Opsin gene expression plasticity and spectral sensitivity as mechanisms for search image formation in mate-searching male damselflies

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ABSTRACT

Odonata (damselflies and dragonflies) are a largely diurnal, colourful, and strongly visually guided group of insects with visual systems influenced by many opsin genes that form the primary light-sensitive eye photopigments. Heritable (genetic) female-limited colour polymorphisms are also common in Odonata, with one female morph typically exhibiting male-like (androchrome) colouration and one or two morphs exhibiting female-specific colouration (gynochromes). In the Common Bluetail Damselfly (*Ischnura elegans*), androchrome females express blue body colouration similar to that of mature males while gynochrome females express either green or red-brown colouration. This and other colour polymorphisms in damselflies are thought to be maintained by male mating harassment and frequency-dependent sexual conflict, in which males form search images for certain morphs which suffer disproportionally from mating harassment. Here, we investigate the role of opsin sensitivity and expression plasticity in visual mate detection in *I. elegans* by quantifying relative opsin mRNA expression over adult maturation in populations with different female morph frequencies in southern Sweden. We find evidence for opsin-specific plasticity in

relative and proportion opsin expression, suggesting changes in opsin regulation and visual sensitivity over adult maturation. Furthermore, the relative expression of the long-wavelength sensitive opsin LWF2 changed in response to female morph frequencies. The highest relative expression levels were found in populations with either a high or low proportion of androchrome females. *In vitro* results indicate that long-wavelength sensitive opsins in *I. elegans* provide a good visual match to the colouration of green gynomorph females and could in principle confer male colour discrimination between female morphs. We discuss these results in relation to frequency dependent selection, male sensory adaptations, plastic search images and mate searching costs. We suggest that opsin gene expression could play an important role in male search image formation of suitable mates.

INTRODUCTION

Opsins are G-protein coupled receptors that, when bound with a vitamin-A derived chromophore, form a light sensitive visual pigment that is responsible for colour vision in animals (Terakita, 2005). Many insects possess three classes of opsin proteins: ultraviolet sensitive (UVS), short-wavelength sensitive (SWS), and long-wavelength sensitive (LWS) that form visual pigments with maximum sensitivity to wavelengths (λ_{max}) around 350 nm, 440 nm, and 530 nm, respectively (Briscoe & Chittka, 2001; van der Kooi et al., 2021). Differences in spectral sensitivity are caused, in part, by changes in opsin expression, such as opsin gains or losses, sequence changes, or expression level differences (Castiglione et al., 2023; Liénard et al., 2021; Shand et al., 2008; Sharkey et al., 2023). In some cases, changes to the visual system are also shaped by environmental factors, behaviour, and associated selection pressures. For example, loss of functional opsin genes correlates with nocturnal lifestyles in mammals (Jacobs, 2013), while opsin duplications in other species are found to contribute to the regaining of spectral sensitivity (Rossetto et al., 2023; Sharkey et al., 2023) and, for several

insect species, functional changes amongst duplicated opsins likely improves detection and/or discrimination of environmental and sexual signals (Liénard et al., 2021; McCulloch et al., 2017, 2022).

In many cases, opsin gene expression is not fixed, but changes plastically over individual ontogeny (Carleton et al., 2016; Chang et al., 2021; Yilmaz et al., 2016). Opsin gene expression profiles also change in in response to environmental pressures (Fuller et al., 2010; Shand et al., 2008; Wright et al., 2020). For example, changes in opsin expression is associated with light exposure in honey bees and ants (Sasagawa et al., 2003; Yilmaz et al., 2016) and in "choosy" relative to "non-choosy" females of the butterfly *Bicyclus anynana* (Everett et al., 2012). In several species of damselfish (Pomacentridae), opsin gene expression plasticity tunes visual sensitivity to local environmental lighting conditions for individuals inhabiting shallow (< 4 m) versus deep (> 10 m) water (Stieb et al., 2016).

Odonata (damselflies and dragonflies) is an insect order with many colourful species and these insects are also characterized by their large and complex eyes (Corbet, 1999; Córdoba-Aguilar, 2023). Odonates inhabit a wide array of visual environments, having both aquatic and terrestrial life stages, and they use vision for a variety of behaviours, including prey capture and mate detection (Combes et al., 2013; Corbet, 1999; Córdoba-Aguilar, 2023). These features make these insects 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 a remarkable genetic diversity, with between 11 – 30 visual opsin genes identified across several families (Futahashi et al., 2015). Recordings of visual sensitivity in the eyes of some species indicate spectral sensitivity ranging from UV to red light (Horridge, 1969; Huang et al., 2014; Meinertzhagen et al., 1983; Yang & Osorio, 1991). While it is not clear how their abundant opsin copies function in vision, visual stimuli are clearly important for odonate

behaviour (Combes et al., 2013; Corbet, 1999; Miller & Fincke, 1999). For example, comparisons of visual and olfactory cues suggest that vision is primarily used in mate choice in several species of damselflies (Rebora et al., 2018; Winfrey & Fincke, 2017). Colouration is also an important signal indicating female sexual maturity (Bybee et al., 2012; Gosden & Svensson, 2009; Huang et al., 2014; Svensson et al., 2020; Takahashi & Watanabe, 2011; Van Gossum et al., 2011; Willink et al., 2019, 2020). Males can discriminate between immature and mature females, and they direct more mating attempts to mature than immature females (Huang et al., 2014; Van Gossum et al., 2001a, 2011; Willink et al., 2019). Experimental manipulation of age-specific colouration in *Ischnura* damselflies also suggest that vision is the primary cue in males discrimination between immature and mature females (Willink et al., 2019).

In addition to colour changes associated with sexual maturation, many odonates also exhibit heritable (genetic) female-limited colour polymorphisms, with one female morph having male-like colouration (androchrome females) and one or two morphs expressing female-specific colouration (gynochrome females) (Blow et al., 2021; Fincke et al., 2005). Evidence suggests 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 (Fincke, 2004; Miller & Fincke, 1999; Takahashi et al., 2014). According to the male mimicry hypothesis, androchrome females have a negative frequency-dependent advantage in their male-like similarity, which reduces male mating harassment (Robertson, 1985). Alternatively, although not mutually exclusive, according to the learned mate recognition (LMR) hypothesis, males form search images for the most common female morph in the population (Fincke, 2004). The LMR hypothesis further predicts that the ratio of correct (e.g., mature conspecific females) versus incorrect (e.g., other males, heterospecifics) identification of mates should increase with experience. There is partial empirical support for

101 both the male mimicry hypothesis and LMR and, as stated above, these two hypotheses are not
102 mutually exclusive (Gosden & Svensson, 2009; Svensson et al., 2005, 2020). Empirical
103 evidence has shown that males show increased preferences for the most abundant female
104 morph in their population (Takahashi & Watanabe, 2010) or increased preference following
105 exposure to specific female morphs (Miller & Fincke, 1999; Van Gossum et al., 2001b; but see
106 Van Gossum et al. 2011). Moreover, male mate preferences become increasingly non-random
107 with increasing density of certain morphs (Gosden & Svensson, 2009). Female fecundity is
108 also higher in populations with more balanced morph frequencies, presumably because females
109 are then expected to suffer from less per-capita harassment than females in populations with
110 biased morph frequencies as, in the latter case, males should more easily form search images
111 for a single morph (Takahashi et al., 2014). In androchrome-biased populations, there is also
112 an increasing number of male-male interactions, consistent with mistaken mate recognition due
113 to male mimicry and the formation of male search images for the common female phenotype
114 (Gering, 2017). Finally, analyses of time-series of morph frequency fluctuations and population
115 genetic modeling have revealed a clear signature of negative frequency-dependent selection,
116 where the fitness of a given female morph decreases with its frequency in the population (Le
117 Rouzic et al., 2015; Svensson et al., 2005; Takahashi et al., 2010).

118 This growing body of ecological, evolutionary, sensory, and physiological studies,
119 paired with the increasing availability of genomic resources, makes Odonata an excellent
120 system to investigate the molecular basis of colour vision. Here, we characterize opsin spectral
121 sensitivity via heterologous expression and quantify variation in opsin mRNA expression
122 across adult male maturation from populations with variable female morph frequencies in the
123 Common Bluetail Damselfly (*Ischnura elegans*). Functional characterization of 2 SWS and 5
124 LWS rhodopsins found in the ventral eye show absorbance peaks (λ_{max}) in the range of 406 –
125 419 nm and 531 – 548 nm, respectively. We also demonstrate opsin expression changes over

126 adult male development, with increased expression of specific opsin types in response to
127 changes in local female morph frequencies. These novel results suggest that the visual system
128 of male *I. elegans* can rapidly and plastically respond to changes in local morph frequencies
129 that, alongside opsin sensitivities, may aid in mate detection and discrimination. Our results
130 further suggest that opsin expression plasticity may provide a mechanistic proximate link
131 between male mate preferences, mating harassment, and the resulting frequency-dependent
132 sexual conflict, consistent with models for how these polymorphisms are maintained (Fincke,
133 2004; Le Rouzic et al., 2015; Svensson et al., 2005). Plastic male opsin expression profiles
134 could therefore help to regulate colour vision and perception of female colour signals in this
135 dynamic system strongly shaped by visually guided male mating behaviours.
136
137 MATERIALS AND METHODS
138 Study system
139 The Common Bluetail Damselfly (*Ischnura elegans*) is found broadly across Europe,
140 with the northern end of its range limit in Central Sweden (Dijkstra & Lewington, 2006;
141 Dudaniec et al., 2018). Sexually mature male *I. elegans* are monomorphic, exhibiting blue body
142 colouration on the thorax. In contrast, female *I. elegans* are polymorphic, with sexually mature
143 females belonging to three distinct genetically determined colour morphs (Fig. 1A) (Willink et
144 al., 2020). Androchrome (male-like) females exhibit body colouration spectrally similar to
145 male colouration while gynochrome females exhibit spectrally distinct green (infuscans) or
146 brown (infuscans-obsoleta; hereafter referred to as obsoleta) thorax colouration (Henze et al.,
147 2019; Van Gossum et al., 2011). Females exhibit ontogenetic changes in thorax and abdomen
148 colouration (Fig. 1B), with male *I. elegans* preferring body colouration of mature over
149 immature females (Willink et al., 2019). Apart from colour differences, androchrome and
150 gynochrome females also differ in mating rates, parasite resistance, and tolerance to parasites

