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A role for ecology in the evolution of colour variation and sexual dimorphism in Hawaiian damselflies

I. A. COOPER*†, J. M. BROWN‡ & T. GETTY*

*Department of Zoology, Kellogg Biological Station, Michigan State University, Hickory Corners, MI, USA
†Department of Biology, James Madison University, Harrisonburg, VA, USA
‡Department of Biology, Grinnell College, Grinnell, IA, USA

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Abstract

Variation in traits that are sexually dimorphic is usually attributed to sexual selection, in part because the influence of ecological differences between sexes can be difficult to identify. Sex-limited dimorphisms, however, provide an opportunity to test ecological selection disentangled from reproductive differences between the sexes. Here, we test the hypothesis that ecological differences play a role in the evolution of body colour variation within and between sexes in a radiation of endemic Hawaiian damselflies. We analysed 17 *Megalagrion* damselflies species in a phylogenetic linear regression, including three newly discovered cases of species with female-limited dimorphism. We find that rapid colour evolution during the radiation has resulted in no phylogenetic signal for most colour and habitat traits. However, a single ecological variable, exposure to solar radiation (as measured by canopy cover) significantly predicts body colour variation within sexes (female-limited dimorphism), between sexes (sexual dimorphism), and among populations and species. Surprisingly, the degree of sexual dimorphism in body colour is also positively correlated with the degree of habitat differences between sexes. Specifically, redder colouration is associated with more exposure to solar radiation, both within and between species. We discuss potential functions of the pigmentation, including antioxidant properties that would explain the association with light (specifically UV) exposure, and consider alternative mechanisms that may drive these patterns of sexual dimorphism and colour variation.

Introduction

In his explanation for sexual dimorphism in The Origin of Species, Darwin (1859) noted, 'When the males and females of any animal have the same general habits of life, but differ in structure, colour, or ornament, such differences have been mainly caused by sexual selection... Yet, I would not wish to attribute all sexual differences to this agency.' Both Darwin and Wallace thought that ecological selection is also an important mechanism leading to sexual dimorphism, although they disagreed on how much of a role it plays (Darwin, 1871; Wallace, 1889; Kottler, 1980; Cronin, 1992; Andersson, 1994; Punzalan & Hosken, 2010). Despite this history, ecological selection has not been as thoroughly studied in sexual dimorphism studies, perhaps because sexually dimorphic traits are always correlated with distinct reproductive roles. Sex-limited (or within-sex) dimorphisms can, however, help to reveal the ecological mechanisms that select for variation both within and between sexes, as they can disentangle the association between sexually dimorphic traits and traits involved directly in reproduction.

Sexual dimorphism in colour is common in many insect groups, but many species also display a sex-limited dimorphism, in which some members of one sex exhibit traits typical of the other sex. Such dimorphisms may be male-limited or female-limited. Male-limited dimorphism, in which some males contain female-like colouration, is strongly associated with variation in mating strategy, particularly 'sneaking', and is found where there is strong competition for access to females

Correspondence: Idelle A. Cooper, Biology Department, James Madison University, Harrisonburg, VA 22807, USA.
Tel.: 540 568 6008; fax: 540-568-3333; e-mail: cooperia@jmu.edu

(Dominey, 1981; Forsyth & Alcock, 1990; Sætre & Slagsvold, 1996; Tsubaki *et al.*, 1997; Plaistow & Tsubaki, 2000; Whiting *et al.*, 2009). Relatively less well-studied are female-limited dimorphisms, in which some females that look like males (andromorphs) coexist with typically coloured females (gynomorphs) (Robertson, 1985; Andrés *et al.*, 2000; Svensson *et al.*, 2005).

Most hypotheses for female-limited dimorphism assume a cost of sexual interactions to females due to male harassment (Fincke, 1994; Van Gossum *et al.*, 2001; Svensson *et al.*, 2005). There is some support for these hypotheses. In some species, males prefer the most common morph (Van Gossum *et al.*, 2001; Gosden & Svensson, 2009). It is unclear whether heightened harassment of the preferred morph reduces female fecundity (Sirot & Brockmann, 2001; Gosden & Svensson, 2007, 2009), though Iserbyt *et al.* (2013) found evidence of frequency-dependent selection on egg number and clutch mass in one *Nahelennia* damselfly species. A recent time-series analysis of *Ischnura elegans* also found a signature of frequency-dependent selection on female morph frequencies: morph frequencies were more stable than expected from drift, which suggests that selection is maintaining a stable polymorphism (Le Rouzic *et al.*, 2015). Thus, frequency-dependent sexual conflict may maintain such polymorphisms via male scramble competition over females, particularly in non-territorial species such as *Nahelennia* and *Ischnura*, in which there may be more sexual conflict.

To understand the evolution of within-sex dimorphism and sexual dimorphism more fully and in a wider array of species, particularly in territorial species where males and females use habitats differentially, the alternative hypothesis of ecological selection warrants consideration. Here, we evaluate whether differences between the sexes in their 'habits in life' and distributions of species over ecological clines could explain variation in sexual dimorphism within and between species.

