

Continental variation in wing pigmentation in *Calopteryx* damselflies is related to the presence of heterospecifics

Wing pigmentation in *Calopteryx* damselflies, caused by the deposition of melanin, is energetically expensive to produce and enhances predation risk. However, patterns of melanisation are used in species identification, greater pigmentation is an accurate signal of male immune function in at least some species, and there may be a role for pigment in thermoregulation. This study tested two potential hypotheses to explain the presence of, and variation in, this pigmentation based on these three potential benefits using 907 male specimens of *Calopteryx maculata* collected from 49 sites (34 discrete populations) across the geographical range of the species in North America: (i) pigmentation varies with the presence of the closely related species, *Calopteryx aequabilis*, and (ii) pigment increases at higher latitudes as would be expected if it enhances thermoregulatory capacity. No gradual latitudinal pattern was observed, as might be expected if pigmentation was involved in thermoregulation. However, strong variation was observed between populations that were sympatric or allopatric with *C. aequabilis*. This variation was characterised by dark wings through allopatry in the south of the range and then a step change to much lighter wings at the southern border of sympatry. Pigmentation then increased further north into the sympatric zone, finally returning to allopatry levels at the northern range margin. These patterns are qualitatively similar to variation in pigmentation in *C. aequabilis*, meaning that the data are consistent with what would be expected from convergent character displacement. Overall, the results corroborate recent research that has suggested sexual selection as a primary driver behind the evolution of wing pigmentation in this group.

10 INTRODUCTION

11 The pigment melanin plays a key role in both colouration of the insect integument and in defence
12 against pathogens. In vertebrates, there is evidence for a cost of carotenoid – but not melanin –
13 based pigmentation ([Badyaev & Young 2004](#); [McGraw & Hill 2000](#)). However, condition
14 dependence of invertebrate melanin-based colouration indicates an energetic cost in this group
15 ([Hooper et al. 1999](#); [Talloe et al. 2004](#)), suggesting that there may be a strong effect of taxon on
16 the costs of melanin production ([Stoehr 2006](#)). In addition to biochemical synthesis the presence
17 of additional melanin in the cuticle may result in a cost due to increased predation risk due to
18 higher conspicuousness ([Svensson & Friberg 2007](#)).

19 Pigmentation has also been linked with thermoregulation. For example, *Colias* butterflies living
20 at higher altitudes possess darker wings which enable greater absorption of solar energy ([Watt](#)
21 [1968](#)) and thermoregulation has been proposed as a selective pressure which drove the early
22 evolution of insect wings ([Kingsolver & Koehl 1985](#)). The dragonfly *Aeshna caerulea* (Ström)
23 holds its wings close to the ground in such a way as to create a pocket of warm air close to its
24 body, as well as undergoing temperature-mediated physiological colour change ([Sternberg 1996](#),
25 [1997](#)). There has been some suggestion that the body colouration of coenagrionid damselflies
26 reflects the limits of their thermal tolerance ([Hilfert-Rüppell 1998](#)). Some equivocal evidence
27 exists for a negative relationship between temperature and the degree of wing pigmentation at a
28 population level in *Calopteryx* damselflies ([Outomuro & Ocharan 2011](#)), while broader
29 comparative analyses of the Calopterygidae show a strong association of pigmentation with
30 species at higher latitudes ([Svensson & Waller 2013](#)). An interspecific comparison of *C. virgo*
31 (darkly-pigmented) and *C. splendens* (less-pigmented) showed that *C. virgo* emerged earlier in
32 the year and maintained a higher body temperature at lower ambient temperatures ([Svensson &](#)
33 [Waller 2013](#)). Thus individuals inhabiting cooler regions or emerging during cooler parts of the

34 flight period may benefit from possessing melanin to assist with the absorption of solar radiation
35 to aid thermoregulation, resulting in a positive correlation between pigmentation and latitude (or
36 a negative correlation with temperature). However, field studies suggest that pigmented wings
37 are frequently cooler than the body temperature, suggesting that no heat transfer is occurring
38 ([Tsubaki et al. 2010](#)).

39 Pigmentation is also thought to play a role in species discrimination, influencing both
40 antagonistic conflict between males, and mate choice in both sexes. Two species-pairs of the
41 damselfly genus *Calopteryx*, one pair in Europe and one pair in North America, have received
42 particular attention with respect to interspecific interactions. *C. virgo* and *C. splendens* overlap
43 greatly in their distributions in Europe, with *C. virgo* extending slightly further south into the
44 Iberian peninsula and north into Scandinavia, while *C. splendens* is more common in eastern
45 Europe ([Dijkstra & Lewington 2006](#)). Reciprocal hybridisation has been documented in this pair
46 ([Tynkkynen et al. 2008](#)) and species isolation is based on both male and female mate
47 discrimination on wing pigmentation ([Svensson et al. 2007](#)). Interactions between males of the
48 two species tend to be dominated by *C. virgo*, and there is evidence that these interspecific
49 interactions may result from poor species recognition in *C. virgo* which mistake large-spotted *C.*
50 *splendens* for conspecifics ([Tynkkynen et al. 2004](#)). A decline in the size of the *C. splendens*
51 wing spot was found in association with greater *C. virgo* density ([Tynkkynen et al. 2004](#)) and
52 where the two species were sympatric ([Honkavaara et al. 2011](#)). Thus this species pair may
53 exhibit agonistic character displacement ([Grether et al. 2009](#)). This selection for smaller wing
54 spot size in territorial encounters may be traded-off against a selection for larger wing spot size
55 by female mate choice in *C. splendens* ([Siva-Jothy 1999](#)).

