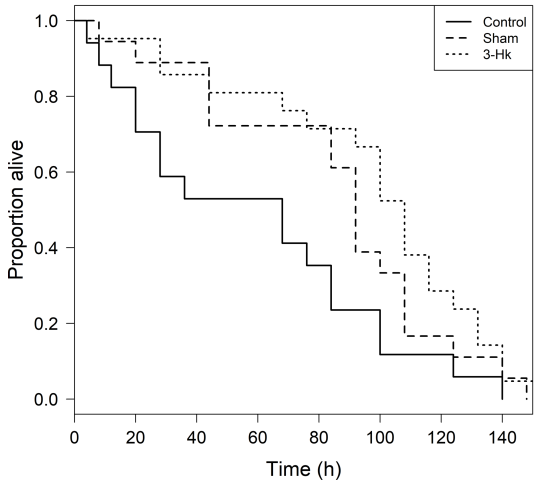


Figure 3. Survival time of *Hetaerina americana* males treated with 3-Hk, sham or control treatments.

The mitigation of 3-Hk toxic effects by deposition of ommochromes in RWS was observed in males treated with the LC_{50} of 3-Hk, since they obtained similar survival to sham males ($z=1.38$, $P=0.167$; Fig. 3); control males survived less than sham ($z=2.33$, $P=0.020$) or 3-Hk treated males ($z=3.71$, $P<0.001$), possibly because distilled water, the vehicle for experimental injections rehydrated both, males injected with 3-Hk and sham males.

In combinations with this survival result, we found that males treated with 3-Hk had higher values of *Rc* than males from the control or the sham treatments (Figure 4). Moreover, there was a significant effect of treatments in interaction with survival time and in interaction with RWS area (Sup. Table 1). The interaction between treatment and survival shows that while red chroma was positively related to survival time in control males, red chroma was negatively related to survival time in sham males, and there was no relationship for 3-Hk treated males (see supplementary figure S4a). The interaction between treatment and RWS area indicated that even though RWS area was positively related to red chroma in all treatments, the slope was steeper in sham males than in control or 3-Hk treated males (see supplementary figure S4b).

On the other hand, mean values of *Rf* showed a slight difference between groups, with higher values in 3-Hk-treated males (1.09±0.17) than sham (0.79±0.18) and control males (0.84±0.20). Nevertheless, the only statistically significant selected predictor for *Rf* was RWS area, as males with larger red areas had higher values of *Rf* ($F_{1,28}=19.0$, $P<0.001$; see supplementary figures S5a, S5b).