The endemic Hawaiian damselflies provide an ideal opportunity to investigate the role of ecological selection in body colour dimorphism. Cooper's (2010) study of habitat and body colour variation in one species, *Megalagrion calliphya,* found that red body pigmentation is more prevalent in habitats with exposure to high solar radiation. All males, who defend territories around open water in exposed pools, are red; females are red in high-elevation populations but are green at low elevation. At low elevation, females can move into canopy cover away from the mating habitat for protection, but at high elevation, the forest stature is diminished, leading to little protection via the canopy cover. Under this ecological selection hypothesis, female-dimorphic populations (at intermediate elevations) are a result of gene flow from the monomorphic populations at the elevational extremes. The red pigment, likely an ommochrome, as in other Odonates (Futahashi *et al.*, 2012), is an antioxidant that may function to protect both male and female damselflies in exposed conditions from the damaging effects of free radicals created by UV exposure (Cooper, 2010). If the function of red pigmentation for an individual damselfly is related to the environment, independent of sex, and if ecological variation is an important determinant of colour variation more broadly, then we predict the same correlation in the other 22 species of *Megalagrion*, in which body colour varies from red to blue.

To test this ecological hypothesis, we measured colour and habitat differences in 25 island-species combinations of *Megalagrion* (17 species in total, including island measures of species that inhabit multiple islands). In doing so, we discovered three species in addition to *M. calliphya* with female dimorphism, all of which have green and red female morphs: *M. blackburni*, *M. hawaiiense* and *M. paludicola*. We used methods of phylogenetic linear regression over a posterior distribution of inferred trees to test the ecological selection hypothesis that greater light exposure is correlated with redder pigmentation within and between sexes, populations and species. Specifically, we expected that greater light exposure predicts redder pigmentation in males and females and that the magnitude of habitat differences between the sexes predicts the degree of sexual dimorphism in colour.

Materials and methods

We measured the association between body colour and light habitat in 17 of the 22 extant *Megalagrion* damselflies, with a total of 25 island species. For each population (Table S1), we measured the following traits for live males, gynomorph females and andromorph females (when present): mean thoracic hue, saturation, brightness (HSB); reflectance spectra; and canopy cover. Data for colour and canopy comparisons were collected between 2006 and 2013, and reflectance spectra were measured during 2011–2013. The within-species comparisons of canopy cover and body colour were conducted on *M. calliphya*. Besides adding 16 species to the phylogenetic comparison, this study expands our previous research on *M. calliphya* (Cooper, 2010) by measuring canopy cover directly (rather than elevation), measuring populations across this species' distribution (three islands, rather than one) and measuring colour as reflectance spectra in addition to HSB.

For each measure, we calculated the degree of sexual dimorphism as female score minus male score and the degree of female-limited dimorphism as the difference in scores between the female morphs (gynomorph minus andromorph). The direction of these differences was chosen simply to result mostly in positive numbers, but the direction does not affect statistical calculations. (Note that ratios, such as those used for quantifying sexual size dimorphism, are inappropriate for a colour

scale.) For the female-dimorphic *M. calliphya*, the morph frequency for each population was determined by a larger sample size (Cooper, 2010) than we used in this study. To get more accurate measures of mean female colour in a population, we weighted the mean colour of each morph by its frequency as determined in the larger sample size.

Study system

The Hawaiian *Megalagrion* damselflies are widespread throughout the Hawaiian archipelago and are ecologically diverse. A molecular phylogenetic analysis (Jordan *et al.*, 2003) estimates that *Megalagrion* arrived in Hawaii 10 million years ago and radiated through two speciation patterns: (i) interisland speciation through colonization of new volcanoes and (ii) rapid radiation into specialized larval habitats. The result is that *Megalagrion* fit the classic definition of adaptive radiation on archipelagos, as they colonized environments missing several dominant orders of aquatic insects (e.g. Ephemeroptera, Megaloptera, Trichoptera and Plecoptera: Williams, 1936). In particular, species have diversified into a spectrum of larval aquatic environments that exceeds the range found across families of continental damselflies: ponds and pools of various sizes; seeps, moist rock faces and waterfalls; phytotelmata (water held in plant axils); fast streams; brackish pools; and damp vegetation mats, a remarkable transition to a fully terrestrial larval environment. As adults, the species are found at a range of elevations, from brackish pools near sea level to over 2000 m, and in forests that vary in stature and thus canopy cover. Although some low-elevation populations have been extirpated by exotic fish (Englund, 1999; Englund & Polhemus, 2001), species can be found essentially in all environments that have predictable moisture, and thus are exposed to a maximum breadth of light environments. Like many species on archipelagos, geographic barriers between populations – both dry environments and ocean channels – have led to substantial differentiation of populations within and between islands, as measured by neutral genetic markers (Jordan *et al.*, 2007). This system provides ample opportunity for differentiation between populations to evolve in colour and other morphological characteristics.

