What doesn't kill you makes you stronger: detoxification ability as an honest sexually selected signal.

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Abstract

Sexual selection maintains colourful signals that increase sexual attractiveness and dominance. Some sexually selected, colourful signals are pigments synthesized from ingested amino acids. The underlying metabolic pathways for these pigments often release toxic byproducts that can reduce individual survival. However, rather than discarding these otherwise harmful byproducts, animals may use them by integrating them into sexually selected traits. We tested this idea using males of the damselfly *Hetaerina americana*, which bear a red-pigmented wing spot that is sexually selected through male-male competition for mating territories. First, by using chromatography and confocal microscopy, we determined that the red wing spots are generated by ommochrome pigments derived from tryptophan metabolism. Second, we injected a group of males with the toxic precursor of these ommochromes, 3-hydroxy-kynurenine (3-Hk), confirming the toxicity of this compound in adult males. Finally, by using spectrophotometry and confocal microscopy, we showed that adult males injected with a LC_{50} of 3-Hk had more ommochromes in their wing spots than controls but similar survival, suggesting that the deposition of ommochrome pigment in the wing detoxifies the tryptophan metabolism process. Thus, we report for the first time that sexually selected pigmented signals involve the biochemical treatment of excreted compounds that could otherwise have lethal effects, a hypothesis we call “detoxifying ability signalling”. Our results provide new insights about the origin and maintenance of sexual signals, elucidating a mechanism for the evolution of honest indicators of quality 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 [1]. 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 [2]. This deposition creates the impression of bird droppings, which looks distasteful for predators, decreasing predation and thus increasing larval survival [2]. 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 [3]. 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 [4]. Interestingly, genes responsible for colouration interact pleiotropically with other genes that can be involved in important physiological functions such as thermoregulation, photoprotection,

83 desiccation resistance, immunoregulation, antioxidation and excretion of toxic
84 compounds that may result from metabolism and immune response [5–7]. Therefore,
85 pigmentation that reflects efficient metabolism may be subject to strong sexual selection
86 if they honestly reflect individual genetic and physiological condition [6,8].
87
88 Storage excretion as a mechanism of sexual pigmentation has not been discussed and
89 examined in detail despite providing a clear physiological and evolutionary explanation
90 of the origin and maintenance of many sexual signals. Storage excretion can be
91 particularly important when the synthesis of such pigments is involved in physiological
92 pathways that generate waste products that are difficult to eliminate or inactivate.
93 Moreover, the assumption that the trait provides honest information about the quality of
94 the bearer is upheld, since the use of toxic byproducts to generate visible colouration
95 could be doubly informative: pigments signal the bearer's ability to deal with toxic
96 metabolites that could otherwise impair their survival, as well as the genetic and/or
97 physiological condition since for example, because only individuals with better diets or
98 protein metabolism will produce a large enough amount of the byproducts to require
99 storage excretion of the pigments (as suggested by [6]).
100 Red pigments are common in several invertebrates, and most of their underlying
101 components are ommochromes [9]. In insects, ommochromes give colour to structures such
102 as the eyes and the wings of flies, butterflies and damselflies [10–12]. These red pigments
103 result from the metabolism of tryptophan, an essential amino acid that is consumed from
104 food [12,13]. Ommochrome pigments are synthetized from the tryptophan metabolite 3-
105 hydroxykynurenine (3-Hk), which together with tryptophan, are considered neurotoxic

106 compounds that cause paralysis, altered mating behaviour, aging and high mortality in
107 adult insects [14–17]. In fact, wild type flies fed with inhibitors of tryptophan-kynurenine
108 metabolism show increased survival compared to control flies [17]. 3-Hk is present at high
109 concentrations during metamorphosis when protein breakdown releases high quantities of
110 this tryptophan product [12]. Given the noxious effects of 3-Hk, an important way in which
111 insects can deal with excessive neurotoxic byproducts is via the synthesis of
112 ommochromes, which can be eliminated in the meconium during pupation in
113 holometabolous insects, in the excreta or by storage excretion in the form of cuticular
114 pigmentations, a hypothesis proposed by Linzen in 1974 and called "the tryptophan-
115 detoxification hypothesis" [5,9,18]. In fact, both 3-Hk and ommochrome pigments are found
116 at high concentrations in the meconium of lepidopterans, the waste product from the
117 pupal stage [19].
118 Ommochromes can function as sexually selected signals and may be considered honest
119 indicators of individual quality, since only well-fed individuals will accumulated high
120 enough amounts of these metabolites to develop conspicuous signals [20].
121 Here, we propose that ommochromes as component of male sexual traits can be used for
122 detoxification, providing a new mechanism of sexual signal production and fulfilling the
123 honesty principle. We tested these ideas using males of the rubyspot damselfly *Hetaerina*
124 *americana*. This species is a classic model in sexual selection studies: males bear a red
125 wing spot (RWS; Fig. 1a) whose size is an honest indicator of individual condition, and
126 which provides an advantage during male-male competition [21,22]. We (1) determined the
127 presence of ommochromes in the wing spot, (2) tested the toxic effect of 3-
128 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.

Results

All biochemical test 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 460 nm with relative absorbances of 2.49, 0.52 and 0.74 respectively. The red pigment dissolved in phosphate buffer (pH 7.0) displayed peaks at two different wavelengths: 235 and 429 nm 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 [23]. 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 [23]). We also found a slight third band (Fig. 1b), which may correspond to 3-Hk (Rf=0.52 [23]), although it was very faint.

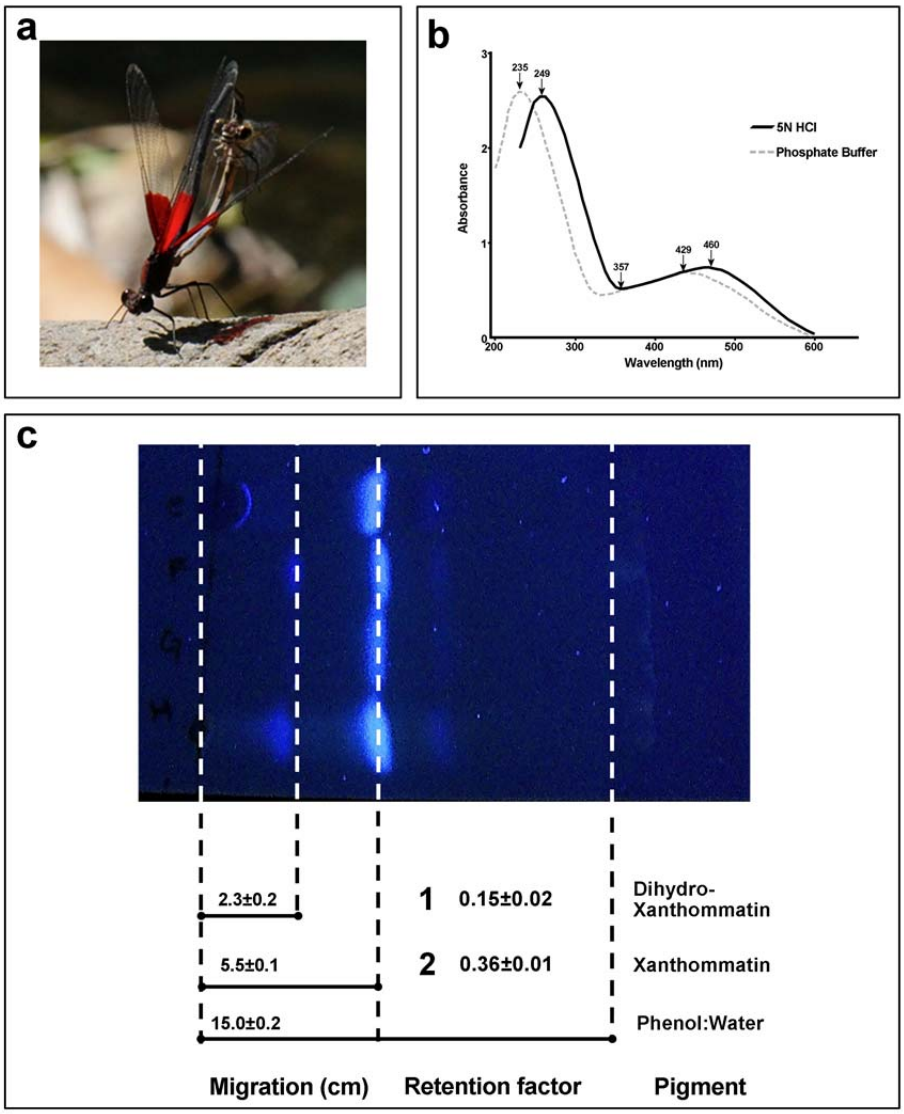


Figure 1. (a) *Hetaerina americana* male showing his red wing spot (RWS) during mating. (b) Spectral absorbance analysis showing the presence of ommochrome pigments in the RWS. The red powder dissolved in 5N HCl (solid black line) or phosphate buffer (dashed grey line) show a typical spectral behaviour 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 561 nm wavelength light. We excited separately with 405, 488, 561 and 647 nm wavelengths and found two main types of fluorescence: the most conspicuous corresponds to ommochrome pigments, which are distributed along the wing veins (Fig. 2a and d) and spread over the wing tissue in the red pigmented area (Fig. 2d and 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 1a, 1b and 1c). 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 1a, 1b and 1c) and shows a characteristic periodicity on its distribution pattern.

