

# 1 **Extraocular photoreception mediates adaptive colour change and** 2 **background choice behaviour in peppered moth caterpillars**

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11 **Light sensing by tissues distinct from the eye occurs in diverse animal groups, enabling**  
12 **circadian control and phototactic behaviour. Extraocular photoreceptors may also**  
13 **facilitate *rapid* colour change in cephalopods and lizards, but little is known about the**  
14 **sensory system that mediates *slow* colour change in arthropods. We previously reported**  
15 **that slow colour change in twig-mimicking caterpillars of the peppered moth (*Biston***  
16 ***betularia*) is a response to achromatic and chromatic visual cues. Here we show that the**  
17 **perception of these cues, and the resulting phenotypic responses, does not require ocular**  
18 **vision. Caterpillars with completely obscured ocelli remained capable of enhancing their**  
19 **crypsis by changing colour and choosing to rest on colour-matching twigs. A suite of**  
20 **visual genes, expressed across the larval integument, likely plays a key role in the**  
21 **mechanism. To our knowledge, this is the first evidence that extraocular colour sensing**  
22 **can mediate pigment-based colour change and behaviour in an arthropod.**

23 *Dermal photoreception*, the ability to perceive photic information through the skin  
24 independently of eyes, has evolved a number of times to serve a variety of functions <sup>1-4</sup>. It is  
25 best known for its involvement in shadow reflexes, phototaxis, and orientation in response to  
26 light <sup>5</sup>. More recently, dermal photoreception (more generally referred to as *extraocular*  
27 *photoreception*) has been proposed to mediate the rapid (*physiological*) colour change  
28 observed in cephalopods <sup>6,7</sup>, fish <sup>8</sup>, and reptiles <sup>9</sup>, through the rearrangement of pigment  
29 granules or reflective platelets within specialised cells called chromatophores. Slow  
30 (*morphological*) colour change, occurring over hours to weeks, is common in arthropods <sup>10,11</sup>.  
31 Several studies have demonstrated that substrate characteristics <sup>12</sup> and the wavelength of light  
32 <sup>13</sup> influence pupal colour in a variety of butterfly species <sup>14</sup>, on the assumption that they use  
33 their eyes to perceive the colour stimuli. Pioneering experiments by Victorian entomologist  
34 Edward Bagnall Poulton on the control of pupal colour in the small tortoiseshell butterfly,  
35 *Aglais urticae*, were the first to provide evidence for extraocular photoreception in colour-  
36 changing arthropods <sup>15</sup>. Only recently have researchers revisited the possibility that  
37 extraocular photoreception is involved in slow colour change of arthropods <sup>16</sup>. Given the  
38 prevalence of slow colour change, research is needed to examine the importance of  
39 extraocular photoreception in this category of colour change and to characterise the  
40 physiological basis of this under-investigated biological phenomenon.

41 The peppered moth (*Biston betularia*) has evolved to be highly cryptic to visual predators,  
42 both in the adult and larval stages. Crypsis is achieved through contrasting mechanisms in  
43 each stage. The adult colour pattern polymorphism (melanism) is genetically determined <sup>17,18</sup>,  
44 while the larvae camouflage through a combination of twig-mimicking masquerade <sup>19</sup> and  
45 colour plasticity <sup>20</sup>. Colour change in these polyphagous larvae is a continuous reaction norm  
46 in response to colour cues from the twigs in the larvae's immediate surroundings rather than  
47 the leaves they eat <sup>20</sup>. The precision of this colour and pattern response is at odds with the  
48 simple larval ocelli <sup>21</sup>, and the distal position of the head relative to the twig when larvae are

49 in the resting pose. We conjectured that the larvae could be using an additional visual sense.  
50 Here we report the results of morphological, behavioural, and gene expression experiments to  
51 investigate the role of extraocular photoreception in colour-changing *B. betularia* larvae.

52 We reared 321 larvae from 4 families in replicated groups of 25 individuals, inside  
53 transparent plastic boxes containing inter-crossing artificial twigs (painted dowels), on  
54 stalkless fresh leaves of the grey willow, *Salix cinerea* (see Methods). We painted over the  
55 caterpillars' ocelli with black acrylic paint with the aid of a microscope (Figure 1). This  
56 obstruction to ocular vision or 'blindfolding' started at late 2<sup>nd</sup> to early 3<sup>rd</sup> instar, which is the  
57 earliest stage at which larvae can be effectively blindfolded, and is prior to a strong colour  
58 response. To overcome the problem of caterpillars shedding the blindfold in the process of  
59 molting between instars, we checked caterpillars twice daily for early signs of head capsule  
60 slippage. Head capsule slippage takes approximately 12-18 hrs to complete, during which  
61 time we held these individuals separately and singly overnight in opaque white boxes without  
62 any dowels. Fresh paint was applied to the new head capsule, thus preventing the caterpillars  
63 from receiving any dowel colour signal, and the caterpillars were returned to their group  
64 enclosure. We used four different dowel colours, with one colour per enclosure: brown, green,  
65 black, and white (see Methods). The first pair of colours differed in chroma and luminance;  
66 the second pair differed only in luminance. The spectral reflectance of each caterpillar's  
67 integument was measured at the final (6<sup>th</sup>) instar using a spectrophotometer (six non-  
68 overlapping measurements). We used a computational model of visual perception to quantify  
69 larval colour and luminance as it would be perceived by a visually hunting avian predator, the  
70 blue tit, *Cyanistes caeruleus*<sup>22</sup>. We calculated how green the caterpillars appeared to a  
71 predator as the ratio of the medium and long wavelength cone responses; the luminance of  
72 each caterpillar as the double dorsal cone responses; and the discriminability of the larvae as  
73 units of *just noticeable differences* (see Methods).