Colour measurements

We used quantitative measures of colour, rather than categorical designations, which allowed us to capture the variation within the red hue as well as between red and other colours. HSB was measured from digital photographs of live damselflies taken in a controlled light environment using Adobe Photoshop (Cooper, 2010) (CS5 v. 10.0 × 64; Adobe Systems, San Jose, CA, USA). To ensure consistency of exposure, we included four background colour standards in the photographs of damselflies and compared mean HSB (Fig. S1 and Table S2). There is a significant effect of photograph date on saturation, but there is no difference in the background colour standards between species or populations, so variation due to photography methods causes some noise, but it does not cause patterns between species or populations. To measure damselfly colour, we averaged hue, saturation and brightness of the entire thorax side using Adobe Photoshop (Cooper, 2010) (CS5 v. 10.0 × 64; Adobe Systems). The HSB colour scale is from 1 to 360, like the degrees of a circle, and red hues are near values of 1 as well as 360 on that scale. To make red hue a continuous measure, we subtracted 360 from the values approaching 360 (in those cases, most values were near 1, so values near 360 were clearly different), which converted the observed range of red values to approximately −10 to 40. Table S3 lists the quantitative values for hue, which includes a substantial amount of variation outside of red hues. 'Red' constitutes about 20 per cent of our data range from −6.25 to 203.17. By using these quantitative values of colour, we could detect evolutionary colour changes at a fine scale in the phylogeny, both within the red section of the spectrum and throughout the rest of the visual colour range.

Although human-vision methods such as the HSB measurements described above are often acceptable approaches for some colour systems, they are best used in combination with a method that can detect UV reflectance, such as spectrometry (Andersson & Prager, 2006). Therefore, we also measured colour through spectral reflectance using a spectrometer (USB 4000, Ocean Optics, Dunedin, FL, USA) running SPECTRASUITE software. The fibre optic probe was held at a constant distance (< 1 cm) from the surface of the thorax with a custom-made probe attachment. On smaller insects, the area measured included most of the thorax and was therefore similar to the area measured in photographs. On larger insects, it was only the middle of the thorax, which is a limitation of using a single-point measurement (Delhey *et al.*, 2014). A constant light source, a PX-2 Pulsed Xenon lamp, illuminated the thoraces over a spectral range of 200–800 nm. The software averaged the reflectance values over five consecutive measurements at the same point to make a final spectrum that showed relative reflectance compared to a white standard (Certified Reflectance Standard, Labsphere, North Sutton, NH, USA). There were no reflectance peaks in the UV range, so analysis was confined to 350–800 nm. The spectral data contained measurements at 0.21-nm intervals, so to reduce the number of variables, we integrated reflectance in 30 bins of 15-nm width. These data were reduced further into three variables in a principal component analysis (Fig. S2 and Table S4), which explains 92.91% of the variance.

Habitat canopy cover

We estimated radiation exposure in male and female habitat by measuring canopy cover with a concave densitometer (as in Cooper, 2010). For the species in which larvae are terrestrial and males do not defend territories, *M. kauaiense* and *M. koelense*, canopy for males and females were measured only where individuals were sighted. For other, territorial species, we measured additional female habitat because females are seen infrequently when not at the mating site, yet spend most of their lives away from the mating location (pers. obs.). In these cases, canopy cover of female habitat was measured at chest height at a location 2 m from the edge of the streambed in a perpendicular direction away from the territorial male (as in Cooper, 2010). The 2 m readings for canopy cover were chosen to be random, conservative measures of habitat that is away from the male habitat. This method provides a single mean value for female or male canopy cover at each population, and therefore, female canopy cover dimorphism was possible to calculate only when more than one population was sampled for a given species. Mean female canopy cover dimorphism was then calculated in the same way as mean morph colour, by weighting the canopy measurements by the frequency of each morph in the population. Where only one female is collected for a species with a known female dimorphism, the other female morph value was coded as missing data, which occurred in *M. blackburni* for Maui and *M. hawaiiense* for Oahu. Canopy cover dimorphism for a species was calculated as the mean of population values of dimorphism, which is the same approach we used to calculate colour dimorphism above.

Correlated character evolution

To test for phylogenetic influence on character evolution, we estimated a phylogeny for all sampled species based on aligned data set S1004 (TreeBase [www.treebase.org]) (Jordan *et al.*, 2003) of two gene sequences: 1287 bp of mitochondrial COII, A6, A8, and lysine and aspartic acid tRNAs; 1039 bp of nuclear elongation factor-1α. For each island species, we chose a random exemplar sequence (avoiding those with missing data), leaving a data set of 25 sequences. From these, we inferred a phylogeny with Bayesian methods using BEAST 1.7.5 (Drummond *et al.*, 2012). Bayes factor tests determined model assumptions, which resulted in two data partitions (mtDNA and nuclear) with distinct models (mtDNA GTR+I+Γ and nuclear HKY+I+Γ) and a strict molecular clock. We used a Yule process for speciation likelihoods and appropriate priors that resulted in stable runs for parameters after burn-in and high ESS values. We used BEAST's Logcombiner 1.7.5 to combine 9000 post-burn-in samples from two runs (each run 10 000 000 states, sampled every 1000), from which we derived a maximum clade credibility tree using TREEANNOTATOR 1.7.5. This tree (Fig. S3) did not differ in topology from the ML analysis of the same gene sequences by Jordan *et al.* (2003), with the exception of the basal nodes describing the branching order among the three major subclades of the radiation. We reconstructed presence of female-limited dimorphism with the MAKE.SIMMAP function in the R package PHYLOTOOLS (Revell, 2011), using an ARD (all-rates-different) model of character change.