56 *C. maculata* (Beauvois) and *C. aequabilis* (Say) overlap to a lesser extent in North America. *C.*
57 *maculata* is found in the south east of the continent while *C. aequabilis* occupies a band
58 stretching east-west across the northern part of the continent. There is currently no evidence of
59 hybridisation between *C. maculata* and *C. aequabilis* ([Mullen & Andrés 2007](#)), despite the
60 readiness of *C. maculata* males to form tandems with *C. aequabilis* females ([Waage 1975](#)). This
61 readiness decreases as *C. aequabilis* female wing pigmentation decreases, rendering it less
62 similar to the *C. maculata* female ([Waage 1975](#)). *C. aequabilis* exhibits considerable variation in
63 the size of the pigmented area of the hind wing in males while female hind and fore wings
64 become lighter in populations that are sympatric with *C. maculata* ([Waage 1979](#)). Thus this
65 species pair may exhibit reproductive character displacement ([Waage 1979](#)).

66 Previous studies on patterns of pigmentation in *Calopteryx* damselflies have focused on its role in
67 specific processes such as antagonistic interactions, sexual signalling and immunology. Studies
68 of geographical variation in pigmentation have been conducted, but these were restricted to the
69 small proportion of the range in the southern UK for *Calopteryx splendens/virgo* ([10 populations,](#)
70 [Honkavaara et al. 2011](#)) and the northeast corner of the range of *C. maculata/aequabilis* ([26](#)
71 [populations, Waage 1979](#)). The broadest study of *Calopteryx maculata/aequabilis* was still
72 focused on the zone of sympatry between the two species, with few populations sampled far
73 beyond that zone ([15 populations, Mullen & Andrés 2007](#)). This study provides a
74 comprehensive description of variation in pigmentation which permits the comparison of
75 competing hypotheses at a continental scale using 907 *C. maculata* males from 34 sites across the
76 entire range. With these data, two potential hypotheses for geographical variation in the wing
77 pigmentation of *C. maculata* males are tested: (i) pigmentation is positively related to latitude (or
78 negatively related to temperature) as predicted by a thermoregulatory mechanism, and (ii)

79 pigmentation varies with range overlap with *C. aequabilis* as would be expected from character
80 displacement.

81 METHODS

82 A total of 907 male specimens of *C. maculata* were collected from 49 sites across the entire range
83 of *C. maculata*. Of these 49 sites, a number of samples were consolidated where sites were <4km
84 apart ([the maximum dispersal distance recorded for a *Calopteryx* species, by the congener *C.*
85 *virgo* Stettmer 1996](#)) and, hence, not independent, to give 34 samples (Figure 1, for details, see
86 Table S1). Collections took place between 13 May and 7 August 2010 and mean sample size from
87 each site was 26.7 ± 2.9 (SE). Information on the body size of these specimens can be seen in a
88 previous paper ([Hassall 2013](#)). Specimens were taken from stream sites where almost all
89 individuals are reproductively mature adults ([note, however, that very occasional younger
90 individuals occupying stream sites may have less-pigmented wings which could add noise to the
91 data, Kirkton & Schultz 2001](#)). Wings were dissected from the body as close to the thorax as
92 possible and the right hind leg was removed. The four wings were mounted on transparent,
93 adhesive tape (Scotch Matte Finish Magic Tape). Wings were scanned using the slide scanner on
94 an Epson V500 PHOTO flatbed scanner with fixed exposure at 1200dpi. The slide scanner
95 contains a light source on the opposite side of the object to the scanner and, hence, measures
96 transparency rather than reflectance.

97 Due to differences between individuals in the area of wing obscured by the thorax and the
98 accuracy of dissections, all wing images were modified to omit the arculus and all regions before
99 the first cross-veins (Figure 2). Wing pigmentation was measured as the average greyscale value
100 of the wing. Greyscale varies between 0 (black) and 255 (white), hence greater values
101 correspond to lighter, more transparent wings. This value was calculated for each pixel on the

102 image, with an average of 503,647 pixels (± 1202 SE) on the fore wing and 496,122 pixels
103 (± 1260) on the hind wing. All measurements were carried out in ImageJ ([Rasband 1997-2007](#)).
104 During measurements, any damage to wings was noted and those measurements which could not
105 be accurately quantified were excluded. This resulted in the exclusion of 140 fore wing and 116
106 hind wing pigmentation measurements (see Table S1 for sample sizes).

107 Mean monthly temperature was extracted for each of the 34 sampling sites in ArcGIS (v9.2)
108 ([ESRI 2006](#)) using the WORLDCLIM gridded "current conditions" dataset ([Haylock et al. 2008](#)).
109 The spatial resolution was 10 arc-minutes and only data for the months in which specimens were
110 collected at each site (May, June, July or August) were used. Sympatry was determined using
111 distributional records from Odonata Central ([Abbott 2007](#)) (Figure 1). A sample from Ohio
112 which is the most-southern population in the sympatric zone may not be sympatric with *C.*
113 *aequabilis* at a local level despite lying within the area bounded by populations of *C. aequabilis*.
114 However, the population is included as sympatric for the analysis. Ordinal date (days since 1st
115 Jan) was used to measure time of season, with which pigmentation has been shown to vary in
116 *Calopteryx dimidiata* (Burmeister) ([Johnson 1973](#)).