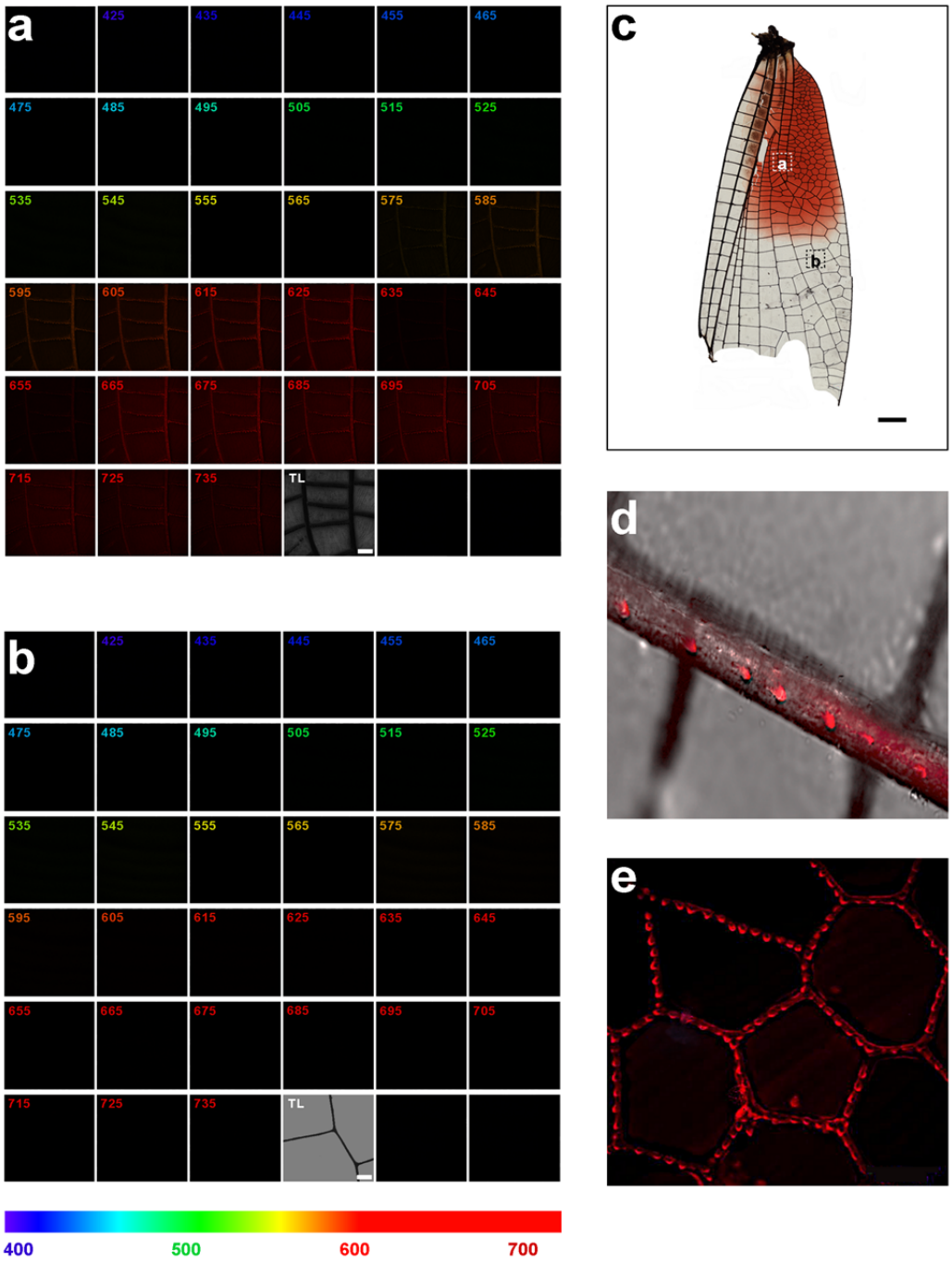


Figure 2. Fluorescence XYZλ scans (425-735 nm) of *H. americana* wings excited at 561 nm. Maximum intensity projection images of (a) a sub-region of the red-pigmented area, and (b) a sub-region of the transparent area, depicted in (c). Each frame in (a) and (b) represent a specific wavelength image, 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 100 µg mL^{-1}) to death for the highest concentrations (1000 and 10000 µg 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 (1 µg 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}).

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 ($z=1.38$, $P=0.167$; Figure 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. Survival was not dependent on body size, since this variable was not retained by the best-supported model selected by Akaike Information Criterion methods (AIC).

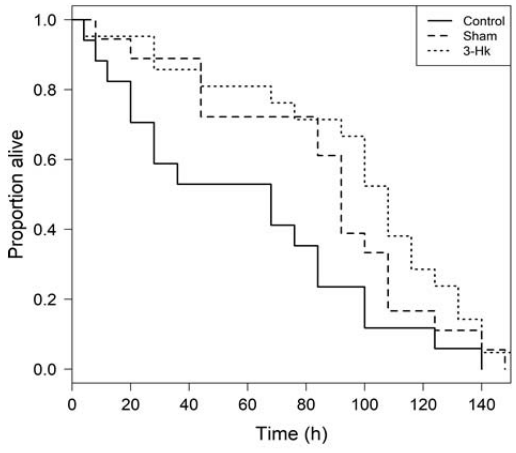
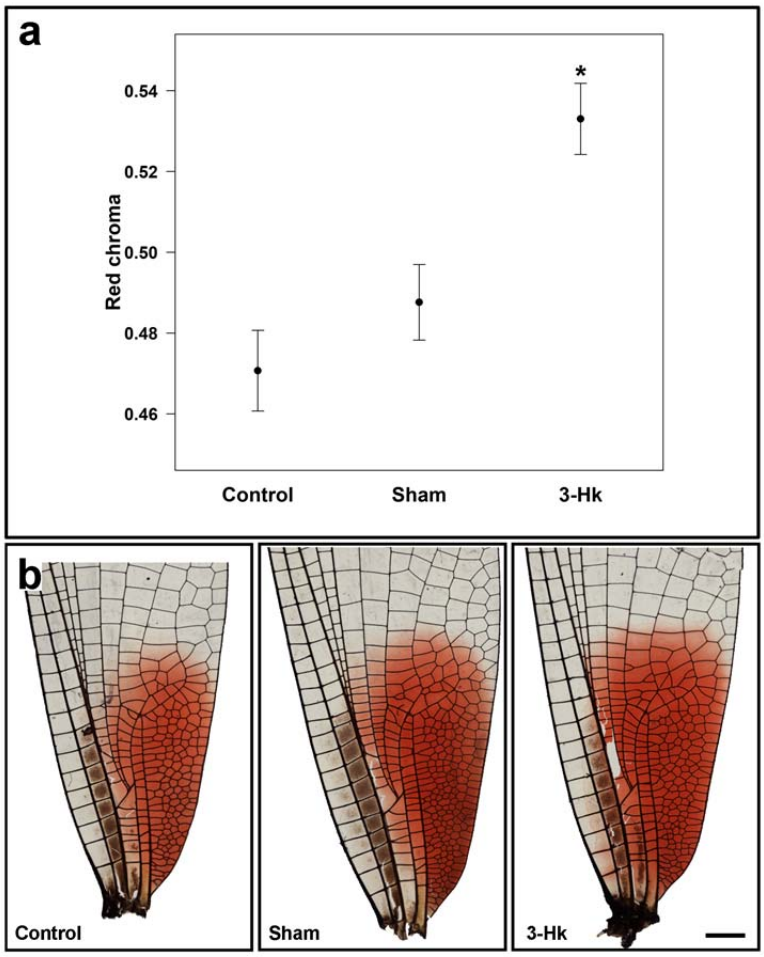


Figure 3. Survival time of *Hetaerina americana* males treated with 3-Hk, sham or control treatments.