151 (Willink & Svensson, 2017) as well as resistance to mating attempts, and fecundity (Gosden &
152 Svensson, 2009).
153
154 Quantification of female morph frequencies between sites and field collection of males
155 We quantified female morph frequencies from eight sites in southern Sweden (Fig. 1C)
156 as part of an ongoing long-term field population study of *I. elegans* (see Le Rouzic et al. 2015
157 and Svensson et al. 2020 for general methodology and details about this long-term study). All
158 study populations were visited and re-visited at regular intervals (1-2 weeks) between 19 May
159 – 1 August 2021 and 16 May – 31 July 2022. Taking into account both immature and mature
160 females, all populations were trimorphic except for one (Höje å 6) in 2022, which had
161 experienced large overall population declines that year (Table S1). However, since male *I.*
162 *elegans* are known to prefer colouration of mature over immature females (Willink et al.,
163 2019), we only considered females that displayed mature adult colouration to calculate the
164 relative abundance of each morph within the current study. The frequencies of the three mature
165 female morphs differed between sites and years (Fig. 1D-E). Androchrome females comprised
166 the majority of females across all sites, ranging from 57.1– 85.7% (mean ± sd: 72.6 ± 9.7%) of
167 females in 2021 and 65.9 – 83.9% (72.3 ± 5.8%) in 2022. Infuscans females were the next most
168 common morph, ranging from 14.3 – 39.1% (24.1 ± 8.8%) of females in 2021 and 16.1 – 30.7
169 (24.9 ± 5.3%) in 2022. Mature obsoleta females were rare, making up 0.00 – 5.5% (3.3 ± 1.8%)
170 of females in 2021 and 0.00 – 7.8% (2.8 ± 2.8%) in 2022.
171 Male *I. elegans* used for opsin quantification were caught from these populations
172 between 28 June – 13 July 2021 and 9 June – 20 July 2022 using sweep nets (Table S2). For
173 all collected individuals, we recorded sexual maturity ("immature" vs. "mature") by evaluating
174 wing stiffness (Corbet, 1999). We also recorded whether males were found in copula with a
175 female or singly ("couple" vs "single") and, in 2022, we recorded the morph of the female that

176 was found in copula with each collected male. In addition to immature and mature males, we
177 also collected males that had newly emerged from the aquatic nymph stage ("tenerals") from
178 two field sites and from a semi-naturalistic mesocosm experiment at the Stensoffa Field Station
179 at Lund University (Table S2). Teneral males are characterized by their extremely soft wing
180 and body tissues and lack of body pigment. These teneral males allowed us to assess opsin
181 gene expression in males that have not had any significant visual experience and have not
182 mated with sexually mature females. All collected males were immediately euthanized by
183 cutting off their head using cleaned RNAse-free dissection tools, placed into individual vials
184 filled with RNA*later* (Ambion, Inc., Austin, TX, USA) at field sites, and then stored at -20°C
185 until RNA extraction.
186
187 Opsin heterologous expression
188 We retrieved visual opsin open reading frame (ORF) sequences for *I. elegans* (UV,
189 SWb1, SWb2, LWA1, LWA2, LWC1, LWD1, LWE1, LWF1, LWF2, LWF3, and LWF 4, see
190 Supplementary File S1) by mining whole genome data from the genome assembly
191 iolscEleg1.1. using complete sequences previously reported from the closely related species
192 *Ischnura asiatica* (Futahashi et al., 2015) as our reference set. Opsin transcript and protein
193 sequences were predicted with AUGUSTUS (Keller et al., 2011) followed by sequence
194 annotation in HMMER v3.1b2 (http://hmmer.org). Opsin ORFs flanked with suitable
195 restriction sites for subcloning in the pcDNA5-FLAG-T2A-mruby2 expression cassette were
196 synthesized for human codon optimization, overexpressed by transient transfection following
197 the PaSHE procedure (Liénard et al., 2021, 2022) followed by purification and ultraviolet-
198 visible spectroscopy. Briefly, we first performed small scale transfections to verify that each
199 final pcDNA5-Opsin construct produced detectable protein levels (Fig. S1). For this,
200 HEK293T cells were seeded at a density of 0.6 x 10^6 cells in DMEM medium (Gibco) in 6-

201 well plates. The next day cells were transfected with a mixture of 4 ug plasmid DNA and a 1:3
202 ratio of PEI (1mg/mL) in Opti-Mem (Gibco). Forty-eight hours later, cells were visualized
203 under a fluorescent microscope to verify expression of mRuby2 and then harvested in cold D-
204 PBS (Sigma-Aldrich). Cells were resuspended in cold Ripa buffer supplemented with 1% n-
205 Dodecyl-ß-D-maltoside and complete ethylenediaminetetraacetic acid (EDTA)-free protein
206 inhibitors (Sigma-Aldrich), and incubated at 4°C for 1 hour, followed by western blot analysis.
207 For large scale opsin purifications, fifteen HEK293T cell dishes containing 2 x 10^6 cells
208 were transfected with 24 ug DNA and 72 uL PEI each. Five micromolar 11,*cis*-retinal was
209 added 6hr post-transfection with fresh culture medium. Cells were collected under dim red
210 light 48h post-transfection and decanted in cold Hepes wash buffer (3 mM MgCl2, 140 mM
211 NaCl, 50 mM Hepes, EDTA-free protein inhibitors) followed by incubation at 4°C for 1 hour
212 in presence of 40 µM 11,*cis*-retinal. Cell pellets were collected by ultracentrifugation and
213 membrane proteins were solubilized for 1hr at 4°C in ice-cold extraction buffer (3 mM MgCl2,
214 140 mM NaCl, 50 mM Hepes, 20% glycerol vol/vol, 1% n-dodecyl β-D- maltoside, complete
215 EDTA-free protein inhibitors) and ultracentrifuged. The crude extract (supernatant) was
216 incubated in the dark with FLAG-resin on a rotator at 10 rpm until the next day. The crude
217 extract containing resin-bound FLAG-epitope rhodopsin complexes was loaded onto a Pierce
218 centrifuge purification column, washed with 3x 3 mL wash buffer (3 mM MgCl2, 140 mM
219 NaCl, 50 mM Hepes, 20% glycerol vol/vol, 0.1% n-dodecyl β-D- maltoside) and eluted using
220 FLAG-peptide. The eluate was concentrated to 100-120 microliters using an Amicon Ultra-2
221 10 kDa Amicon centrifugal filter for 50 min at 4°C and UV-visible absorption spectra (200 to
222 800 nm) of dark-adapted purified proteins were measured in the dark from 1.5-µL aliquots
223 using a NanoDrop 2000/2000c UV-VIS spectrophotometer (Thermo Fisher). For each
224 rhodopsin, we obtained estimates of lambda max through nonlinear least-square fitting to the
225 absorbance data according to a visual template (Govardovskii et al., 2000) and performed 1000

226 bootstrap replication to calculate lambda max predictions and confidence intervals (Liénard et
227 al., 2022) in R v.3.6.6.(*R Core Team*, 2021) using the packages rsample and tidymodels (Frick
228 et al., 2022; Kuhn & Wickham, 2020).
229
230 Quantitative PCR analysis of *I. elegans* opsin expression levels
231 We extracted RNA from the heads of N = 87 male *I. elegans* (Table S2) using a
232 Monarch Total RNA Miniprep Kit protocol (Thermo Fisher Scientific, Waltham, MA, USA)
233 following the manufacturer's instructions and including a genomic DNA clean-up. cDNA was
234 synthesized for each sample using a GoScript™ Reverse Transcriptase kit (Promega, Madison,
235 WI, USA). The *Ischnura*-specific RL14 and GADPH gene sequences were annotated as
236 described above for opsin sequences and used as reference for mRNA quantification.
237 For quantitative PCR analysis, two sets of gene-specific primers were designed in
238 Geneious Prime v.2022.91 (Biomatters, Auckland, New Zealand) for both housekeeping genes
239 and all opsins expressed in the adult ventral eye (UV, SWb1, SWb2, LWA2, LWF1 – F4) and
240 the ocelli opsins (LWD1 and LWE1). We did not include LWA1 or LWC1 since these opsin
241 types are primarily expressed in larval tissues in other Odonate species (Futahashi et al., 2015).
242 We tested the efficiency of designed primers by generating a standard curve following a 4-fold
243 dilution of *I. elegans* cDNA and selected one primer pair for each opsin based on amplification
244 efficiency (Table S3). For the ocelli-specific opsins, we focused further analysis on the LWE1
245 opsin due to more consistent amplification rates relative to LWD1. The threshold detection
246 cycle (Ct) values of selected primers were plotted against the log of cDNA dilutions and the
247 efficiency percentage values were assessed to ensure that they were within acceptable ranges
248 (94 – 117%) and R^2 (0.95 – 1.00). PCR amplicons were run by electrophoresis on a 2% agarose
249 gel to ensure the absence of primer-dimers and PCR samples for each primer pair-opsin target
250 were purified using Exonuclease (EXO) and Shrimp alkaline phosphatase (SAP) (NEB) prior

251 to Sanger Sequencing to confirm gene-specific amplification. Hereafter, we use abbreviated
252 names for the opsins SWb1, SWb2, LWA2, and LWE1, which have been shortened to SW1,
253 SW2, LWA, and LWE, respectively.