We then performed phylogenetic linear regression (PGLS) on colour traits vs. canopy cover using either the *pgls* function in the R package CAPER (Orme *et al.*, 2013) or the PHYL.RESID function in PHYTOOLS (Revell, 2011). PGLS simultaneously estimates Pagel's (1999) lambda (a measure of phylogenetic signal) and the regression coefficients using ML (Revell, 2010). We evaluated the effect of phylogenetic uncertainty on these values by estimating them over 1000 of the 180000 post-burn-in trees and calculating HPDs. As many colour or habitat traits showed no significant phylogenetic signal (i.e. lambdas near zero), we also compared these results to those using ordinary linear regression of species-island means (both trait means and $\log_{10}$-transformed means). If differences in light habitats drive general colour evolution, we expect a positive relationship of canopy cover to hue (e.g. more blue and less red with higher canopy cover). If degrees of dimorphism, both sexual dimorphism and within-female dimorphism, are also driven by this adaptive mechanism, we expect a positive relationship between differences in colour or reflectance and canopy cover differences.

Results

Phylogenetic reconstruction of body colour and canopy cover revealed recent and rapid colour evolution in both males and females (Fig. 1), which resulted in a lack of phylogenetic signal (Pagel's lambda near zero) on many colorimetric and spectrophotometric measures of reflectance (Table 1). This was true even when the analysis considered populations of three species found on multiple islands as separate taxa, as they also vary in colour and show varying degrees of genetic differentiation. The significance of the regressions were similar for ordinary least squares regressions and phylogenetic linear regressions (Table 1), which is not surprising for characters showing little effect of phylogenetic history, but was also true for those that had nonzero lambdas.

In support of our ecological selection hypothesis, canopy cover was a significant predictor of variation in hue, saturation and reflectance spectra for males and hue in females (Table 1). Among populations of *M. calliphya*, redder colour was associated with low canopy cover (high exposure) (Fig. 2a, Table S5) (only hue is

