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Opsin gene expression plasticity and spectral sensitivity in male damselflies could mediate female colour morph detection

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The visual systems of Odonata are characterized by many opsin genes, which form the primary light-sensitive photopigments of the eye. Female-limited colour polymorphisms are also common in Odonata, with one morph typically exhibiting male-like (androchrome) coloration and one or two morphs exhibiting female-specific coloration (gynochromes). These colour polymorphisms are thought to be maintained by frequency-dependent sexual conflict, in which males form search images for certain morphs, causing disproportionate mating harassment. Here, we investigate opsin sensitivity and gene expression plasticity in mate-searching males of the damselfly *Ischnura elegans* during adult maturation and across populations with different female morph frequencies. We find evidence for opsin-specific plasticity in relative and proportional opsin mRNA expression, suggesting changes in opsin regulation and visual sensitivity during sexual maturation. In particular, expression of the long-wavelength-sensitive opsin LWF2 changed over development and varied between populations with different female morph frequencies. UV-Vis analyses indicate that short- and long-wavelength opsins absorb wavelengths of light between 350 and 650 nm. Assuming opponency between photoreceptors with distinct short- and long-wavelength sensitivities, these sensitivities suggest male spectral visual discrimination ability of androchrome and gynochrome females. Overall, our results suggest that opsin sensitivity and expression changes contribute to visual tuning that could impact conspecific discrimination.

1. Introduction

Opsins are G-protein-coupled receptors that, when bound with a vitamin-A-derived chromophore, form light-sensitive visual pigments responsible for colour vision in animals [1]. Many insects possess three classes of opsin proteins: ultraviolet sensitive (UVS), short-wavelength sensitive (SWS) and long-wavelength sensitive (LWS) with maximum sensitivity to wavelengths (λ_{max}) around 350, 440 and 530 nm, respectively [2,3]. Photoreceptor spectral sensitivity depends mainly on the absorption spectrum of expressed opsins, which can vary due to opsin gains or losses, structural changes or expression level differences [4–6]. Spectral sensitivity can also be modified by screening and filtering mechanisms, which are relatively widespread in insects [3]. The visual system is also shaped by environmental factors, behaviours and associated selection pressures. For example, loss of functional opsin genes correlates with nocturnal lifestyles in mammals [7], while opsin duplications in other species contribute to the regaining of spectral sensitivity [6,8]. In several insect species, functional changes among duplicated opsins likely

improve detection and/or discrimination of environmental and sexual signals [5,9].

Opsin gene expression is typically not fixed, but changes plastically over individual ontogeny or in response to environmental factors and lighting conditions [10–12]. For example, changes in opsin expression have been associated with light exposure in honeybees and ants [13,14], and in 'choosy' relative to 'non-choosy' females of the butterfly *Bicyclus anynana* [15].

Damselflies and dragonflies (Insecta: Odonata) are colourful diurnal flying insects that possess large conspicuous eyes comprising thousands of ommatidia (figure 1A). Odonates inhabit a wide array of visual environments, having both aquatic and terrestrial life stages, and they strongly rely on vision for a variety of behaviours including predator avoidance, prey capture and mate searching [17–19]. These insects are therefore excellent model organisms to study evolutionary and functional aspects of colour vision. Like many other insects, odonates express UVS, SWS and LWS opsins in their eyes. However, duplications of the SWS and LWS opsin classes have resulted in remarkable genetic diversity, with between 11 and 30 visual opsin genes identified across several families [20]. Electrophysiological recordings of visual sensitivity in the eyes of some species indicate photoreceptor spectral curves ranging from UV to red light [17]. While it is not clear how their abundant opsin copies function in vision, visual stimuli are clearly important for odonate behaviour. For example, comparisons of visual and olfactory cues suggested that vision is primarily used in mate choice in several species of damselflies [21,22].

In addition to colour changes associated with sexual maturation, many odonates exhibit heritable (genetic) female-limited colour polymorphisms, with one female morph having male-like coloration (androchrome females) and one or two morphs expressing female-specific coloration (gynochrome females) [23,24]. Observational and experimental field studies suggest that female colour polymorphisms function to reduce the negative fitness effects of excessive male sexual harassment by disrupting males' ability to efficiently form search images for mates [25–27]. According to the male mimicry hypothesis, androchrome females have a negative frequency-dependent advantage in their male-like similarity, which reduces male mating harassment [28]. Alternatively, although not mutually exclusive, the learned mate recognition (LMR) hypothesis suggests that males form a search image for the most common female morph in the population, allowing them to better detect the most locally abundant female morph, resulting in a rare-morph advantage [25]. The LMR hypothesis further predicts that the ratio of correct (e.g. mature conspecific females) versus incorrect (e.g. other males, heterospecifics) identifications should increase with experience [29].

There is partial empirical support for both the male mimicry and LMR hypotheses and, as stated above, these two hypotheses are not mutually exclusive. In support of the LMR hypothesis, males of the damselflies *Enallagma civile* and *I. elegans* show increased preference for androchrome or gynochrome females following previous exposure to these morphs [26,30,31]. Furthermore, naive *Enallagma* damselflies react sexually to both female morphs, but rarely to other males, contrary to expectations of the male mimicry hypothesis [30]. However, experiments in *Ischnura ramburi* revealed more male–male interactions in androchrome-biased settings, consistent with both mistaken mate recognition due to male mimicry and the formation of male search images for the common female phenotype [32]. Similarly, male mating harassment in *I. elegans* increases with increasing morph density for gynochrome females, indicative of search-image formation, but not for androchrome females [33].

