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Evolution of opsin expression in birds driven by sexual selection and habitat

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Theories of sexual and natural selection predict coevolution of visual perception with conspecific colour and/or the light environment animals occupy. One way to test these theories is to focus on the visual system, which can be achieved by studying the opsin-based visual pigments that mediate vision. Birds vary greatly in colour, but opsin gene coding sequences and associated visual pigment spectral sensitivities are known to be rather invariant across birds. Here, I studied expression of the four cone opsin genes (*Lws*, *Rh2*, *Sws2* and *Sws1*) in 16 species of New World warblers (Parulidae). I found levels of opsin expression vary both across species and between the sexes. Across species, female, but not male *Sws2* expression is associated with an index of sexual selection, plumage dichromatism. This fits predictions of classic sexual selection models, in which the sensory system changes in females, presumably impacting female preference, and co-evolves with male plumage. Expression of the opsins at the extremes of the light spectrum, *Lws* and *Uvs*, correlates with the inferred light environment occupied by the different species. Unlike opsin spectral tuning, regulation of opsin gene expression allows for fast adaptive evolution of the visual system in response to natural and sexual selection, and in particular, sex-specific selection pressures.

1. Introduction

The striking colours of birds, and especially the large differences in colour across males of sexually dimorphic species, are thought to be a result of sexual selection [1]. Theories about how sexual selection drives the evolution of colour fall into three general classes. The null hypothesis is that colours evolve to exploit an invariant receiver's sensory system [2,3]. For example, colours can appear relatively conspicuous or cryptic against different backgrounds [4,5] or, as initially suggested by Darwin [6], novelty *per se* may be attractive to females. In one alternative hypothesis, colour perception and colourful displays co-evolve even if populations occupy similar environments. This is exemplified by Fisherian models of runaway sexual selection, in which female preferences for a colour become genetically correlated with colour [7–9], as well as in models in which a male trait indicates some sort of benefit to a female (e.g. good parental ability), and thus colour preferences are under direct selection to increase [10]. In a second alternative hypothesis, the visual system evolves in response to natural selection pressures [11–14]. For example, finding ripe fruit has been suggested to be an important selective factor leading to the evolution of trichromatic colour vision in primates [15,16] and a changing light environment with water depth has been related to many features of fish vision [11,12,17–19]. The resulting changes in visual sensory perception may then affect preferences for conspecific colours, driving colour evolution [20,21].

Direct behavioural measurements of colour perception in animals are difficult, as they require extensive training of laboratory animals and testing with multiple wavelength stimuli to quantify what an animal sees [22]. Here, I employ an alternative approach, quantifying differences in the visual pigments, the light-catching molecules that populate the rod and cone photoreceptors of the retina. Visual pigments consist of an opsin protein bound to a light-sensitive chromophore, and in the case of cone opsins, are known to belong to different spectral classes sensitive to light of different wavelengths [23]. Visual pigment

evolution has been linked to the light environment in species in which their spectral sensitivities are shifted to improve visual performance in different light environments (reviewed in [3]). But additionally, as sensory receptors, visual pigments are at the basis of perceptual biases that direct female preferences for male traits [3,7,12]. In fishes and butterflies, variation in visual pigments has been implicated in mate choice, thereby shaping the evolution of male colours [12,19,24]. Birds are a notably colourful group, and have been the subject of many classic studies of sexual selection [1,25], that have shown plumage colour is used as a mating signal. To date, associations between birds' visual system and the evolution of plumage colour have been rarely studied ([26,27], reviewed in [2]). This may be in part because substitutions in opsin genes are few and have small effects on the spectral sensitivity of the resulting visual pigments, leading to the prevailing view that bird colour vision is quite invariant [2,22,28].

Opsin gene expression is expected to show greater evolutionary lability than opsin coding sequence, making this a logical trait to look for signatures of natural and sexual selection. Assuming opsin mRNA levels reflect protein abundance [29–32], differential expression of the opsin genes should result from differences in either the abundance of the photoreceptor type in which they are expressed, or in the packing of the visual pigment into each photoreceptor type. Both of these factors could impact vision: photoreceptor abundances have been suggested to determine colour discrimination thresholds in vertebrates [33] and the density at which visual pigments are packed in the membranes of photoreceptors is known to affect both sensitivity and speed of response [34]. I compare patterns of opsin gene expression across a group of colourful birds, the New World warblers (Parulidae). This group has varying degrees of sexual dimorphism in colour, a commonly used measure of the intensity of sexual selection [1], and occupies a range of light environments, making it possible to evaluate the two alternative hypotheses for the evolution of colour perception.