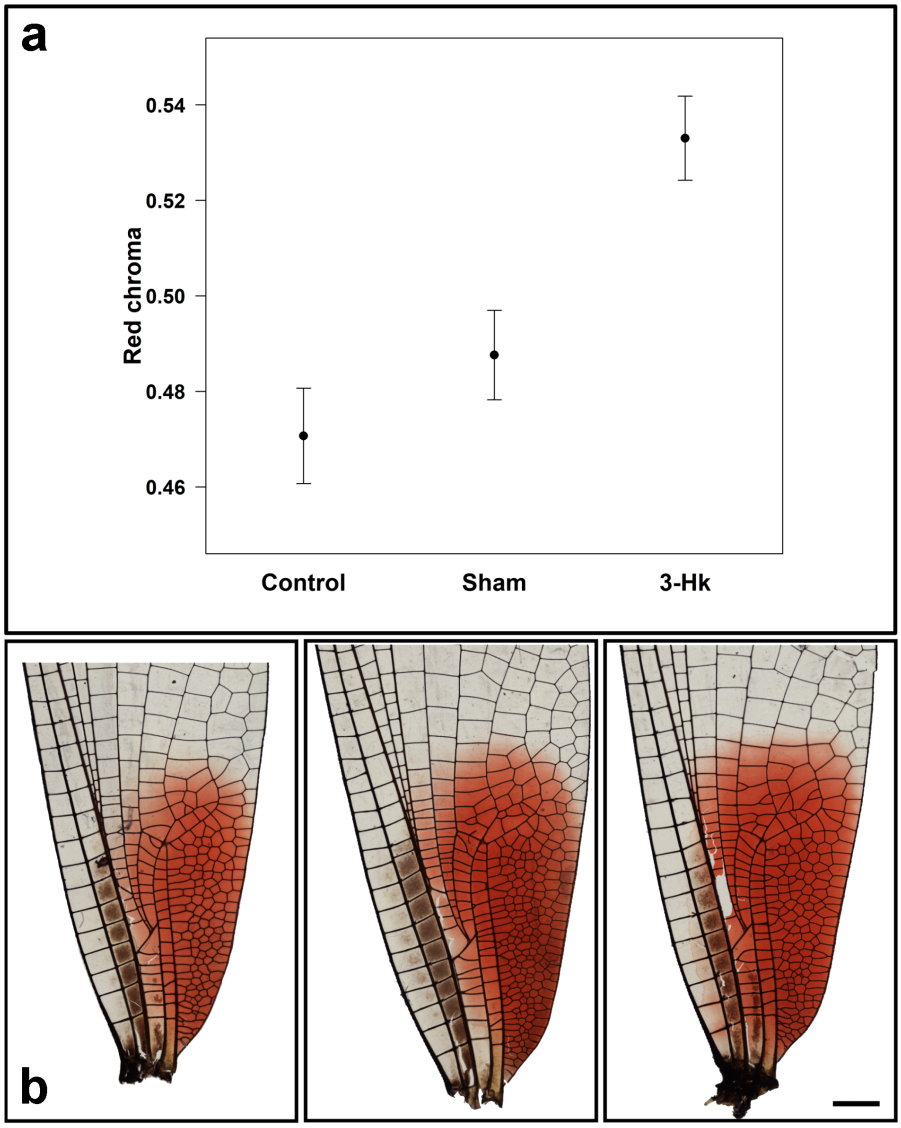


Figure 4. (a) Red chroma from RWS of *Hetaerina americana* males. Adult males injected with the toxic metabolite 3-Hk had higher values of red chroma than sham (i. e. PBS injected), and control (i. e. non-manipulated) males. (b) Bright field tile scan images of control, sham and 3-Hk treated males. Although the distribution of the wing veins is the same for all groups, qualitatively 3-Hk treatment shows stronger, diffuse red staining within the wing when compared with control and sham treatments. Scale bar: 1 mm.

Discussion

Ommochrome pigments were confirmed through all four of the biochemical properties we evaluated of the wings. Also, in support of this determination, TLC and spectral behaviour analyses indicated that xanthommatin and its reduced form, di-hydroxanthommatin, are the autofluorescent ommochromes distributed along the wing spot veins (as shown by confocal microscopy; Fig.2d and 2e). It is possible that ommatin D or rhodommatin are also present but that our pigment extraction method caused these pigments to spontaneously degrade into xanthommatin (Linzen 1974; Nijhout 1997). TLC also revealed the presence of 3-Hk in the RWS. The participation of 3-Hk as a reddish pigment in insects has been previously reported in red wing regions of lepidopterans (Nijhout 1997) and in the cuticle of *Bombyx mori* (Meng *et al.* 2009). Nonetheless, its presence in *H. americana* wings was so dim that a slight band was hardly discernible (Fig.2b).

To our knowledge this is the first time that ommochromes have been observed by confocal microscopy techniques, taking advantage of the ommochromes' property of autofluorescence when excited at a certain wavelength (561nm). This approach to reveal insect ommochromes could be complementary with other microscopy techniques previously used to observe these pigments, such as electronic microscopy, which has actually allowed the identification of the special organelles where ommochromes are produced, known as ommochromasomes (Figon & Casas 2019).

In odonates, ommochromes have been found in the cuticle of some members of the dragonfly genera *Sympetrum* and *Crocothemis,* where these compounds are related to sexual dimorphism, maturation patterns and antioxidant capacity (Futahashi *et al.* 2012).

Ommochromes in other taxa are also versatile pigments involved in several functions and not only colour production. Ommochromes are found in the eyes of insects (Figon & Casas 2019) and to a lesser extent, in other structures such as the wings of some lepidopterans (Nijhout 1997). Functionally, ommochromes are crucial in processing visual information in the insect compound eye (Linzen 1974), and they are suitable for transporting electrons or reacting with oxidants, reducers and free radicals (Needham 1974). Notwithstanding, as evidenced by our survival experiment, tryptophan and its metabolites (including 3-Hk) are toxic at high concentrations in all insects (Linzen 1974; Manoukas 1981). This dangerous situation can occurs due to important phenotypic changes during insects' life cycles, for example, during molting (Linzen 1974; Manoukas 1981). Ommochromes are also thought to be end-products of tryptophan detoxification (Linzen 1974). While some of these hemimetabolous insects, such as locusts, get rid of the toxic tryptophan by converting it to ommochromes and excreting it in faeces (Chapman 1998) storage of toxic byproducts in the form of ommochromes could be of particular relevance for detoxification in insects lacking pupation (i.e. hemimetabolous insects) such as our study animal and allied taxa. Nevertheless, this idea needs further testing.

Despite that our survival experiment shows that 3-Hk impairs survival when we estimated the LC_{50}, this tryptophan metabolite affected the expression of the sexual trait in our animal model, resulting in an increment of red pigmentation in wings of male treated with 3-Hk compared to Control and Sham males. This result in combination with the lack of survival differences between Sham and 3-Hk males support the idea that wing ommochromes may mitigate the toxicity of 3-HK. The increase in red pigmentation

found in males treated with 3-Hk can be explained by several enzymatic and spontaneous reactions that may participate in a few metabolic processes, such as kynurenine-3-monooxygenase, which takes place during 3-Hk formation, or phenoxazone synthase, and has a role in ommochrome formation. Nonetheless, the physiological and molecular mechanisms that underlie the metabolic pathways from tryptophan metabolites to ommochromes are poorly known in biochemical terms to clarify ommochrome biosynthesis (Figon & Casas 2019). Interestingly, our experimental manipulation with 3-Hk did not impact the area of the RWS, which correlates with male energetic condition during aerial contests over mating territories (Grether 1997; Contreras-Garduno *et al.* 2008). Thus, one explanation for our results is that by making the spot appear red, serves as the information signal that a territorial male need to convey to his conspecific and heteroespecific rivals during territorial tenure. This is compatible with a previous experiment in which the red spot was manipulated to appear blue (without manipulating spot area) in male territory holders (González-Santoyo *et al.* 2014). This change elicited high levels of aggression by rivals towards "blue-spotted" males, showing that it is red coloration but not spot area alone that is perceived as a the first signal of territoriality (González-Santoyo *et al.* 2014).