In combinations with these results, 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 (showed by the best AIC model) of treatments in interaction with survival time and in interaction with the RWS area (Sup. Table1). 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 2a). 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 2b).



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216 **Figure 4.** (a) Red chroma from RWS of *Hetaerina americana* males. Adult males
217 injected with the toxic metabolite 3-Hk had higher values of red chroma than sham (i. e.
218 Distiller water injected), and control (i. e. non-manipulated) males. (b) Bright field tile
219 scan images of control, sham and 3-Hk treated males. Although the distribution of the
220 wing veins is the same for all groups, qualitatively 3-Hk treatment shows stronger,
221 diffuse red staining within the wing when compared with control and sham treatments.
222 Scale bar: 1 mm.
223 ***** Significance at level of $p < 0.01$
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227 On the other hand, mean values of *Rf* showed a slight difference between groups, with
228 higher values in 3-Hk-treated males (1.09±0.17) than sham (0.79±0.18) and control males
229 (0.84±0.20). Nevertheless, the only statistically significant selected predictor for *Rf* was
230 RWS area, as males with larger red areas had higher values of *Rf* ($F_{1,28}$=19.0, P<0.001;
231 see supplementary figures 3a and 3b).

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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 [9,23]. TLC also revealed the presence of 3-Hk in the RWS. The participation of 3-Hk as a reddish pigment in insects is possible, as it was previously reported in red wing regions of lepidopterans [23] and in the cuticle of *Bombyx mori* [24]. Nonetheless, its presence in *H. americana* wings was so dim that a slight band was hardly discernible (Fig.1b).

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 [18].

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 [10]. Ommochromes in other taxa are also versatile pigments involved in several functions and not only colour

257 production. Ommochromes are found in the eyes of insects [18] and to a lesser extent, in
258 other structures such as the wings of some lepidopterans [23]. Functionally, ommochromes
259 are crucial in processing visual information in the insect compound eye [9], and they are
260 suitable for transporting electrons or reacting with oxidants, reducers and free radicals [25].
261 Notwithstanding, as evidenced by our survival experiment, tryptophan and its metabolites
262 (including 3-Hk) are toxic at high concentrations in all insects [9,26]. This dangerous
263 situation can occurs due to important phenotypic changes during insects' life cycles, for
264 example, during molting [9,26]. Ommochromes are also thought to be end-products of
265 tryptophan detoxification [9]. In fact, both 3-Hk and ommochrome pigments are found at
266 high concentrations in the meconium of holometabolous insects, a waste product from the
267 pupal stage [19]. While some of these hemimetabolous insects, such as locusts, get rid of
268 the toxic tryptophan by converting it to ommochromes and excreting it in faeces [12]
269 storage of toxic byproducts in the form of ommochromes could be of particular relevance
270 for detoxification in insects lacking pupation (i.e. hemimetabolous insects) such as our
271 study animal and allied taxa. Nevertheless, this idea needs further testing.
272 Despite that our survival experiment shows that 3-Hk impairing survival when we
273 estimated the LC_{50}, this tryptophan metabolite affected the expression of the sexual trait
274 in our animal model, resulting in an increment of red pigmentation in wings of male
275 treated with 3-Hk compared to Control and Sham males. This result in combination with
276 the lack of survival differences between Sham and 3-Hk males may support the idea that
277 wing ommochromes may mitigate the toxicity of 3-HK. The increase in red pigmentation
278 found in males treated with 3-Hk can be explained by several enzymatic and spontaneous
279 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 [18]. Interestingly, our experimental manipulation with 3-Hk did not impact the area of the RWS. Previous works in our study species have provided evidence that this feature correlates with male energetic condition during aerial contests over mating territories [21,27]. 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 [28]. This change elicited high levels of aggression by rivals towards "blue spot" males, showing that it is red coloration but not spot area alone that is perceived as a the first signal of territoriality [28].

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 [29,30]. Food, parasites and free radicals are the main drivers of these signals [30–33]. 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

303 therefore gives them an advantage in acquiring and maintaining a mating territory. In this
304 sense, the detoxification mechanism could have initially evolved because the production
305 of ommochromes was an effective mechanism for dealing with metabolic waste products,
306 and then became co-opted by sexual selection when these ommochromes were allocated
307 to wings and indicated male nutritional condition to rivals. It is not that rivals assess the
308 detoxification ability, but rather how much energy a rival has to utilize during aerial
309 contests for territories. Given these metabolic processes and sexual selection
310 mechanisms, individuals cannot "cheat" the system. This hypothesis—which we call here
311 detoxifying ability signalling—should be tested in other pigments that are used in sexual
312 selection contexts (one example is the case of butterfly pteridines [34]). Interestingly,
313 ommochromes in their reduced form are important antioxidants [35] and may combat
314 oxidative stress, a situation that was recently shown in our study species [36]. Thus, by
315 reducing ommochrome toxicity, males may also benefit in terms of dealing with
316 oxidative stress. This three-fold function – detoxification, antioxidant ability, and sexual
317 signalling – provide support for a metabolic efficiency mechanism that could be informed
318 through the expression of theses colourful pigments.
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Methods

Animals

Adult male *H. americana* were captured in the riverine areas of the Tetlama River, Morelos, Mexico (18° 45' 55''N, 99° 14' 45'' W) with a butterfly net between 10.00 and 16.00h, the time at which males are most active [37]. Males were aged according to three visual categories [38]: 1) juvenile males, bearing soft, dorso-ventrally flexible, undamaged wings with a small and incompletely formed RWS; 2) sexually mature males, with less flexible wings, fully fixed red wing spot and no signs of thoracic pruinescence (Fig. 1a), and 3) old males, with abundant thoracic pruinescence and inflexible, damaged or worn wings with the 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 [28], we only used sexually mature males.

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

1.1.Pigment extraction

To determine whether ommochrome pigments are present in the RWS, we sacrificed 30 adult males by freezing for 20 minutes at -20°C soon after collection. These animals were then dried in a drying oven (Heratherm-ThermoFisher) for 24 hours at 30°C. The four wings were removed from each dried male, but only the RWS from the forewings was used. To extract the red pigment from the forewings we used the methodology proposed by Nijhout[23], with some modifications from Riou and Christidès [11]. All spots were pooled and homogenized in methanol at 4°C and centrifuged for 5 minutes at 14,000 g. Then, the precipitate was washed twice with 99% methanol and three times with ethyl ether by

repeated suspension and centrifugation (5 minutes at 14,000g). After the ethyl ether was evaporated from the final precipitate, 4 ml of acidic methanol (100% methanol plus 0.5% hydrochloric acid) were added to the residue and the suspension was centrifuged for 10 minutes at 14,000 g. After centrifugation, the supernatant was concentrated to one-quarter of its volume using a vacuum concentrator (SpeedVac-Thermo®, Mod. ISS110). We added 2 ml of distilled water and SO gas to this solution and, after incubating overnight for 8 hours at 2 °C, the red pigment obtained was precipitated by centrifugation for 10 min at 14,000 g and washed with a 2 mL 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.

1.2.*Pigment determination*

We performed four complementary methods to evaluate presence of ommochromes via their biochemical properties: Redox behaviour, spectral absorbance, thin layer chromatography and autofluorescence by confocal microscopy.