This growing body of ecological, evolutionary, sensory and physiological studies, combined with the increasing availability of genomic resources [34], makes Odonata an excellent system to investigate the molecular basis of colour vision. Here, we focus on the well-studied Common Bluetail Damselfly (*I. elegans*) and first characterize opsin spectral sensitivity via heterologous expression in HEK293T cells. We next quantify variation in opsin mRNA expression across adult male maturation, in populations with variable female morph frequencies. We then use visual modelling to predict the effect of shifts in visual sensitivity on female morph detection and discrimination. Results suggest that opsin expression plasticity may provide one mechanistic proximate link between male vision, morph detection/discrimination and the resulting frequency-dependent sexual conflict, consistent with models for how these genetic polymorphisms are maintained in natural populations [25,35,36]. Further experiments coupling behavioural differences in mate preferences will be interesting to link our results of ontogenetic and plastic variation in male opsin expression profiles to colour vision preferences and perception of female colour signals in this dynamic system strongly shaped by visually guided male mating behaviours.

2. Material and methods

(a) Study system

Sexually mature male *I. elegans* are monomorphic, exhibiting blue body coloration on the thorax. In contrast, female *I. elegans* are polymorphic, with sexually mature females belonging to three distinct genetically determined colour morphs (figure 1B) [16]. Androchrome (male-like) females exhibit body coloration spectrally similar to male coloration, while gynochrome females exhibit spectrally distinct green (*infuscans*) or brown (*infuscans-obsoleta*) thorax coloration [37,38]. Females exhibit ontogenetic changes in thorax and abdomen coloration (figure 1C), with male *I. elegans* known to prefer body coloration of mature over immature females [39]. Apart from colour differences, androchrome and gynochrome females also differ in resistance and tolerance to ectoparasites [40] and in mating rates, resistance to mating attempts and fecundity [33].

(b) Quantification of female morph frequencies between sites and field collection of males

We quantified female morph frequencies from eight sites in southern Sweden (figure 1D) as part of an ongoing long-term field population study of *I. elegans* (see [35] and [16] for general methodology). Studied sites have both low genetic differentiation and high allelic diversity, indicating recent divergence and/or high gene flow [41]. All study populations were visited and

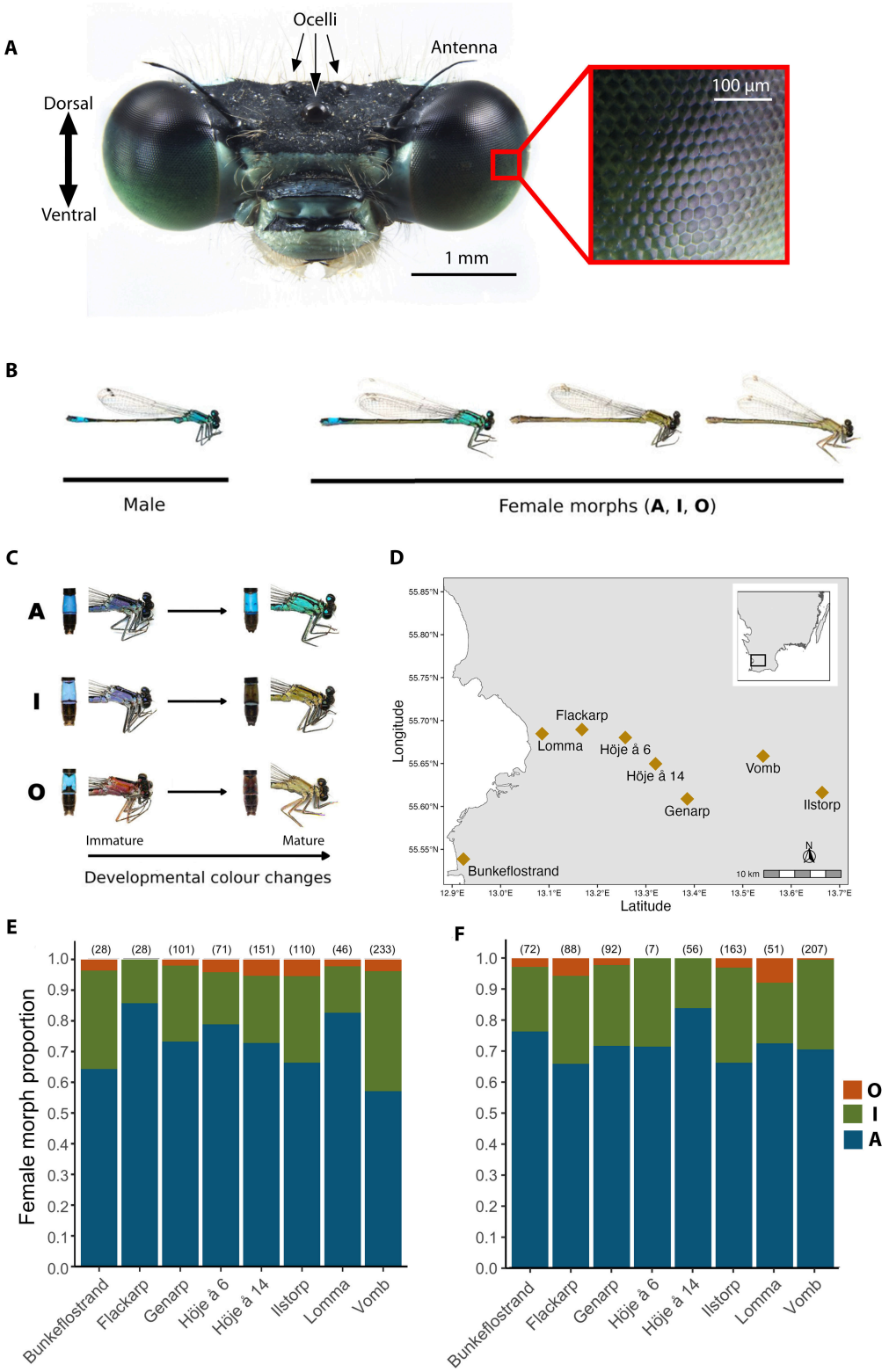

Figure 1. Head and eyes, colour polymorphism and female morph frequencies from eight populations in southern Sweden for the damselfly *Ischnura elegans*. (A) Head of male *I. elegans*, showing the large compound eyes and three dorsally situated ocelli. Box shows magnified view of the compound eye depicting individual ommatidial units. Photo credit: M. Bok. (B) Mature male *I. elegans* and the three female-limited colour morphs in their sexual mature colour phases. (C) Females undergo ontogenetic colour changes in the distal abdomen segments (left photo) and thorax (right photo) between immature and sexually mature adult stages. (D) Sampling locations of *I. elegans* used in the current study with inset showing the region sampled. (E,F) Female morph frequencies per site in 2021 (E) and 2022 (F). Numbers in parentheses above each bar indicate sample size. (B,C) Modified from Willink *et al*. [16]. A = androchrome, I = *infuscans*, O = *infuscans-obsoleta*.