The honest signalling theory indicates only individuals in good condition (e.g. healthy) are able to afford the costs of generating and maintaining sexual signals (Zahavi 1975; Hamilton & Zuk 1982). Food, parasites and free radicals are the main drivers of these signals (Hamilton & Zuk 1982; Sheldon & Verhulst 1996; Montoya *et al.* 2016; Olzer *et al.* 2018). In this sense, our study indicates that ommochrome-pigmented sexual traits in *H. americana* males could act as honest indicator of nutritional condition, which is

correlated with excretion ability. According to this, only males that acquire sufficient protein in their diets to require tryptophan detoxification and are able to use an effective detoxification mechanism to convert and excrete it as red ommochrome pigment will produce sufficient amounts of metabolic products to develop the red signal, which indicates this quality to rivals, and therefore gives them an advantage in acquiring and maintaining a mating territory. In this sense, the detoxification mechanism could have initially evolved because the production of ommochromes was an effective mechanism for dealing with metabolic waste products, and then became co-opted by sexual selection when these ommochromes were allocated to wings and indicated male nutritional condition to rivals. It is not that rivals assess the detoxification ability, but rather how much energy a rival has to utilize during aerial contests for territories. Given these metabolic processes and sexual selection mechanisms, individuals cannot "cheat" the system. This hypothesis—which we call here detoxifying ability signalling—should be tested in other pigments that are used in sexual selection contexts (one example is the case of butterfly pteridines (Tigreros *et al.* 2014)). Interestingly, ommochromes in their reduced form are important antioxidants (Futahashi 2016) and may combat oxidative stress, a situation that was recently shown in our study species (Martínez-Lendech *et al.* 2018). Thus, by reducing ommochrome toxicity, males may also benefit in terms of dealing with oxidative stress. This three-fold function – detoxification, antioxidant, and sexual signalling – provide support for a metabolic efficiency mechanism that could be informed through the expression of these colourful pigments.

Acknowledgements: We are grateful to Ana Rivas-Caicedo for her advice on the microscopy techniques implemented in the present study. We thank Roberto Arreguín Espinosa to his advice on pigment determination. We thank Fernanda Baena for helpful discussion and fieldwork. We also thank Lynna Marie Kiere for her style revision and thoughtful comments, and Osiris Gaona for helpful discussion. This project was funded by UNAM-PAPIIT [grant numbers IA207019 and IN204610] and CONACYT Ciencia Básica [grant number 241744].

References:

Abramoff, M., Magalhaes, P. & Ram, S. (2004). Image processing with ImageJ. *Biophotonics Int.*, 11, 36–42.

ANDERSSON, M. (2019). Sexual Selection: *Sex. Sel.*, 433–444.

Bolognese, A., Liberatore, R., Riente, G. & Scherillo, G. (1988). Oxidation of 3-hydroxykynurenine. A reexamination. *J. Heterocycl. Chem.*, 25, 1247–1250.

Cerstiaens, A., Huybrechts, J., Kotanen, S., Lebeau, I., Meylaers, K., De Loof, A., *et al.* (2003). Neurotoxic and neurobehavioral effects of kynurenines in adult insects. *Biochem. Biophys. Res. Commun.*, 312, 1171–1177.

Chapman, R. (1998). *The Insects: Structure and Function*. 4th edn. Cambridge University Press, New York.

Cochran, D. (1975). *Insect biochemistry and function*. Springer-Science business media, New York.

Connolly, K., Burnet, B. & Sewell, D. (1969). Selective Mating and Eye Pigmentation: An Analysis of the Visual Component in the Courtship Behavior of *Drosophila melanogaster*. *Evolution (N. Y).*, 23, 548.

Contreras-Garduno, J., Buzatto, B.A., Serrano-Meneses, M.A., Najera-Cordero, K. & Cordoba-Aguilar, A. (2008). The size of the red wing spot of the American rubyspot as a heightened condition-dependent ornament. *Behav. Ecol.*, 19, 724–732.

Contreras-Garduño, J., Canales-Lazcano, J. & Córdoba-Aguilar, A. (2006). Wing pigmentation, immune ability, fat reserves and territorial status in males of the rubyspot damselfly, *Hetaerina americana*. *J. Ethol.*, 24, 165–173.

Córdoba-Aguilar, A. & González-Tokman, D.M. (2014). The Behavioral and Physiological Ecology of Adult Rubyspot Damselflies (*Hetaerina*, Calopterygidae, Odonata). *Adv. Study Behav.*

Crawley, M.J. (2012). *The R Book Second Edition*. 2nd edn. Wiley, Sussex, U.K.

Figon, F. & Casas, J. (2019). Ommochromes in invertebrates: biochemistry and cell biology. *Biol. Rev.*, 94, 156–183.

Futahashi, R. (2016). Color vision and color formation in dragonflies. *Curr. Opin. Insect Sci.*, 17, 32–39.

Futahashi, R., Kurita, R., Mano, H. & Fukatsu, T. (2012). Redox alters yellow dragonflies into red. *Proc. Natl. Acad. Sci. U. S. A.*, 109, 12626–12631.