254 qPCR was performed in a CFX384 Real-Time System (BioRad Laboratories, Inc.,
255 Hercules, CA, USA). Reaction mixes were prepared that contained 1x SYBR Green, 1uM
256 primer mix, and 0.1 ng cDNA. The program was designed as follows: denaturation at 95°C for
257 2min, followed by 39 cycles at 95°C for 10s and 60°C for 10s, with melting curve analysis
258 performed from 65.5°C to 89.5°C at 0.6°C increments to confirm the specificity of PCR
259 products. Two independent technical replicates were processed for each sample and each
260 sample was run in triplicate within each technical replicate. We averaged triplicates within
261 each technical replicate for a total of n = 2 Ct values per male sample.

262 We calculated expression of opsin genes relative to the housekeeping genes and
263 normalized against the UV opsin gene using the $(1 + E)^{-\Delta\Delta CT}$ method where E equals the PCR
264 efficiency for each primer (Livak & Schmittgen, 2001; Pfaffl, 2001). We also calculated the
265 proportion opsin expression relative to total opsin expression for opsins by dividing relative
266 expression for each individual opsin by the sum of total opsin expression, excluding the ocelli
267 opsin LWE. Results of relative opsin expression indicated differential regulation of opsin
268 genes, while proportional measures of opsin expression are suggested to be better for making
269 inferences about colour vision (Fuller & Claricoates, 2011). For proportion opsin expression,
270 we focused only on opsins expressed in the compound eye since this is where colour
271 discrimination primarily occurs within the insect visual system.

272

273 Statistical analysis

274 We performed a Multivariate Analysis of Variance (MANOVA) to assess the effect of
275 maturity on log relative opsin gene expression of all eight opsin genes for male *I. elegans.* Log

276 relative opsin expression for each individual male was averaged between replicates to maintain
277 the assumption of independence. We assessed the dataset for outliers and compared result
278 outputs performed before and after their removal to determine whether this affected results.
279 The inclusion of outliers did not qualitatively change the general results, and we therefore
280 report results with outliers included. Further analysis to account for repeated measures on the
281 full dataset were performed using linear mixed modelling (see below).
282
283 *General protocol for model selection*
284 We used linear mixed modeling using the lme4 package (Bates et al., 2015) in R v 4.1.1
285 to test the effects of population morph frequency and its squared component, maturity level,
286 and opsin type on log relative opsin expression in male *I. elegans*, with site, male ID, year, and
287 qPCR replicate as random effects. Model selection followed methods in Zuur (Zuur et al.,
288 2009), in which we first selected the random effect structure for the model by comparing
289 Akaike information criterions (AICs) between models fitted using restricted maximum
290 likelihood (REML) estimation that included all possible fixed effects and relevant interactions.
291 Following selection of the random effect structure, we used backward model selection with
292 maximum likelihood (ML) estimation to determine the optimal fixed effect structure by testing
293 the significance of each interaction or term. After selection of the optimal random and fixed
294 effect structures, the final model was run using REML estimation and the model output and
295 results of post-hoc analysis were obtained using the lmerTest and emmeans packages
296 (Kuznetsova et al., 2017; Lenth, 2022), respectively.
297
298 *Opsin mRNA expression in teneral, immature, and mature males across sites*
299 We tested the effect of maturity stage (factor with 3 levels: teneral, immature, mature),
300 opsin type (factor with 8 levels: SW1, SW2, LWA, LWF1 – LWF4, LWE), and their interaction

301 on log relative opsin expression. Model selection indicated a significant interaction between
302 maturity level and opsin type (likelihood ratio test: $\chi2$ (14) = 163.9, $p < 0.0001$). The final
303 model therefore included year, site, and male ID as random effects with fixed effects of
304 maturity stage, opsin type, and their interaction to explain log relative opsin expression in male
305 *I. elegans*.

306 To investigate how proportional opsin gene expression changes over development, we
307 again used maturity stage, opsin type, and their interaction as fixed effects, but now only
308 focused on opsins found in the compound eye (factor with 7 levels: SW1, SW2, LWA, LWF1
309 – LWF4). Data exploration suggested that opsin type violated the homogeneity of variance
310 assumption for this dataset. We therefore allowed for differing variance structure in the term
311 *opsin* using the varIdent function in the nlme package (Pinheiro et al., 2021). We included only
312 male ID as a random effect since inclusion of additional random effects did not improve model
313 AIC. Model selection showed a significant interaction between opsin type and maturity stage
314 (likelihood ratio test: $\chi2$ (12) = 268.7, $p < 0.0001$), making the final model the interaction of
315 maturity stage and opsin type on proportion opsin expression.

316 Preliminary analysis suggested differences in relative opsin expression between teneral
317 males from the two field populations and those from outdoor mesocosm tanks at Stensoffa
318 Field Station (Table S4). Despite these differences, the results with and without males from the
319 mesocosm tanks were largely comparable. We therefore retained the teneral males from the
320 mesocosm tanks in our main analyses. Results of log relative expression and proportion opsin
321 expression without the mesocosm males are presented in Supplementary Material (Tables S5-
322 S7, Fig. S2).

323

324 *Opsin mRNA expression in populations with different female morph structure*

325 To assess the effect of local female morph frequency on male opsin expression, we used
326 the frequency of androchrome females for each site and year to describe local population morph
327 frequency. We used the proportion of androchrome females within a site for each year
328 separately, instead of generating a site average, since a primary aim of the current study was to
329 assess if opsin expression changes in response to current local conditions. Therefore,
330 "population morph frequency" refers to the local morph frequency in a given year, with two
331 population morph frequencies per site.

332 We followed the same modeling procedure to test the effect of maturity stage, opsin
333 type, the proportion of androchrome females, and their interactions on relative opsin
334 expression. We included the proportion of androchrome females also as a quadratic term to
335 account for any non-linear effect of androchrome frequencies. In this analysis, we focused only
336 on immature and mature males since data for teneral males was not available for most sites.
337 Backward model selection of fixed effect structure indicated that the three-way interaction
338 between opsin type, maturity stage, and androchrome proportion was significant (likelihood
339 ratio test: $\chi 2$ (14) = 43.9, $p < 0.0001$). The final model therefore accounted for a quadratic
340 relationship between androchrome proportion, opsin type, maturity stage, and their interaction
341 as fixed effects with year, male ID, and replicate as random effects.

342

343 *Opsin expression in response to mating outcomes*

344 We recorded the female morph of all males collected in copula in 2022 to assess
345 whether relative opsin expression differed between males collected in copula with
346 androchrome or infuscans females. The selected random effect structure included male ID and
347 replicate. We considered maturity level, opsin type, and female morph (factor with 2 levels:
348 androchrome, infuscans), and their interactions as fixed effects. Backwards model selection
349 resulted in a final model with opsin as the only fixed effect, suggesting that there is no

350 significant effect of female morph type on a males' relative opsin expression in the current
351 study. However, it is possible this is due to relatively small sample size, with n = 8 males
352 collected in copula with an androchrome female and n = 7 males collected in copula with an
353 infuscans female.
354
355 RESULTS
356 *Opsin functional expression and spectral sensitivity*
357 To determine the spectral sensitivity of opsins expressed in the ventral eye and ocelli,
358 we first subcloned codon-optimized individual opsin open reading frames for two short-
359 wavelength opsins (SW1 and SW2), as well as seven long-wavelength opsins including five
360 expressed in the ventral eye (LWA, LWF1-F4) and two ocelli-specific opsins (LWE and LWD)
361 (Futahashi et al., 2015) in a recently developed expression cassette (Liénard et al., 2021). Small
362 scale assays were performed to verify transcriptional activity via fluorescent microscopy of a
363 co-transcribed mRuby marker, and via western-blot visualization (Fig. S1). We then transiently
364 expressed each opsin construct in large scale HEK293T cell cultures. Active rhodopsin
365 complexes were reconstituted by addition of 11, *cis*-retinal in the dark and purified to obtain
366 dark adapted rhodopsin eluates prior to ultraviolet-visible spectroscopy analyses. First, we
367 found that the SWS opsins SWb1 and SWb2 absorb maximally at λ_{max} of 406 and 419 nm (Fig.
368 2A-B). We then assayed the seven LWS opsins and measured their individual absorbance
369 spectrum (Fig. 2C-I). The LW opsin types expressed in the ventral eye display maximal
370 sensitivity to approximately 530 – 550 nm with the following λ_{max}: LWA2 = 548 nm, LWF1 =
371 531 nm, LWF2 = 543 nm, LWF3 = 545 nm, and LWF4 = 541 nm (Fig. 2C-G). Finally, we find
372 that the ocelli-specific opsins, LWD1 and LWE1 absorb maximally at 541 nm and 533 nm,
373 respectively (Fig. 2H-I).