re-visited at regular intervals (1–2 weeks) between 19 May and 1 August 2021, and 16 May and 31 July 2022. Including both immature and mature females, all populations were trimorphic except for one (Höje å 6) in 2022, which had experienced large overall population declines that year (electronic supplementary material, table S1). The frequency of each morph in the current study was calculated based on females that displayed mature adult coloration.

Male *I. elegans* were caught from sampled populations between 28 June and 13 July 2021, and 9 June and 20 July 2022 using sweep nets (electronic supplementary material, table S2). For all collected individuals, we recorded sexual maturity

('immature' versus 'mature') by evaluating wing stiffness [18]. In 2022, when males were caught in copula with a female, we also recorded the females' morph identity. These results are, however, not presented in the main text because analysis indicated no significant effect of female morph on opsin expression (see supplemental methods). In addition to immature and mature males, we also collected males that had newly emerged from the aquatic nymph stage ('tenerals') from two field sites and from a semi-naturalistic mesocosm experiment at the Stensoffa Ecological Field Station at Lund University (electronic supplementary material, table S2). Teneral males are characterized by their extremely soft wing and body tissues and lack of body pigment. Including teneral males allowed us to assess opsin gene expression in males that have not had any significant visual experience or mated with sexually mature females. All collected males were immediately euthanized by cutting off their head using cleaned RNAse-free dissection tools, placed into individual vials filled with RNA*later* (Ambion, Inc., Austin, TX, USA) at field sites and then stored at −20°C until RNA extraction.

(c) Functional expression in HEK293T cells and homology modelling

For functional expression, we selected SWS and LWS visual opsin types whose orthologues in *Sympetrum frequens* and *Ischnura asiatica* have been shown to be expressed in the adult ventral compound eye (SWb1, SWb2, LWA2, LWF1—F4) and LWE1 to test for function of an opsin whose orthologue in *I. asiatica* is primarily expressed in ocelli [20]. We included *Apis mellifera* SWS opsin as a positive control and SWb2 mutated at the retinal binding site (K335E) as a negative control. Homology modelling was performed for the SWb1 and LWF1 opsin amino acid sequences, aligned against the invertebrate jumping spider rhodopsin crystal structure (PDB 6i9k) [42]. Heterologous opsin expression and purification procedures, western blot analyses and protein modelling are presented in supplemental methods.

(d) Quantitative PCR analysis of *I. elegans* opsin expression levels

We quantified whole-head opsin mRNA expression via quantitative polymerase chain reaction (qPCR) in $n = 87$ male *I. elegans* (8 teneral, 36 immatures, 43 matures; electronic supplementary material, table S2) for UV, SWb1 (or SW1), SWb2 (or SW2), LWA2 (or LWA), LWF1-F4 and LWE1 (or LWE) opsins. Detailed procedures for complementary DNA (cDNA) synthesis using oligo-(dT), qPCR optimization steps and analysis are presented in supplemental methods.

Expression of opsin genes was calculated relative to two housekeeping genes and calibrated against the UV opsin gene using the field standard $(1 + E)^{-\Delta\Delta CT}$ method, taking into account that E equals primer efficiency for each opsin [43,44] (see electronic supplemental methods). We also calculated the proportion of opsin expression relative to total opsin expression for opsins likely expressed in the main compound eye in other odonatans by dividing relative expression for each individual opsin by the sum of total opsin expression.

(e) Visual modelling of female morph detection and discrimination

To assess how observed differences in LWS opsin expression might impact detection or discrimination of female androchrome and *infuscans* morphs, we calculated just noticeable differences (JNDs) for three parsimonious variations of Odonate visual systems. The first modelled visual system follows electroretinogram (ERG) spectral sensitivity values from *Ischnura heterosticta* [45]. The second and third visual models were based on *I. elegans* opsin spectral sensitivity corresponding to maximal opsin absorbance spectra for *I. elegans* LWF1 and LWF2 opsins, for which relative and proportional gene expression decreased and increased, respectively, in mature versus immature males (see §3). Equations used in visual model calculations are presented in electronic supplementary material, methods.

(f) Statistical analysis

We performed a multivariate analysis of variance (MANOVA) to assess the effect of maturity on log relative opsin gene expression of all eight opsin genes for male *I. elegans*. Log relative opsin expression for each individual male was averaged between replicates to maintain the assumption of independence.

We used linear mixed modelling to test the effects of population morph frequency, maturity stage and opsin type on log relative and proportion opsin expression in male *I. elegans*, with site, male ID, year and qPCR replicate as possible random effects. Model selection followed methods in Zuur *et al.* [46] to identify the random and fixed effects included in each model. Model 1 assessed changes in relative opsin expression for the main effects of maturity stage and opsin type, including opsins likely expressed in both the compound eyes and ocelli. Model 2 assessed changes in proportion opsin expression for opsins with likely expression in only the compound eye, with the main effects of maturity stage and opsin type. Model 3 tested the effects of maturity stage, opsin type, population morph frequency and its squared component on relative opsin expression. Detailed modelling methods are presented in the supplemental methods.