## 74 Results

75 **Colour change.** We found a striking whole-body colour change in the absence of visual  
76 information from the eyes, whereby caterpillars not only changed colour to resemble the  
77 dowel colour in their enclosure, but they did so to the same degree as non-blindfolded  
78 controls. This is evident to the human eye (Figures 2A, D), and is also apparent by  
79 comparison of the spectral reflectance curves in the visible wavelength range (Figures 2C, E).  
80 However, the more critical and ecologically relevant assessment is through the prism of an  
81 avian predator's perception, which we have quantified through psychophysical modelling.  
82 Viewed through this lens, *B. betularia* larvae reared in white dowel enclosures were  
83 significantly brighter than those reared on black dowels, when measured as the double cone  
84 responses of the avian retina ( $F_{1, 127} = 177.4$ ,  $P < 0.0001$ ; Figure 2B), but there was no  
85 significant effect of blindfolding on the luminance of larvae from black or white treatments  
86 ( $F_{1, 127} = 0.28$ ,  $P = 0.6$ ). Larvae reared in green dowel enclosures were significantly greener to  
87 an avian predator than larvae from brown treatments ( $F_{1, 169} = 451.2$ ,  $P < 0.0001$ ; Figure 2E).  
88 Moreover, blindfolding had no significant effect on the greenness of larvae in the green or  
89 brown treatments ( $F_{1, 169} = 0.67$ ,  $P = 0.4$ ), and the distribution of greenness was similar  
90 between blindfolded and control larvae across both treatments (Figure 2E). Using a  
91 complementary approach to quantify the ability of an avian predator to distinguish between  
92 two stimuli<sup>23</sup>, we find that birds would not be able to discriminate between blindfolded and  
93 control larvae, whether reared on achromatic ( $F_{1, 127} = 2.64$ ,  $P = 0.1$ ; Supplementary Figure  
94 1A) or chromatic dowels ( $F_{1, 169} = 1.01$ ,  $P = 0.3$ ; Supplementary Figures 1B and 2).

95 **Background choice.** To further evaluate *B. betularia* caterpillar's capacity for extraocular  
96 colour perception, we tested background choice behaviour using two designs of background

97 choice arena: a transparent plastic cube containing two diagonally crossing dowels, each  
98 painted with a single colour (bright green vs. dark brown); and a transparent horizontal tube  
99 with a single horizontally suspended dowel, one half painted green and the other brown (see  
100 Methods). These two designs allowed us to test for the consistency of background choice in  
101 different contexts. For each trial, final instar larvae from blindfolded and control groups of the  
102 green and brown treatments were placed equidistant from each dowel colour. Because  
103 predation risk increases the likelihood of behavioural background matching, we simulated  
104 predation by gently poking larvae on the dorsal surface with tweezers (following methods in  
105 <sup>24</sup>). For horizontal dowel chambers, to eliminate any positional preferences, two trials were  
106 conducted per larva. In one trial, the brown end of the dowel was at the far end of the  
107 chamber; in the other trial, the direction of the dowel was reversed (the order of trials was  
108 randomised). Individual larvae were left for 12h (7h dark: 5h light), after which the dowel  
109 colour that each caterpillar was resting on was recorded. In both types of arena (and both  
110 dowel orientations in the horizontal arena), larvae were able to maximise camouflage by  
111 selecting dowel colours that more closely matched their own body colours (Figure 3). On  
112 average, 75-80% of brown larvae chose to rest on a brown dowel, and 70-80% of green larvae  
113 chose to rest on a green dowel. In the diagonal chamber design, there was no effect of  
114 blindfolding ( $Z = -0.22$ ,  $P = 0.83$ ) or larval colour ( $Z = -0.87$ ,  $P = 0.39$ ) on matching success.  
115 In the horizontal chamber, there was also no effect of blindfolding ( $Z = -1.24$ ,  $P = 0.21$ ),  
116 larval colour ( $Z = 0.82$ ,  $P = 0.41$ ), or dowel position ( $Z = -1.72$ ,  $P = 0.08$ ) on matching  
117 success.

118 **Visual gene expression.** To investigate the molecular basis of the morphological and  
119 behavioural responses, we analysed the expression of key genes involved in visual perception  
120 in head (including eyes) and dermal tissue of *B. betularia* larvae and adults. Opsins are light-  
121 sensitive proteins that mediate the conversion of a photon of light into an electrochemical  
122 signal, necessary for vision and photoreception <sup>25</sup>. We identified opsins sensitive to ultraviolet  
123 (two splice variants UVA and UVB), blue (two splice variants BIA and BIB), long  
124 wavelength (two gene copies LW1 and LW2), and *melanopsin* (two splice variants MelA and  
125 MelB) (Supplementary Figures 3-5). We also determined the coding sequence for visual  
126 *arrestin-1* (Arr-1; Supplementary Figure 6) and *retinal degeneration B* (RDB; Supplementary  
127 Figure 7), which are essential components of phototransduction <sup>26,27</sup>. Using end-point RT-  
128 PCR, we detected expression of these genes not only in the eyes (head), but also in all  
129 segments of the whole body epidermis, both in larvae and adults (Figure 4A, Supplementary  
130 Figure 8). Subsequent quantitative assessments using RT-qPCR revealed that in the head  
131 tissue, expression levels for several of the genes tested are orders of magnitude higher in  
132 adults than in larvae (Figure 4A;  $t_{71} = -5.33$ ,  $P < 0.0001$ ). This likely reflects the relative size  
133 of the compound vs the simple eyes compared to the head of the two life stages. Dermal tissue  
134 expression for all genes, averaged across all three body segments, is similar across larvae and  
135 adults (Figure 4A;  $t_{69} = -1.15$ ,  $P = 0.26$ ). Within life stages, dermal expression levels are  
136 similar among body segments for most genes (Supplementary Figure 9). In larvae  
137 (Supplementary Figure 9A), RDB expression is higher in claspers, and BIB expression is  
138 much lower in the abdomen; in adults (Supplementary Figure 9B), RDB expression is lower  
139 in the genitalia segment, and UVA expression is somewhat higher in the thorax.

140 The ratio of gene expression in the epidermis to that in the head provides a measure of the  
141 contribution of putative photoreceptors in the larval epidermis to the total light-sensing  
142 capacity of a caterpillar. By this measure, dermal expression of photoreception genes is  
143 significantly higher in larvae, compared to adults ( $Z_{11} = 0.22$ ,  $P < 0.0001$ ), with LW2 as the  
144 only gene showing relatively higher dermal expression in adults (Figure 4B). In larvae,  
145 expression of RDB, BIB and LW1 is upregulated in dermal tissue to similar levels of that in

146 the head. The strongest contrast in relative dermal expression between larvae and adults is for  
147 Arr-1, BlB, MelB and LW1.