1.2.1. Redox behaviour and spectral absorbance

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 [9]. Hence, we evaluated whether our red powder changed colour when it was subjected to reductant or oxidant conditions. The red spot powder was dissolved in phosphate buffer (0.067 M, pH 8.5) containing 10 µL of 1% ascorbic acid as a reductant component to observe the resulting colour. Next, we added 10 µL of 1% $NaNO_2$ as an

372 oxidant component and the colour changes were observed. Finally, we again added 10 µL
373 of 1% ascorbic acid to test whether the colour reverted to its previous state.
374 Complementary with this Redox behaviour, all ommochromes have a particular spectral
375 behaviour, which is characterized by 2 to 4 spectral peaks, distributed throughout the UV
376 and visible ranges of the spectrum [9,39]. These spectral peaks have been traditionally
377 characterized when ommochromes are dissolved in both phosphate buffer (pH 7.0) and
378 5N HCl [23,40]. Therefore, to determine whether our red powder showed the typical spectral
379 behaviour of ommochromes, we measured the spectral peaks when the red powder was
380 dissolved in these solvents. To accomplish this, we prepared two different solutions using
381 20 µg of the red powder and 100 µL of phosphate buffer or 5N HCl. Given that spectral
382 peaks of the ommochrome xanthommatin are the best characterized by previous studies
383 [9,18], we processed the samples to convert any ommochromes that could be present in the
384 RWS powder (e.g. ommatin D or dihydroxanthommatin) into xanthommatin. This
385 conversion allows us to ensure the specificity in the resulting peaks. For this purpose, in
386 the case of the phosphate buffer, the solution was refrigerated at 4°C for 12 hours, while
387 the 5N HCl solution was kept at 25 °C in a drying oven (Heratherm-ThermoFisher) for 5
388 days [40]. After this period, both solutions were centrifuged (14,000 g for 5 min) and the
389 absorbance of the supernatant was measured from 220-600 nm wavelength using a
390 spectrophotometer (Hitachi, Mod. U-3900). Finally, we compared the spectral peaks
391 obtained in both solvents with those described by Nijhout [23], who used the same solvents
392 to evaluate spectral peaks of ommochromes extracted from the ventral hind wing pattern
393 of the *linea* and *rosa* forms of the lepidopteran *Precis coenia* [23].
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395 *1.2.2 Thin layer chromatography (TLC)*

396 TLC is a technique that allows the separation and characterization of ommochromes from
397 insects when these pigments are present [23]. For this purpose, we used a 10x20 cm silica
398 gel plate (Merck, Darmstadt, Germany, Silica Gel 60 F254). This plate was divided into 4
399 channels (2 cm between channels). The division was made from the narrower side of the
400 plate (10 cm) and a thin base line was drawn with a pencil at 1 cm from the bottom of the
401 plate in each channel. At this base line, we added 10 µL from the wing spot red powder
402 that was previously diluted in acidic methanol (1 mL [23]), and the plate was left in a
403 vertical position in a chromatographic chamber (Aldrich). The chamber was previously
404 saturated with a developing solvent of phenol:water (3:1 by volume) using a small piece
405 of filter paper (JM 3639, JoyLab) around the chamber. The silica gel plate was left inside
406 the chamber for 2 hours to allow the complete separation of pigments. After this time, we
407 identified the bands formed along the plate in both ultraviolet and visible light spectrum
408 using an UV/White transilluminator (Safe Imager 2.0, Invitrogen). Then, we measured
409 the distance (mm) at which these bands were located with respect to the base line as well
410 as the distance travelled by the developing solvent in each channel, and we obtained an
411 average value for each band and for the solvent. These distances were used to obtain the
412 retention factor (R_f) for each pigment found. Finally, to determine whether any R_f
413 obtained in our sample corresponded to ommochrome pigments, these values were
414 compared with the R_f of authentic synthetic ommochromes previously reported from the
415 same silica gel/phenol TLC system [23], since there are no commercially available
416 ommochrome standards available to date [18].
417

1.2.2. Autofluorescence analysis

The presence of indole groups in the chemical structure of all ommochromes gives them the property of autofluorescence at certain wavelengths [9]. This biochemical feature is a helpful way to determine the nature of certain colours in insects, since only red pigments derived from ommochromes and aphins (i.e. a pigment only present in aphids and not in any other insects [41]) will show this property [9,42]. Therefore, one last complementary test to determine whether ommochromes are present in the RWS of *H. americana* was to observe autofluorescence induced by excitation with specific laser lines and evaluated with a spectral detector using multiphoton microscopy. To accomplish this, five forewings from adult males were carefully mounted onto pre-cleaned microscope slides (Lauka, CDMX, México) and covered with 0.17mm thick 180 x 180 mm coverslips. Then, the edges of the coverslip were carefully sealed to the slide with nail hardener and slides were kept at room temperature until imaging. Bright field tile imaging was carried out with an upright microscope (Olympus BX51-WI, Olympus Corporation, Tokyo, Japan) equipped with an XYZ motorized stage (MAC6000, Ludl Electronic Products, Ltd., Hawthorne, NY, USA), an RGB CCD camera (MBF CX9000, MBF Bioscience, Williston, VT, USA) and StereoInvestigator software (v. 9.0.1, MBF Bioscience). Imaging was done using a UPLAN FL N 10X N.A. objective. To visualize the autofluorescence produced by the red wing spot, 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, Tokyo, Japan) equipped with a motorized stage (TI-S-E, Nikon). We thus excited the samples using four lasers of different wavelengths: 405 (2mW), 488 (70mW),

561 (1.4mW) and 647 nm (1.25mW). We evaluated the resulting signals using a 32-channel spectral detector (10 nm resolution, from 425 to 735 nm wavelength) plus a transmitted light detector. The pinhole value was set at 29.4 µm 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.

Finally, fluorescence single plane tile imaging of the forewings 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).

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

Given that 3-Hk has been reported to be fatal to insects [15], 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. For this, 3-Hk (Sigma-Andrich; Catalogue number: 2147-61-7) was dissolved in distilled water using a vortex mixer for 10 minutes. Five different 3-Hk concentrations were injected into 75 adult males (15 per group): 0 (only distilled water), 1, 100, 1000 and 10,000 µg 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 (10 µL, Hamilton 80330; Hamilton, Reno, Nevada). Individual manipulation lasted no more than 1 min. Males were then placed individually into transparent plastic 5-mL assay tubes (Simport, Canada) with a piece of

464 wood as a perch, moist pieces of cotton to provide humidity and a temperature of 26 ◦C
465 inside the tubes. Males were not fed during the experiment and conditions of captivity
466 were always the same. Twenty-four hours after injection, male mortality was recorded for
467 each of the five groups. This survival experiment concluded that 3-Hk is toxic for adult
468 males of *H. Americana*; the LC_{50} we determined for this species which was estimated at
469 368.69µg mL^{-1} (C.I 95%: 78.5- 1729.9µg mL^{-1}; See results section for further details).
470

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

472 To evaluate whether males are able to counteract the toxic effect of 3-Hk by depositing
473 ommochromes into their wings, forming the red wing spots, we performed another
474 captivity experiment administrating 3-Hk in males, then determining whether the
475 ommochromes were subsequently deposited into their RWS. To accomplish this, 54 adult
476 males were captured and randomly allocated to three different treatments: 1) 3-Hk
477 treatment (N=20 males injected with 363µg of 3-Hk diluted in 4 µL of PBS 1x—the
478 previously estimated LC_{50}), 2) Sham treatment (N=17 males injected with 4 µL of PBS
479 1X), and 3) Control treatment (N=17 males with no manipulation). After manipulation,
480 males were monitored in captivity every 4 h to record the time to death, and the
481 experiment ended when the last male died. Captivity conditions were the same as those
482 used to calculate the LC_{50}. After the last male died, the anterior wings from all
483 individuals were removed from the body. The effect of 3-Hk on wing red pigmentation
484 was evaluated by two complementary techniques: 1) quantification of red chroma (*Rc*) by
485 spectrophotometry (see also [21]), and 2) the relative fluorescence *(Rf)* by confocal
486 microscopy. The red chroma of RWS from the different treatments was calculated as the

proportion of total reflectance (from 360 to 740 nm) occurring in red wavelengths (600-700 nm) using a spectrophotometer (MINOLTA CR-200, Konica Sensing Inc., Osaka, Japan; for a similar procedure see [28]).

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, 3 µm 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 20.43 µm, 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 [43], 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 [43]). Finally, *Rf* value of all Z- projections were averaged to obtained a unique *Rf* value per individual.

4. Statistical analyses

To determine whether ommochromes are responsible for the male RWS we qualitatively analyzed the biochemical properties previously mentioned: redox behaviour, spectral absorbance, *Rf* of purified ommochromes, and autoflourescence. To evaluate whether 3-Hk, the precursor of ommochrome pigments, is a toxic tryptophan metabolite for adult males, we performed a survival analysis to calculate the LC_{50} for these males through the

Trimmed Spearman-Karber method [44]. We included in the model the following predictors: treatment (3-Hk, Sham, and Control), wing length, RWS area, and their interactions. Analysis was done in R [45] using the tsk package [46].

To evaluate whether males of *H. americana* are able to 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 [28]. This model was simplified based on AIC values to obtain the best supported model (i.e. the model with the lowest AIC-value).

To determine differences in *Rc* and *Rf* we used linear mixed and linear models respectively. For *Rc* 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 the left and right forewings of each damselfly, individual identity was included as a random effect in this analysis. For the case of *Rf* we used the same predictors as for *Rc.* 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 [45] according to Crawley [47] and Zuur and collaborators [48].