González-Santoyo, I., González-Tokman, D.M., Munguía-Steyer, R.E. & Córdoba-Aguilar, A. (2014). A mismatch between the perceived fighting signal and fighting ability reveals survival and physiological costs for bearers. *PLoS One*, 9, e84571–e84571.

González-Tokman, D.M., Munguía-Steyer, R., González-Santoyo, I., Baena-Díaz, F.S. & Córdoba-Aguilar, A. (2012). Support for the immunocompetence handicap hypothesis in the wild: Hormonal manipulation decreases survival in sick damselflies. *Evolution (N. Y).*, 66.

Grether, G.F. (1997). Survival cost of an intrasexually selected ornament in a damselfly. *Proc. R. Soc. London. Ser. B Biol. Sci.*, 264, 207–210.

Hamilton, M.A., Russo, R.C. & Thurston, R. V. (1977). Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. *Environ. Sci. Technol.*, 11, 714–719.

Hamilton, W. & Zuk, M. (1982). Heritable true fitness and bright birds: a role for parasites? *Science (80-.).*, 218, 384–387.

Hooper, R.E., Tsubaki, Y. & Siva-Jothy, M.T. (1999). Expression of a costly, plastic secondary sexual trait is correlated with age and condition in a damselfly with two male morphs. *Physiol. Entomol.*, 24, 364–369.

Insausti, T.C. & Casas, J. (2008). The functional morphology of color changing in a spider: development of ommochrome pigment granules. *J. Exp. Biol.*, 211, 780–789.

Kayser, H. (1979). Ommochrome formation and kynurenine excretion in *Pieris brassicae*: Relation to tryptophan supply on an artificial diet. *J. Insect Physiol.*, 25, 641–646.

Linzen, B. (1974). The Tryptophan → Ommochrome Pathway in Insects. *Adv. In Insect Phys.*

Manoukas, A. (1981). Effect of excess levels of individual amino acids upon survival, growth and pupal yield of *Dacus oleae* (Gmel.) larvae. *Zeitschrift für Angew. Entomol.*, 91, 309–315.

Martínez-Lendech, N., Golab, M.J., Osorio-Beristain, M. & Contreras-Garduño, J. (2018). Sexual signals reveal males' oxidative stress defences: testing this hypothesis in an invertebrate. *Funct. Ecol.*, 32, 937–947.

McGraw, K.J. (2005). The antioxidant function of many animal pigments: are there consistent health benefits of sexually selected colourants? *Anim. Behav.*, 69, 757–764.

Meng, Y., Katsuma, S., Mita, K. & Shimada, T. (2009). Abnormal red body coloration of the silkworm, *Bombyx mori*, is caused by a mutation in a novel kynureninase. *Genes*

to Cells, 14, 129–140.

Monaghan, P., Metcalfe, N.B. & Torres, R. (2009). Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. *Ecol. Lett.*, 12, 75–92.

Montoya, B., Valverde, M., Rojas, E. & Torres, R. (2016). Oxidative stress during courtship affects male and female reproductive effort differentially in a wild bird with biparental care. *J. Exp. Biol.*, 219, 3915–3926.

Needham, A. (1974). *The Significance of Zoochromes*. Springer, Berlin, Heidelburg, New York.

Nijhout, H. (1997). Ommochrome pigmentation of the linea and rosa seasonal forms of *Precis coenia* (Lepidoptera: Nymphalidae). *Arch. Insect Biochem. Physiol.*, 36, 215–222.

Ogawa, H. & Hasegawa, K. (1980). Kynureninase and its activity during metamorphosis of the silkworm, *Bombyx mori*. *Insect Biochem.*, 10, 589–593.

Olzer, R., Ehrlich, R.L., Heinen-Kay, J.L., Tanner, J. & Zuk, M. (2018). Reproductive behavior. In: *Insect Behavior: from mechanisms to ecological and evolutionary consequences*. pp. 189–202.

Oxenkrug, G.F. (2010). The extended life span of *Drosophila melanogaster* eye-color (white and vermilion) mutants with impaired formation of kynurenine. *J. Neural Transm.*, 117, 23–26.

Oxenkrug, G.F., Navrotskaya, V., Voroboyva, L. & Summergrad, P. (2011). Extension of life span of *Drosophila melanogaster* by the inhibitors of tryptophan-kynurenine metabolism. *Fly (Austin).*, 5, 307–309.

Plaistow, S. & Siva-Jothy, M. (1996). Energetic constraints and male mate-securing tactics in the damselfly *Calopteryx splendens* xanthostoma (Charpentier). *Proc. R. Soc. London. Ser. B Biol. Sci.*, 263, 1233–1239.

Riou, M. & Christidès, J.-P. (2010). Cryptic Color Change in a Crab Spider (*Misumena vatia*): Identification and Quantification of Precursors and Ommochrome Pigments by HPLC. *J. Chem. Ecol.*, 36, 412–423.

von Schantz, T., Bensch, S., Grahn, M., Hasselquist, D. & Wittzell, H. (1999). Good genes, oxidative stress and condition-dependent sexual signals. *Proceedings. Biol. Sci.*, 266, 1–12.

Shamim, G., Ranjan, S.K., Pandey, D.M. & Ramani, R. (2014). Biochemistry and biosynthesis of insect pigments. *Eur. J. Entomol.*, 111, 149–164.

Sheldon, B.C. & Verhulst, S. (1996). Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends Ecol. Evol.*, 11, 317–321.