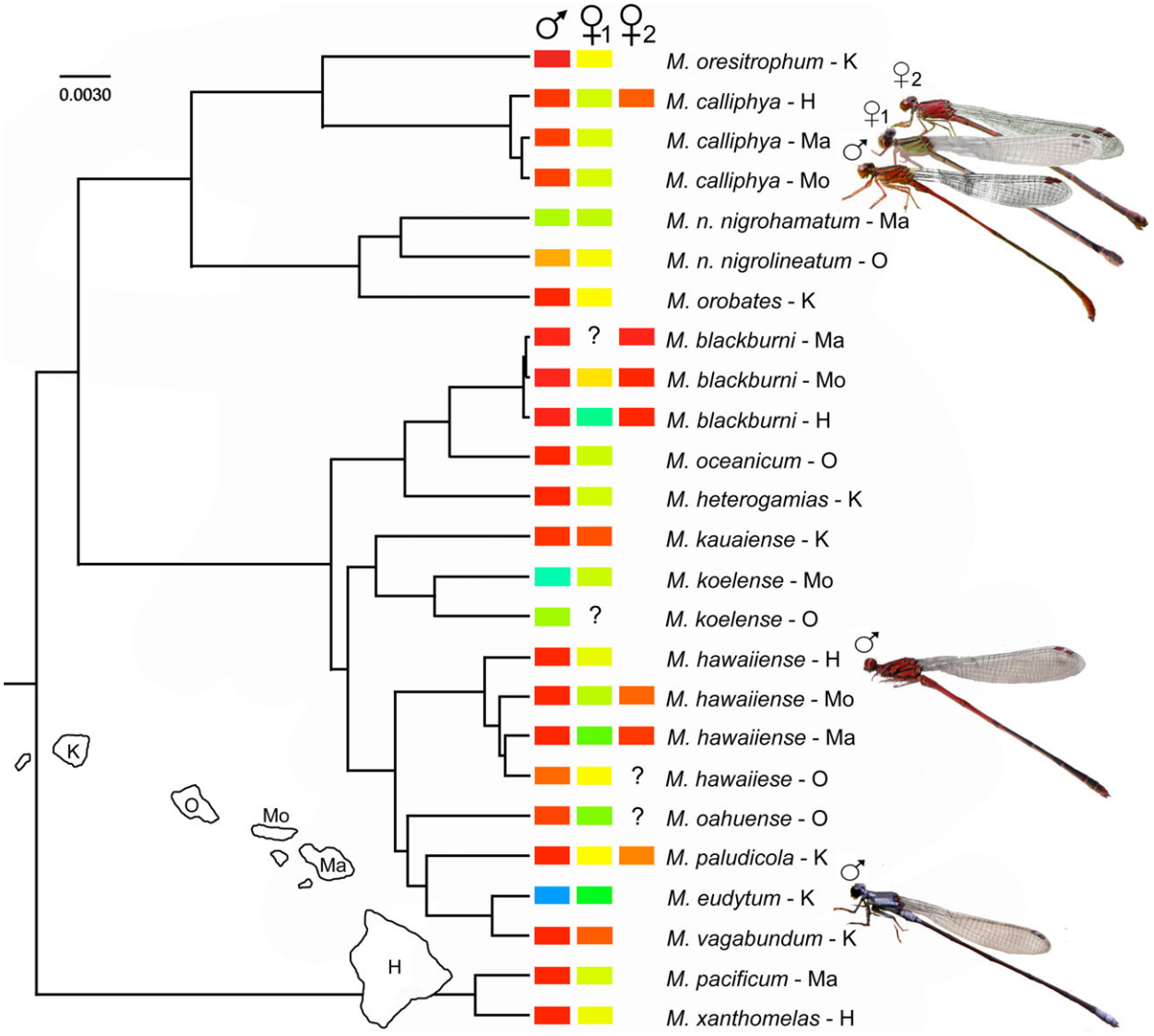

Fig. 1 Mean body hue of males and females, including second female morphs, on the MCC *Megalagrion* tree. Hue is shown in coloured blocks, but see Table S4 for the hue numbers. The *Megalagrion* species names are indicated, with their Island location (Kauai, Hawaii, Maui, Molokai or Oahu, also shown on map). *M. n.* refers to *Megalagrion nigrohamatum*. The question marks denote unknown values. Most species show sexual dimorphism in hue, and four species contain a female-limited dimorphism on at least one island (*M. calliphya, M. blackburni, M. hawaiiense and M. paludicola*). Images of damselflies to depict colour variation are shown for *M. calliphya* (male, green female, red female), *M. hawaiiense* male and *M. eudytum* male.

shown Fig. 2, though we also saw a significant pattern in saturation for males). When all of the *Megalagrion* species were included in the comparison as species means, we saw similar patterns of low canopy cover habitat being associated with redder colouration, for both males and females (Fig. 2b). Although HSB colour measures showed more significant relationships with canopy cover than did reflectance PC values, the patterns were similar (Table 1). We illustrate the relationships between colour and canopy cover here in scatterplots because there was no significant phylogenetic signal for most traits (Table 1), though the trait changes in males and females can also be visualized over the phylogenetic tree (Fig. S4). We also mapped the three relationships that had significant nonzero lambda values, which were canopy cover with male saturation, sexual dimorphism in hue and sexual dimorphism in saturation (Fig. S5).

The degree of habitat sexual dimorphism predicted the degree of colour sexual dimorphism, both among

Table 1 Species-level associations between canopy and colour, using ordinary least squares regressions (OLS) (separate tests for HSB and reflectance for each sex) as well as phylogenetic linear regressions (PGLS).

	OLS		PGLS		
Measure	Beta	*P*	Lambda (HPD)	Beta (HPD)	*P* for beta
Male canopy					
Hue	**1.230**	**0.009**	0*	**1.23***	**0.009**
Saturation	**−0.431**	**0.010**	0.69 (0.59,0.82)	**−0.44 (−0.41, −0.45)**	**0.005**
Brightness	0.003	0.969	0.65 (0.49,0.77)	0.021 (0.013,0.027)	0.721
Reflectance pc1	**0.021**	**0.027**	0*	**0.021***	**0.027**
Reflectance pc2	−0.004	0.617	0*	−0.004*	0.617
Reflectance pc3	**0.034**	**0.049**	0*	**0.034***	**0.048**
Female 1 canopy					
Hue	0.105	0.730	0*	0.105*	0.730
Saturation	−0.111	0.554	0 (0.000,0.033)	−0.111 (−0.110, −0.112)	0.554
Brightness	0.038	0.564	0.24 (0.21,0.29)	0.029 (0.031,0.026)	0.640
Reflectance pc1	0.011	0.292	0*	0.011*	0.292
Reflectance pc2	0.005	0.600	0*	0.005*	0.600
Reflectance pc3	0.007	0.607	0*	0.007*	0.607
Female 2 canopy					
Hue	**0.632**	**0.011**	0*	**0.63 ***	**0.011**
Saturation	−0.234	0.096	0*	−0.23 *	0.096
Brightness	0.032	0.553	0.10 (0.00, 0.18)	0.028 (0.032,0.025)	0.587
Reflectance pc1	0.005	0.512	0*	0.005*	0.512
Reflectance pc2	−0.004	0.494	0*	−0.004*	0.494
Reflectance pc3	0.007	0.430	0*	0.007*	0.430
Male-female canopy					
Hue	1.050	0.074	0.71 (0.00,0.77)	**1.199 (1.24, 1.05)**	**0.028**
Saturation	**−0.505**	**0.018**	0.9 (0.00,0.92)	**−0.47 (−0.45, −0.50)**	**0.009**
Brightness	0.038	0.519	0*	0.038*	0.519
Reflectance pc1	0.008	0.477	0.195 (0.17,0.21)	0.008*	0.463
Reflectance pc2	−0.004	0.589	0*	−0.004*	0.589
Reflectance pc3	0.001	0.936	0.137 (0.10,0.15)	0.001*	0.924
Female diff canopy					
Hue	**1.415**	**0.004**	0.031 (0.0,0.078)	**1.41 (1.415, 1.406)**	**0.004**
Saturation	**−0.573**	**< 0.001**	0*	**−0.573***	**< 0.001**
Brightness	0.022	0.518	0*	0.022*	0.518
Reflectance pc1	0.001	0.595	0*	0.001*	0.595
Reflectance pc2	**−0.016**	**0.039**	0*	**−0.016***	**0.039**
Reflectance pc3	0.007	0.104	0*	0.007*	0.104