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534

535

536 **Data availability**

537 All data are available from the corresponding author on reasonable request.

538

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547

548 **Author contributions**

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

553

554 **Competing interest.**

555 We declare that the authors have no competing interests as defined by Nature Research or
556 other interests that might be perceived to influence the results and/or discussion reported
557 in this paper.
558
559 **Additional information**
560 Supplementary information is available for this paper upon correspondence and requests
561 for materials should be addressed to I.G.-S or A.C.-A.
562
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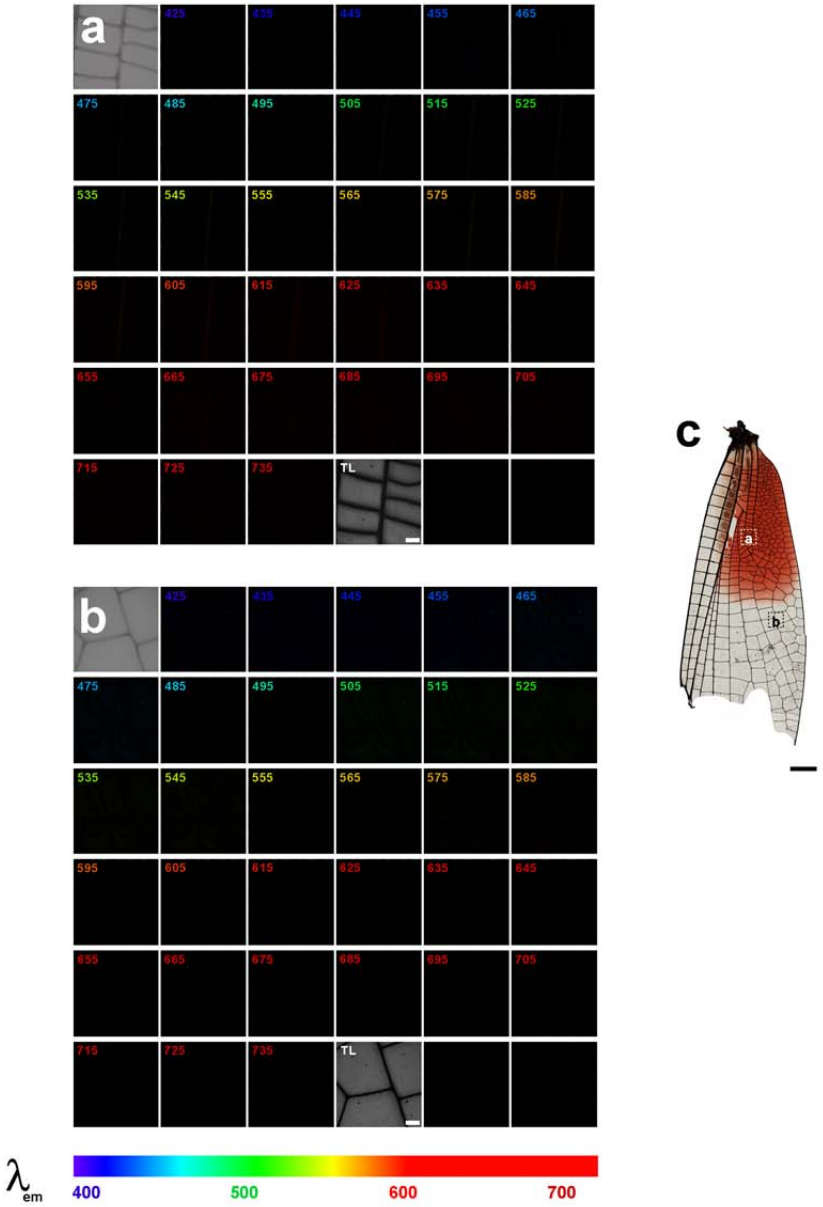
693 **Supplementary figures**



694 **Figure S1a.** Fluorescence XYZ scans (425-735 nm) of *Hetaerina americana* wing
695 excited at 405 nm. Maximum intensity projection images of (a) a sub-region of the red-
696 pigmented area, and (b) a sub-region of the transparent area, depicted in (c). A relatively
697 weak fluorescence intensity was found in wing veins of the anterior region from 505 to
698 725 nm (a). In contrast, no apparent signal is present in wing veins but in the wing
699 between 465-615 nm in the medial region of dragonfly wing (b). Scale bar: 100 m.

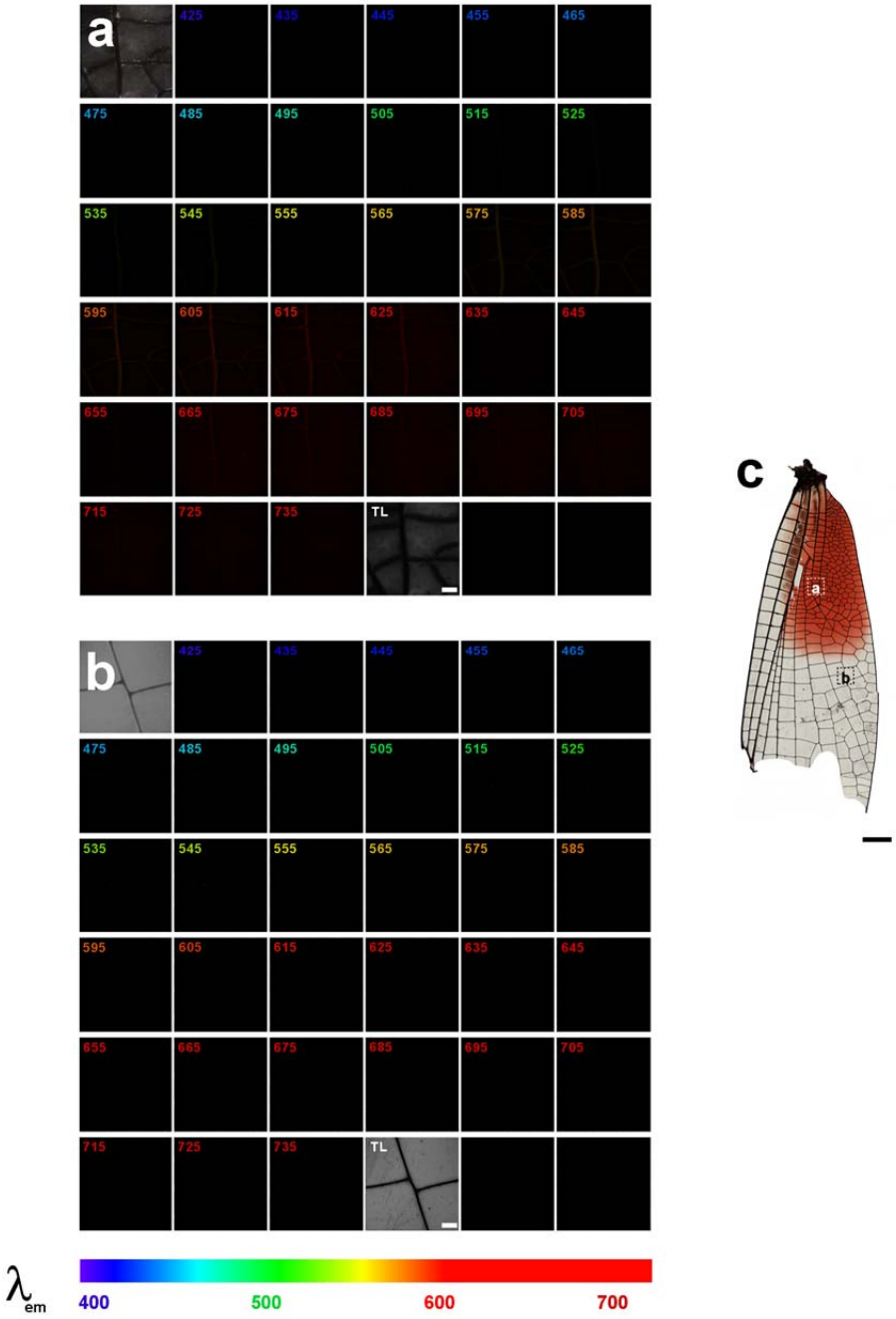


Figure S1b. Fluorescence Zλ scans (425-735 nm) of *Hetaerina americana* wing excited at 488 nm. Maximum intensity projection images of (a) a sub-region of the red-pigmented area, and (b) a sub-region of the transparent area, depicted in (c). A relatively