117 Variables were transformed for normality where appropriate after assessment using Shapiro-Wilk
118 tests. Generalised linear models weighted by the square root of the sample size were constructed
119 in R ([R Development Core Team 2013](#)) with fore wing and hind wing pigmentation as response
120 variables. It is unclear (i) how atmospheric temperature relates to the temperature experienced by
121 aquatic larvae and (ii) what aspect of temperature variation might influence odonate
122 pigmentation. As a result, latitude was used as a general proxy for temperature variation.
123 Latitude, ordinal date on which specimens were collected, and sympatry with *C. aequabilis* were
124 included as predictors in the GLMs. An interaction between latitude and sympatry was also

125 included to evaluate the effect of range overlap on latitudinal trends. Where specimens were
126 collected across multiple dates, the average of those dates was taken for the sample. Similarity
127 between specimens from different geographical locations may be as much a function of their
128 proximity as of any underlying patterns. This should be corrected for using spatial autoregressive
129 models, but only if the residuals of the uncorrected models are spatially autocorrelated ([Diniz-](#)
130 [Filho et al. 2003](#)). The residuals of all models were tested for spatial autocorrelation using SAM
131 v3.0 ([Rangel et al. 2010](#)).

132 Supplementary data on the degree of wing pigmentation in *C. maculata* females and *C.*
133 *aequabilis* females ([Waage 1979](#)). Mean transparency of the dark regions of female *C. maculata*
134 and *C. aequabilis* wings, and the proportion of the wing that was pigmented in *C. aequabilis*
135 males were extracted from Waage ([1979](#)), see Figure 4A,B,D. Data extraction was carried out
136 using the GetData Graph Digitizer v2.24 ([Fedorov 2008](#)). Waage used a densitometer to quantify
137 relative transparency between samples and so his absolute measurements are not directly
138 comparable to the present study. However, the data still permit a qualitatively comparison of
139 variation between the species.

140 RESULTS

141 I found substantial variation in the wing pigmentation of male *C. maculata*. The 34 sites varied
142 significantly in both fore wing and hind wing pigmentation intensity (ANOVA, fore wing:
143 $F_{33,733}=14.761$, $p<0.001$, hind wing: $F_{33,757}=13.264$, $p<0.001$; Figures 1, 3 and 4C). When
144 individual data were averaged across sites there was a highly significant correlation between hind
145 and fore wing pigmentation ($n=34$, $r=0.985$, $p<0.001$) and so only fore wing data are shown in
146 Figures 1, 3 and 4C.

147 In the allopatric zone, male *C. maculata* showed consistently dark wings (Figures 1 and 3). The
148 exception was a single population in Texas in which individuals had lighter wings. Where the
149 range of *C. aequabilis* overlaps the range of *C. maculata*, wings suddenly become lighter and
150 then darken further north in the sympatric zone. There is a significant correlation between
151 latitude and mean population transparency for populations of *C. maculata* when sympatric with
152 *C. aequabilis* (n=21, $r=-0.624$, $p=0.003$ – note that this negative correlation means increasing
153 pigmentation with latitude) but not for allopatric populations (n=13, $r=-0.197$, $p=0.519$) or
154 populations as a whole (n=34, $r=0.182$, $p=0.302$). The increase in transparency does not appear
155 to result from a continuation of any trends from the allopatric region but occurs suddenly.

156 The results from GLMs demonstrate that sympatry with *C. aequabilis* and latitude influenced
157 variation in fore and hind wing pigmentation but that the effect of latitude was dependent upon
158 sympatry (Table 1). This was confirmed by removing sympatry from the models which led to
159 latitude being non-significant and the model having negligible explanatory power (fore wings:
160 date, $F=0.039$, $p=0.845$, latitude, $F=1.526$, $p=0.226$, $R^2_{adj}=-0.041$; hind wings: date, $F=0.005$,
161 $p=0.942$, latitude, $F=1.526$, $p=0.226$, $R^2_{adj}=-0.014$). The significance of latitude in the full model
162 presumably stems from the linear decline in pigmentation with increasing latitude in the
163 sympatric zone, as noted above, which only becomes statistically relevant when sympatry is
164 taken into account. Residuals of these models were not spatially autocorrelated as revealed by
165 SAM and so no control for spatial autocorrelation was necessary.

166 The relative wing transparency between geographical locations that has previously been reported
167 for *C. maculata* females, *C. aequabilis* females, and *C. aequabilis* males ([Waage 1979](#)) appears to
168 follow the same geographic pattern as that found in the present study for *C. maculata* males
169 (Figure 4). In allopatry, wings tend to be more pigmented (lower transparency). At the southern

170 margin of the sympatric zone wings tend to be less pigmented (higher transparency) and
171 pigmentation increases towards the northern margin of the sympatric zone in all four groups.
172 Note that only qualitative comparisons are possible between the groups due to variation in the
173 methods used to obtain the data, but that these comparisons suggest a consistent pattern. All data
174 for the *C. maculata* males used in this study are available as electronic supplementary
175 information (Table S1 for site summaries, Table S2 for individual measurements).