Bird colour vision is thought to be mediated by four types of opsin-based visual pigments [28]: long-wavelength sensitive (LWS), medium-wavelength sensitive (RH2), short-wavelength sensitive type 2 (SWS2) and short-wavelength sensitive type 1 (SWS1), all of which are found in single cones. SWS1 can be one of two types in birds, with peaks of maximum absorbance (λ_{max}) either in the 'violet' region of the spectrum (VS type) or in the 'ultraviolet' region of the spectrum (UV type) [35,36]. As in most passerines whose spectral sensitivity has been measured, the New World warblers possess an SWS1 that peaks deep in the ultraviolet (λ_{max} 365 nm, [37]) and is here termed UVS. LWS pigments are also expressed in a fifth cone type, the double cones, which are believed to be involved in various achromatic processes, such as texture and motion detection, but not in colour vision [38,39]. I use quantitative real-time PCR (qRT-PCR) to determine the relative mRNA expression levels for the four cone opsins associated with bird visual pigments across 16 species. I then compare differences in opsin expression to measures of the light environment and the degree of sexual dimorphism in each species to assess factors driving the evolution of opsin gene expression [1,40,41]. I found support for a role for sexual and natural selection in driving opsin gene expression. Female *Sws2* expression is strongly correlated with sexual dichromatism and the expression of two other opsins, *Uvs* and *Lws* correlates with the inferred light environment.

2. Material and methods

All individuals for this study died as a result of building collisions in Chicago (USA) and were collected during spring and autumn migration seasons between 2008 and 2010. New World warblers migrating through Chicago establish breeding grounds anywhere from northern Illinois to northern Canada depending on the species [42], and as such probably represent individuals from multiple populations. This diverse sample is an appropriate strategy to detect genetic differences in patterns of opsin expression across species. I obtained retinas for 16 species of New World warblers belonging to six different genera: *Setophaga caerulescens*, *Setophaga castanea*, *Setophaga coronata*, *Setophaga fusca*, *Setophaga magnolia*, *Setophaga palmarum*, *Setophaga pensylvanica*, *Setophaga striata*, *Setophaga virens* and *Setophaga ruticilla*, *Geothlypis trichas and Geothlypis philadelphia*, *Oreothlypis ruficapilla*, *Mniotilta varia*, *Seiurus aurocapilla*, *and Parkesia noveboracensis*. When possible, I collected retinas from two males and two females in each of these 16 species. All birds were collected immediately after death, between 5.00 and 9.00, to avoid variation in opsin levels that could occur throughout the day.

I isolated total mRNA from the retinas of 64 individuals and reverse transcribed it into cDNA. I performed qRT-PCR for each opsin and the endogenous control, β-actin, in parallel for all samples. To obtain estimates of the relative expression of each opsin gene, I measured initial fluorescence and normalized it against that of the endogenous control. I calculated relative expression as:

$$\text{normalized opsin } R_0 = \frac{\text{opsin gene } R_0}{\text{β-actin control } R_0}.$$

Then I multiplied it by 100 to express all normalized opsin expression as a percentage of β-actin expression. Initial fluorescence (R_0) for each reaction was calculated in DART-PCR [43] using the Amplification Plot method to estimate amplification efficiencies (E), and averaged across replicates as described in the electronic supplementary material. R_0 was calculated using the average efficiency from all reactions for each gene to avoid introducing random variation (as recommended in [43]).

To evaluate the evolutionary significance of the expression differences, I used data on plumage sexual dimorphism from Shutler & Weatherhead [40]. In this study, plumage dichromatism was calculated as the per cent of body area where plumage colour differed between the two sexes. Additionally, I used two measures of habitat use: (i) one of general habitat, following classifications in [42], according to which the New World warblers in this study are classified as occupying coniferous, deciduous, shrub or open habitats; and (ii) foraging height as it has been shown to follow an important axis of light quality variation in forests [44]. I used a discrete measure of foraging height as recommended in [45], classifying birds as ground (less than 1 m) or arboreal foragers. Data can be found in the electronic supplementary material, table S7. I used linear models to assess the association of differences in the degree of sex bias and relative expression with plumage dimorphism and environmental measures. For each test, I performed the corresponding phylogenetic least-squares regression based on the New World warbler phylogeny in [46] (details of models and corresponding phylogenetic correction can be found in the electronic supplementary material).