*Indicates an HPD band that is below the significant digit resolution shown.
Statistically significant measures (at 0.05 *P*-value) are shown in bold.

populations of *M. calliphya* (Fig. 3a) and among species (Fig. 3b). Males and females were more different in colour where they were also more different in canopy cover. For all *M. calliphya* populations and in most *Megalagrion* species, males had lower hue values (are more red) than females and were also located in more exposed habitat.

There are four *Megalagrion* species that contain female-limited dimorphism, and in those species, there is variation in the presence of the second female morph between islands. The female-dimorphism reconstruction was well supported and indicated that female-limited dimorphism evolved independently in the four species (Fig. S6). Similar to the pattern of sexual dimorphism described above, female colour morphs were more different where females were in more different canopy cover. As in the pattern of sexual dimorphism, andromorphs had lower hue values (more red) than gynomorphs and were found in more exposed habitat (Table 1). Female colour dimorphism was captured best in reflectance PC2, which shows red–green variation (Table 1, Fig. S2).

Discussion

The association of colour and habitat that we documented previously in one species, *M. calliphya*, is repeated throughout the *Megalagrion* radiation. Canopy cover is a significant predictor of variation in hue, saturation and reflectance spectra (Table 1). Importantly, the associations are the same across males and females, and the degree of sexual dimorphism in a species is correlated with the degree of their habitat differences, both among *M. calliphya* populations (Fig. 2a) and among species (Fig. 2b). Redder, more saturated colour is associated with low canopy cover (high solar exposure)

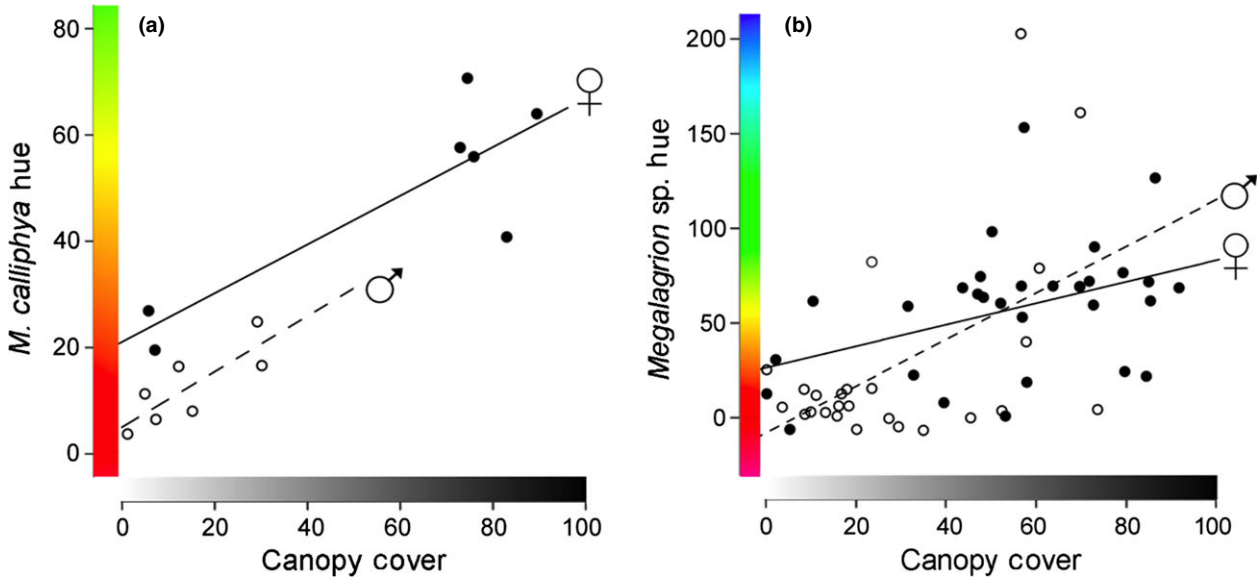

Fig. 2 Regression of body hue and canopy cover (a) within *M. calliphya* and (b) among *Megalagrion* island species for males (open circles and dashed line) and females (closed circles and solid line). The x-axes are per cent canopy cover, and the y-axes are average hue from red at low numbers to blue at high (with a coloured bar showing hue). (a) *M. calliphya* population means for males ($R^2 = 0.66$, $P = 0.027$, $y = 5.14+0.49x$) and females ($R^2 = 0.72$, $P = 0.015$, $y = 21.26+0.43x$) on Hawaii Island (see Table S5). (b) *Megalagrion* species averages, including island variation for species found on multiple islands, for males ($R^2 = 0.26$, $P = 0.009$, $y = -8.21+1.23x$) and females ($R^2 = 0.17$, $P = 0.011$, $y = 26.81+0.63x$). The sample sizes are 25 island species for males, and 30 for females because some populations contained two female morphs (see Fig. 1).