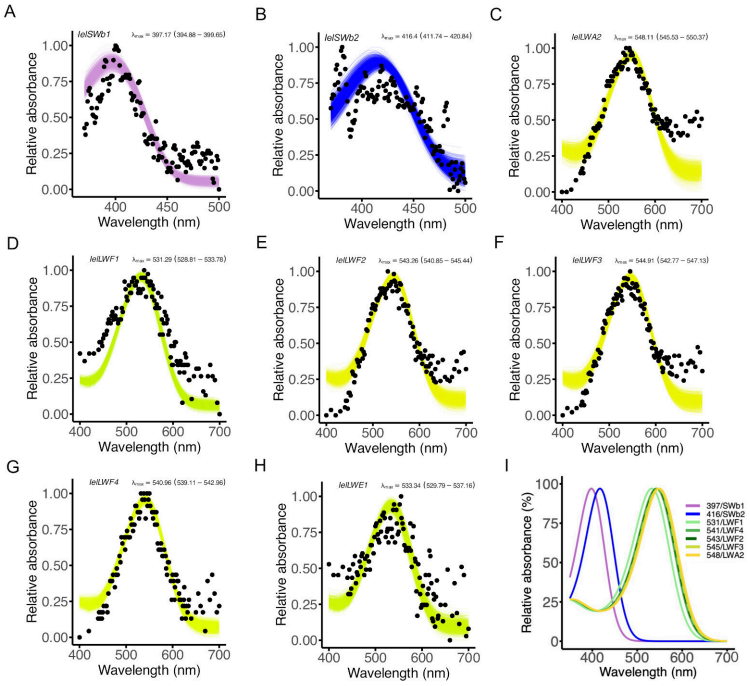

Figure 2. Functional expression of *Ischnura elegans* short-wavelength (SWS) and long-wavelength (LWS) opsins. Ultraviolet-Visible (UV-Vis) absorbance analyses of dark-adapted rhodopsin visual pigments reconstituted and purified from HEK293T cell cultures in the dark in the presence of 11-*cis*-retinal. The black dots represent mean absorbances at a given wavelength. (A) Difference spectrum for SWb1 ($n = 6$) (electronic supplementary material, figure S2D–F), (B) difference spectrum for SWb2 ($n = 4$) (electronic supplementary material S2G–I), (C) LWA2 ($n = 2$), (D) LWF1 ($n = 2$), (E) LWF2 ($n = 5$), (F) LWF3 ($n = 6$), (G) LWF4 ($n = 1$) and (H) LWE1 ($n = 6$), where n is the number of measurements of protein aliquots with active rhodopsin complexes. (I) Combined absorbances for SWS and LWS opsins, excluding LWE1, which is expressed in the ocelli in other odonates. Relative absorbance data are fitted to a visual template with polynomial function analyses computed in R to obtain the best estimates following 1000 bootstrap analysis of lambda max (λ_{max}) for each rhodopsin. The coloured curves represent λ_{max} of the fitted visual template. Confidence intervals are indicated in parentheses. UV-Vis absorbance data are available in electronic supplementary material, dataset S1.

3. Results

(a) Female morph frequencies

The frequencies of the three mature female morphs differed between sites and years (figure 1E,F). Androchrome females (A) were the majority female morph at all sites, and their frequency ranged from 57.1 to 85.7% (mean ± s.d.: 72.6 ± 9.7%) in 2021 and 65.9 to 83.9% (72.3 ± 5.8%) in 2022. The *infuscans* female morph (I) was the next most common morph, ranging from 14.3 to 39.1% (24.1 ± 8.8%) in 2021 and 16.1 to 30.7 (24.9 ± 5.3%) in 2022. Finally, the third morph (*infuscans-obsoleta*, O) was rare, making up only between 0.00 and 5.5% (3.3 ± 1.8%) of mature females in 2021 and between 0.00 and 7.8% (2.8 ± 2.8%) in 2022. Only one of the eight populations differed significantly in morph frequencies between both years (Vomb; see electronic supplementary material, table S3 for statistical comparisons for all sites).

(b) Opsin spectral sensitivity determined using HEK293 cell heterologous expression

Expression of the *A. mellifera* SWS positive control corroborated pigment absorption at 430 nm (electronic supplementary material, figure S2A-C, S2M), in line with previous evidence [47,48]. Difference spectra indicated that SWb1 and SWb2 opsins absorb maximally at λ_{max} of 397 and 416 nm (figure 2A,B, electronic supplementary material, figure S2D–I), whereas the SWb2 opsin mutated to a glutamine at the retinal binding lysine site (K335E) did not show activity upon light exposure (electronic supplementary material, figure S2J–L). The LWS opsin types display maximal sensitivity between 530 and 550 nm with the following λ_{max}: LWA2 = 548 nm, LWF1 = 531 nm, LWF2 = 543 nm, LWF3 = 545 nm, LWF4 = 541 nm, LWE1 = 533 nm (figure 2C–H, electronic supplementary material, figure S1, dataset S1). Spectral sensitivity functions of SWS and LWS opsins overlap with spectral reflectance of female thorax coloration of androchrome and *infuscans* females (electronic supplementary material, figure S3).

We ran homology modelling of SWb1 and LWF1 opsins against the spider rhodopsin (PDB: 6i9k) and mapped sites interacting with the chromophore in PyMOL. Of the 71 amino acid substitutions between SWb1 and SWb2 (81.8% amino acid identity), we identified 23 sites predicted to be within 5 Å of the binding pocket (electronic supplementary material, table S4). Among these, Y136 (TM3) and Y294 (TM6) residues located at the top and bottom of the binding pocket, respectively, are substituted by phenylalanine (F136 and F294) in SWb2 (electronic supplementary material, figure S4A). For LWS opsins, which share between 88.9 and 91.2% aa identity (i.e. 33–42 variant residues), we obtained the predicted LWF1 opsin structure based on 6i9k and mapped 24 sites predicted to interact with the *cis*-retinal, all of which are conserved for all four LWF opsins (electronic supplementary material, table S4, figures S4B, S5). The LWF2 protein shares from 89.9 to 90.5% identity with LWF1, LWF3 and LWF4, translating to 36–38 residue substitutions, and 67.4% identity (123 amino acid residue differences) with LWA2. LWA2