374 We ran homology modeling of SWb1 against the spider rhodopsin (PDB: 6i9k) and
375 mapped sites interacting with the chromophore in Pymol. Of the 71 amino acid substitutions
376 between SWb1 and SWb2 (81.8% aa identity), we identified 23 sites predicted to be within 5Å
377 of the binding pocket (Table S8). Among these, Y136 (TM3) and Y294 (TM6) residues located
378 at the top and bottom of the binding pocket, respectively, are substituted by phenylalanine
379 (F136 and F294) in SW2 (Fig. S3A). For LWS opsins, which share between 88.9 - 91.2% aa
380 identity (i.e., 33 - 42 variant residues), we obtained the predicted LWF1 opsin structure based
381 on 6i9k and mapped 24 sites predicted to interact with the cis-retinal, all of which are conserved
382 for all four LWF opsins (Table S8; Fig. S3B). LWA2 is more divergent in sequence, 67.5 -
383 68.7% aa identity with 119 - 124 aa differences with LWF opsins. Of the 24 chromophore-
384 interacting residues, its binding pocket exhibits two potential spectral variant residues with
385 LWF1 (A131G in TM1 and S319A in TM7).
386 Finally, sensitivity of the SWS and LWS opsins expressed in the ventral compound
387 provides sensitivity to body reflectance of androchrome and infuscans females (Fig. 3).
388 Particularly, sensitivity of the LWA and LWF2 – LWF4 opsins provides a close
389 correspondence to infuscans female thorax colouration (Fig. 3B).
390
391 *Opsin expression in teneral, immature, and mature males*
392 Results from the MANOVA show a significant effect of male maturity stage on overall
393 opsin gene expression ($F_{16,146} = 3.2$, $p < 0.0001$; Fig. 4). To further assess how relative and
394 proportion opsin expression differed between teneral, immature, and mature male *I. elegans*,
395 we performed mixed modeling with maturity stage, opsin type, and their interaction as main
396 effects.
397 There was a significant effect of opsin type and maturity stage × opsin interaction on
398 log relative opsin expression in male *I. elegans* (Table 1, Tables S9-S10). For teneral males,

399 relative opsin expression was lowest for the SW1, SW2, and the ocelli LWE opsin, increased
400 in LWA – LWF3 opsins, and was significantly highest for LWF4 (Fig. 5A). In immature males,
401 the ocelli specific LWE opsin had significantly lower relative expression than the opsins found
402 within the compound eye, as expected. Among visual opsins, SW2 and LWF1 opsins had the
403 lowest relative expression, followed by the SW1 and LWF3 opsins, then LWA, LWF4, and
404 finally, LWF2 which was expressed at significantly higher levels (Fig. 5B). In mature males,
405 relative expression of LWE is also significantly lower than the opsins within the compound
406 eye, followed by increasing expression for LWF1, SW2, SW1 and LWF3, LWA, LWF4 and
407 LWF2 (Fig. 5C).

408 We next compared relative opsin expression between teneral, immature, and mature
409 males, pooling sites, to assess whether developmental changes affect relative opsin expression
410 uniformly or whether there is an opsin-specific effect on relative expression, suggestive of
411 differential plasticity in opsin expression. We found that relative opsin expression changed
412 over sexual maturation in an opsin-specific manner (Fig. 6). There was a significant increase
413 in relative expression of the SW1 opsin between teneral and mature males (teneral – mature: t
414 = -2.7, df = 396, $p = 0.02$) and between all maturity categories for relative LWF2 expression
415 (teneral – immature: t = -5.1 df = 401, $p < 0.0001$; teneral – mature: t = -6.9, df = 396, $p <$
416 0.0001; immature – mature: t = -3.0, df = 419, $p = 0.009$). In contrast, we saw decreases in
417 opsin expression over maturity between all maturity categories for relative LWF1 expression
418 (teneral – immature: t = 4.7, df = 402, $p < 0.0001$; teneral – mature: t = 6.1, df = 399, $p <$
419 0.0001; immature – mature: t = 2.4, df = 433, $p = 0.04$) and between teneral males with
420 immature and mature adult males for relative LWE expression (teneral – immature: t = 3.4, df
421 = 401, $p = 0.002$; teneral – mature: t = 3.9, df = 400, $p = 0.0004$).

422 To decouple the effect of LWF1 and LWF2 per site and assess the variation in the
423 dataset, we further assessed differences over maturity separately for each site (Table S11, Fig.

424 S4). While there were some sites that did not show a significant difference in relative
425 expression over maturation, three sites, Bunkeflostrand, Ilstorp, and Flackarp showed a
426 significant interaction between maturity stage and opsins found in the ventral eye. Differences
427 between this analysis and the large comparative metapopulation analysis likely arise due to
428 reduced sample size in each site, but nevertheless highlight consistent results for three
429 independent sites, corroborating the robustness of the pooled analysis that supports specific
430 opsin expression changes over maturity.

431 Comparing proportional opsin expression levels, we again observe a significant
432 interaction between maturity stage and opsin type (Table 2). For teneral males, LWF4
433 expression accounts for over half of opsin expression in the compound eye (0.523 ± 0.01) while
434 in immature and mature males, LWF2 expression makes up the largest proportion of
435 expression, accounting for 0.524 ± 0.03 of expression in immature and 0.599 ± 0.02 of
436 expression in mature males (Fig. 7). Post-hoc analysis show that proportional expression levels
437 differed significantly between all LWS opsins for teneral and immature males and between all
438 LWS and the SW2 opsin for teneral and mature males. There were also significant differences
439 between immature and mature males in proportional expression of the SW2 and LWF1 opsins
440 (Table S12).

441

442 *Opsin expression in populations with different female morph frequencies*

443 We performed mixed modeling to assess whether relative opsin expression differs
444 between immature and mature male *I. elegans* that had experienced different population morph
445 frequencies (here measured as proportion of androchrome females). There was a significant
446 effects of opsin type, maturity stage, frequency of androchrome females and their interaction
447 on log relative opsin expression (Table 3). For immature males, the proportion of androchrome
448 females in a population had a relative weak linear effect on relative opsin expression. However,

449 in mature males, there was a significant quadratic relationship of the proportion of
450 androchrome females on relative LWF2 expression. The highest LWF2 expression in mature
451 males were found in the populations with the lowest and highest proportion of androchrome
452 females (Fig. 8).
453
454 DISCUSSION
455 Vision is an important sensory modality in Odonata, functioning in a wide array of
456 behaviours ranging from flight stabilization to mate choice (Rebora et al., 2018; Wang et al.,
457 2013; Winfrey & Fincke, 2017). The prevalence of female-limited colour polymorphisms in
458 Odonata have made these insects powerful model systems to study how and why
459 polymorphisms emerge and persist over micro- and macroevolutionary time scales (Blow et
460 al., 2021; Le Rouzic et al., 2015; Willink et al., in press). Here, we combined ecological,
461 molecular, and functional approaches to investigate the peripheral visual system in male *I.*
462 *elegans* of different maturation stages. Specifically, we investigated the role of opsin
463 sensitivities, opsin gene regulation, and proportional opsin expression over male ontogeny and
464 across populations with different female morph frequencies. Our results suggest that the
465 peripheral visual system of *I. elegans* is sensitive to body colouration of the two most abundant
466 female morphs from our sampled sites (Fig. 3). Further, plastic changes in opsin expression
467 over male ontogeny, particularly during sexual maturation (Figs. 4 - 7), as well as changing
468 male opsin expression profiles in response to local female morph frequencies (Fig. 8) suggest
469 a role of opsin expression in male mate detection and/or mate preference. Taken together, these
470 novel results suggest that tuning of the peripheral visual system in response to local morph
471 abundances could provide a mechanistic link between male search image formation and the
472 negative-frequency dependent selection that maintains female polymorphism within the tested
473 sites (Fincke, 2004; Le Rouzic et al., 2015; Svensson et al., 2005).

474

475 *Overlapping opsin spectral sensitivities confer broad colour vision in the ventral eye*

476 The many opsin duplications present in Odonata are fairly unique among insects. Other
477 insect species typically express only three – four opsins, while over 30 visual opsins genes have
478 been identified in some species of dragonflies and 12 visual opsin genes have been identified
479 from the damselfly *Ischnura asiatica* (Futahashi et al., 2015). Here, we confirm the expression
480 of 10 opsin genes that have been predicted to be expressed in adult ventral eyes or ocelli from
481 the heads in adult male *I. elegans*. These opsins include 1 UVS, 2 SWS, and 5 LWS opsins that
482 are primarily expressed in the compound eye and 2 LWS opsins that are primarily expressed
483 in ocelli. Our functional analyses of reconstituted active rhodopsin pigments followed by visual
484 template fit for all five ventrally expressed LWS opsins show broad sensitivity to green
485 wavelengths of light, produced from opsins with λ_{max} between 530 nm and 540 – 550 nm (Fig.
486 2). The observed overlap in long-wavelength sensitivities provides the ventral eye in principle
487 with spectral discrimination capacity ranging from 450 nm to 640 nm (Figs. 2 - 3). In addition,
488 functional data of SWS opsins shows two peaks of sensitivity in blue wavelengths of light,
489 with λ_{max} of 406 and 419 nm, which in principle allows *I. elegans* damselflies to readily
490 distinguish wavelengths in the ultraviolet – blue spectrum from 350 nm to circa 490 nm (Figs.
491 2 - 3). These results demonstrate that spectral sensitivities derived from the multiple SWS and
492 LWS opsin types known to be expressed in the ventral eye of Odonata, and in particular
493 *Ischnura* species, allows for capture of a remarkably broad light spectrum from short ultraviolet
494 to long-wavelengths above 650 nm.