Stone, B.R. (2012). tsk: Trimmed Spearman-Karber Method.

Team, R.C. (2019). R: A Language and Environment for Statistical Computing.

Tigreros, N., Mowery, M.A. & Lewis, S.M. (2014). Male mate choice favors more colorful females in the gift-giving cabbage butterfly. *Behav. Ecol. Sociobiol.*, 68, 1539–1547.

Timmerman, S. & Berenbaum, M. (1999). Uric acid deposition in larval integument of black swallowtails and speculation on its possible functions. *J. Lepid. Soc.*, 53, 104–107.

Umebachi, Y. & Uchida, T. (1970). Ommochromes of the testis and eye of *Papilio xuthus*. *J. Insect Physiol.*, 16, 1797–1812.

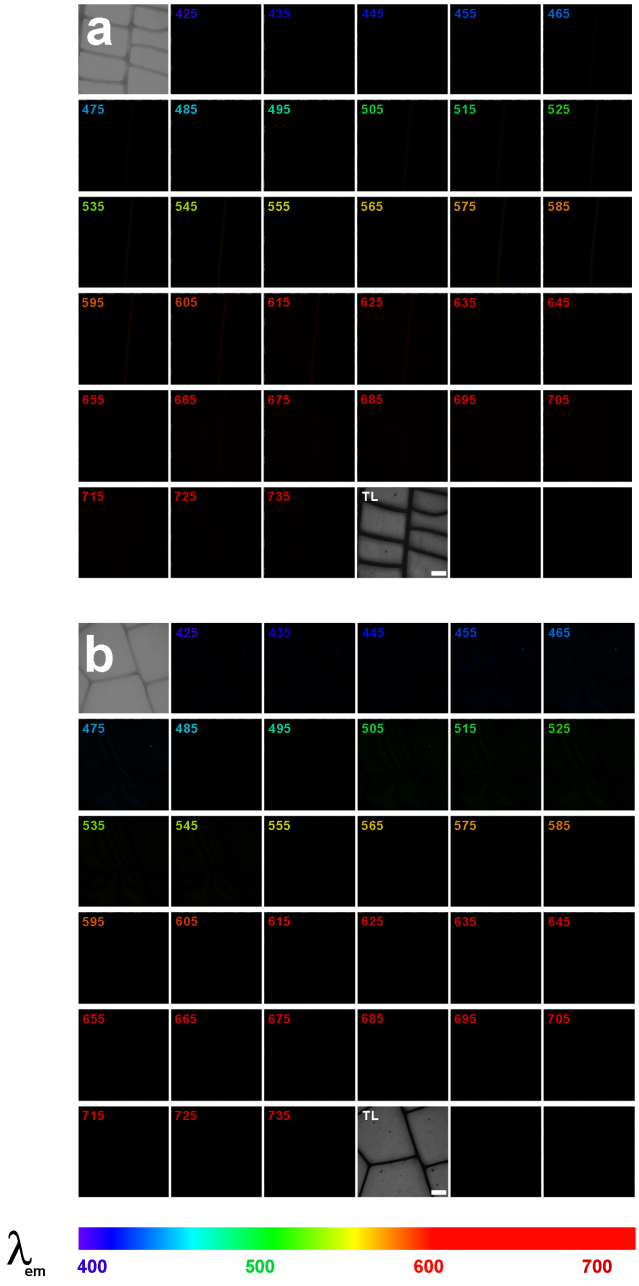
Vukusic, P., Chittka, L. & Chapman, R.F. (2013). Visual signals: color and light production. In: *The Insects:structure and function* (eds. Chapman, R.F., Simpson, S. & Douglas, A.). Cambridge University Press, New York, pp. 793–823.

Zahavi, A. (1975). Mate selection—A selection for a handicap. *J. Theor. Biol.*, 53, 205–214.

Zera, A.J. & Harshman, L.G. (2001). The physiology of life history trade-offs in animals. *Annu. Rev. Ecol. Syst.*, 32, 95–126.

Zuur, A.F., Ieno, E.N., Walker, N., Saveliev, A.A. & Smith, G.M. (2009). Mixed effects models and extensions in ecology with R. *Stat. Biol. Heal.*

Supplementary figures



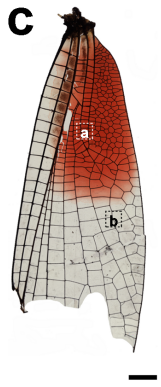


Figure S1. Fluorescence XYZ□ scans (425-735 nm) of *Hetaerina americana* wing excited at 405 nm. Maximum intensity projection images of (a) a sub-region of the red-pigmented area, and (b) a sub-region of the transparent area, depicted in (c). A relatively weak fluorescence intensity was found in wing veins of the anterior region from 505 to 725 nm (a). In contrast, no apparent signal is present in wing veins but in the wing between 465-615 nm in the medial region of dragonfly wing (b). Scale bar: 100 □m.