To estimate cone abundances, I collected eyes from males and females of *Seiurus aurocapilla* and *Geothlypis trichas* and prepared retinal whole-mounts following [44,47,48]. I used coloured oil droplets counts to estimate the relative abundance of the different cone types from photographs of eight retinal quadrants taken with a stereomicroscope at 600× magnification.

More methodological details as well as primer sequences and data used in the analysis can be found in the electronic supplementary material.

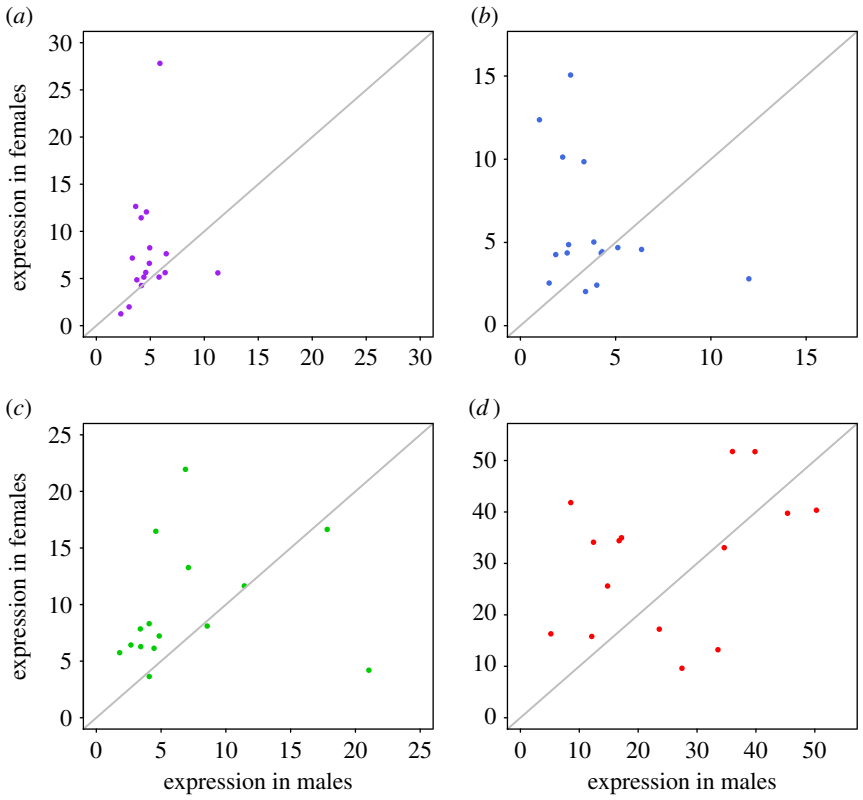

Figure 1. Male versus female normalized expression for (*a*) *Uvs* (*b*) *Sws2* (*c*) *Rh2* and (*d*) *Lws*. Opsin initial fluorescence (R_0) was used as a proxy for expression and normalized to an endogenous control, β-actin, as $R_{0N} = R_{0\text{opsin}}/R_{0\beta\text{-actin}}$ (see the electronic supplementary material for additional information). Each point corresponds to each species mean male and female expression. Note differences in axis scales. Grey 1 : 1 lines correspond to male = female expression, thus any species above the line have higher expression in females than males, and species below the line have higher male expression. Intraspecific variation and mean values can be found in the electronic supplementary material, figure S1 and table S1. (Online version in colour.)

3. Results

(a) Species and sex differences in opsin expression

Opsin expression levels averaged across species reveal *Uvs* and *Sws2* are expressed at similar, low levels: mean expression normalized to β-actin is 6.5 ± 4.8 s.d., $N = 16$ species for *Uvs* and 4.9 ± 3.4 s.d. for *Sws2* (all values of mean normalized expression are ratios of initial fluorescence (R_0) normalized to β-actin and multiplied by 100 to be expressed as a percentage of β-actin fluorescence; figure 1; electronic supplementary material, figure S1 and table S1). The relatively low expression of the *Uvs* and *Sws2* opsins is expected, given the UVS and SWS2 cones are the least abundant cone types in birds [23,47]. *Rh2* is expressed at higher levels than *Uvs* and *Sws2* (8.5 ± 5.6 s.d.) and *Lws* has the highest relative expression of all the opsins (27.4 ± 14.0 s.d.), which is also expected because *Lws* is expressed in both single and double cones [49] and, in all birds studied so far, double cones are the most abundant cone cells in the retina, comprising around 50% of the cone population [23]. I controlled for the possible effects of collection season and time and found that the expression of cone opsins does not vary significantly with either of these factors (electronic supplementary material, table S2).