495 Given our findings of developmental regulation of opsin expression and overlapping
496 spectral sensitivities of LWS opsins expressed in the ventral eye, and previously identified
497 differences in expression patterns between the dorsal and ventral eye in several Odonate
498 species (Futahashi et al., 2015; Labhart & Nilsson, 1995), it is likely that the multiple tandem

499 LWS duplicate opsins in *I. elegans* exhibit some degree of spatial regionalization in expression
500 patterns throughout the retina. Regional retinal patterning and spectral mosaics occur in a
501 number of insects (Rister & Desplan, 2011) and opsin co-expression in other insects are
502 hypothesized to provide broadband spectral sensitivities (reviewed in Arikawa, 2017; van der
503 Kooi et al., 2021; Wernet et al., 2015). In Odonata, the eyes of the dragonfly *Hemicordulia*
504 *tau* have three short-wavelength sensitive zones (i.e., the dorsal acute zone, the dorsal rim area
505 and the frontal acute zone) and display ventral spectral band patterning maximally sensitive to
506 either short-, middle-, or long-wavelengths (Lancer et al., 2020). Electroretinogram (ERG)
507 recordings in the ventral eye region showed that the dragonfly *Sympetrum frequens* exhibit a
508 wide variety of sensitivities from UV to red (Futahashi et al., 2015). Similarly, early ERG
509 recordings from *Ischnura heterosticta* show UV and blue-sensitive photoreceptor types with
510 peak sensitivities (λ_{max}) at 360 nm and 450 nm, respectively, and a green-sensitive
511 photoreceptor type (λ_{max} = 525 nm) with broad sensitivity to light between 520 – 540 nm and
512 decreasing sensitivity up to 650 nm (Huang et al., 2014). It is therefore also possible that the
513 broad sensitivity to green and red light recorded from other odonate species results from several
514 ommatidial types expressing combinations of LWS opsins. Analysis of opsin expression via
515 gene-specific antibody staining or *in situ* hybridization, along with *in vivo* ERG quantification
516 of visual sensitivity, will be valuable next steps to determine how opsin spatial distribution and
517 co-expression patterns contribute to overall visual spectral sensitivity in *I. elegans.*

518 Finally, we mapped two strong candidate spectral tuning sites at positions 177 and 300
519 between *I. elegans* duplicate SWb1 (λ_{max} 406 nm) and b2 (λ_{max} 419 nm) opsins (Fig. S3). A
520 comparison of duplicate SWb opsins across five *Ischnura* species, *I. cervula*, *I. verticalis*, *I.*
521 *hastata* and *I. asiatica,* shows that Y294F (H6) is present only in *I. cervula* whereas Y136F
522 (H3) is conserved across all species. Although we have not tested the effects on these mutations
523 in the present study, repeated homologous Y/F changes have been functionally shown to cause

524 convergent spectral shifts in SWS insect opsins, including a 5nm shift to longer wavelengths
525 caused by Y195F in *Limenitis* Blue butterfly opsins (Frentiu et al., 2015), a 4nm shift towards
526 longer wavelengths caused by Y177F between *Pieris* violet and blue opsins (Wakakuwa et al.,
527 2010), a 25nm shift towards long wavelength (Y177F) in combination with A116S in *Eumaeus*
528 blue opsin duplicates (Liénard et al., 2021), and a 3-nm shift to shorter wavelengths involving
529 the reverse substitution F285Y in bruprestid beetle UV opsins (Sharkey et al., 2023).
530 Accumulated evidence for the role of Y to F substitutions in providing longer wavelength-
531 sensitive SWS invertebrate opsins is consistent with the gain of F136 in *I. elegans* SWb2,
532 possibly underlying the change in paralog SWS sensitivity (Fig. 2). The conserved Y136F
533 substitution presumably also influences spectral tuning across other *Ischnura* SWb1 and b2
534 opsin paralogs, although possibly to varying degrees, as species-specific variation in spectral
535 sensitivity can be expected between orthologous native rhodopsins, as observed with the
536 orthologous opsins of *Sympetrum frequens*: LWA2 (λ_{max} 557 nm) and LWD1 (λ_{max} 542 nm)
537 (Liénard et al., 2022) compared to the *Ischnura* orthologs (LWA2 λ_{max} 548 nm; LWD1 λ_{max}
538 541 nm) characterized here.
539
540 *Opsin expression plasticity as a mechanism underlying learned male mate choice*
541 One major goal of this study was to investigate the potential role of peripheral visual
542 system tuning in a species characterized by dynamic, female-limited polymorphisms and
543 sexual conflict. In such systems with rapidly changing female morph frequencies, males are
544 expected to rapidly respond to the challenges associated with mate detection by developing
545 plastic search images shaped by the local frequencies of female morphs and their intrinsic
546 fecundities (Fincke, 2004; Gosden & Svensson, 2009; Verzijden et al., 2012). There is
547 increasing interest in the role of plasticity in evolution (West-Eberhard, 2003), including
548 plasticity in mate preferences of both males and females (Verzijden et al., 2012). Such plastic

549 or learned mate preferences can have profound consequences for sexual selection, sexual
550 conflict, the maintenance of sexually selected polymorphisms, population divergence, and
551 speciation (Svensson et al., 2010; Verzijden et al., 2012; Westerman et al., 2012). Our results
552 suggest that opsin gene expression profiles could provide a proximate, mechanistic link
553 between learned or plastic mate preferences, the visual system, and ultimate evolutionary
554 theories about polymorphism maintenance.

555 In this study, we quantified opsin mRNA levels across adult *I. elegans* maturation from
556 eight sites across two years, allowing for opsin expression to be quantified across a continuum
557 of androchrome female frequencies and over male ontogony. Patterns of opsin plasticity over
558 adult male development and opsin expression profiles of adult males were consistent in some
559 ways with predictions of the LMR hypothesis, namely that increased sexual experience should
560 tune the visual system for successful mate identification and in response to local female morph
561 abundances; however, our results suggest a more complicated effect of local female morph
562 abundances on opsin expression.

563 We identified opsin-specific plasticity in relative expression over male ontogeny (Figs.
564 4 - 7), consistent with age-specific selection pressures likely acting on males within each
565 maturity stage. Teneral males, which are sexually immature, are not expected to express opsin
566 genes important in mate detection, under the assumption that such expression is costly. In line
567 with this assumption, we found no significant differences in relative expression of SWS opsins
568 and few significant differences between LWS opsins for teneral males (Fig. 5A). In contrast,
569 there were many differences in relative expression between SWS and LWS opsin classes for
570 sexually immature and sexually mature males (Fig. 5B, C), which could indicate gradual tuning
571 of the visual system corresponding to the increasing relevance of sexual signals in mate-
572 searching males. Between maturity stages, significant increases in relative expression of the
573 SW1 and LWF2 opsins occurred over maturation, while LWF1 and the ocelli opsin LWE

574 showed significant decreases in relative expression (Fig. 6), consistent with opsin-specific
575 regulation over male sexual maturation. Further, proportional opsin expression, indicative of
576 changes in visual sensitivity, differed between developmental stages, with significant increases
577 in proportion expression of the LWF2 opsin in immature and mature males relative to teneral
578 males (Fig. 7). *In vitro* measurements of opsin sensitivity show that the LWF2 opsin provides
579 the ability to perceive wavelengths of light reflected by infuscans females for a finely tuned
580 visual match to infuscans body colouration (Fig. 3). Lastly, assessing the effect of local female
581 morph abundances on relative opsin gene expression suggest that mature males, which are
582 likely to have had more sexual encounters than immature and teneral males, have opsin
583 expression profiles well-suited to detect the relatively more common infuscans females in
584 populations with low frequencies of androchrome females (Fig. 8).

585 These results are broadly in agreement with the predictions of the LMR hypothesis.
586 However, unexpectedly, LWF2 expression was also increased in mature males in settings with
587 high frequencies of androchrome females, with no evidence that SWS opsins were significantly
588 upregulated in androchrome-biased settings (Fig. 8). Our previous research has revealed that
589 androchrome females have the lowest mating rates of the three female morphs in *I. elegans*
590 (Gosden & Svensson, 2009; Willink et al., 2019). This could increase mating competition
591 between males in populations with high frequencies of androchrome females. Experiments in
592 *Ischnura ramburii* have revealed that males exposed to higher frequencies of androchrome
593 females experience more antagonistic male-male interactions, had lower body condition, and
594 higher mortality rates than males from gynochrome-biased populations (Gering, 2017). Given
595 these potential costs of increased androchrome encounters and the intrinsic fecundity
596 differences between female morphs, with as much as 30% higher relative fecundity estimated
597 for infuscans females (Svensson & Abbott, 2005; Willink & Svensson, 2017), male *I. elegans*

598 from high androchrome populations might gain direct fitness benefits by tuning their visual
599 system to be better able to identify infuscans females.

600 Overall, our results suggest opsin expression can be tuned in the male visual system in
601 *I. elegans* in a manner consistent with a role in mate choice; however, we would ideally link
602 the expression differences documented in the current study to behavioural differences in mate
603 identification or preferences. While the link between opsin expression and behaviour is weak
604 in some systems (Fuller et al., 2010; Wright et al., 2020), in other cases, differences in opsin
605 expression does correspond with behaviour (Sakai et al., 2018; Seehausen et al., 2008). For
606 example, female guppies from low-predator populations prefer males with more orange/red
607 colouration and have higher expression of opsins that are sensitive to orange and red
608 wavelengths compared to females from high-predator populations (Sandkam et al., 2015).
609 Future work directly assessing the effect of specific opsin expression profiles on mating
610 outcomes will provide valuable insights into the function of opsin plasticity in mate choice and
611 the maintenance of female polymorphism across Odonata.