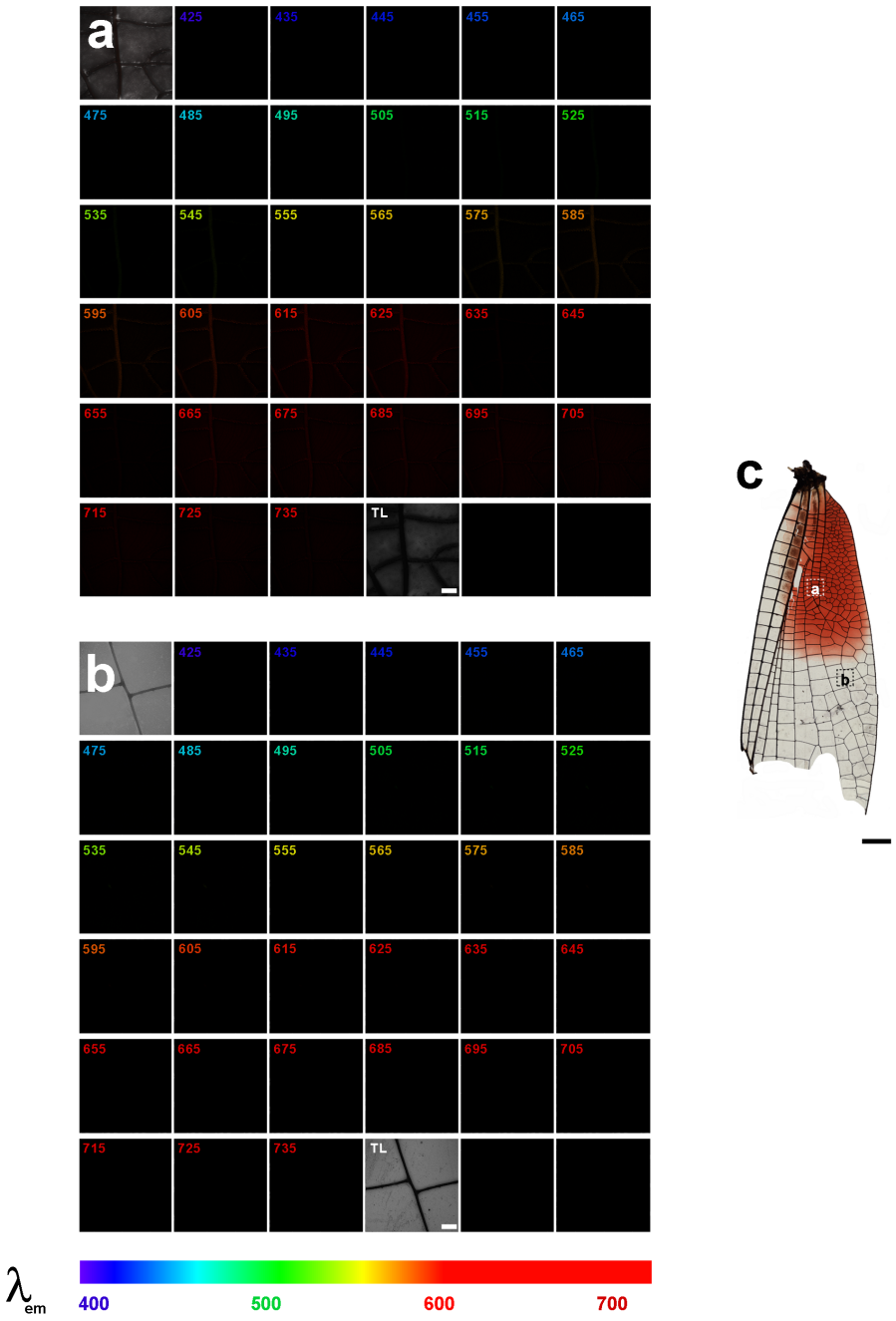


Figure S2. Fluorescence Zλ scans (425-735 nm) of *Hetaerina americana* wing excited at 488 nm. Maximum intensity projection images of (a) a sub-region of the red-pigmented area, and (b) a sub-region of the transparent area, depicted in (c). A relatively weak fluorescence intensity was found in the wing veins of the anterior region from 505 to 735 nm (a). In contrast, no apparent signal is present in the medial region of the damselfly wing (b). Scale bar: 100 μm.