## 148 Discussion

149 *Biston betularia* larvae that were prevented from receiving light input through their ocelli  
150 changed colour in response to luminance and colour cues, and also maximised the benefits of  
151 this plastic masquerade by actively selecting twigs of similar colour. Experimental and  
152 control larvae were equally able to change appearance and choose the appropriate resting  
153 background, demonstrating that they are capable of spectrally sensitive extraocular  
154 photoreception, and implying that the ocelli play a secondary role in these responses. Our  
155 results contrast those of similar blindfolding experiments in other arthropods<sup>10,28</sup>, where the  
156 characteristics of the blindfolding paint, rather than the background colour, affected colour  
157 change. The necessity for extraocular photoreception in *B. betularia* may relate to the angled  
158 twig-posture of the larvae during the daytime, which places the ocelli away from the twig  
159 perch (Supplementary Figure 10). In this position, as well as during feeding on leaves,  
160 photoreceptors across the larval skin could receive more accurate colour and pattern  
161 information on the resting twig than the ocelli.

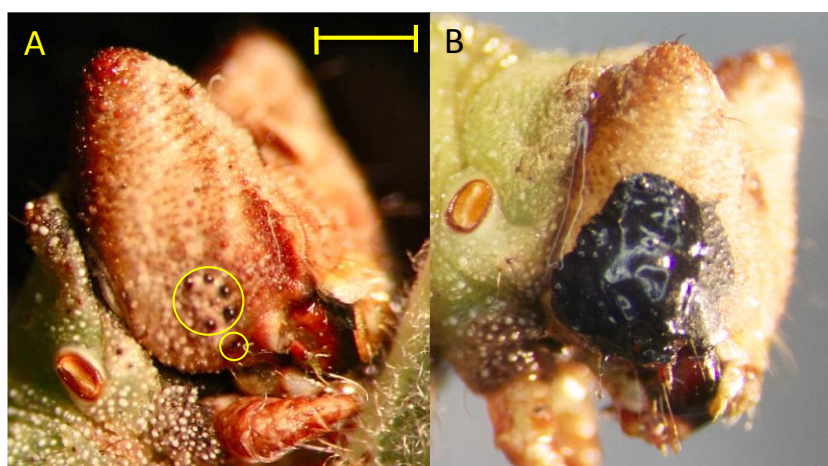
162 The potential role of extraocular photoreceptors in colour change *via* pigment-production was  
163 first suggested by Poulton<sup>15,29</sup>, working on the determination of pupal colour in *A. urticae*. By  
164 means of individual partitioned chambers (i.e., not relying on the occlusion or destruction of  
165 the ocelli), in which the head end of a larva was exposed to a contrasting colour than the  
166 remainder of the body, he showed that the resulting pupal colour was determined by the  
167 background colour to which the greatest surface area of skin had been exposed. Over one  
168 hundred years later, Kato *et al.*<sup>30</sup> showed that the pupal greenness of the Japanese oak  
169 silkmoth, *Antheraea yamamai*, was determined by the intensity of white light and was  
170 unaffected by cauterization of the larval ocelli. Although many other species of caterpillar  
171 change colour to better match their resting background<sup>31</sup>, no other research on arthropods has  
172 distinguished the role of ocular *vs* extraocular photoreceptors.

173 The ability to choose a colour-matching background could be considered redundant in colour-  
174 changing animals, such as peppered moth caterpillars, which gain additional protection from  
175 predation by masquerading as twigs<sup>32</sup>. However, as colour change in *B. betularia* is a slow  
176 process, and the twig colour environment inhabited by these caterpillars is often  
177 heterogeneous, background choice matching offers added flexibility and responsiveness. The  
178 equivalent strategy of choosing to rest on matching backgrounds in visually heterogeneous  
179 environments in species that are also capable of colour change has evolved in flatfish, larval  
180 newts, and salamanders<sup>33-35</sup>.

181 Epidermal opsin expression associated with achromatic light perception has been reported in  
182 cnidarians<sup>36</sup>, cephalopods<sup>37</sup>, arthropods<sup>38</sup>, and vertebrates<sup>9</sup>. Given what is known about  
183 their primary function, and the energetic cost of gene expression<sup>39</sup>, the relatively high  
184 abundance of a whole suite of phototransduction gene transcripts in the larval epidermis  
185 suggests that they constitute part of the extraocular photoreceptor machinery. Whether this is  
186 also true for the adult moths, that also show appreciable levels of visual gene expression in all  
187 segments of their epidermal tissue, is an open question. Precise background matching has  
188 been reported for adults of another geometrid moth<sup>40</sup>, but the evidence for *B. betularia*,  
189 which occur as a melanic series of genetically-determined morphs<sup>41</sup> is equivocal<sup>42</sup>. To our  
190 knowledge, our study provides the first evidence for extraocular opsin expression potentially  
191 capable of detecting colour in an arthropod, linked to functional changes in appearance and  
192 behaviour.

193 The identity and precise location of the extraocular photoreceptors remains to be determined.  
194 Based on the uniformity and fine grain of the colour change (which is a composite of different  
195 epidermal layers; Supplementary Figure 11), together with the even expression of  
196 phototransduction genes across body sections, we speculate that they are distributed more or  
197 less evenly within a layer of the larval dermis, rather than in a few spatially restricted  
198 specialised cells<sup>38</sup>. Extraocular photoreceptors, resembling light-sensitive *phaosome* cells in  
199 earthworm skin, have been described in the genitalia of swallowtail butterflies, proposed to  
200 aid in mate choice and oviposition<sup>43</sup>. Whilst the colour response of blindfolded *B. betularia*  
201 larvae could, in principle, be produced by a highly compartmentalised physiological  
202 mechanism, the background matching behaviour suggests the integration of diffuse  
203 information from the epidermis, not only about the twig colours but also resemblance to self.  
204 It is therefore likely that the nervous and endocrine systems have a combined role in the  
205 colour and background choice responses.