Expression of *Rh2*, *Sws2* and *Uvs* differs significantly across species and between sexes. Using individuals as replicate in an ANOVA, differences across species were highly significant: *Rh2* $F_{15,29} = 6.18$, $p < 0.0001$; *Sws2* $F_{15,29} = 2.59$, $p = 0.01$; *Uvs* $F_{15,29} = 5.77$, $p < 0.0001$. The same was true for opsin expression differences between sexes: *Rh2* $F_{1,29} = 30.27$, $p < 0.0001$; *Sws2* $F_{1,29} = 14.56$, $p = 0.0007$; *Uvs* $F_{1,29} = 4.65$, $p = 0.04$. In these models, the interaction term was always significant, indicating the degree to which opsin expression differs between females and males varies across species (*Rh2* $F_{15,29} = 5.43$, $p < 0.0001$; *Sws2* $F_{15,29} = 5.90$, $p < 0.0001$; *Uvs* $F_{15,29} = 2.48$, $p < 0.05$; see figure 1; electronic supplementary material, figure S2). For *Lws*, I found no significant differences in expression between species or sexes, which may reflect high intraspecific variation, more so in females than males (electronic supplementary material, figure S1). A larger sample size than the one used in this study would be necessary to determine whether the variation observed here reflects consistent differences in *Lws* expression across species and between sexes.

(b) Comparative tests

The finding of opsin expression differences across the warblers rejects the null model of an invariant visual system. Using phylogenetically controlled tests, I consider the two general alternatives for why visual sensory systems might vary, as outlined in the Introduction: first, visual perception and traits may coevolve without any necessary adaptation to environmental features and second, visual perception may evolve as a result of selective pressures imposed by the environment. I studied correlates of expression levels with: (i) the degree of plumage dichromatism, a widely used index of the strength of sexual selection [1,40], as independently assessed for Parulid warblers by Shutler & Weatherhead [40]; (ii) habitat occupied (deciduous forest, coniferous forest, shrub or open habitats [42]); and (iii) foraging height, (dichotomized into the binary variable

ground versus arboreal [45]), as these two measures of habitat use reflect changes in the light environment each species experiences. Because opsin expression differed significantly between sexes, I evaluated the relationship between opsin expression and each of these measures separately in each sex. To control for the false discovery rate associated with multiple testing, I performed a step-up Benjamini–Hochberg procedure [50] correcting for 24 tests (three explanatory variables—plumage dichromatism and two habitat measures—for four opsins, each separately in males and females). A list of the tests performed and associated false discovery rate procedure can be found in the electronic supplementary material, table S4.

(c) Strength of sexual selection and opsin expression

Plumage dichromatism and the height at which birds forage in the forest are thought to be correlated in neotropical rainforest birds [4] and this association is also present in the current dataset ($F_{1,14} = 7.07$, $p = 0.019$). Therefore, when testing for an association between opsin expression and plumage dichromatism, I used multiple regression to control for effects of habitat. Plumage dichromatism and *Sws2* expression in females are strongly correlated (figure 2*a*; controlling for foraging height: $F_{1,13} = 29.3$, $p = 0.0001$; phylogenetic control using phylogenetic least-squares regression $p < 0.0001$, model details can be found in the electronic supplementary material, Methods and table S5). This relationship was absent in males ($F_{1,13} = 1.54$, $p = 0.24$; phylogenetic control, $p = 0.52$; figure 2*b*). The relatively high *Sws2* expression in females of the more dichromatic species results in a strong correlation between sex-bias in *Sws2* expression (female/male expression ratio) and plumage dichromatism (electronic supplementary material, figure S4). Plumage dichromatism is not significantly correlated with the expression of the three other opsin genes in either sex, although *Rh2* expression follows the same trend as *Sws2*, which is significant after removal of two outliers (electronic supplementary material, table S5).