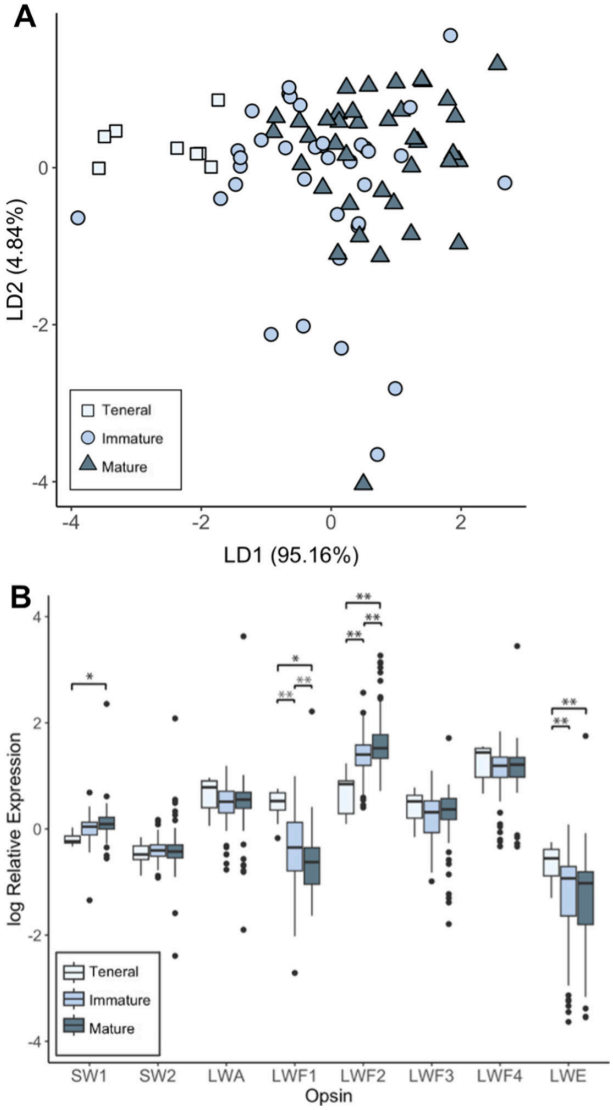

Figure 3. Relative opsin expression in teneral, immature and mature male *I. elegans*. (A) Linear discriminate analysis showing the relationship between relative expression of all tested opsins expressed in teneral, immature and mature male *I. elegans*. The percent of variation described by each linear discriminate factor (LD1 and LD2) is shown in parentheses. (B) Relative opsin expression between maturity stages. Teneral males are shown in light blue, immature adult males in medium blue and mature males in dark blue. Bars in box and whisker plots show medians, boxes indicate upper and lower quartiles, whiskers show sample minima and maxima and open circles show outliers. Expression data are normalized against housekeeping genes and calibrated against UVS opsin expression levels. Single asterisks indicate comparisons that are significant at alpha < 0.05 and double asterisks represent comparisons that are significant at alpha < 0.01 following Tukey corrections for multiple comparisons within opsin types. Test statistics and *p*-values for all comparisons are presented in electronic supplementary material, table S7; single population analyses are presented in electronic supplementary material, figure S7.

is more divergent in sequence, 67.5–68.7% aa identity with 119–124 aa differences with LWF opsins (electronic supplementary material, figure S5). Of the 24 chromophore-interacting residues, its binding pocket exhibits two potential spectral variant residues with LWF1 (A131G in TM1 and S319A in TM7).

(c) Opsin expression in teneral, immature and mature males

We compared the expression levels of two SWS and six LWS opsins, relative to UV opsin expression, across adult male developmental stages in *I. elegans* from eight sites. Our results from the MANOVA showed a significant effect of male maturity stage on overall opsin gene expression ($F_{16,146} = 3.2$, $p < 0.0001$; figure 3A).

For relative opsin expression (model 1), we found a significant interaction between maturity stage and opsin with increasing differences in relative expression from teneral males to immature and mature males (electronic supplementary material, figure S6, tables S5–S7). For teneral males, the highest relative opsin expression was for LWF4, while LWF2 has the highest relative expression for immature and mature males. Results also show that, for immature and mature males, the opsin LWE has significantly lower relative expression relative to other opsin types, consistent with expression levels observed in *S. frequens* and *I. asiatica* [20].

Comparing teneral, immature and mature males, results showed opsin-specific changes over maturation (figure 3B). Increases in relative expression occurred for the SW1 (teneral–mature: t = −2.7, d.f. = 396, $p = 0.02$) and LWF2 opsins (teneral–immature: t = −5.1, d.f. = 401, $p < 0.0001$; teneral–mature: t = −6.9, d.f. = 396, $p < 0.0001$; immature–mature: t = −3.0, d.f. = 419,