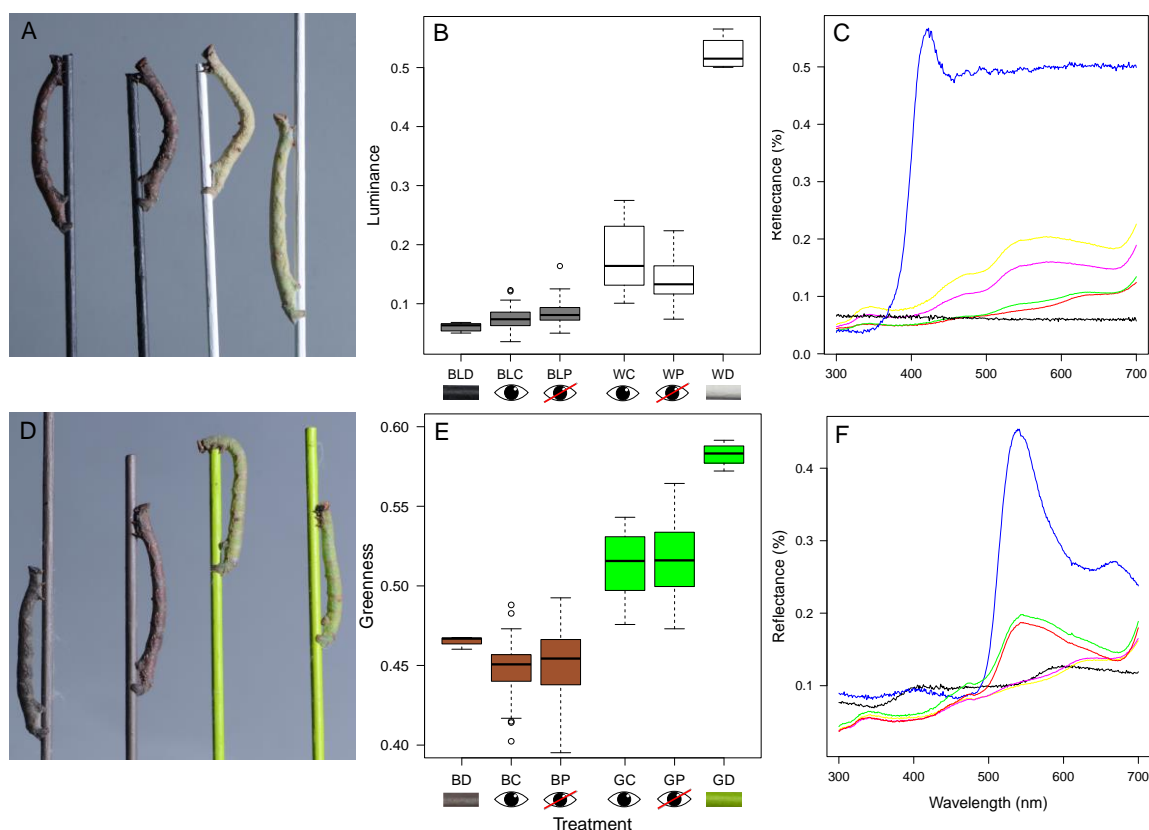
206 The expression profiles of visual genes in *B. betularia*, combined with morphological and  
207 behavioural evidence, lead us to propose that larvae of *B. betularia* possess photoreceptors  
208 distributed throughout the epidermis. Their function is to provide more complete information  
209 on colour and pattern than can be achieved with the ocelli alone – not only of the resting twig,  
210 but also of the match between self and twig. The detailed and composite nature of the  
211 caterpillar's colour pattern suggests a complex signal-processing cascade that initiates,  
212 controls, and coordinates the production of multiple pigments in different cell types. Our  
213 results significantly expand the current view of dermal light sense to include slow colour  
214 change, raising intriguing questions about the evolutionary sequence of pathway recruitment  
215 and modification that has culminated in this sophisticated system of extraocular  
216 photoreception and phenotypic plasticity, driven by a predator-prey evolutionary arms race.  
217

218 **Figures**

219

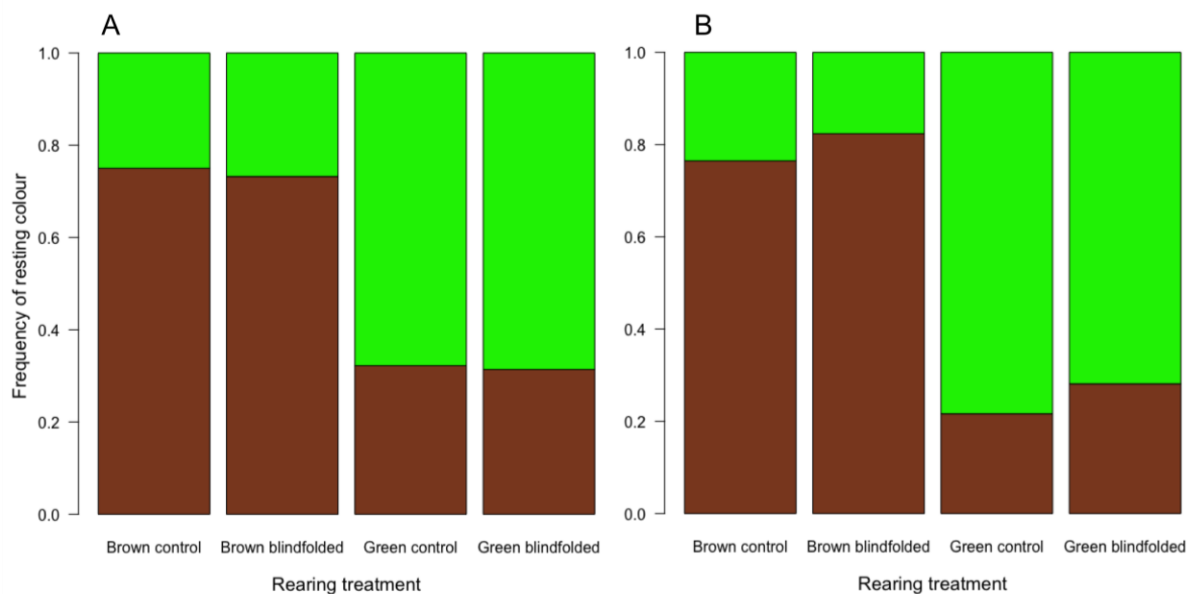
220 **Figure 1.** Blindfolding of *B. betularia* larvae. (A) Final (6<sup>th</sup>) instar *B. betularia* control  
221 caterpillar showing ring of five ocelli circled in yellow, and 6<sup>th</sup> ventral ocellus circled  
222 separately. (B) Example of a final instar larva with ocelli obscured by opaque black acrylic  
223 paint. Scale bar represents 1 mm.