(d) Opsin relative expression in different light environments

The expression of *Rh2* and *Sws2* does not differ significantly between species that occupy different habitats (electronic supplementary material, figure S1 and table S1), but both *Lws* and *Uvs* expression correlates with species habitat use. *Lws* expression is higher in species found in the two darker forest environments (deciduous and coniferous) than in the more open habitats outside the forest (shrub and open habitats; figure 3). Again this relationship is much stronger in females ($F_{3,11} = 7.97$, $p = 0.004$, phylogenetic correction $p = 0.0007$), than in males ($F_{3,11} = 1.46$, $p = 0.28$; phylogenetic correction $p = 0.07$).

Species that forage low in the forest tend to have lower *Uvs* expression than species that forage higher up (figure 4). After removing one outlier in each sex that had unusually high *Uvs* expression, this correlation is present in both sexes, but stronger in females after phylogenetic correction (females: $F_{1,13} = 7.89$, $p = 0.015$, phylogenetic correction $p = 0.0005$; males: $F_{1,13} = 8.72$, $p = 0.011$, phylogenetic correction $p = 0.02$). Species that forage lower in the forest experience environments that are relatively poor in low-wavelengths [44] (figure 4), implying differences in *Uvs* expression are in the direction expected, given the way light composition changes with

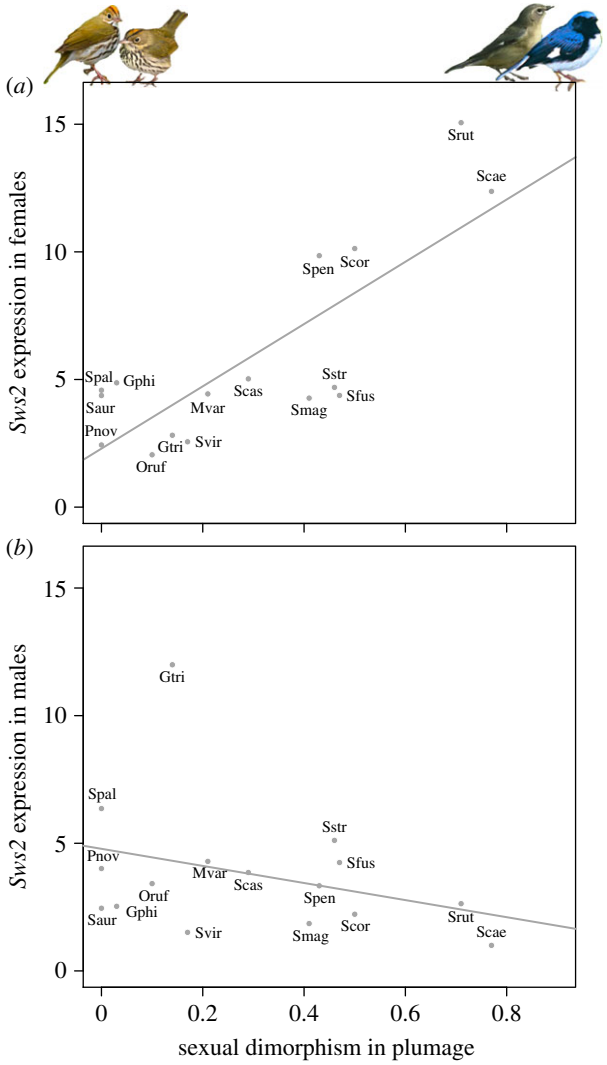

Figure 2. Relationship between *Sws2* expression and sexual dimorphism in plumage in (*a*) females, $y = 14.50x + 3.43$ (phylogenetically corrected $p < 0.0001$) and (*b*) males, $y = -3.45x + 4.73$ (phylogenetically corrected $p = 0.52$). Species names are abbreviated as the first letter of the genus and the three first letters of the species. See Methods for full species names. Bird illustrations: male and female of *Seiurus aurocapilla*, a species sexually monomorphic for plumage (left) and *Setophaga caerulescens* (right). These are the species with the least and most sexually dichromatic plumage in the study. (Online version in colour.)

foraging height in a forest [51]. The significance of this association is not robust to the false discovery rate, and thus results may be treated more cautiously; the low within species sample sizes in this study do not allow for a definite conclusion. But similar results are apparent for a continuous, independent, classification of foraging height (electronic supplementary material, figure S5) and the relationship is present in both sexes. The outlier removed from the analysis, *G. trichas* has unusually high *Uvs* expression and high intraspecific variation (electronic supplementary material, figure S1). Data for an additional sample indicate the higher expression, and variability in this species is not the result of experimental problems but a reflection of a unique feature of *G. trichas* opsin expression profile.