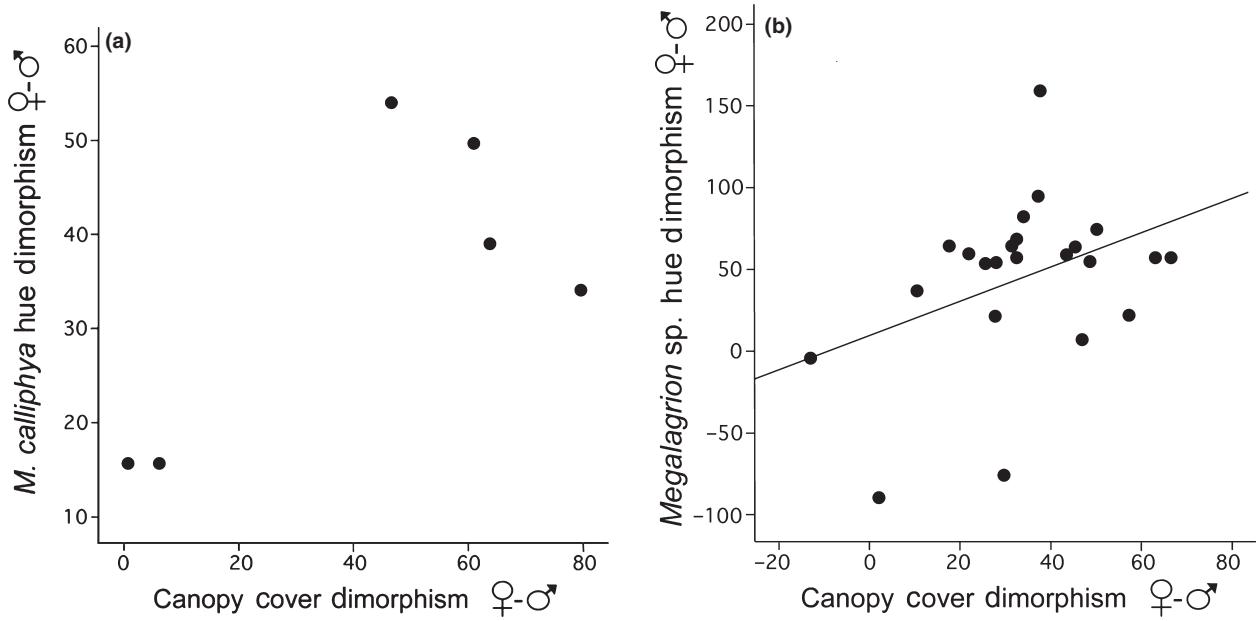

Fig. 3 Regressions of body hue sex differences on canopy cover sex differences for (a) population means of *M. calliphya* and (b) species means of *Megalagrion*. (a) Populations in which female *M. calliphya* are under similar canopy cover to males contain little sexual dimorphism in hue, whereas populations in which males are under less canopy cover contain sexual hue dimorphism in which male hue is lower, or redder ($R^2 = 0.52$, $P = 0.053$, $y = 19.01+0.37x$). (b) Throughout the *Megalagrion* radiation, sexual dimorphism in hue is greater where the sex difference in habitat is larger ($R^2 = 0.15$, $P = 0.074$, $y = 9.77+1.05x$).

among populations of *M. calliphya* as well as among *Megalagrion* species (hue shown in Fig. 2). Similarity of colouration throughout the radiation is not primarily due to shared ancestry (Fig. 1, Table 1), but to shared light environment (Table 1).

Colour may be correlated with an ecological variable because it is under ecological selection. Thermoregulation (Watt, 1968; Ellers & Boggs, 2003; Lacey *et al.*, 2010) and free radical inhibition (Caldwell *et al.*, 1998; Pék & Helyes, 2010) are two potential functions of colour. Colour can affect cellular conditions and functions as a consequence of differential reflection and absorption of light of varying wavelengths and energy levels. The role of pigments in protection against the harmful effects of light, particularly UV, is known from a variety of studies in plants and animals (Caldwell *et al.*, 1998). Our predicted function of the red pigmentation throughout the *Megalagrion* radiation is as an antioxidant, as it is in *M. calliphya* (Cooper, 2010), but further study is required to test this hypothesis.