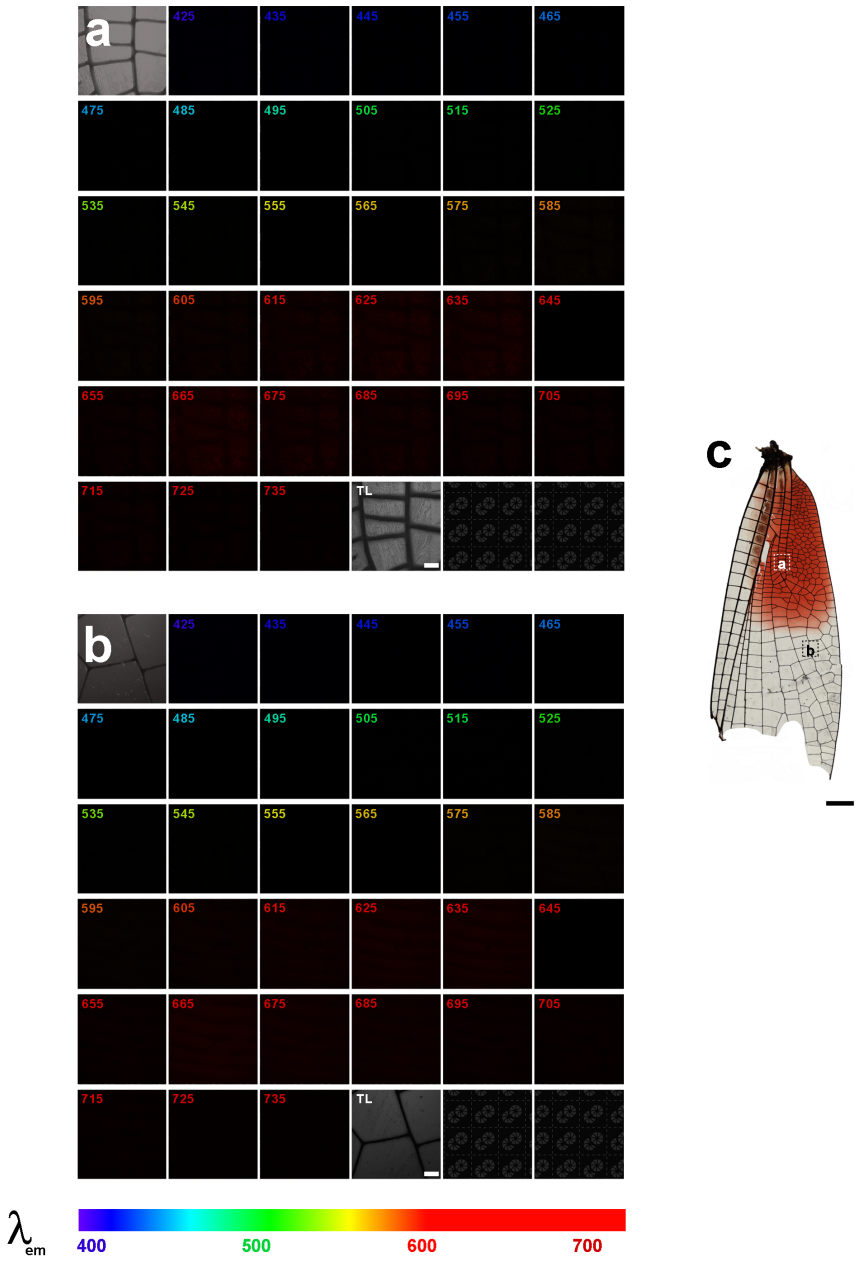


Figure S3. Fluorescence Zλ scans (425-735 nm) of *Hetaerina americana* wing excited at 646 nm. Maximum intensity projection images of (a) a sub-region of the red-pigmented area, and (b) a sub-region of the transparent area, depicted in (c). There was no apparent signal in wing veins but curiously a relatively weak fluorescence intensity was found in the wing of both anterior (a) and medial (b) regions from 575 to 735 nm. Scale bar: 100 μm.

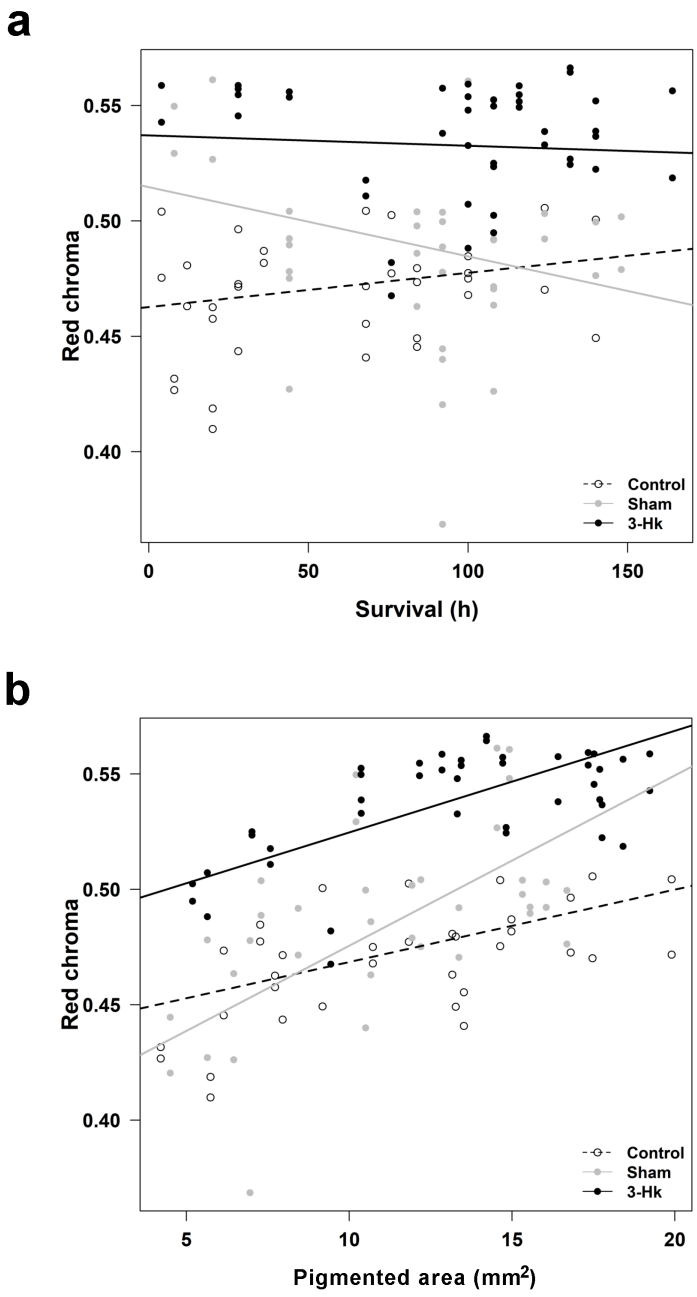


Figure S4 (a) Relationship between survival time and red chroma in each experimental treatment. While in males treated with 3-Hk(continuous black line) red chroma was not affected by survival time, control males (black dotted line) showed a positive relationship. In sham males (gray line), chroma was also affected by survival but in a negative direction. (b) Red chroma was also affected by RWS area. All treatments showed a positive relationship, but with different slopes.