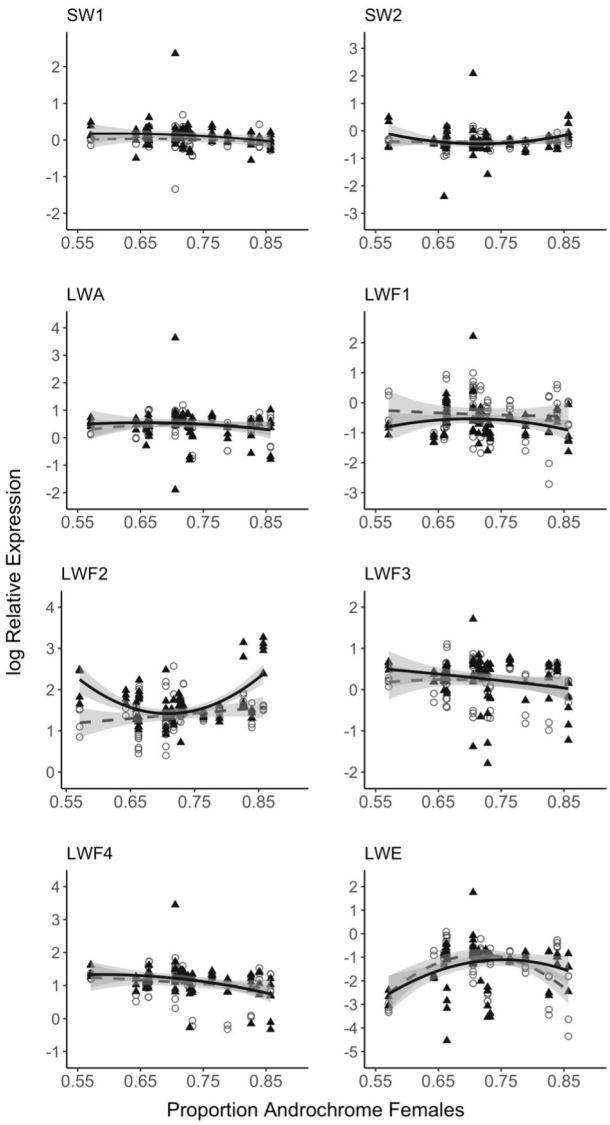

Figure 4. Opsin expression changes relative to proportions of androchrome females. Log relative opsin expression in immature (open circles, dashed line) and mature (black triangle, solid line) male *I. elegans* from populations with different proportions of androchrome females. Grey shading around trend lines indicates 95% confidence intervals.

$p = 0.009$). Conversely, decreases in relative expression occurred for the LWF1 (teneral–immature: t = 4.7, d.f. = 402, $p < 0.0001$; teneral–mature: t = 6.1, d.f. = 399, $p < 0.0001$; immature–mature: t = 2.4, d.f. = 433, $p = 0.04$) and LWE opsins (teneral–immature: t = 3.4, d.f. = 401, $p = 0.002$; teneral–mature: t = 3.9, d.f. = 400, $p = 0.0004$). For results for each independent population, see electronic supplementary material, tables S8, figure S7.

Analysing proportional opsin expression (model 2) showed a significant interaction between maturity stages and opsin type (electronic supplementary material, table S9). For all LWS opsins, except LWF1, we found a significant difference between teneral males and both immature and mature males, but not between immature and mature males (electronic supplementary material, table S10). For LWF1, there was a significant difference between all maturity stages tested (teneral–immature: t = 12.4, d.f. = 84, $p < 0.001$; teneral–mature: t = 15.2, d.f. = 84, $p < 0.001$; immature–mature: t = 4.4, d.f. = 84, $p < 0.001$). Relative and proportional opsin expression was thus largely consistent, with a decrease in LWF1 expression and increase in LWF2 expression over maturity, although differences in proportional LWF2 expression were not significant between immature and mature males (t = 2.1, d.f. = 84, $p = 0.10$), as with relative expression (electronic supplementary material, table S10). LWF4 expression, which did not significantly differ in relative expression over development (figure 3B), made up the largest proportion expressed for teneral males (0.523 ± 0.01). In immature and mature males, LWF2 made up the largest proportion opsin expressed, accounting for 0.524 ± 0.03 and 0.599 ± 0.02 of expression, respectively (electronic supplementary material, figure S8).

(d) Opsin expression across populations with different female morph frequencies

Comparing variation in opsin expression in immature and mature males including the effect of local female morph frequency (model 3), we find a significant interaction between opsin type, maturity stage and the squared component of androchrome frequency (electronic supplementary material, table S11). Plotting log relative expression for each opsin type revealed a

quadratic relationship between LWF2 expression and androchrome frequency in mature, but not in immature, males. We observed the highest LWF2 expression in populations with the lowest and highest proportion of androchrome females. In contrast, androchrome frequency had little effect on relative expression for immature or mature males for the other opsins tested (figure 4).

(e) Visual modelling of female morph detection and discrimination

Visual modelling results suggest that the LWF2 visual model outperformed the LWF1 visual model for all comparisons ($\Delta JND_{LWF2\text{-}LWF1}$ = 0.36–0.57) except for detection of *infuscans* females against brown and green vegetation, where JNDs were similar ($\Delta JND_{LWF2\text{-}LWF1}$ = −0.02 and 0.07). Comparing the LWF1 and LWF2 visual models to the model using ERG sensitivity from *I. heterosticta* showed that the LWF2 model had similar or larger JNDs for detecting androchrome females against vegetation (brown: $\Delta JND_{LWF2\text{-}ERG}$ = 0.43; green: $\Delta JND_{LWF2\text{-}ERG}$ = 0.00) (electronic supplementary material, table S12).

4. Discussion

Vision is an important sensory modality in Odonata and the prevalence of female-limited colour polymorphisms makes the damselfly genus *Ischnura* a powerful model system to study how and why polymorphisms emerge and persist over both micro- and macro-evolutionary time scales [23,34,35]. Here, we combined ecological, molecular and functional approaches to investigate opsin gene expression in male *I. elegans* of different maturation stages and across populations with varying female morph frequencies. Our measures of *in vitro* opsin absorbance evidence that the photosensitive opsin receptors forming the peripheral visual system of *I. elegans* perceive wavelengths that overlap with reflectance intensity (brightness) and chromatic body coloration cues of the two most abundant female morphs, namely androchrome and *infuscans*, from field sites (electronic supplementary material, figure S3). Furthermore, recurrent directional changes in opsin expression over male ontogeny, particularly during sexual maturation (figure 3), as well as changing male gene expression profiles in response to local female morph frequencies (figure 4) suggest a role for opsin expression plasticity in the *Ischnura* system.

Vision is used for many tasks in Odonates, and ecological factors might select for additional differences that may not necessarily be attributable to conspecific search and morph discrimination. The spatial organization of yet unknown ommatidial types possibly expressing a subset of the multiple SWS and LWS opsins across the retina may also influence how male *I. elegans* perceive conspecific body cues. However, *I. elegans* is equipped with numerous functional SWS and LWS visual opsins activated maximally by wavelengths of light highly reflected by female morphs (electronic supplementary material, figure S3). In light of known behavioural male preference for female colour morphs and dynamically changing morph frequencies [31,49], opsin-based spectral sensitivity across short and long wavelengths likely contributes to visual detection and discrimination of local female morphs.