612

613 CONCLUSION

614 The results of the current study show that opsin gene expression, specifically the long-
615 wavelength sensitive LWF2 opsin, is plastic over male ontogeny and changes in response to
616 local female morph frequencies in mate-searching male *I. elegans*. Functional
617 analyses revealed that the range of spectral sensitivity of *I. elegans* long-wavelength opsins
618 closely corresponds to infuscans female body reflectance, with subsequent opsin-specific
619 expression changes likely contributing to efficiently detect infuscans females over the male-
620 mimicking androchrome females. Males that likely encounter proportionally more infuscans
621 females showed increased relative expression of LWF2, suggestive of a mechanism at the
622 peripheral visual system level to improve detection of common female morphs. In contrast,

623 higher relative LWF2 expression in males from high androchrome populations does not support
624 visual tuning for identification of common female morphs. In this case, other behavioural and
625 physiological differences between androchrome and gynochrome females (e.g., aggression or
626 fecundity differences) may incur additional costs or benefits that could explain observed
627 patterns of opsin gene expression in the current study. Future work exploring how these
628 dynamic patterns of opsin expression correspond to differences in male mating behaviour have
629 the potential to reveal the proximate mechanisms underlying morph detection and mate choice.
630

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640

641 DATA ACCESSIBILITY

642 Datafiles and code associated all the analyses in this paper will be uploaded to the Dryad Digital
643 Data Repository upon acceptance.

644

645 AUTHOR CONTRIBUTIONS

All authors contributed to conception of the experimental design. M.A.L performed opsin heterologous expression and analysis of opsin function sensitivity data, N.S.R collected field data and performed qPCR and statistical analyses with support from E.I.S and M.A.L. N.S.R wrote the manuscript with support from E.I.S and M.A.L. All authors reviewed the final manuscript.

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TABLES & FIGURES

Table 1. Final model and model output showing the effect of maturity stage and opsin type on log relative opsin expression in teneral, immature, and mature male *I. elegans*. Significant differences are shown in italics.

log Relative Opsin Expression ~ Maturity × Opsin + (1|ID) +(1|Replicate) + (1|Year)			
	df	F	*P*
Maturity	2	0.2	0.82
Opsin	7	292.3	*< 0.0001*
Maturity × Opsin	14	12.3	*< 0.0001*

Table 2. Final model and model output showing the effect of maturity stage and opsin type on proportion opsin expression in teneral, immature, and mature male *I. elegans*. Significant differences are shown in italics.

Proportion Opsin Expression ~ Maturity × Opsin + (1|ID)			
	df	F	*P*
Maturity	2	10.8	*< 0.0001*
Opsin	6	415.6	*< 0.0001*
Maturity × Opsin	12	29.8	*< 0.0001*

Table 3. Final model and model output showing the effect of the proportion of androchrome females in a population, opsin type, and maturity stage on log relative opsin expression for immature and mature male *I. elegans*. Significant differences are shown in italics.

log Relative Opsin Expression ~ Opsin × Maturity × (Prop. Androchrome + Prop. Androchrome^2) + (1|ID) + (1|Replicate) + (1|Year)			
	df	F	*P*
Opsin	7	18.7	*< 0.0001*
Maturity	1	1.3	0.27
Prop. Androchrome	1	0.2	0.66
Prop. Androchrome^2	1	0.2	0.67
Opsin × Maturity	7	3.1	*< 0.01*
Opsin × Prop. Androchrome	7	17.1	*< 0.0001*
Opsin × Prop. Androchrome^2	7	17.3	*< 0.0001*
Maturity × Prop. Androchrome	1	1.3	0.26
Maturity × Prop. Androchrome^2	1	1.3	0.25
Opsin × Maturity × Prop. Androchrome	7	3.3	*< 0.01*
Opsin × Maturity × Prop. Androchrome^2	7	3.6	*< 0.001*

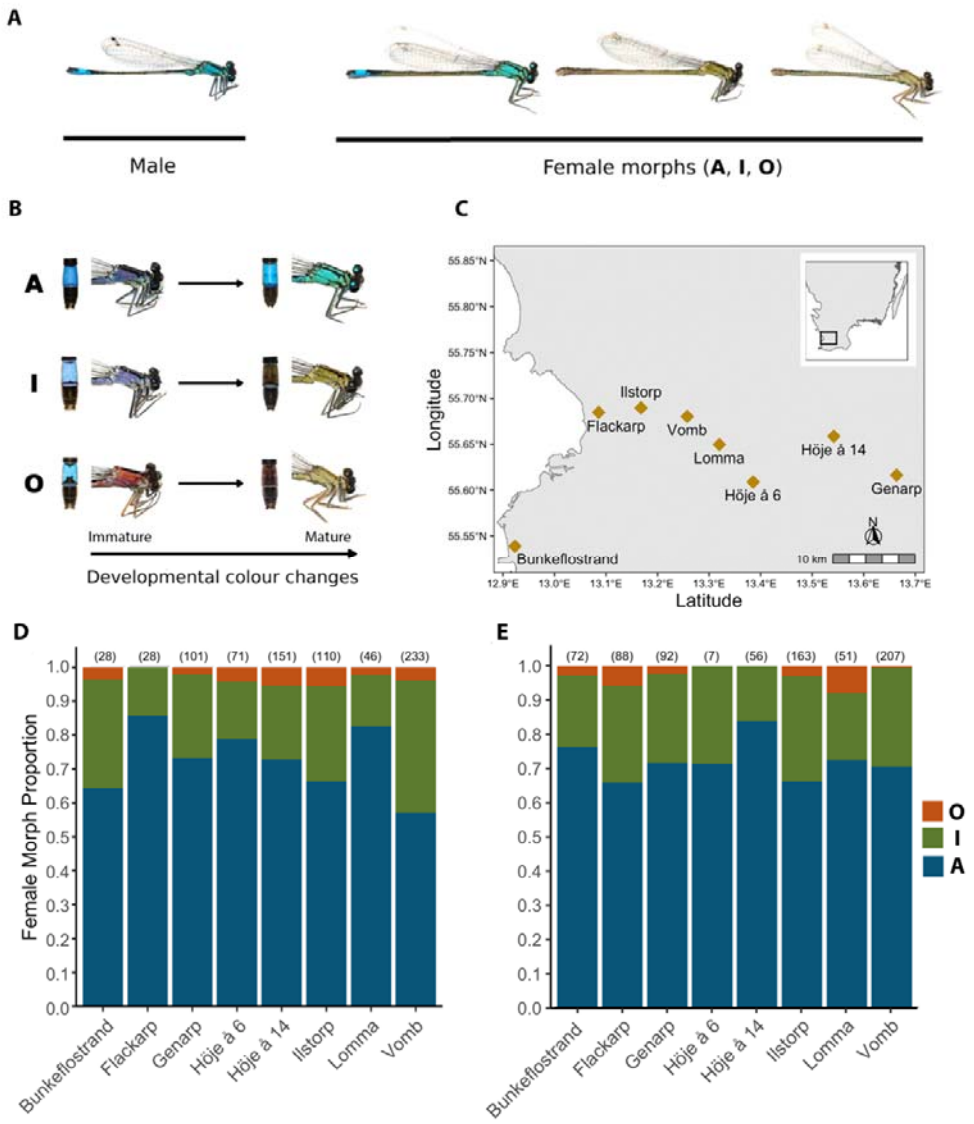

Figure 1. Colour polymorphism and female morph frequencies in the damselfly *I. elegans* from eight populations from sites in the region of Skåne, southern Sweden for the damselfly *Ischnura elegans*. (A) Mature male *I. elegans* and the three female-limited colour morphs in their sexual mature colour phases. (B) Females undergo ontogenetic colour changes in the distal abdomen segments (left photo) and thorax (right photo) between immature and sexually mature adult stages. A = androchrome, I = infuscans, O = infuscans-obsoleta. (C) Sampling locations of *I. elegans* used in the current study with inset showing the area of southern Sweden sampled. (D) Female morph frequencies per population in 2021 and I 2022. Numbers in parentheses above each bar indicate sample size. (A, B) modified from Willink et al. (2020).