(e) Opsin expression and cone counts

The mechanisms by which opsin expression differences could ultimately affect vision depend on whether opsin expression

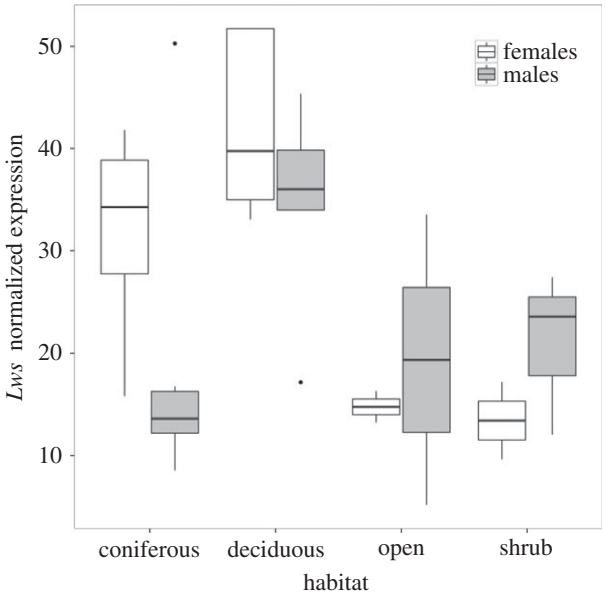

Figure 3. *Lws* expression is higher in darker forest habitats than in brighter open and shrub habitats. Distribution of *Lws* relative expression values for males (grey) and females (white) of species occupying various habitats ($N = 16$ species). Boxes correspond to the first and third quartiles (the 25th and 75th percentiles). Whiskers extend to the lower and higher values and lines indicate the median. Points beyond the inter-quartile range are outliers. Habitat classifications for each species can be found in the electronic supplementary material, table S7.

reflects variation in cone abundance or the density at which opsins are packed into the cones [22,34]. In birds, cone relative abundances can be estimated because each cone type is associated with a different colour oil droplet, that can be differentiated using retinal whole-mounts microscopy (shown in the electronic supplementary material, figure S3, [47]). I estimated cone abundances in two warbler species, *Sei. aurocapilla* and *G. trichas* (electronic supplementary material, table S3). I excluded *Lws* from this analysis as the expression of this opsin in single LWS cones and double cones cannot be discriminated. For these two species, the relative density of cone types in the retina does not match relative opsin expression for *Uvs*, *Sws2*, *Rh2* ($r = -0.02$, $N = 12–3$ cone/opsin types, in two sexes for two species—data in electronic supplementary material, tables S1 and S3). This lack of correspondence is more generally apparent, because the UVS cones have been consistently shown to be the rarest type of cone in birds (between 4.7 and 7.3% in Passerines [47]), as well as in my own cone counts (around 6%; electronic supplementary material, table S3), whereas in some species *Uvs* expression is higher than *Sws2* expression (electronic supplementary material, figure S1 and table S1). With the caveat that it was not possible to obtain both relative opsin expression and cone abundances from the same individual bird, these findings imply that differences in the expression of each opsin reflect more than just relative cone abundances and could be owing to differences in the amount of opsin packed in each cone type.

4. Discussion

The contribution of gene regulation to phenotypic evolution has been discussed extensively during the past decade [52,53]. In other groups, such as *Drosophila*, gene expression patterns are known to be evolutionarily labile [54,55] and gene expression differences have been shown to be at the basis of adaptive, phenotypic evolution [55,56]. In birds, differential gene expression has also been associated with sexual differentiation [57]. Here, I found large differences in opsin expression among New World warbler species as well as between the sexes, which can be related to both measures of sexual selection and the light environment.

New World warbler species have accumulated few substitutions in their opsin genes and their visual pigment spectral sensitivities have evolved slowly [58]. Thus in warblers, opsin expression regulation is evolving faster than opsin structural gene mutations. Expression variation should also more easily lead to the evolution of sex limited traits than structural gene mutations, which are likely to simultaneously affect both sexes [59]. In this way, opsin expression should respond more readily to sex-specific selective pressures. In birds, the only previous demonstration of sexual dimorphism in the visual system comes from a study of cowbirds, *Molothrus ater*, where males and females differ in their cone photoreceptor densities and associated behaviour in gathering visual information [48]. Sexual dimorphism in the visual system across birds may be much more widespread than is currently appreciated.