(a) Overlapping opsin spectral sensitivities likely confer broad wavelength discrimination

A total of 12 expressed visual opsin genes have been identified in the damselfly *I. asiatica* [20], a gene repertoire that we find conserved in the genome of *I. elegans* (electronic supplementary material, figure S5A). From the *I. elegans* opsin gene set, we quantitatively assessed whole-head adult expression levels of two SWS (SWb1, SWb2) and five LWS (LWA, LWF1-F4) opsins for which orthologues in *I. asiatica* and *Sympetrum frequens* are primarily expressed in the ventral eye [20], a region implicated in conspecific search [50]. For comparison, we also quantified expression levels of *I. elegans* LWE1 opsin, which is restricted to ocelli in other odonates [20]. The *I. elegans* LWE1 opsin, which is sensitive to green wavelength light (figure 2), consistently exhibited the lowest monitored expression levels across opsin types (figure 3B, electronic supplementary material, figure S6), suggesting a restricted ocelli-specific expression pattern and function. In contrast, SWS and the other LWS opsins exhibited higher baseline expression levels compared with LWE1, as observed in *I. asiatica*, therefore supporting a role as visual opsins in the main compound eye.

The SWS opsins confer sensitivity to light in the violet-blue spectrum (λ_{max} = 397 and 416 nm) and the five visual LWS opsins show broad sensitivity to green wavelengths of light, with λ_{max} between 530 nm and 540–550 nm (figure 2). This forms a key component defining the genetic basis of light perception and functionality of multiple opsin duplicates, as part of several complementary mechanisms that certainly contribute to modulate visual sensitivity and capture a remarkably broad light spectrum from ultraviolet to long wavelengths above 600 nm. While achromatic vision of intensity-related cues can be directly modulated by light capture of single opsins [51], chromatic discrimination involves at least two distinct but overlapping sensitivities in a given ommatidial unit [52,53]. ERG recordings from other odonates show broad sensitivity of the green photoreceptor type which may indicate the presence of multiple opsin genes expressed within a single photoreceptor [45,54]. Irrespective of how many LWS opsins may be expressed in a single photoreceptor or ommatidium, overlap between short- (UV and violet-blue) and at least one long-wavelength opsin sensitivities could provide fine-tuned trichromatic discrimination capacity across a broad spectral range in *I. elegans* (figure 2, electronic supplementary material, figure S3), with additional filtering mechanisms common in arthropods potentially further increasing spectral channels [55,56].

Our results show a high degree of overlap in the recorded sensitivities of the LWS opsins, which suggest that changes in expression of any single opsin might not have a large impact on spectral sensitivity if several LWS opsins are co-expressed. This also suggests that there may be no opponency unless combining LWS opsins with 10–20 nm differences between peak spectral

sensitivity [57]. However, substantial increases and decreases in opsin expression levels for opsin types with λ_{max} differences greater than 10 nm (e.g. LWF1 λ_{max} = 531 nm and LWF2 λ_{max} = 543 nm) may affect the underlying photoreceptor spectral visual sensitivity if these specific opsins are also spatially segregated across the retina.

Spatial differences in opsin expression patterns have been identified in other insects [58] including other species of Odonata [19,20], which suggests that *I. elegans* LWS opsin duplicates could have regionalized expression patterns throughout the retina. For example, the eyes of the dragonfly *Hemicordulia tau* seem to have three SWS zones and display ventral spectral band patterning maximally sensitive to either short, middle or long wavelengths [50]. Likewise, ERG recordings from the dragonfly *S. frequens* show regional differences in spectral sensitivity coinciding with dorso-ventral opsin expression levels and broad green spectral sensitivity [20]. Future analysis of LWS opsin spatial distribution via antibody staining or *in situ* hybridization, along with *in vivo* ERG quantification of visual sensitivity, will be valuable next steps to determine how opsin spatial distribution and co-expression patterns may contribute to finely tune visual spectral sensitivity in *I. elegans*.

(b) Candidate spectral tuning sites between SWS and LWS opsin duplicates

Homology modelling indicated that the residues predicted to interact with the retinal chromophore are conserved between all tested LWF opsins. It is therefore possible that variant residues at positions outside the canonical binding pocket play a role in fine spectral tuning, causing the observed narrow spectral shifts.

For SWS opsins, we mapped two strong candidate spectral tuning sites at positions 136 and 294 between *I. elegans* duplicate SWb1 and SWb2 opsins (electronic supplementary material, figure S4). A comparison of duplicate SWb opsins across additional *Ischnura* species indicates that Y136F (H3) is conserved in *I. cervula*, *I. verticalis* and *I. hastata* whereas Y294F (H6) is present only in *I. cervula*. Repeated homologous Y/F changes have been functionally shown to cause convergent spectral shifts in SWS insect opsins [5,59,60]. Accumulated evidence for the role of Y to F substitutions in providing longer wavelength-sensitive SWS invertebrate opsins is consistent with the gain of F136 in *I. elegans* SWSb2, possibly underlying the change in paralogue SWS sensitivity (figure 2). The conserved Y136F substitution presumably also influences spectral tuning across other *Ischnura* SWb1 and SWb2 opsin paralogues, although possibly to varying degrees, as species-specific variation in spectral sensitivity can be expected between orthologous native opsins, as observed with the orthologous opsin of *S. frequens*: LWA2 (λ_{max} 557 nm) [61] compared with the *Ischnura* orthologue (LWA2 λ_{max} 548 nm) characterized here.

(c) Opsin expression plasticity as a potential mechanism underlying morph detection and discrimination

In this system with rapidly changing female morph frequencies, males are expected to rapidly develop plastic search images, which may be shaped by local frequencies of female morphs and their intrinsic fecundities [25,33]. The visual system and peripheral opsin receptors are known major evolutionary targets [62–64] with the potential to fine-tune wavelength light capture and colour vision, in particular in altering opsin gene evolution, function and expression. There is also increasing empirical evidence for a key role of phenotypic plasticity in evolution, including in shaping the development of both male and female mate preferences [65,66]. Such learned mate preferences may impact sexual selection, sexual conflict, the maintenance of sexually selected polymorphisms, population divergence and ultimately speciation [65,67,68].