703 weak fluorescence intensity was found in the wing veins of the anterior region from 505
704 to 735 nm (a). In contrast, no apparent signal is present in the medial region of the
705 damselfly wing (b). Scale bar: 100 μm.
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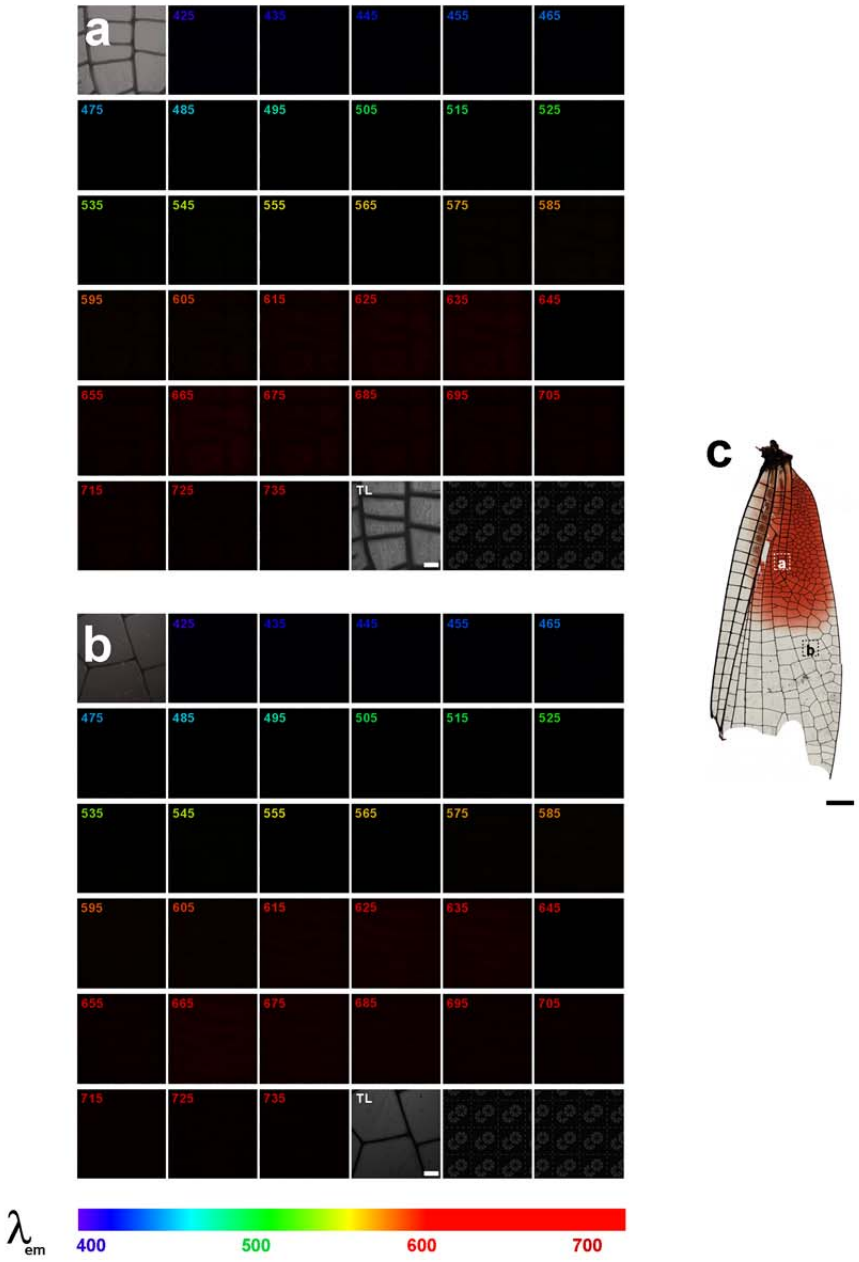
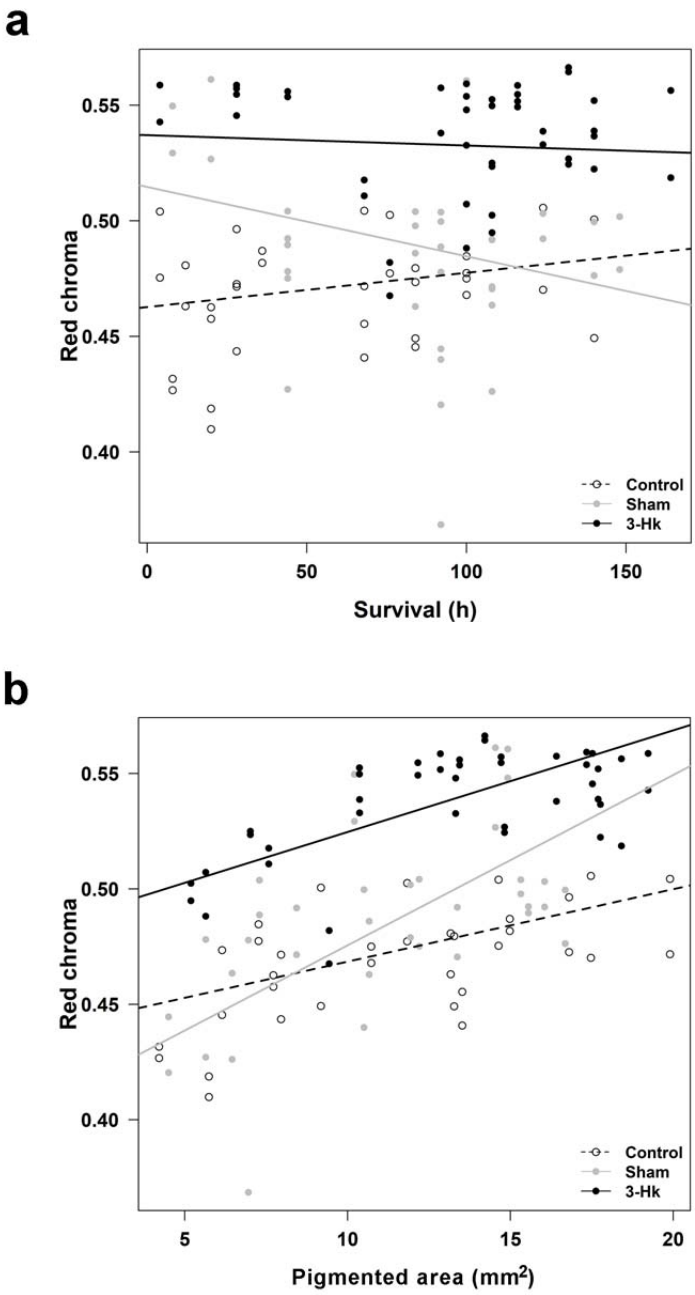


Figure S1c. Fluorescence Zλ scans (425-735 nm) of *Hetaerina americana* wing excited at 646 nm. Maximum intensity projection images of (a) a sub-region of the red-pigmented area, and (b) a sub-region of the transparent area, depicted in (c). There was no apparent signal in wing veins but curiously a 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.



724

725 **Figure S2** (a) Relationship between survival time and red chroma in each experimental
726 treatment. While in males treated with 3-Hk(continuous black line) red chroma was not
727 affected by survival time, control males (black dotted line) showed a positive
728 relationship. In sham males (gray line), chroma was also affected by survival but in a
729 negative direction. (b) Red chroma was also affected by RWS area. All treatments
730 showed a positive relationship, but with different slopes.
731