176 **DISCUSSION**

177 I demonstrate that, contrary to previous assumptions, males of *C. maculata* vary greatly in their
178 pigmentation and this variation coincides with the presence of a congener, *C. aequabilis*.
179 Qualitatively similar patterns of pigmentation have been observed in females of the same species
180 and in females of the heterospecific *C. aequabilis*. Across the entire range, no gradual latitudinal
181 patterns are present in wing pigmentation which would be expected if a covariate of latitude (be it
182 temperature or another variable) was influencing pigmentation. However, within the zone of
183 sympatry with *C. aequabilis*, wings become progressively darker as latitude increases. This
184 finding not only provides another important component of a well-studied evolutionary system
185 (the *C. maculata/aequabilis* species pair) but also constitutes a thorough test of intraspecific
186 variation in pigmentation with changing latitude at a continental scale.

187 It has previously been stated that *Calopteryx maculata* "...has dark wings and shows little
188 geographic variation in the wing pattern" ([Mullen & Andrés 2007](#)) and that a "...sympatric shift in
189 wing pigmentation was exhibited by females of [*C. maculata* and *C. aequabilis*] but only by
190 males in *C. aequabilis*" ([Honkavaara et al. 2011](#)). This originated in a misinterpretation of the
191 work of Waage ([1979](#)) who focused only female pigmentation in *C. maculata* and did not
192 measure that in males, stating that "extent of the dark area in *C. maculata* males was not

193 measured as their wings are 95-100% pigmented" and "...wings of *C. maculata* are entirely dark
194 and vary only in size... among the populations sampled" ([Waage 1979, p106 and 108,](#)
195 [respectively](#)) but did not measure the intensity of pigmentation. However, from a broader
196 comparison of sites, the degree of variation is marked (see the comparison of wing pigmentation
197 levels in Figure 3) and varies depending on sympatry or allopatry with *C. aequabilis*. No
198 evidence exists for latitudinal patterns in pigmentation apart from a latitudinal decline in
199 pigmentation through the sympatric zone.

200 From a previous study it has been shown that *C. aequabilis* reduces its pigmentation
201 progressively in populations that are located deeper inside the range of *C. maculata* ([wing](#)
202 [pigmentation is significantly correlated with latitude, see Table 1 in Waage 1979](#)) and it has been
203 assumed that this was to enhance species recognition where dark-winged *C. maculata* were
204 present. However, it appears that the northward increase in pigmentation within the sympatric
205 zone is also present in *C. maculata*, with qualitatively similar trends in increasing pigmentation
206 further north in both sexes of both *C. maculata* and *C. aequabilis*. This pattern is consistent with
207 the existence of convergent character displacement ([Grant 1972](#)) with male wing pigmentation
208 changing to match variations in female *C. aequabilis* pigmentation intensity and male *C.*
209 *aequabilis* wing spot size (see Figure 4 for a comparison with Waage's ([1979](#)) data). However, a
210 notable difference between the two trends is that the decline in pigmentation in *C. maculata*
211 males is found in both wings, while this is only true for the hind wing in *C. aequabilis* ([Waage](#)
212 [1979](#)). This difference may relate to variations between species in the "cross-displays" performed
213 by males to court females, which result in the more prominent display of hind wings in *C.*
214 *aequabilis* ([Waage 1973](#)).

215 It is important to note that the previous demonstration of character displacement involved
216 behavioural differences between species. The key observation was that *C. maculata* males
217 exhibit a greater ability to discriminate between conspecific and heterospecific females when in
218 sympatry than when in allopatry ([Waage 1975](#)). This can be taken as evidence of a cost of
219 confused mating by *C. maculata* which leads to a selective pressure acting on the reinforcement
220 of species identification. The present study provides a description of apparent convergence in a
221 character that is key to discriminating between species, which appears to render both sexes of
222 both species less discriminable. This observation runs counter to what would be expected given
223 this selection pressure. Furthermore, the pattern cannot be explained by correlations with latitude
224 or temperature, which might be predicted based on the fact that melanin can play a role in
225 thermoregulation at higher latitudes (leading to a positive correlation between latitude and
226 pigmentation), as the levels of pigmentation return to allopatric levels (dark pigmentation) at the
227 northern-most sites (Figures 1, 3 and 4).

228 The pattern of results effectively rule out temperature and latitude as causal factors in the broader
229 trend, although these have been suggested to influence geographic variation in odonate colour
230 ([Hilfert-Rüppell 1998](#)) and are strongly associated with the occurrence of wing pigmentation
231 across the Calopterygidae as a group ([Svensson & Waller 2013](#)). Another mechanism that has
232 been implicated in driving changes in odonate colour is the avoidance of harassment by
233 conspecifics ([Van Gossum et al. 2001](#)) or heterospecifics ([Tynkkynen et al. 2004](#)). Evidence for
234 this playing a role in calopterygid damselflies is present in the diversity of wing morphs exhibited
235 by European species. Males of *Calopteryx exul* (Selys), inhabiting north Africa, have no
236 pigmentation on their wings and so resemble females. *Calopteryx virgo virgo* has no
237 pigmentation at the base or the tip of the wing and resembles *Calopteryx splendens* found in
238 eastern Europe. *Calopteryx splendens balcanica* females have pigmented wings which resemble

239 the male (androchrome). Wing pigmentation polymorphism is also present in males of the
240 calopterygid damselfly *Mnais costalis* (Selys), where clear-winged males exhibit a "sneaker"
241 mating strategy while orange-winged males are territorial ([Plaistow & Tsubaki 2000](#)). Therefore,
242 there is evidence from the Calopterygidae for male-mimicking females, female-mimicking males
243 and, possibly, heterospecific mimicry. Establishing which, if any, of these explanations best fit
244 the *C. maculata*/*C. aequabilis* system would require further experiments along the same lines as
245 those conducted previously ([Tynkkynen et al. 2004](#); [Waage 1979](#)).