The damselfly *I. elegans* is characterized by visually guided mate-searching males who are exposed to female colour polymorphisms and a continuum of androchrome female frequencies across local populations (figure 1). This system allowed us to explore male visual system response dynamics quantitatively in natural field settings. Quantification of opsin mRNA levels throughout adult *I. elegans* revealed consistent plasticity for specific opsin genes over adult male development. Successful visual mate identification has also been predicted to vary in response to local female morph abundances and correlate with increased sexual experience [25,29]. Since opsin expression profiles of adult males were consistent with a gradual increase (i.e. LWF2) and decrease (i.e. LWF1) in opsins with distinct spectral sensitivities, these changes may contribute, at least partly, in fine-tuning the male visual system for mate detection and mate discrimination during sexual maturation.

Moreover, sexually immature teneral males are inexperienced with visually guided behaviours compared with immature and mature adult males. In line with this, we found that opsin gene expression levels, for SWS and LWS visual opsin types, are more homogenous in teneral whole-head tissues, whereas variation in relative expression increased in immature and mature male *I. elegans* (electronic supplementary material, figure S6). Major directional expression changes were found at the metapopulation level, including changes that reliably depend on maturity stage, and target opsin type, with notable decreases in LWF1 and increases in LWF2 expression (figure 3B). The relationship between LWF1 and LWF2 expression appeared to be conserved across all eight sampled populations, and statistical signal is recovered at the population level for LWF1 and LWF2 in several of the individual sites (electronic supplementary material, figure S7), despite lower sampling power. Taken together, these differences in relative expression are consistent with opsin-specific regulation during ontogenic development and male sexual maturation, suggesting that vision changes in immature and mature adult males compared with teneral males.

Proportional opsin expression also differed between developmental stages, with significantly more abundant LWF2 opsin expression and significantly less abundant expression of other LWS opsin types in immature and mature males relative to teneral males (electronic supplementary material, figure S8). Visual modelling, informed by *in vitro* measurements of opsin sensitivity, indicated that visual systems with maximal LWS opsin sensitivity set as the LWF2 λ_{max} (i.e. 543 nm) would likely outperform chromatic visual systems modelled with LWF1 λ_{max} values (i.e. 531 nm) in discriminating between thorax coloration of androchrome and *infuscans* females, and for detecting androchromes against vegetation. Additionally, males may respond to achromatic cues, with a nearly twofold difference in the overall brightness of androchrome and *infuscans* females

at the descending midpoint limb of absorbance of LWS opsins around 600 nm (electronic supplementary material, figure S3). These results suggest that abundantly expressed LWS opsins sensitive to longer wavelengths can provide an ecological advantage for specific visual tasks involved in conspecific detection and likely discrimination between brightness and/or chromatic cues.

For mature males across all sites, the LWF2 opsin expression pattern exhibited a strong positive quadratic relationship, meaning that LWF2 expression was significantly greater in populations with either very low (<0.6) or very high (>0.8) female androchrome frequencies (figure 4). We established that the long wavelength shifted spectral sensitivity of LWF2 can, in principle, improve chromatic discrimination of androchrome and *infuscans* females and improve detection of androchrome females against vegetation, which could benefit males in populations where androchrome females are more or less abundant compared with other sites (although still the majority morph in all populations studied here). Further behavioural experiments will be important to causally establish if and how female morph frequencies might impact male behavioural choice.

Links between opsin expression and behaviour, while weak in some cases [11,69], have been established in several biological systems [70,71]. Such studies include female guppies from low-predator populations which have been shown to prefer males with more orange/red coloration, a preference linked to higher expression of opsins sensitive to orange and red wavelengths compared with females from high-predator populations [72]. Future work directly assessing the effect of mating outcomes on specific opsin expression profiles will provide valuable insights into the function of opsin plasticity in mate detection, mate choice and female morph preference in Odonata.

5. Conclusion

Our study shows that the visual system of *I. elegans* is characterized by multiple SWS and LWS opsin genes, and that their absorbance spectra confer broad spectral sensitivity. We found striking variation in opsin gene expression profiles between male maturation stages and across populations with varying female morph frequencies. Specifically, the LWS LWF2 opsin showed expression profiles that consistently increased over male ontogeny and showed expression changes that correlated with local female morph frequencies encountered by mate-searching mature male *I. elegans*. Conversely, the relative and proportional expression of another LWS opsin, LWF1, decreased over male ontogeny. Functional analyses revealed that the range of SWS and LWS spectral sensitivity of *I. elegans* likely allows males to discriminate androchrome and *infuscans* female body reflectance, with visual modelling indicating that long-wavelength sensitivity characterized by LWF2 would improve detection of androchromes against vegetation and discrimination between the two most common morph types. Other behavioural and physiological differences between androchrome and gynochrome females, such as aggression or fecundity differences, or ecological factors such as local lighting environments that are not necessarily related to mating behaviours may also play a role in modulating male opsin gene expression profiles. Overall, our new results suggest that directional changes in opsin expression may reflect visual plasticity that might aid males in mate recognition, morph detection and/or discrimination. Future work should aim to complement these findings from natural populations with experimental manipulations to determine if there is a causal role in mate choice in modulating expression profiles.

Ethics. As an invertebrate, *I. elegans* is not protected by the EU Directive 2010/63/E on the use of animals in scientific research, to which the Swedish legislation was adjusted in 2013.

Data accessibility. Supplemental datasets including data associated with analysis and R scripts can be accessed in Dryad [73].

Supplementary material is available online [74].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. N.S.R.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, writing—original draft; E.I.S.: conceptualization, methodology, project administration, resources, supervision, writing—review and editing; M.A.L.: conceptualization, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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