These data do not eliminate a potential role of sexual selection in the evolution of sexual dimorphism in this system. Unfortunately, little is known about *Megalagrion* colour vision, except that they do have a single long wavelength (LW) opsin in addition to a UV and blue opsins (Bybee *et al.*, 2012). Although *Ischnura elegans* have two long wavelength opsins (Chauhan *et al.*, 2014), this appears to vary among species, as Huang *et al.* (2014) report from electrophysiological studies that the LW ('green cell') of *Ischnura heterosticta* has peak sensitivity at 525–560 nm, and the spectral sensitivity overlaps extensively with the 'blue cell' which peaks at 450 nm. Colour discrimination requires differences in sensitivity ratios of two receptors; in *I. heterosticta*, andromorphs (immature females) and males are blue, gynomorphs (sexually mature females) are green, and males can thus perceive the difference in these colours. If *Megalagrion* species likewise have only a single green opsin that is sensitive from the green to red wavelengths, it is not clear that they are able to discriminate between green and red females, though we are currently testing this in field experiments.

Although the strength and type of sexual selection may also vary over an ecological cline (Miller & Svensson, 2014), clinal variation in sexual conflict cannot alone explain why all low-elevation females are green (the ancestral female condition), and all high-elevation females red, in all four species that have female-limited dimorphism. In the absence of ecological selection, the presence of monomorphic red females at high elevations, in four species, would have to be due to chance effects (drift). As an explanation of patterns that we document here, sexual selection only seems logical in combination with ecological selection.

Inherent sexual difference is certainly at the base of our hypothesis: males spend more time (and many defend territories) near oviposition sites where they can find mates, whereas females can use other habitats to forage or avoid severe environmental conditions. In this system, the simplest explanation of colour evolution involves sex differences in habitat that vary among species and populations, followed by sexes diverging in colour in response to that selection. In high-elevation populations of *M. calliphya* on Hawaii Island, males and females are in different locations, with males at breeding pools and females elsewhere except to mate and oviposit, but they are the same colour. Colour does not vary because, despite these habitat differences, male and female habitats have the same radiation exposure at high elevation. This condition is not easily explained if colour differences evolved first, followed by sexes diverging in response to habitat differences.

This ecological explanation for the female dimorphism may not apply to other damselfly species in which females experience greater harassment. Females of territorial species, such as some species of *Megalagrion*, may avoid most harassment when away from the oviposition sites. In nonterritorial species such as in *Ischnura* and *Enallagma*, however, females may experience more harassment because there is scramble competition and males may search for females away from the oviposition habitat (Fincke, 1986). There is variation in territoriality among *Megalagrion* species, however, which would be a valuable trait to quantify and add to additional comparative studies of the degree of sexual dimorphism.

Among *Megalagrion* species, variation in sexual dimorphism appears to be driven primarily by variation in ecological differences between male and female habitats; female dimorphisms are a consequence of clines in the degree of habitat difference combined with gene flow between populations. We are currently examining the causal links between damselfly colour variation and selection in field studies. A better understanding of how such selection acts in diversification could also result from understanding the plasticity of colour development, the role of crypsis in different habitats and light environments, and the effects of geographic barriers and gene flow across ecological clines in the maintenance of colour variation.

Other recent findings call for a re-evaluation of some assumptions that are widespread about the evolution of sexual dimorphism, including (i) viewing sexual dimorphism as discrete rather than a continuous variable and (ii) assuming that levels of sexual dimorphism necessarily reflect levels of sexual selection (see review in Price, 2015). There is a growing body of research on ecological selection driving sexual dimorphism (Batesian mimicry in butterflies: Kunte, 2009; Aardema & Scriber, 2013; thermoregulation in fiddler crabs: Darnell & Munguia, 2011; size in lizards: Stamps *et al.*, 1997; Butler *et al.*, 2007), and the recognition of the function of colour in physiological protection should lead us to consider alternative mechanisms to explain the diver-

sity of sexual differences and variation within sexes in these and other traits. These surprising patterns are also a reminder that the function of colours may not always be primarily visual, even when our visual experience of them is striking.

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Supporting information

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

Figure S1 Mean background standard values for hue, saturation, and brightness.

Figure S2 PC 1-3 loadings of visible light reflectance.

Figure S3 MCC tree of *Megalagrion* species, with labels from Jordan *et al.* (2003) data.

Figure S4 Male and Female 2 hue and canopy cover for each species mapped onto the phylogeny using *phytools* continuous trait mapping (contMap) function.

Figure S5 Relationships with significant non-zero values include (a) male saturation and canopy cover ($\beta = -0.44$, $P = 0.005$, $\lambda = 0.69$), (b) sexual dimorphism in hue and canopy cover ($\beta = 1.12$, $P = 0.028$, $\lambda = 0.71$), (c) and sexual dimorphism in saturation and canopy cover ($\beta = -0.47$, $P = 0.009$, $\lambda = 0.9$) (see also Table 1).

Figure S6 Posterior probability of having female-limited dimorphism (state 1) for nodes and ancestors, using the *make.simmap* function in the R package PHYTOOLS (Revell, 2011), using an ARD (all-rates-different) model and nsim = 500.

Table S1 Hawaiian damselflies examined in this study.

Table S2 Multivariate ANOVA results on mean background hue (H), saturation (S), and brightness (B) of photographs.

Table S3 Mean hue values for each island-species and morph in the phylogenetic reconstruction.

Table S4 PC values and percent variance in the data explained by the axes.

Table S5 Linear regressions of *M. calliphya* color on canopy cover were performed for each color variable: hue (H), saturation (S), and brightness (B).

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