246 A conclusive demonstration of convergent character displacement requires knowledge of both the
247 selection pressures and the evolutionary processes that are causing the convergence of traits.
248 Clearly a case for convergent displacement cannot be made purely on the basis of the
249 observations described here, although the patterns resemble those that would be expected from
250 such a process. Very few examples of convergent character displacement have been documented,
251 despite being theoretically plausible ([Abrams 1996](#)). Among these examples, Leary ([2001](#)) found
252 that the nature of the calls given by male toads (*Bufo* sp) to prevent prolonged amplexus by
253 conspecific and heterospecific males converged when in sympatry. This convergence may
254 facilitate interspecific communication to reduce wasteful energy expenditure and exposure to
255 predation. Following the invasion of American mink (*Mustela vison* (Schreber)) to Belarus, the
256 larger invading species decreased in size while the native European mink (*M. lutreola* (L))
257 increased in size ([Sidorovich et al. 1999](#)). On the other hand, many examples of divergent
258 character displacement have been documented ([Dayan & Simberloff 2005](#)).

259 The importance of thermoregulatory behaviour has been noted in a number of Odonata ([for a](#)
260 [review see Hassall & Thompson 2008](#)), and a number of adaptations are present. There has been
261 a suggestion that variations in body pigmentation with latitude in *Orthetrum cancellatum* (L)

262 contribute to enhanced absorption of solar energy ([Hilfert-Rüppell 1998](#)). Similarly, a melanic
263 form of *Sympetrum striolatum* (Charpentier) found only at the northern range margin where such
264 pigmentation could assist in thermoregulation was previously described as a separate species,
265 *Sympetrum nigrescens*, before molecular studies demonstrated that the two were synonymous
266 ([Pilgrim & Von Dohlen 2007](#)). The only other study of range-wide variation in wing
267 pigmentation in a damselfly (*Calopteryx splendens*) also demonstrated no consistent patterns with
268 latitude ([Sadeghi et al. 2009](#)), and this has been attributed to different wing morphs being
269 genetically distinct gene pools with intermediates resulting from hybridisation ([Sadeghi et al.](#)
270 [2010](#)). This study similarly shows no unequivocal evidence of a latitudinal cline, despite the
271 wide climatic range over which the species occurs. However, the increased pigmentation at
272 higher latitudes within the sympatric zone is consistent with a potential role in thermoregulation
273 within this region.

274 It has been demonstrated that the extent of pigmentation in male wings is an honest indicator of
275 immune function in a number of calopterygid damselflies ([Hetaerina americana](#), [Contreras-](#)
276 [Garduño et al. 2006](#); [Calopteryx splendens](#), [Rantala et al. 2000](#); [Calopteryx splendens](#)
277 [xanthostoma](#), [Siva-Jothy 2000](#)). As such, variation in pigmentation may be under indirect
278 selective pressures acting on immune function. Parasites increase in diversity and abundance
279 closer to the equator ([Poulin & Morand 2000](#)) and there is some evidence that virulence follows a
280 similar pattern ([Møller et al. 2009](#)). This selection may result in greater immune function at
281 lower latitudes (e.g. [Ardia 2007](#)). Thus we may expect to observe greater pigmentation at lower
282 latitudes where immune challenge is at its highest. However, damselflies emerging later in the
283 season exhibit greater immune responses ([Yourth et al. 2002](#)) which are associated with higher
284 temperatures ([Robb & Forbes 2005](#)). The melanotic encapsulation involved in the immune
285 response follows the same biochemical pathways as those involved in melanisation of the cuticle

286 ([Marmaras et al. 1996](#)). Thus, a negative correlation between pigmentation and latitude (or a
287 positive correlation with temperature) is predicted by both parasite-mediated selection and plastic
288 responses to temperature, but there is no evidence of this pattern in the present study.

289 The results presented here fill a gap in the knowledge of geographical patterns of wing
290 pigmentation in a well-studied two-species system. In so doing, two key results present
291 themselves. First, there is geographical pattern in wing pigmentation that would suggest
292 variation in thermoregulatory potential across the range. Second, there is strong evidence for
293 variation in wing pigmentation in male *C. maculata* in relation to its co-occurrence with a
294 congener, *C. aequabilis*. This pattern of variation is qualitatively similar to patterns seen in *C.*
295 *maculata* females, *C. aequabilis* females, and *C. aequabilis* males. This apparent convergence of
296 a character that is important in species identification may contribute to the divergent character
297 displacement observed in species recognition behaviour in the same system ([Waage 1975](#)).