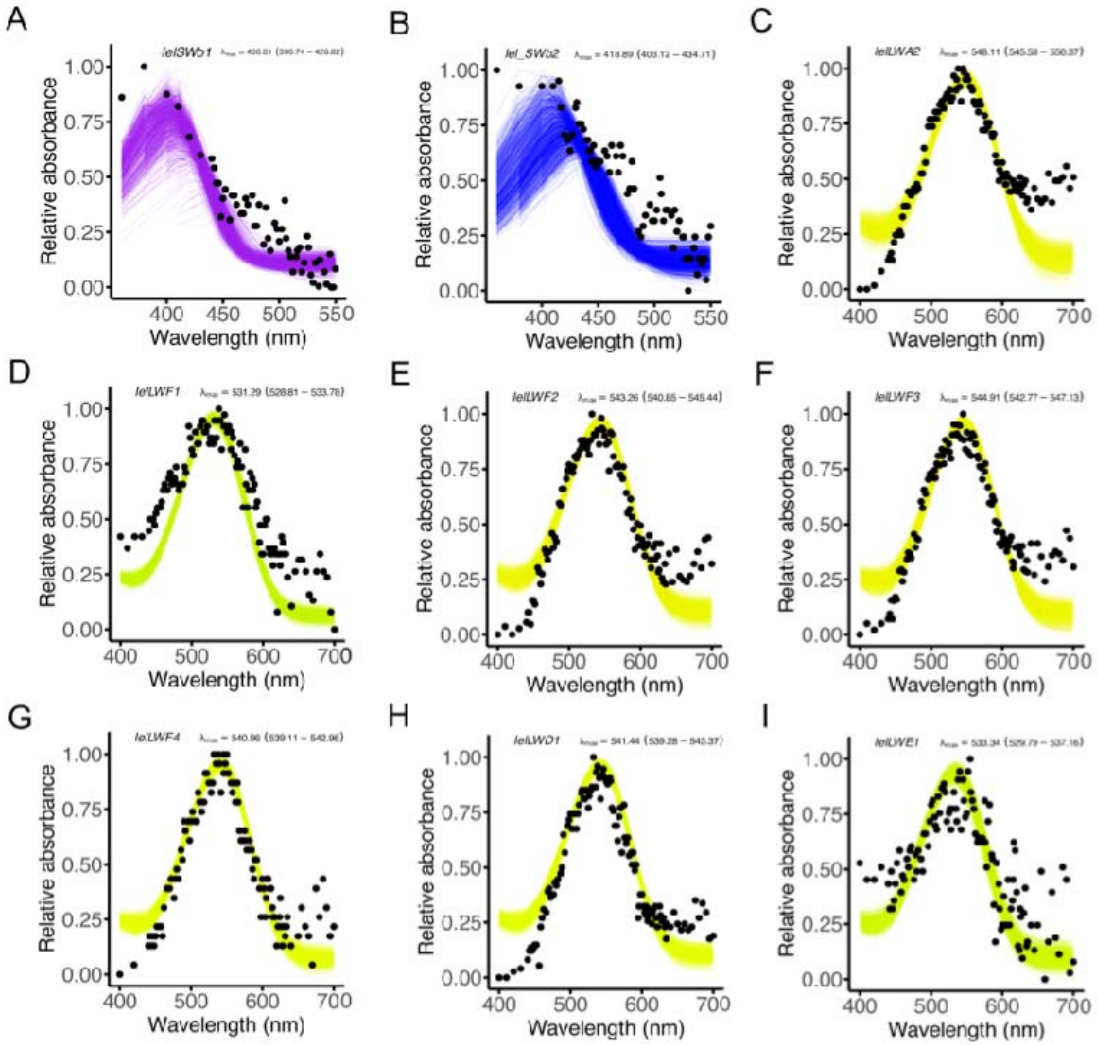

Figure 2. Functional expression of *Ischnura elegans* short-wavelength (SW) and long-wavelength (LW) opsins. Ultraviolet-visible (UV-VIS) absorbance analyses of dark-adapted rhodopsin visual pigments reconstituted and purified from HEK293T cell cultures in the dark in the presence of 11-*cis*-retinal. The black dots represent mean absorbances at a given wavelength. (A) SWb1 (n = 7), (B) SWb2 (n = 3), (C) LWA2 (n = 2), (D) LWF1 (n = 2), I LWF2 (n = 5), (F) LWF3 (n = 6), (G) LWF4 (n = 1), and opsins expressed in the ocelli: (H) LWD1 (n = 3) and (I) LWE1 (n = 6), where n is the number of measurements of protein aliquots with active rhodopsin complexes. Absorbance at 380 nm in A and B is due to residual unbound *cis*-retinal. 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 for each rhodopsin (Liénard et al., 2022). Confidence intervals are indicated in parentheses.

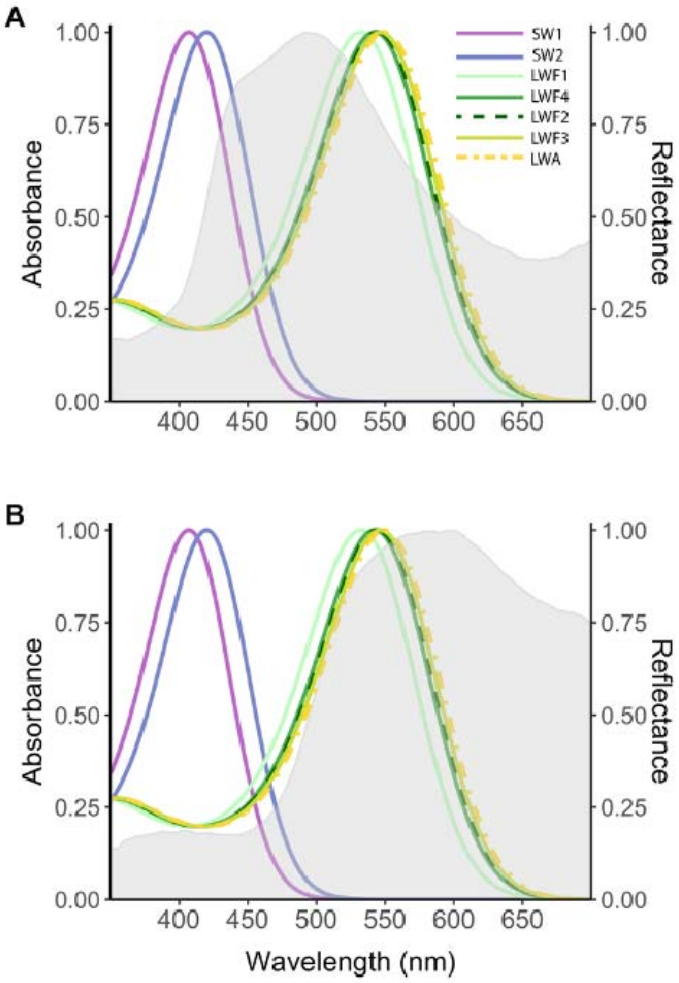

Figure 3. Absorbance spectra of opsins and female body reflectance in *I. elegans*. Sensitivity of opsins expressed in the ventral compound eye of male *I. elegans* are represented by coloured visual fit curves. Body reflectance for (A) androchrome and (B) infuscans female body colouration is represented by the shaded gray area. Body reflectance data from Henze et al. (2019).

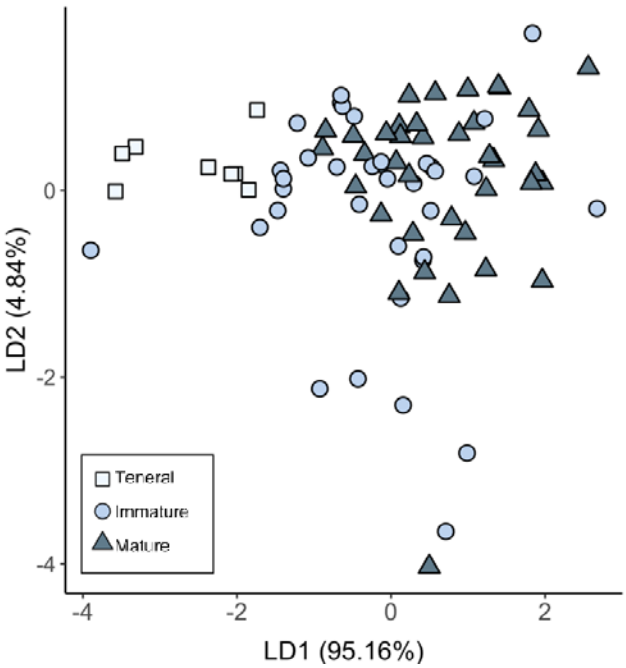

Figure 4. Linear discriminate analysis showing the relationship between relative expression of eight opsins expressed within the ventral eye or ocelli of teneral, immature, and mature male *I. elegans* for the first two linear discriminate factors (LD1 and LD2; percentages shown on the x- and y -axes).

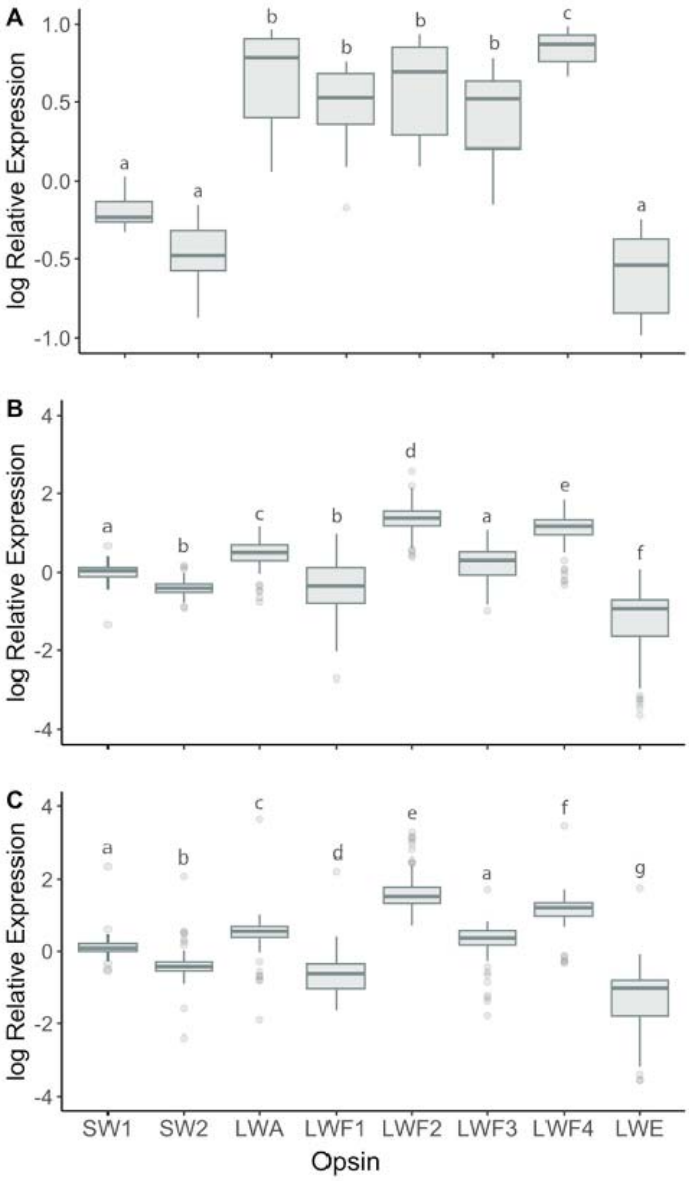

Figure 5. Relative opsin expression for (A) teneral, (B) immature, and (C) mature male *I. elegans.* Bars in box and whisker plots show medians, boxes indicate upper and lower quartiles, whiskers show sample minima and maxima, and open circles show outliers. Letters above each bar show significant differences between opsin types within each maturity level following Tukey corrections for multiple comparisons. Test statistics and p-values for all comparisons are presented in Table S8.