224



225

226 **Figure 2.** Blindfolded and control *B. betularia* larvae from achromatic and chromatic dowel  
 227 treatments. (A) Examples of final instar blindfolded (first and third from left) and control  
 228 (second and fourth from left) larvae on black and white treatment dowels. (B) Luminance of  
 229 black and white larvae and dowels, calculated from double dorsal blue tit cone catches, where  
 230 BL=black, W=white, D=dowel, C=control larvae, P= painted or blindfolded larvae. (C)  
 231 Reflectance of black and white larvae (mean and standard error) and dowels in the visible  
 232 wavelength range (300-700 nm, where black = black dowel (BLD), blue = white dowel (WD),  
 233 red = black control larvae (BLC: n = 29), green= black blindfolded larvae (BLP: n = 45),  
 234 yellow = white control larvae (WC: n = 26), magenta = white blindfolded larvae (WP: n =  
 235 49). (D) Examples of final instar blindfolded (two outermost) and control (two innermost)  
 236 larvae on brown and green treatment dowels. (E) ‘Greenness’ of brown and green larvae and  
 237 dowels, calculated as a ratio of mediumwave (MW) to longwave (LW) blue tit cone catches  
 238  $[MW/(MW+LW)]$ , where B= brown, G = green, D= dowel, C= control larvae, P = painted or  
 239 blindfolded larvae. (F) Reflectance of brown and green larvae (mean and standard error) and  
 240 dowels, where black = brown dowel (BD), blue = green dowel (GD), yellow = brown control  
 241 larvae (BC: n = 44), magenta = brown blindfolded larvae (BP: n = 50), green = green control  
 242 larvae (GC: n = 36), and red = green blindfolded larvae (GP: n = 31). n = number of  
 243 biologically independent samples.  
 244

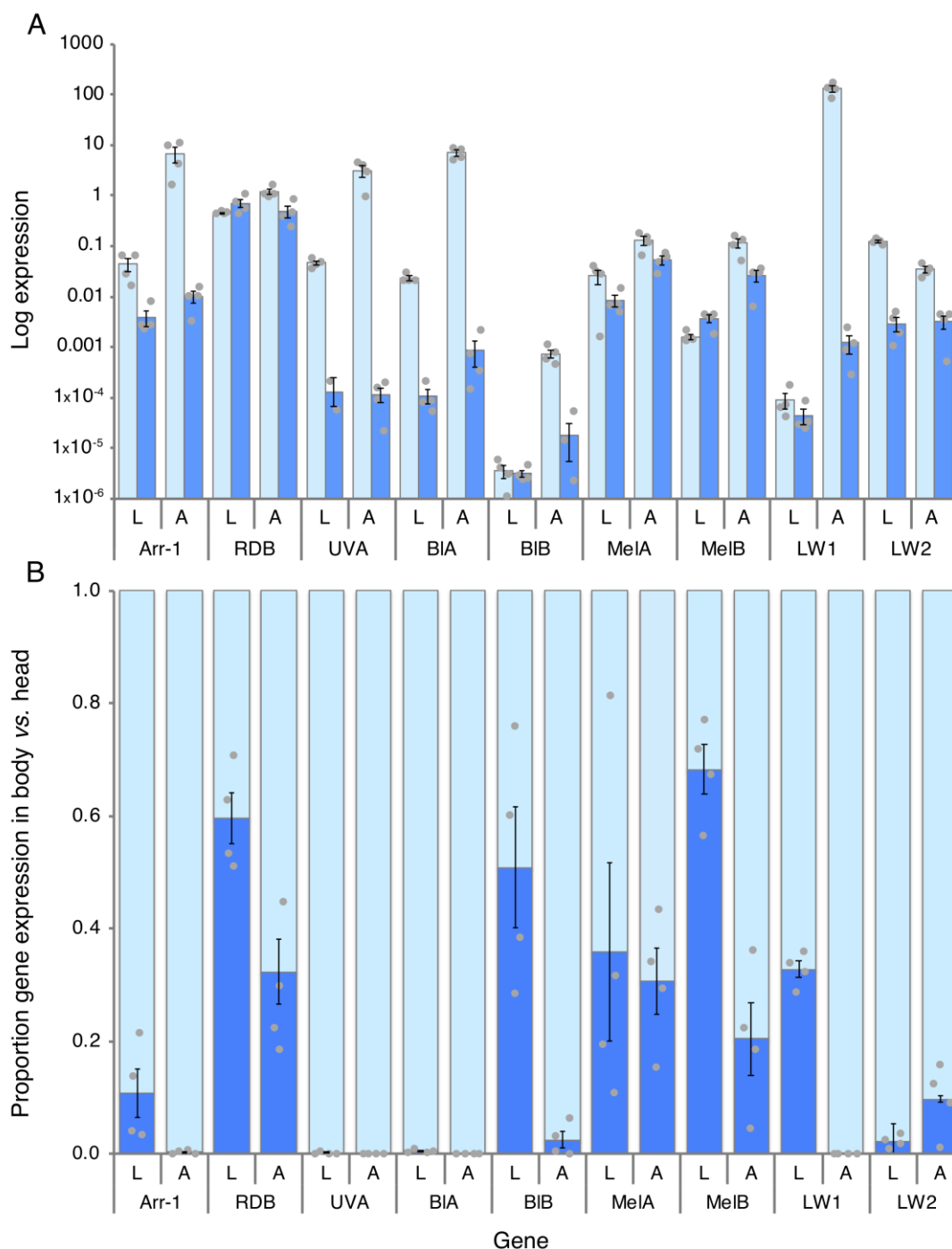


245

246 **Figure 3.** Frequency of resting background colour chosen by *B. betularia* caterpillars. Mean  
 247 frequency, as proportions of final instar *B. betularia* blindfolded and control caterpillars found  
 248 on each dowel colour (contrasting luminance green or brown). Individual larvae from  
 249 blindfolding experiments were placed in either diagonal dowel arenas (A), or horizontal  
 250 dowel arenas (B) and their resting choice was recorded after 12hrs. Sample sizes (number of  
 251 biologically independent replicates) are, for horizontal and crossed dowel experiments,  
 252 respectively: brown control (n = 34 and 60), brown blindfolded (n = 34 and 56), green control  
 253 (n = 37 and 59), green blindfolded (n = 32 and 51).