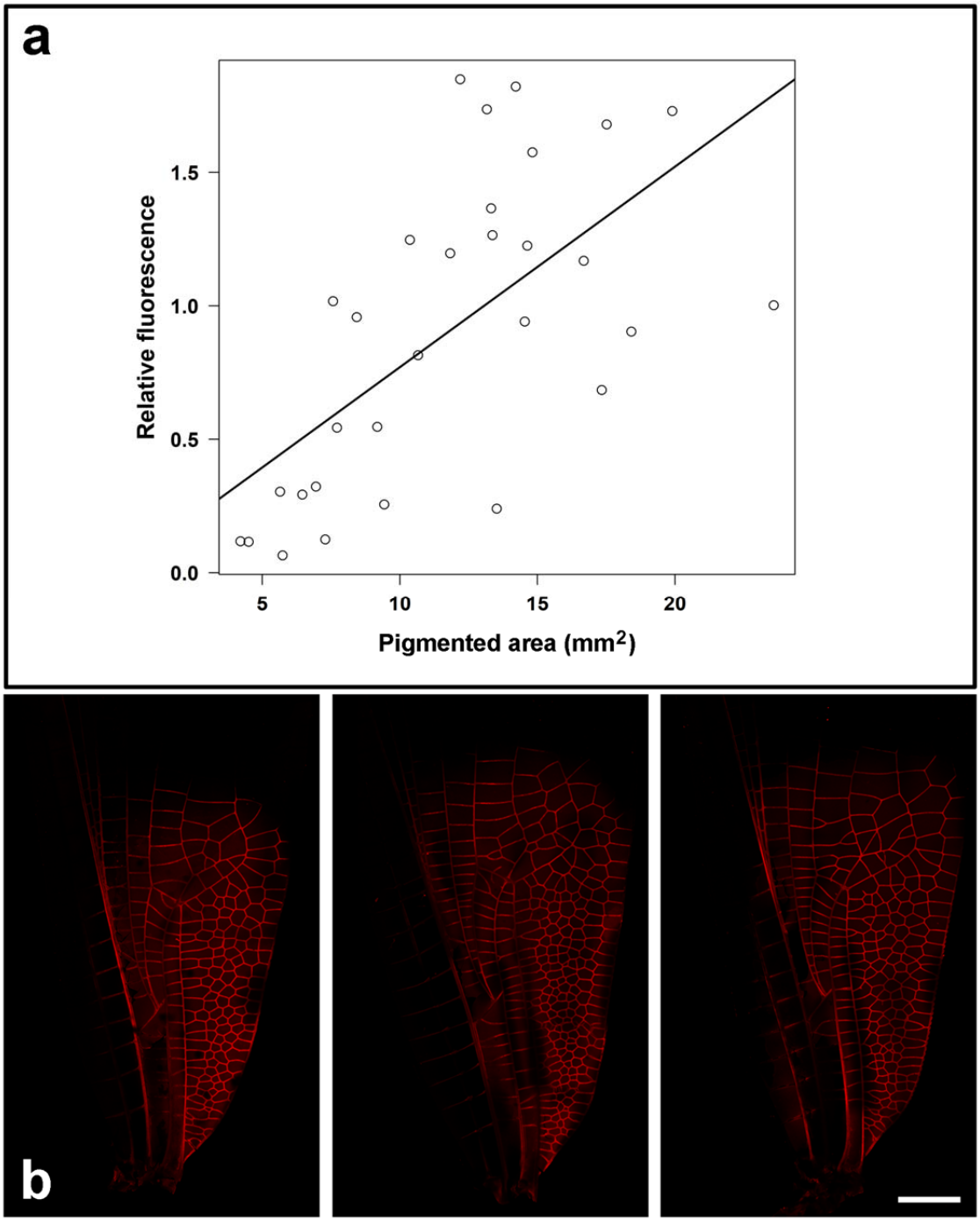
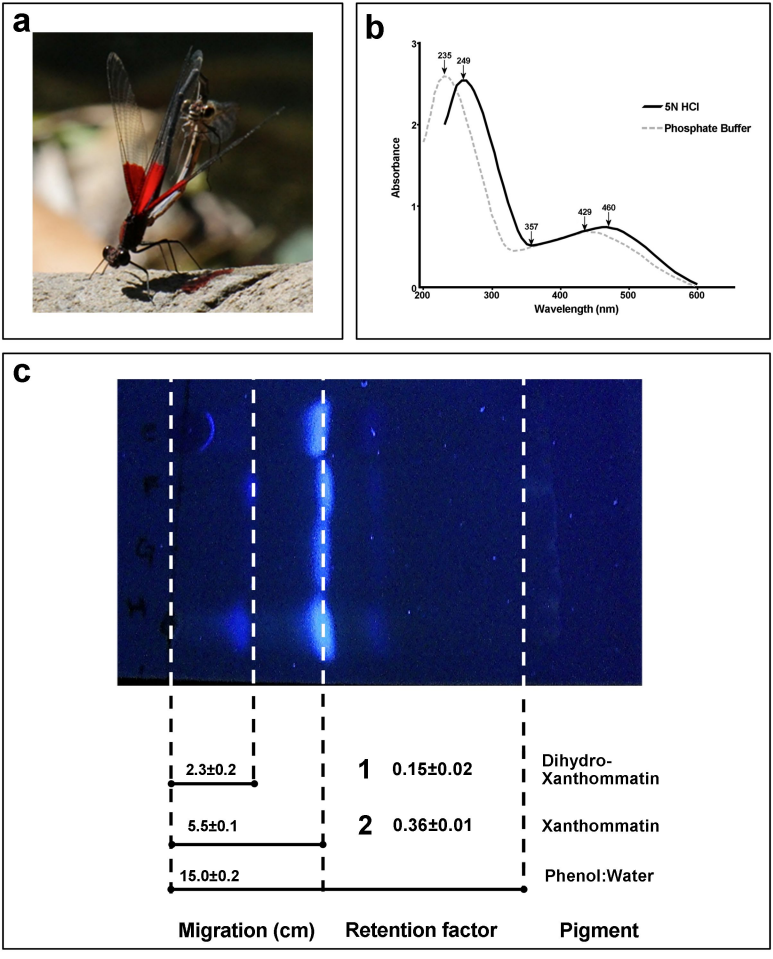


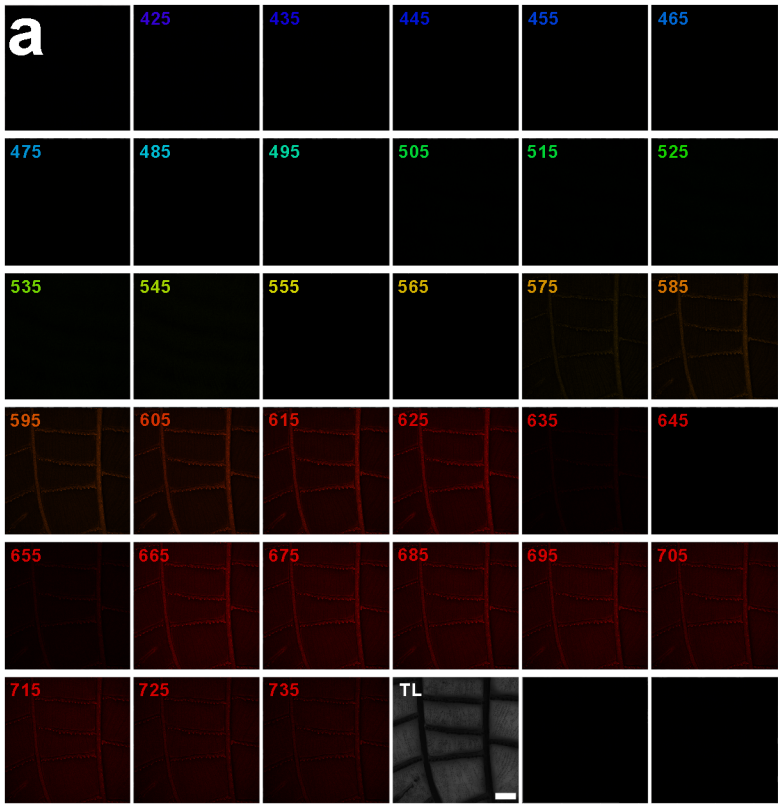
Figure S3 (a) Fluorescence XYZλ scans (425-735 nm) of *H. americana* wings excited at 561 nm of control, sham and 3-Hk males. Although 3-Hk males showed higher fluorescence values, the only significant predictor for this colour property was RWS area, which had a positive relationship in all treatments (b).