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310 REFERENCES

- 311 Abbott JC. 2007. *OdonataCentral: An online resource for the distribution and identification of*
312 *Odonata*. . available at <http://www.odonatacentral.org>. (Accessed: April 01, 2010): Texas Natural
313 Science Center, The University of Texas at Austin.
- 314 Abrams PA. 1996. Evolution and the consequences of species introductions and deletions.
315 *Ecology* 77:1321-1328.
- 316 Ardia DR. 2007. The ability to mount multiple immune responses simultaneously varies across
317 the range of the tree swallow. *Ecography* 30:23-30.
- 318 Badyaev AV, and Young RL. 2004. Complexity and integration in sexual ornamentation: an
319 example with carotenoid and melanin plumage pigmentation. *Journal of Evolutionary Biology*
320 17:1317-1327.
- 321 Contreras-Garduño J, Canales-Lazcano J, and Córdoba-Aguilar A. 2006. Wing pigmentation,
322 immune ability, fat reserves and territorial status in males of the rubyspot damselfly, *Hetaerina*
323 *americana*. *Journal of Ethology* 24:165-173.
- 324 Dayan T, and Simberloff D. 2005. Ecological and community-wide character displacement: the
325 next generation. *Ecology Letters* 8:875-894.
- 326 Dijkstra K-DB, and Lewington R. 2006. *Field Guide to the Dragonflies of Britain and Europe*.
327 Gillingham, UK: British Wildlife Publishing.
- 328 Diniz-Filho JAF, Bini LM, and Hawkins BA. 2003. Spatial autocorrelation and red herrings in
329 geographical ecology. *Global Ecology and Biogeography* 12:53-64.
- 330 ESRI. 2006. *ArcGIS v.9.2*. Redlands: Environmental Systems Research Institute, Inc.
- 331 Fedorov S. 2008. *GetData Graph Digitizer v2.24*: <http://getdata-graph-digitizer.com/>.

- 332 Grant PR. 1972. Convergent and divergent character displacement. *Biological Journal of the*
333 *Linnean Society* 4:39-68.
- 334 Grether GF, Losin N, Anderson CN, and Okamoto K. 2009. The role of interspecific interference
335 competition in character displacement and the evolution of competitor recognition. *Biological*
336 *Reviews* 84:617-635.
- 337 Hassall C. 2013. Time stress and temperature explain continental variation in damselfly body
338 size. *Ecography* 36:894-903.
- 339 Hassall C, and Thompson DJ. 2008. The impacts of environmental warming on Odonata: a
340 review. *International Journal of Odonatology* 11:131-153.
- 341 Haylock MR, Hofstra N, Klein Tank AMG, Klok EJ, Jones PD, and New M. 2008. A European
342 daily high-resolution gridded dataset of surface temperature and precipitation. *Journal of*
343 *Geophysical Research Series D, Atmospheres* 113.
- 344 Hilfert-Rüppell D. 1998. Temperature dependence of flight activity of Odonata by ponds.
345 *Odonatologica* 27:45-59.
- 346 Honkavaara J, Dunn DW, Ilvonen S, and Suhonen J. 2011. Sympatric shift in a male sexual
347 ornament in the damselfly *Calopteryx splendens*. *Journal of Evolutionary Biology* 24:139-145.
- 348 Hooper RE, Tsubaki Y, and Siva-Jothy MT. 1999. Expression of a costly, plastic secondary sexual
349 trait is correlated with age and condition in a damselfly with two male morphs. *Physiological*
350 *Entomology* 24:364-369.
- 351 Johnson C. 1973. Variability, distribution and taxonomy of *Calopteryx dimidiata*. *Florida*
352 *Entomologist* 56:207-222.
- 353 Kingsolver JG, and Koehl MAR. 1985. Aerodynamics, thermoregulation, and the evolution of
354 insect wings: differential scaling and evolutionary change. *Evolution* 39:488-504.
- 355 Kirkton SD, and Schultz TD. 2001. Age-specific behavior and habitat selection of adult male
356 damselflies, *Calopteryx maculata*. *Journal of Insect Behavior* 14:545-556.

- 357 Leary CJ. 2001. Evidence of convergent character displacement in release vocalizations of *Bufo*
358 *fowleri* and *Bufo terrestris* (Anura; Bufonidae). *Animal Behaviour* 61:431-438.
- 359 Marmaras VJ, Charalambidis ND, and Zervas CG. 1996. Immune response in insects: The role of
360 phenoloxidase in defense reactions in relation to melanization and sclerotization. *Archives of*
361 *Insect Biochemistry and Physiology* 31:119-133.
- 362 McGraw KJ, and Hill GE. 2000. Differential effects of endoparasitism on the expression of
363 carotenoid- and melanin-based ornamental coloration. *Proceedings of the Royal Society: Series B*
364 *(Biological Sciences)* 267:1525-1531.
- 365 Møller AP, Arriero E, Lobato E, and Merino S. 2009. A meta-analysis of parasite virulence in
366 nestling birds. *Biological Reviews* 84:567-588.
- 367 Mullen SP, and Andrés JA. 2007. Rapid evolution of sexual signals in sympatric *Calopteryx*
368 damselflies: reinforcement or 'noisy-neighbour' ecological character displacement? *Journal of*
369 *Evolutionary Biology* 20:1637-1648.
- 370 Outomuro D, and Ocharan FJ. 2011. Wing pigmentation in *Calopteryx* damselflies: a role in
371 thermoregulation? *Biological Journal of the Linnean Society* 103:36-44.
- 372 Pilgrim EM, and Von Dohlen CD. 2007. Molecular and morphological study of species-level
373 questions within the dragonfly genus *Sympetrum* (Odonata: Libellulidae). *Annals of the*
374 *Entomological Society of America* 100:688-702.
- 375 Plaistow SJ, and Tsubaki Y. 2000. A selective trade-off for territoriality and non-territoriality in
376 the polymorphic damselfly *Mnais costalis*. *Proceedings of the Royal Society: Series B*
377 *(Biological Sciences)* 22:969-975.
- 378 Poulin R, and Morand S. 2000. The diversity of parasites. *The Quarterly Review of Biology*
379 75:277-293.
- 380 R Development Core Team. 2013. *R: A language and environment for statistical computing*.
381 Vienna, Austria: R Foundation for Statistical Computing.