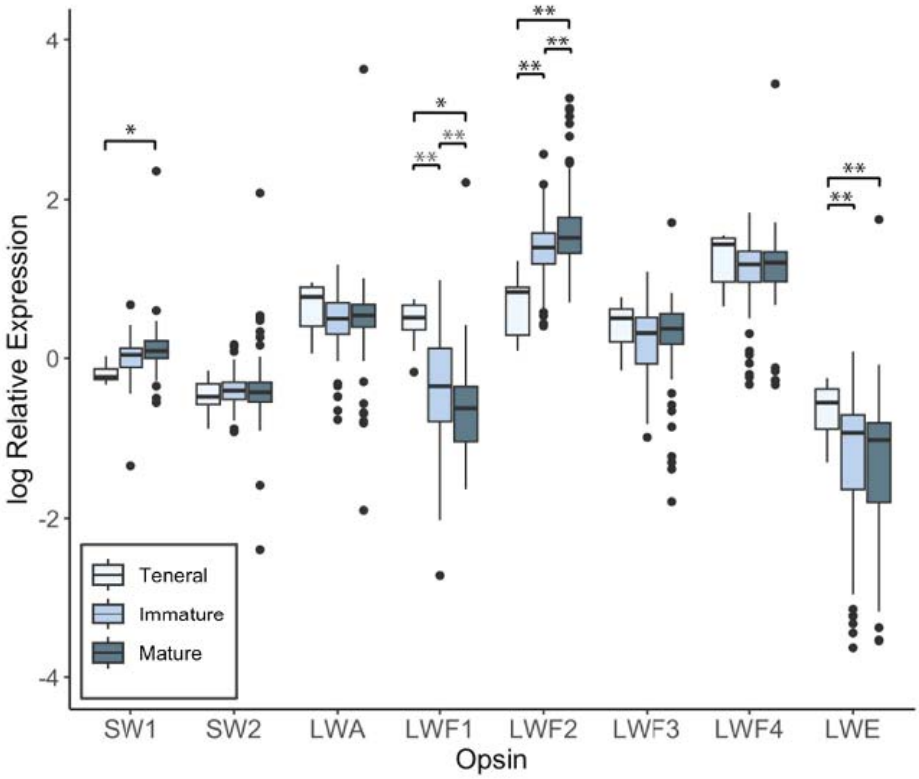

Figure 6. Relative opsin expression across seven opsins expressed primarily in the ventral compound eye (SW1 – LWF4) and one opsin primarily expressed in the ocelli (LWE) for male *I. elegans*. Teneral males are shown in light blue, immature adult males in medium blue, and mature males in dark blue. 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. 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 Table S9.

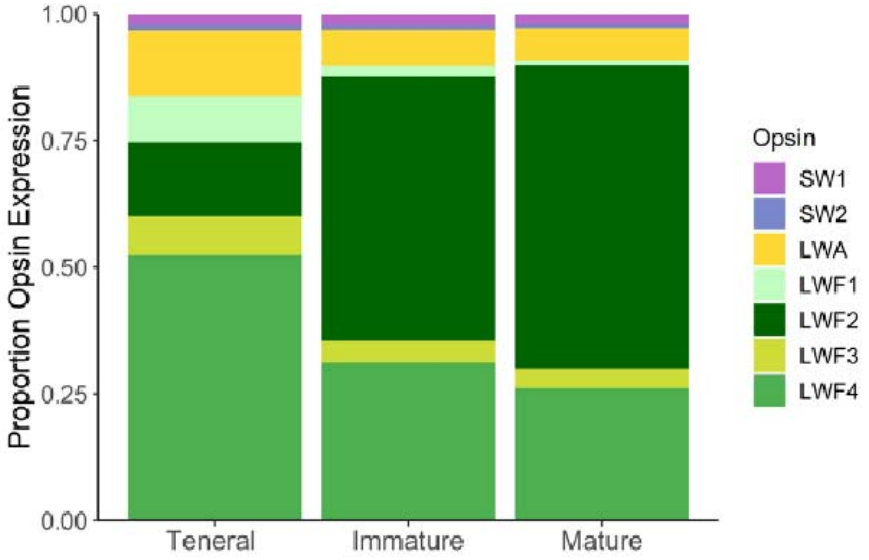

Figure 7. Average proportion expression of opsins found in the ventral compound eye of teneral, immature, and mature male *I. elegans*.

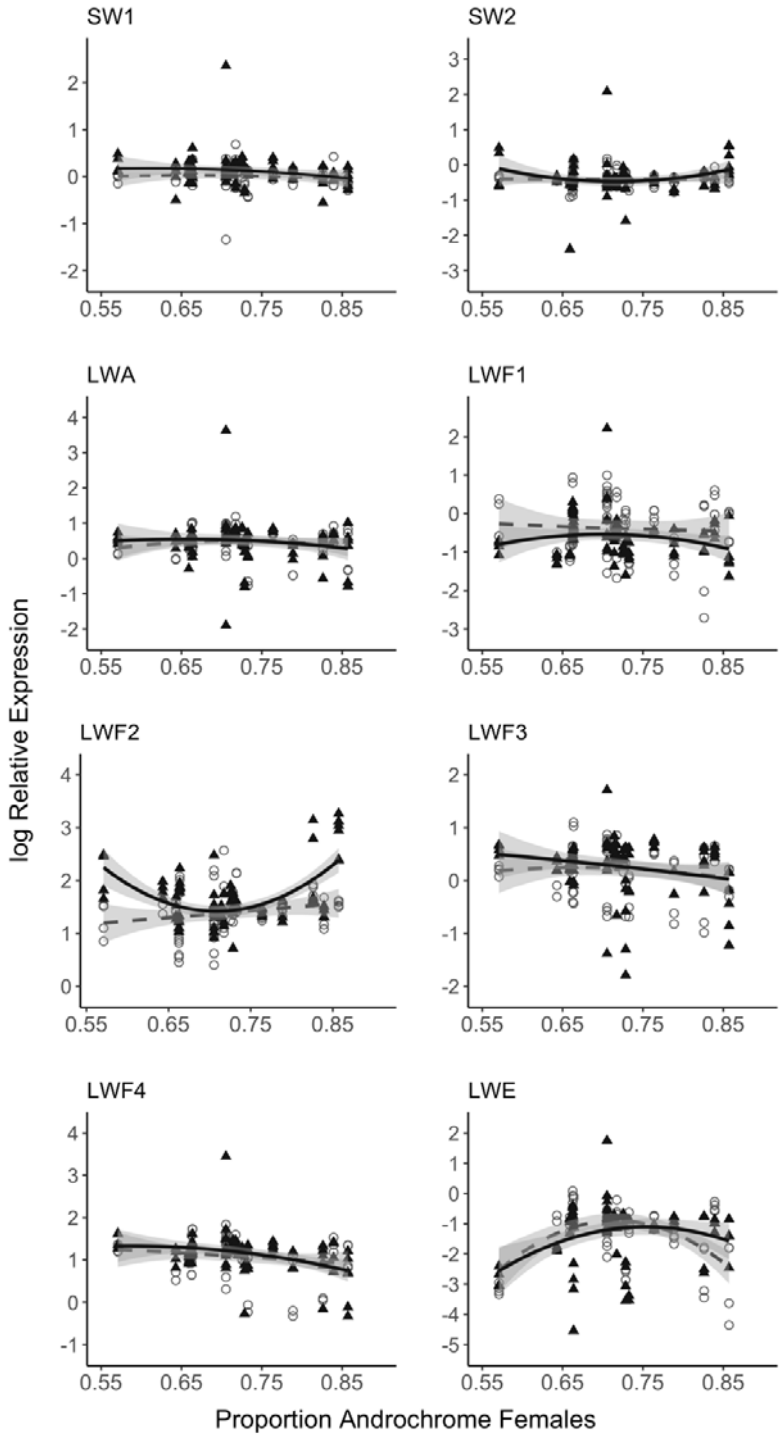

Figure 8. Log relative opsin expression in immature (open circles, dashed line) and mature (black triangle, solid line) males *I. elegans* from populations with different proportions of androchrome females. The opsins SW1, SW2, LWA, and LWF1 – LWF4 are expressed in the compound eye and the opsin LWE is expressed primarily in ocelli. Grey shading around trend lines indicates 95% confidence intervals.