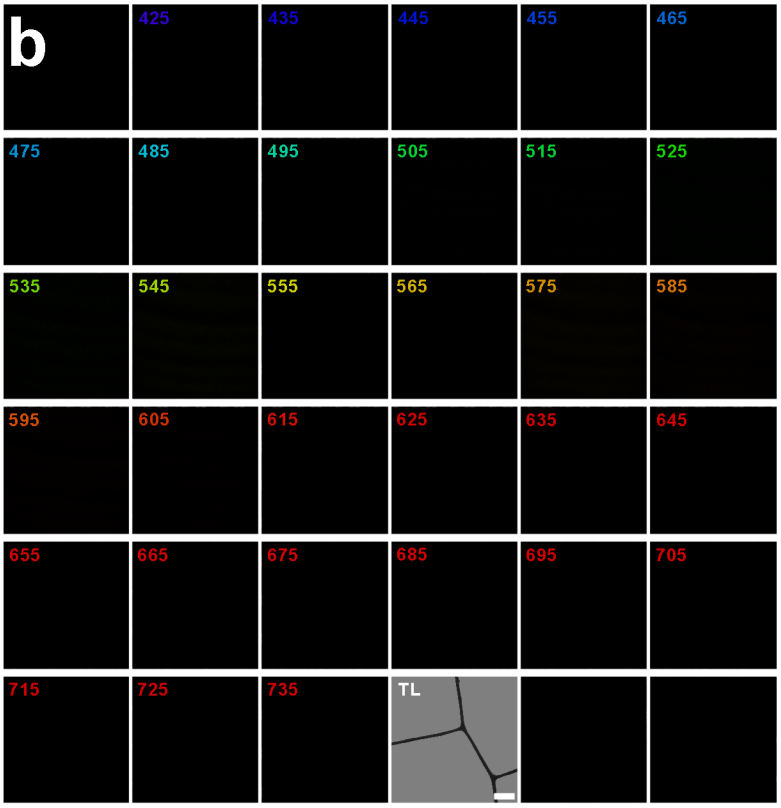
Suplementary tables

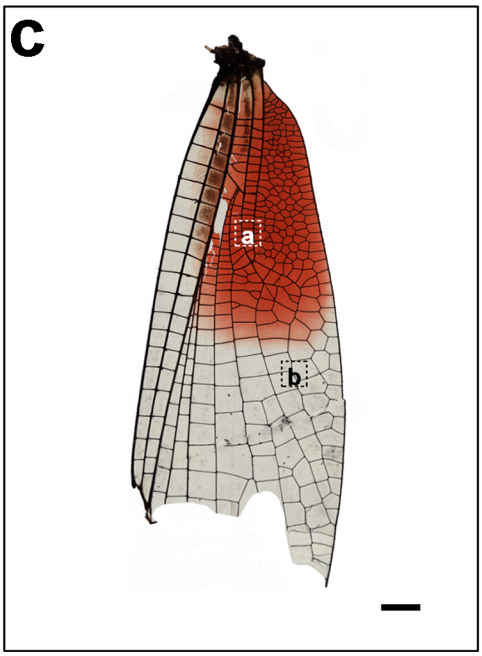
TABLE S1. Linear mixed effect model to explain variation in wing red chroma in *Hetaerina americana* males manipulated with 3-Hk, sham or control treatments. Significant predictors are shown in bold. L. Ratio=Likelihood ratio. NS=Not selected in the best supported model.

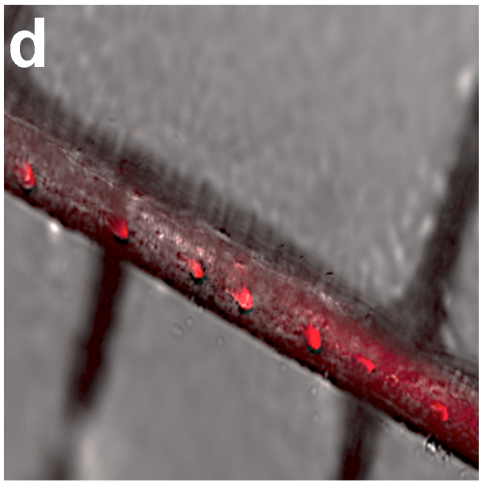
Effect on red chroma	L. Ratio	P-value
Treatment	69.07	**<0.001**
Survival time	8.97	**0.030**
Wing length	9.99	**0.019**
Pigmented area	43.42	**<0.001**
Treatment X Survival	8.29	**0.016**
Treatment X Wing length	NS	NS
Treatment X Pigmented area	8.43	**0.014**

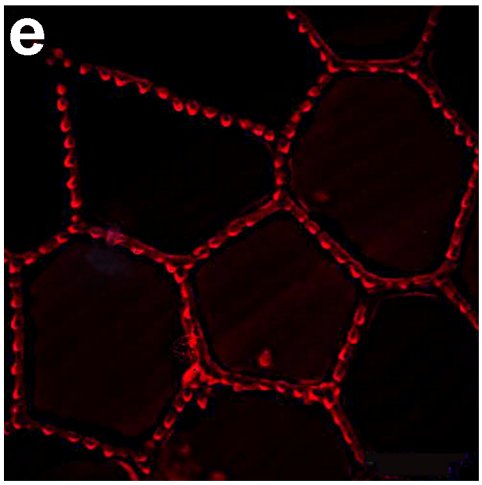

a
b
c
235
249
357
429
460
5N HCl
Phosphate Buffer
Absorbance
Wavelength (nm)
2.3±0.2
5.5±0.1
15.0±0.2
1
0.15±0.02
2
0.36±0.01
Dihydro-
Xanthommatin
Xanthommatin
Phenol:Water
Migration (cm)
Retention factor
Pigment

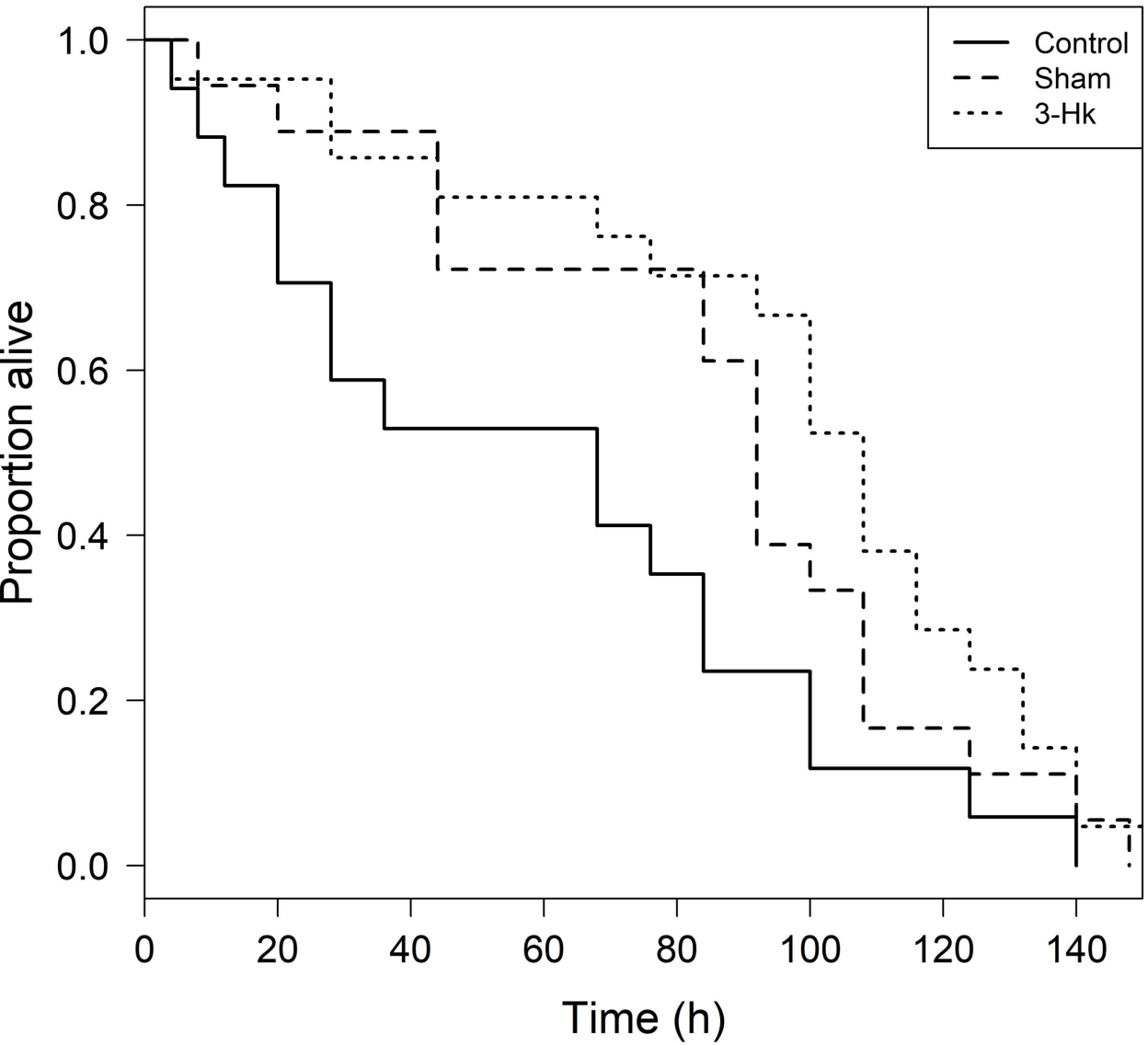











Control
Sham
3-Hk
Proportion alive
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
140
Time (h)