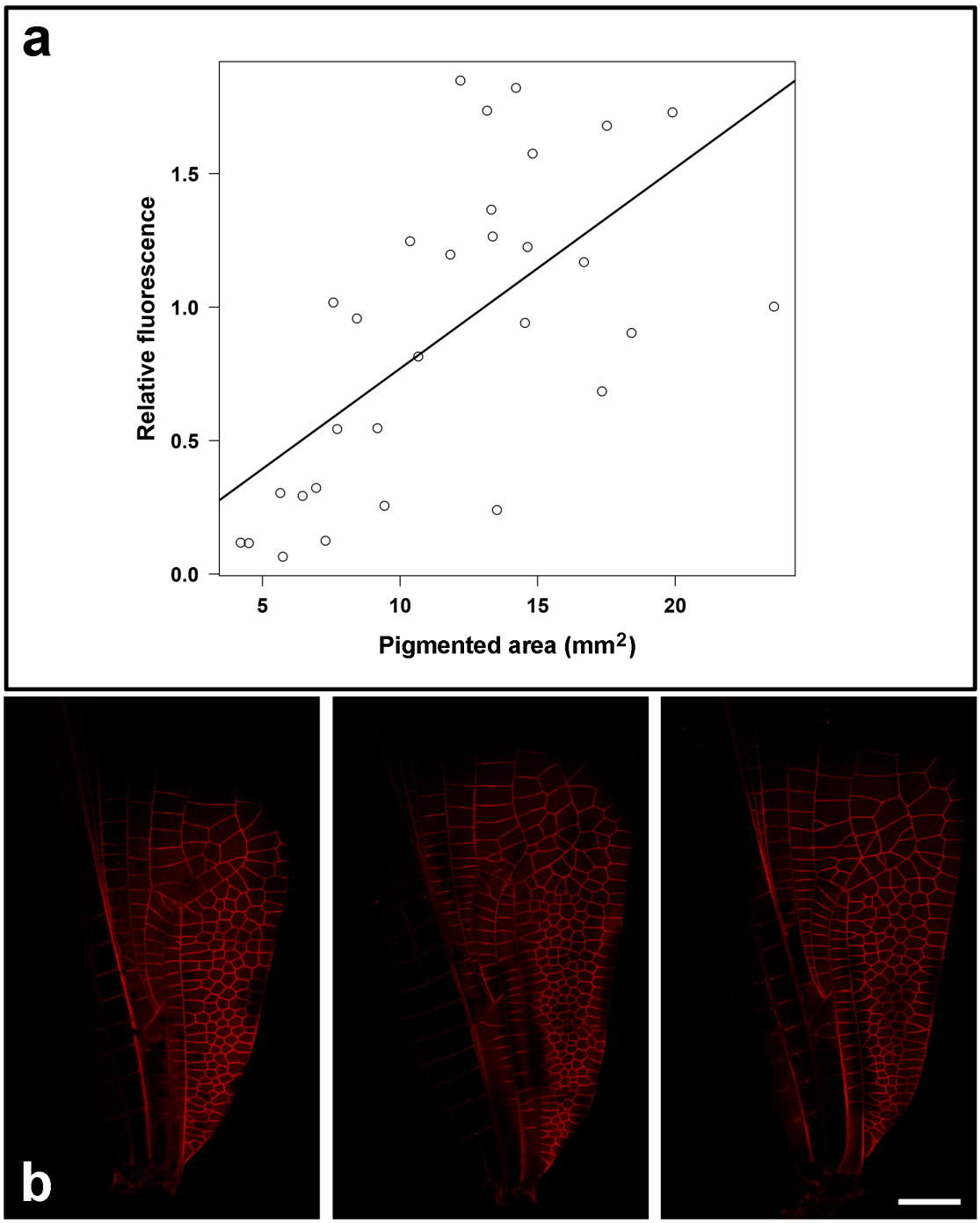


Figure S5 (a) Fluorescence XYZλ scans (425-735 nm) of *H. americana* wings excited at 561 nm of control, sham and 3-Hk males. Although 3-Hk males showed higher fluorescence values, the only significant predictor for this colour property was RWS area, which had a positive relationship in all treatments (b).

Suplementary tables

TABLE S1. Linear mixed effect model to explain variation in wing red chroma in *Hetaerina americana* males manipulated with 3-Hk, sham or control treatments. Significant predictors are shown in bold. L. Ratio=Likelihood ratio. NS=Not selected in the best supported model.

Effect on red chroma	L. Ratio	P-value
Treatment	69.07	**<0.001**
Survival time	8.97	**0.030**
Wing length	9.99	**0.019**
Pigmented area	43.42	**<0.001**
Treatment X Survival	8.29	**0.016**
Treatment X Wing length	NS	NS
Treatment X Pigmented area	8.43	**0.014**