254





255

256 **Figure 4.** Visual gene expression in head and dermal tissues in larval (L) and adult (A) stages  
 257 of *B. betularia*. (A) Expression of nine visual gene isoforms relative to a control gene  
 258 (*spectrin*) in head (light blue) and body tissue (dark blue). (B) Expression of the same visual  
 259 genes in the skin (dark blue) relative to the head (light blue), calculated as [dermal  
 260 expression/(head + dermal expression)]. Bars show standard errors, grey circles individual  
 261 estimates (n = 4 biologically independent replicates for each stage). Gene names: Arr-1 =  
 262 *arrestin-1*, RDB = *retinal degeneration B*, UVA = *ultraviolet wavelength sensitive opsin*  
 263 *isoform A*, BIA = *blue wavelength sensitive opsin isoform A*, BIB = *blue wavelength sensitive*  
 264 *opsin isoform B*, MelA = *melanopsin isoform A*, MelB = *melanopsin isoform B*, LW1 = *long*  
 265 *wavelength sensitive opsin copy one*, LW2 = *long wavelength sensitive opsin copy two*.

266

## 267 **Methods**

### 268 **Dowel experiments**

269 **Rearing.** To control for any potential genetic effects among families in larval colour  
270 responses, the dowel experiments were conducted with a split family design (Supplementary  
271 Table 1). *Biston betularia* were reared from eggs and provided with goat willow (*Salix*  
272 *caprea*) *ad libitum*, with leaves on branches and in the absence of artificial dowels. At second  
273 instar, prior to any strong colour-matching response (Supplementary Figure 12), 25 larvae  
274 were transferred to each treatment arena. Treatment arenas comprised of transparent plastic  
275 boxes measuring 279 x 159 x 102 mm (length x width x depth) lined with plain blue C-fold 1-  
276 ply paper towel, each box contained 20 x 12 cm-long wooden dowels (10 x 5 mm diameter  
277 and 10 x 3 mm diameter) held in position by a chicken-wire frame painted to match the  
278 colours of the dowels used for each experiment (Supplementary Figures 13 and 14;  
279 Supplementary Table 1). Larvae were fed on *S. caprea* leaves stripped from the branches and  
280 stem *ad libitum* and boxes were washed with 10% bleach every three days to reduce infection  
281 risk. Treatment boxes were kept 20 cm apart in a Sanyo Versatile Environment Test Chamber  
282 (model MLR-351), with a 12:12 hour day: night cycle, at 24°C in the day with luminescence  
283 set at 15,000 lux, and 18°C at night for the duration of the experiment, until pupation.

284  
285 **Blindfolding.** Following a pilot study, black acrylic paint (Royal Langnickel Essentials  
286 Acrylic Paint PNTA158 BLACK) was chosen as the most suitable method to occlude light  
287 from ocelli and applied using a Royal Langnickel Sable Hair Detail Brush (Liner 5/ 0,0), with  
288 the aid of a microscope. The paint did not permit light transmission (Supplementary Figure  
289 15). Larvae were checked twice daily for signs of head capsule slippage. Individuals  
290 presenting signs of head capsule slippage were removed from the treatment arena and placed  
291 into small plastic boxes (70mm x 70mm base x 50mm high) covered in opaque white card,  
292 containing only food material (no dowel to rest on). This treatment removed the dowel  
293 stimulus whilst maintaining the normal day/night cycle, albeit at a reduced light intensity  
294 during the day period. Following complete head capsule slippage, the ocelli of these  
295 individuals were re-painted and they were placed back into their designated treatment arenas.  
296 The maximum time taken for complete head capsule slippage from beginning to end is 24  
297 hours<sup>44</sup>. In this experiment, larvae had usually completed 6-12 hours after removal from  
298 dowels. In this way, there was no point at which the ocelli in the blindfolded group could  
299 have received visual information about the dowels. Control larvae were not painted or  
300 transferred to isolation cups. Partial removal of the blindfold was observed only twice out of a  
301 total of 11,480 checks across all experiments; these individuals were removed from the  
302 experiment.

303  
304 **Quantifying the colour response.** Colour quantification and analysis was performed as  
305 described in<sup>20</sup>. The reflectance of final instar larvae (and painted dowels) was measured using  
306 an Ocean Optics USB2000 spectrophotometer, with a DH-2000 halogen deuterium light  
307 source and measured relative to a WS-1 reflectance standard. Larvae were cooled in a fridge  
308 for 2-10 minutes prior to measurement to reduce movement. A total of six measurements  
309 were taken; three from the left and three from the right lateral surfaces of each individual,  
310 always recorded from the 3rd thoracic segment, and the 2<sup>nd</sup> and 6<sup>th</sup> abdominal segments. This  
311 was to prevent overlap in measurements, and because these segments showed no prominent  
312 markings. All spectrometry data was recorded using Overture v.1.0.1.

313 We processed spectra to 1 nm intervals within the visible light spectrum (300-700) using  
314 a program in MATLAB (provided by I. C. Cuthill), and modelled vision in avian colour space  
315 using cone photon catches from the blue tit, *Cyanistes caeruleus*<sup>22</sup>. Cone stimulation values

316 were converted to Cartesian coordinates and plotted in a tetrahedral space using a custom  
 317 written MATLAB script<sup>45</sup>, such that each cone is represented by an axis. This colour space is  
 318 useful because if a colour stimulates only one cone type, then its coordinates lie at the  
 319 appropriate tip of the tetrahedron, and when all four cone types are equally stimulated the  
 320 point lies at the origin (Supplementary Figure 2).

321 To provide a simpler measure of colour, we calculated ‘greenness’ as the ratios between  
 322 the cone catch values of the medium wavelength and long wavelength photoreceptors  
 323 [MW/(MW + LW)], which represent opponent mechanisms, following Arenas & Stevens<sup>46</sup>.  
 324 For the black and white dowel experiment we did not model response to colour, only  
 325 luminance. We therefore analysed only the blue tit double dorsal cone catch, as these cones  
 326 mediate luminance vision<sup>22,47</sup>.

327 We modelled the ease with which an avian predator might discriminate between dowels  
 328 and larvae using just noticeable differences (JND); for mathematics, see Vorobyev & Osorio  
 329<sup>23</sup>. For chromatic contrasts, we used spectral sensitivities of the blue tit through relative cone  
 330 ratios of SW = 0.7111; MW = 0.9926; LW = 1.0 and UV = 0.3704<sup>48</sup>, with a Weber fraction  
 331 of 0.05 and idealised irradiance (D65). To model luminance JNDs, we used blue tit double  
 332 dorsal (DD) cones. JND <1.00 indicate that two stimuli are indiscriminable; stimuli differing  
 333 by 1–3 JND units are only discriminable under good viewing conditions; and stimuli showing  
 334 values above this should be distinguishable with increasing ease<sup>49</sup>.