Opsin expression differs between the sexes and across species in warblers, but the question remains as to how this ultimately affects vision. In other systems, there is evidence that mRNA expression correlates well with protein expression from genome wide studies [31,32] and more specifically for opsin gene expression [60]. Here I assume this is the basis for any adaptive differences between species. The differences may be achieved by altered cone numbers or by differences in the amount of visual pigment packed into each of the various cone types.

Clearly, some differences in opsin expression should be a direct reflection of cone abundance, for the more cones of a particular type, the higher the expression of the corresponding opsin will be. This has been confirmed in studies on fishes and humans [29,30]. Variation in the number of cones of each type can have important consequences for photon capture, especially in dim light when photon capture becomes limiting [33,61]. In an influential model Vorobyev & Osorio [38] proposed the abundance of the different cone types is important to colour discrimination, because it should determine the amount of noise in each receptor channel. My cone counts in two warbler species suggest that opsin expression varies beyond cone abundances and also reflects how much visual pigment is packed into individual cones of different types. Visual pigment densities in the photoreceptor's membranes, together with cone outer axial length affect optical density, i.e. the probability that a photon is captured by the photoreceptor [62]. Higher optical density translates into a broader cone spectral sensitivity curve, impacting colour discrimination by changing the overlap between different photoreceptor's spectral sensitivities and the slope of the spectral sensitivity curve [63,64]. In addition, research on the rods of transgenic mice has shown sensitivity, and the speed of response to light trades off with changing visual pigment densities: when visual pigments are packed at higher densities, photoreceptor sensitivity increases, but this decreases the speed of the response and thus temporal resolution (i.e. precision in detecting an object in motion), as it impedes the free movement of these molecules along the membrane [34]. In face of this

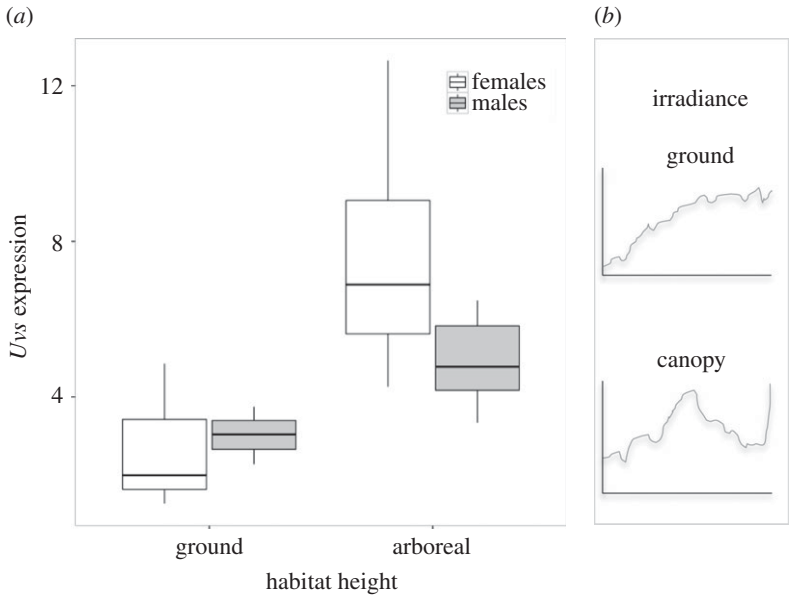

Figure 4. *Uvs* relative expression increases with habitat height. (*a*) Distribution of *Uvs* relative expression values for males (grey) and females (white) of species occupying ground (less than 1 m) and arboreal habitats. Boxes correspond to the first and third quartiles (the 25th and 75th percentiles). Whiskers extend to the lower and higher values and lines indicate the median. One outlier for each sex not shown ($N = 15$ species). Habitat classifications for each species can be found in the electronic supplementary material. See the electronic supplementary material, figure S5, for similar trend with a continuous classification of foraging height. (*b*) Irradiance as a function of wavelength for forest ground and tree canopy adapted from [44], illustrating how the lower habitats in the forests are relatively deprived of short-wavelengths.

trade-off, visual pigment density within a photoreceptor would presumably be optimized by selection. For example, good temporal resolution might be selected for in the double cones of a flycatching bird that needs to catch fast moving prey, whereas sensitivity might be favoured instead in the photoreceptors of species occupying dark forest environments.