- 382 Rangel TFLVB, Diniz-Filho JAF, and Bini LM. 2010. SAM: a comprehensive application for
383 Spatial Analysis in Macroecology. *Ecography* 33:46-50.
- 384 Rantala MJ, Koskimaki J, Taskinen J, Tynkkynen K, and Suhonen J. 2000. Immunocompetence,
385 developmental stability and wingspot size in the damselfly *Calopteryx splendens* L. *Proceedings*
386 *of the Royal Society: Series B (Biological Sciences)* 267:2453-2457.
- 387 Rasband WS. (1997-2007) *ImageJ*. <<http://rsb.info.nih.gov/ij/>>.
- 388 Robb T, and Forbes MR. 2005. On understanding seasonal increases in damselfly defence and
389 resistance against ectoparasitic mites. *Ecological Entomology* 30:334-341.
- 390 Sadeghi S, Adriaens D, and Dumont HJ. 2009. Geometric morphometric analysis of wing shape
391 variation in ten European populations of *Calopteryx splendens* (Harris, 1782) (Zygoptera:
392 Odonata). *Odonatologica* 38:343-360.
- 393 Sadeghi S, Kyndt T, and Dumont HJ. 2010. Genetic diversity, population structure and taxonomy
394 of *Calopteryx splendens* (Odonata: Calopterygidae): An AFLP analysis. *European Journal of*
395 *Entomology* 107:137-146.
- 396 Sidorovich V, Kruuk H, and Macdonald DW. 1999. Body size, and interactions between
397 European and American mink (*Mustela lutreola* and *M. vison*) in Eastern Europe. *Journal of*
398 *Zoology* 248:521-527.
- 399 Siva-Jothy MT. 1999. Male wing pigmentation may affect reproductive success via female choice
400 in a calopterygid damselfly (Zygoptera). *Behaviour* 136:1365-1377.
- 401 Siva-Jothy MT. 2000. A mechanistic link between parasite resistance and expression of a sexually
402 selected trait in a damselfly. *Proceedings of the Royal Society: Series B (Biological Sciences)*
403 267:2523-2527.
- 404 Sternberg K. 1996. Colours, colour change, colour patterns and "cuticular windows" at light traps
405 - their thermoregulatory and ecological significance in some *Aeshna* species. *Zoologischer*
406 *Anzeiger* 235:77-88.

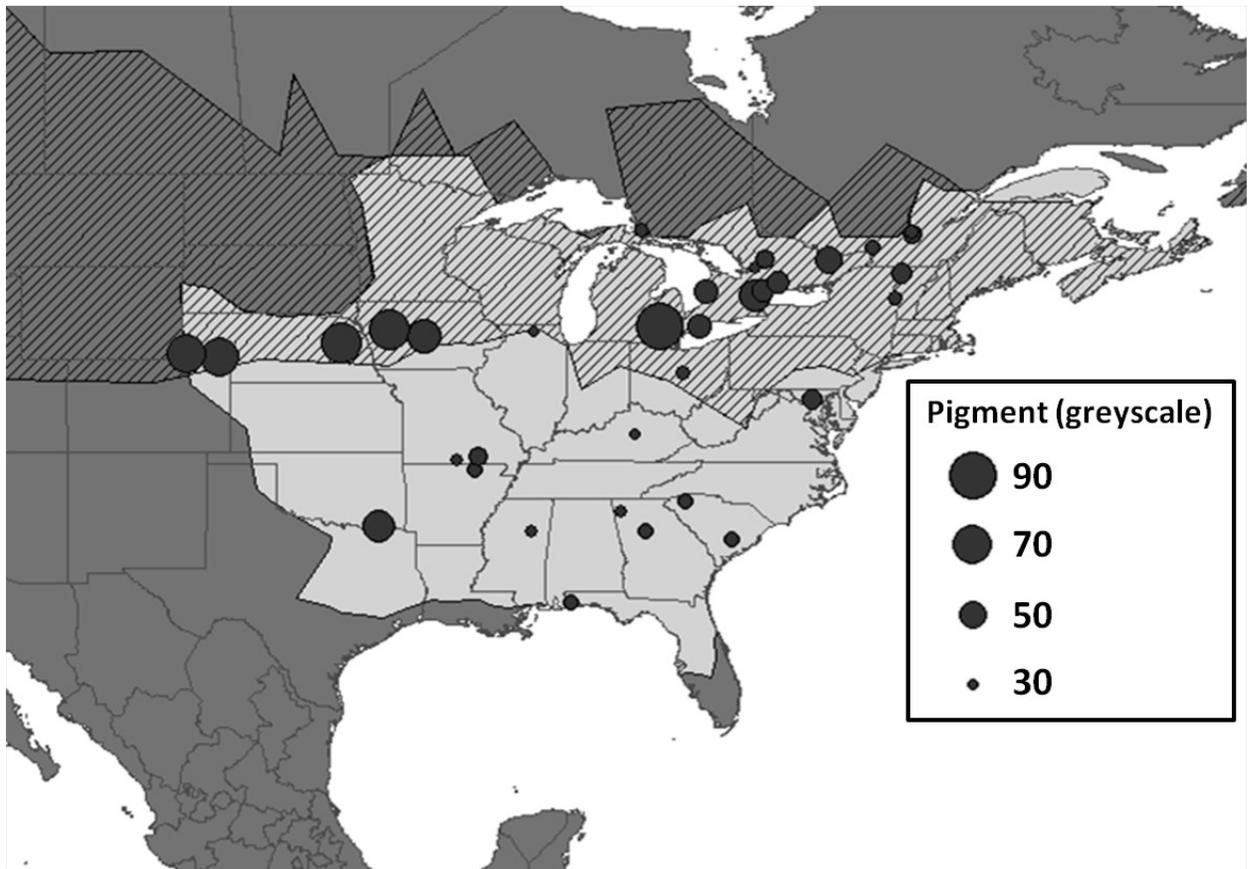
- 407 Sternberg K. 1997. Adaptation of *Aeshna caerulea* (Strom) to the severe climate of its
408 environment (Anisoptera: Aeshnidae). *Odonatologica* 26:439-449.
- 409 Stettmer C. 1996. Colonisation and dispersal patterns of banded (*Calopteryx splendens*) and
410 beautiful demoiselles (*C. virgo*) (Odonata: Calopterygidae) in south-east German streams.
411 *European Journal of Entomology* 93:579-593.
- 412 Stoehr AM. 2006. Costly melanin ornaments: the importance of taxon? *Functional Ecology*
413 20:276-281.
- 414 Svensson EI, and Friberg M. 2007. Selective predation on wing morphology in sympatric
415 damselflies. *The American Naturalist* 170:101-112.
- 416 Svensson EI, Karlsson K, Friberg M, and Eroukhmanoff F. 2007. Gender differences in species
417 recognition and the evolution of asymmetric sexual isolation. *Current Biology* 17:1943-1947.
- 418 Svensson EI, and Waller JT. 2013. Ecology and Sexual Selection: Evolution of Wing
419 Pigmentation in Calopterygid Damselflies in Relation to Latitude, Sexual Dimorphism, and
420 Speciation. *The American Naturalist* 182:E174-E195.
- 421 Talloen W, Van Dyck H, and Lens L. 2004. The cost of melanization: butterfly wing coloration
422 under environmental stress. *Evolution* 58:360-366.
- 423 Tsubaki Y, Samejima Y, and Siva-Jothy M. 2010. Damselfly females prefer hot males: higher
424 courtship success in males in sunspots. *Behavioral Ecology and Sociobiology* 64:1547-1554.
- 425 Tynkkynen K, Grapputo A, Kotiaho JS, Rantala MJ, Väänänen S, and Suhonen J. 2008.
426 Hybridization in *Calopteryx* damselflies: the role of males. *Animal Behaviour* 75:1431-1439.
- 427 Tynkkynen K, Rantala MJ, and Suhonen J. 2004. Interspecific aggression and character
428 displacement in the damselfly *Calopteryx splendens*. *Journal of Evolutionary Biology* 17:759-
429 767.