335  
 336 **Microhabitat choice.** Final instar blindfolded and control larvae that had been reared on  
 337 brown and green dowels were placed into two designs of choice chamber: one with a choice  
 338 of two diagonally crossing dowels; and one with a single horizontal dowel. The rationale for  
 339 using two design was to test larvae under different starting conditions, which may produce  
 340 initial, non-selective escape responses (onto any twig when the larva is placed on a flat  
 341 surface). All microhabitat experiments were conducted using 12 individuals at a time in a  
 342 Sanyo Versatile Environment Test Chamber (model MLR-351) on light level 4 (15,000 lx).

343 The diagonal habitat choice chamber consisted of a transparent plastic cube measuring  
 344 70 x 70 x 80 mm (length x width x depth, including lid), each containing two diagonally  
 345 crossing 100 mm-long dowels painted in the contrasting colours (brown vs green) that larvae  
 346 were reared on during blindfolding experiments (Supplementary Figure 16A). Individual  
 347 larvae were placed either on the base of the diagonal dowel enclosures, equidistant from each  
 348 dowel. Prior to placement, larvae were gently poked with tweezers three times along the  
 349 dorsal surface to simulate predation, as predation risk increases likelihood of microhabitat  
 350 choice<sup>24</sup>. A sticker with larva ID was placed on the side of each chamber. Individuals were  
 351 left for 12h (7h dark: 5h light, chosen to reduce disturbance to the natural circadian rhythm of  
 352 the larvae), after which the dowel colour that each caterpillar was resting on was recorded,  
 353 followed by the larva ID. One recording was taken per larva.

354 The horizontal design was a single 200 mm dowel suspended horizontally inside a  
 355 transparent cylindrical tube measuring 210 x 60 mm length x diameter (Supplementary Figure  
 356 16B). Each half of the dowel was painted with the same pairs of contrasting colours as  
 357 described for the diagonal chamber design. Final instar larvae were draped along the centre of  
 358 the two-tone dowel not facing either colour, after simulating predation. Individuals were left  
 359 for 12h (7h dark: 5h light), as in the diagonal dowels experiments, and the dowel colour that  
 360 each caterpillar was resting on and the larva ID was recorded. If the larva position was found  
 361 to be crossing two colours (<10% of larvae), then the colour that the larva most occupied was  
 362 recorded. Two experiments were conducted per individual, where the position of the dowel  
 363 was switched, so that the brown end was facing the base of the chamber (back of the cabinet)  
 364 for one experiment, and the green end for the other (the order was random). Out of 137  
 365 individuals, 34 (~25%) alternated their colour choice between trials.

366  
367

## Identification and characterisation of visual genes

368 **Visual gene identification.** Predicted coding sequences (CDS) for *ultraviolet wavelength*  
369 *sensitive opsin*, *blue wavelength sensitive opsin*, *melanopsin*, and *long wavelength sensitive*  
370 *opsin* (copy one and two) were obtained (see Supplementary Table 2 for accession numbers)  
371 by aligning contiguous sequence reads from the *B. betularia* whole genome sequence (WGS)  
372 by tBLASTn<sup>50</sup> with homologous *Manduca sexta* sequence (Supplementary Table 2), using  
373 Geneious, v.5.5.6 (Biomatters Ltd). CDS for *retinal degeneration B* and *arrestin-1* genes  
374 were predicted using the same method, with known *Drosophila melanogaster*, *Bombyx mori*,  
375 and *Plutella xylostella* homologs (Supplementary Table 2). These CDS were completed and  
376 confirmed using a *B. betularia* whole genome BAC library (constructed by Amplicon  
377 Express) and a mixture of larval and pupal cDNA from head and dermal tissue. BAC library  
378 clones containing sequences of interest were identified from superpools with primers  
379 designed from the predicted CDS using Oligo v.6.0<sup>51</sup> (Supplementary Table 3), and Sanger  
380 sequenced (ABI 3130xl).

381  
382 **Phylogenetic analysis.** To ensure that visual genes were true homologs, wavelength-  
383 sensitive opsins – ultraviolet (UV), blue (Bl), long wavelength copy one (LW1), and long  
384 wavelength copy 2 (LW2), in addition to *arrestin-1* (Arr-1) and *retinal degeneration B* (RDB)  
385 – were aligned with corresponding genes of closely related Lepidoptera species  
386 (Supplementary Table 2), obtained using a combination of NCBI BLAST using *Biston*  
387 *betularia* sequence as the query sequence. Sequences were aligned manually in MEGA6 v.6.0  
388<sup>52</sup>, and model selection was performed on nucleotide substitutions using the Maximum  
389 Likelihood statistical method for all sites, with complete deletion of gaps/missing data.

390 Phylogenetic trees for each gene were then constructed from nucleotide substitutions  
391 using Maximum Likelihood. The model used was the best-fitting model based on AICc and  
392 BIC values. For *ultraviolet wavelength sensitive opsin* nucleotide sequences, the best model  
393 was the Tamura 3-parameter model with a discrete Gamma distribution used to measure  
394 evolutionary differences among sites. For *blue wavelength sensitive opsin* and *arrestin-1*  
395 nucleotide sequences, the Tamura 3-parameter model was also used, with a discrete Gamma  
396 distribution and 5 rate categories, assuming that a certain fraction of sites are evolutionarily  
397 invariable. For LW sequences, the General Time Reversible model was used, with a discrete  
398 Gamma distribution and 5 rate categories, assuming that a certain fraction of sites are  
399 evolutionarily invariable. For RDB sequences, the General Time Reversible model was used,  
400 with a discrete Gamma distribution. Each phylogeny was constructed using all codon  
401 positions and analysis was run using 2000 bootstrap replications. Trees were constructed in  
402 MEGA6 v.6.0 and edited in Figtree v.1.4.3<sup>53</sup>.