As a framework for this study, I considered three general hypotheses for how colour may be related to colour perception, all derived from sexual selection theory. Evidence from opsin coding sequences suggests that the visual pigments are rather invariant, with colour signals evolving to exploit perceptual biases. However, the finding that opsin expression is labile admits the two alternative possibilities, that colour signals and the visual system co-evolve and that the visual system itself evolves in response to environmental variation. I find evidence for both.

Plumage dichromatism has been found to be a reasonable measure of the strength of sexual selection [65]. Thus, the positive relationship between female *Sws2* opsin expression and plumage dichromatism imply a role for sexual selection linking the evolution of the visual system and plumage. Moreover, the degree of plumage dichromatism is mainly a consequence of male plumage evolution [1], indicating that the female visual system is co-evolving with male plumage evolution. This association fits the predictions of classic models for the evolution of mate choice, in which the female sensory system, and consequently female preference, and male traits coevolve [25,66]. This finding suggests differences in opsin expression could be associated with changes in female preferences through changes in visual perception. Higher expression of *Sws2* in females of species with more colourful males may be associated specifically with detecting colour variants within the wavelengths to which *Sws2* is tuned. Alternatively, strong sexual selection may place a greater premium on distinguishing among conspecific males across the colour spectrum, with *Sws2* expression representing just one component. There is some support for this in that *Rh2* expression shows a similar pattern to that observed in *Sws2*. Few previous examples of an association between colour and colour perception arising apparently independently of the environment have been presented [2], probably because most research has focused on visual pigment structural and functional evolution.

A second mechanism whereby visual systems can evolve is in response to selective pressures imposed by the light environment [14]. Previous studies on visual pigments, including opsin expression patterns in cichlid fishes [51,67,68], suggested that the visual system is adapted to exploit the available quantity and quality of light in an organism's environment [17,19,69]. These have been across steep light gradients, notably water depth in aquatic organisms and the diurnal/nocturnal transition [70]. Aspects of the visual system shown to correlate with the light spectrum include visual pigment spectral sensitivities [12,71,72], intraocular filters [73], cone abundance [74], opsin gene expression [75], cone outer segment length [76] and detection thresholds as measured behaviourally [20]. In the warblers, *Uvs* and *Lws* expression correlate with features of their habitat. First, warblers foraging in lower strata have lower *Uvs* expression than species foraging higher up in the canopy (figure 4 and electronic supplementary material, figure S5). As short-wavelength light is filtered by leaves and trees, lower strata of the forest become relatively poor in short-wavelength light [44] (figure 4*b*). This result implies that the visual system is adapted to light gradients in diurnal terrestrial systems, with changes in the direction that matches the most abundant wavelengths in their environments. Second, warblers in forest environments express *Lws* at higher levels than those in open/shrub habitats, environments that differ dramatically in light availability. In sunny conditions, the intensity of light in darker forest habitats can

differ from open habitats by almost three orders of magnitude (total light intensities averaged over several localities approx. 14 versus approx. 1150 $\mu mol\ m^{-2}\ s^{-1}$, respectively, from table 2 in [44]). Expression differences may reflect activity in the double cones or the LWS single cone, both of which contain the *Lws* pigment [28]. This may affect colour discrimination in dark environments (if the LWS single cone is affected) or increase sensitivity and/or temporal resolution for species in the darker forest habitats (if the changes are mainly in the double cones) [34]. The differences in the way *Lws* expression associates with habitat between males and females could be explained by differences in habitat use between the sexes. We know from the study of a few warbler species that males forage higher and flycatch more often than females [77], and it is entirely possible that intersexual differences in habitat and foraging habits could lead to some of the expression differences reported here.

Studying opsin expression in birds has revealed the visual system of birds is more evolutionarily labile than suggested on the basis of opsin spectral tuning [2,28]. In warblers, the expression of various opsins correlates both with plumage dichromatism and the light environment different species occupy, suggesting avian visual systems respond to both sexual and natural selection pressures through changes in opsin regulation. This study also implies the different visual pigments are responding to different selective pressures: while *Sws2*, and possibly *Rh2* expression, relates to changes in the intensity of sexual selection, the expression of the two opsins at the extremes of the visual spectrum, *Lws* and *Uvs*, correlates with habitat, and hence the light environment. Differences in the expression of all cone opsins apparently evolve both independently and in concert to achieve fine-tuning of the visual system that matches the specific needs of different species. Opsin expression is only one component of colour perception, and when the perceptual system is fully understood, we should have a much clearer understanding of why colours vary so greatly in nature.

Data accessibility. Data used in this study can be found as the electronic supplementary material.

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