403  
404 **Gene expression.** Four final instar larvae and four imagines (two male, two female) were  
405 placed intact (except for gut tissue removal) in 1.5 mL eppendorfs of RNAlater®  
406 (ThermoFisher) and stored at -80°C until required. Larvae were later dissected into head,  
407 thorax, abdomen, and claspers (Supplementary Figure 17A; Supplementary Table 1) and  
408 imagines were dissected into head, thorax, abdomen, and the distal portion of the abdomen  
409 containing the genitalia (Supplementary Figure 17B; Supplementary Table 1). For all  
410 specimens, as much internal tissue as possible was removed from the body, leaving only  
411 dermal tissue intact. RNAlater was removed by pipette and all tissue was placed in a clean 1.5  
412 mL Eppendorf Safe-Lock Tube containing a 3 mm tungsten bead (Qiagen), to which 1 mL of  
413 TRIzol reagent (ThermoFisher) was added. Samples were homogenised with a Qiagen tissue  
414 lyser II, at 25 Hz for 4 minutes. Total RNA was isolated following the TRIzol manufacturer's  
415 guidelines (Invitrogen). Genomic DNA was removed from 6 µL of each RNA sample by

416 DNase I Amplification Grade (1 U/ $\mu$ L), following the manufacturer's protocol. First strand  
 417 cDNA was synthesised from 5  $\mu$ L of DNase-treated RNA using 200 U/ $\mu$ L Superscript III  
 418 Reverse Transcriptase (ThermoFisher), following a modified version of the recommended  
 419 protocol, excluding the RNaseOUT stage and using 0.5  $\mu$ L of 100  $\mu$ M Oligo (dT20) as the  
 420 anchor primer. Reactions were incubated at 50°C for 60 minutes, followed by deactivation at  
 421 70°C for 15 minutes.

422 In total we quantified nine visual genes, including splice variants: UV, Bl (splice variants  
 423 A and B), Mel (splice variants A and B), LW1, LW2, Arr-1, and RDB (Supplementary Figure  
 424 18). We were unable to amplify the alternative splice variant of UV, so only UV splice variant  
 425 A was quantified. End-point PCR reactions were performed in a Veriti (Applied Biosystems)  
 426 96-well thermal cycler with LongAmp® Hot Start Taq DNA Polymerase (New England  
 427 Biolabs) and the following cycling conditions: 2 min at 94°C, 40 cycles of [20 s at 94°C, 30 s  
 428 at 57°C, 1 min at 70°C]. PCR products were loaded onto 2% agarose gel and visualised with 3  
 429  $\mu$ L Midori Green DNA stain (Nippon Genetics) against Hyperladder 50 bp (Biolone).  
 430 Quantitative PCR was performed using KAPA SYBR fast qPCR (2x) mastermix (KAPA  
 431 Biosystems), following the manufacturer's protocol to provide a reaction mixture of 0.5  $\mu$ L  
 432 cDNA template (diluted to 55%), in a final reaction volume of 10  $\mu$ L. Each sample was  
 433 repeated in triplicate and quantified using a Roche Lightcycler 480 II and software v.1.5,  
 434 under cycling conditions: [3 min at 95°C, 45 cycles of 3 s at 95°C, 20 s at optimal annealing  
 435 temperature, 20 s at 72°C]. Melting curves were inspected to ensure single products. Relative  
 436 expression of PCR product was determined as a ratio against a reference gene, *spectrin*  
 437 (Supplementary Table 2), which shows uniform expression across cells in *B. betularia*<sup>18</sup>,  
 438 using  $[(E_{Ref})^{(Cp_{Ref})}] / [(E_{Target})^{(Cp_{Target})}]$ . Here, E = efficiency of PCR reaction  
 439 (assumed to be the idealised value of 2), Cp = crossing point, Ref= reference gene (*spectrin*),  
 440 Target= target gene (visual genes). Primers for all PCR reactions were designed using Oligo  
 441 v.6.0<sup>51</sup> (see Supplementary Table 3 for sequences).

442

#### 443 **Statistics and reproducibility**

444 All statistics were performed using R version 3.3.2<sup>54</sup>.

445

446 **Gene expression.** Comparisons between head and dermal expression of larvae and adults  
 447 were tested by fitting linear models to the  $\log_{10}$  of gene expression values. Deviance from  
 448 normality was checked with qqPlot. To examine the relative contribution of dermal  
 449 expression within life stages, we used the ratio of dermal to head expression, taking the sum  
 450 of all the dermal tissue parts (thorax, abdomen, claspers/genitalia) as a proportion of total  
 451 expression [dermal expression/(head + dermal expression)]. We modelled this ratio using beta  
 452 regression, appropriate for proportional data that follows a beta distribution. We tested stage  
 453 (adult, larvae) as predictors of relative dermal expression across genes. Model residuals were  
 454 checked for normality using qqPlot.

455

456 **Colour response.** To test whether treatment colour and blindfolding affected the colour and  
 457 luminance of larvae, as well as their ability to match dowels (JND between larvae and  
 458 dowels), we used linear models with logged JND, greenness, and luminance values. Deviance  
 459 from normality was checked with qqPlot and by plotting model residuals. Treatment (dowel  
 460 colour) and blindfolding were tested as predictors of larvae luminance (DD) for black and  
 461 white achromatic treatments, larvae greenness for brown and green chromatic treatments, and  
 462 JNDs for chromatic and achromatic treatments.

463

464 **Microhabitat choice.** To test the effects of blindfolding, treatment, and dowel position  
 465 (horizontal dowel chambers only) on dowel colour choice, we performed generalised linear

466 models (family= binomial) with larva colour, blindfolding, and dowel position as predictors  
467 of matching success (0 or 1).

468

#### 469 **Data Availability**

470 Genomic data were submitted to the GenBank database with accession numbers: MH166324-  
471 MH166333 (details in Supplementary Table 2). The source data underlying figures 2-4 are  
472 provided as a Source Data file. All other data supporting the findings of this study are  
473 available from Figshare (DOI: 10.6084/m9.figshare.8108831) and from the corresponding  
474 author upon reasonable request. A reporting summary for this article is available as a  
475 Supplementary Information file.

476

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612 A.E., H.M.R., and I.J.S. designed research; A.E., A.E.v.H., C.J.Y., and N.E. performed  
613 research; A.E. and H.M.R. analysed data; A.E., H.M.R., and I.J.S. wrote the paper.

### 614 **Competing interests**

615 The authors declare no competing interests.

616