- 430 Van Gossum H, Stoks R, and De Bruyn L. 2001. Frequency-dependent male mate harassment and
431 intra-specific variation in its avoidance by females of the damselfly *Ischnura elegans*. *Behavioral*
432 *Ecology and Sociobiology* 51:69-75.
- 433 Waage JK. 1973. Reproductive behavior and its relation to territoriality in *Calopteryx maculata*
434 (Beauvois) (Odonata: Calopterygidae). *Behaviour* 47:240-256.
- 435 Waage JK. 1975. Reproductive isolation and the potential for character displacement in the
436 damselflies, *Calopteryx maculata* and *C. aequabilis* (Odonata: Calopterygidae). *Systematic*
437 *Zoology* 24:24-36.
- 438 Waage JK. 1979. Reproductive character displacement in *Calopteryx* (Odonata, Calopterygidae).
439 *Evolution* 33:104-116.
- 440 Watt WB. 1968. Adaptive significance of pigment polymorphisms in *Colias* butterflies. I.
441 variation of melanin pigment in relation to thermoregulation. *Evolution* 22:437-458.
- 442 Yourth CP, Forbes MR, and Smith BP. 2002. Immune expression in a damselfly is related to time
443 of season, not to fluctuating asymmetry or host size. *Ecological Entomology* 27:123-128.

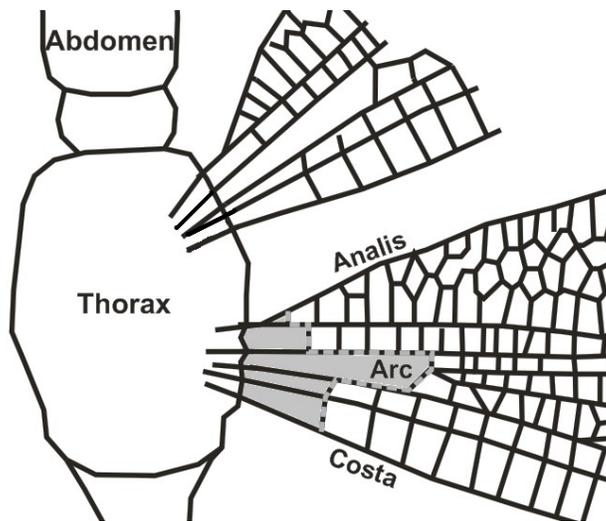
444 **TABLES**

445 Table 1 – Parameter estimates from multiple regression models (weighted by square root of
 446 sample size) describing variation in wing pigmentation in *Calopteryx maculata* males.

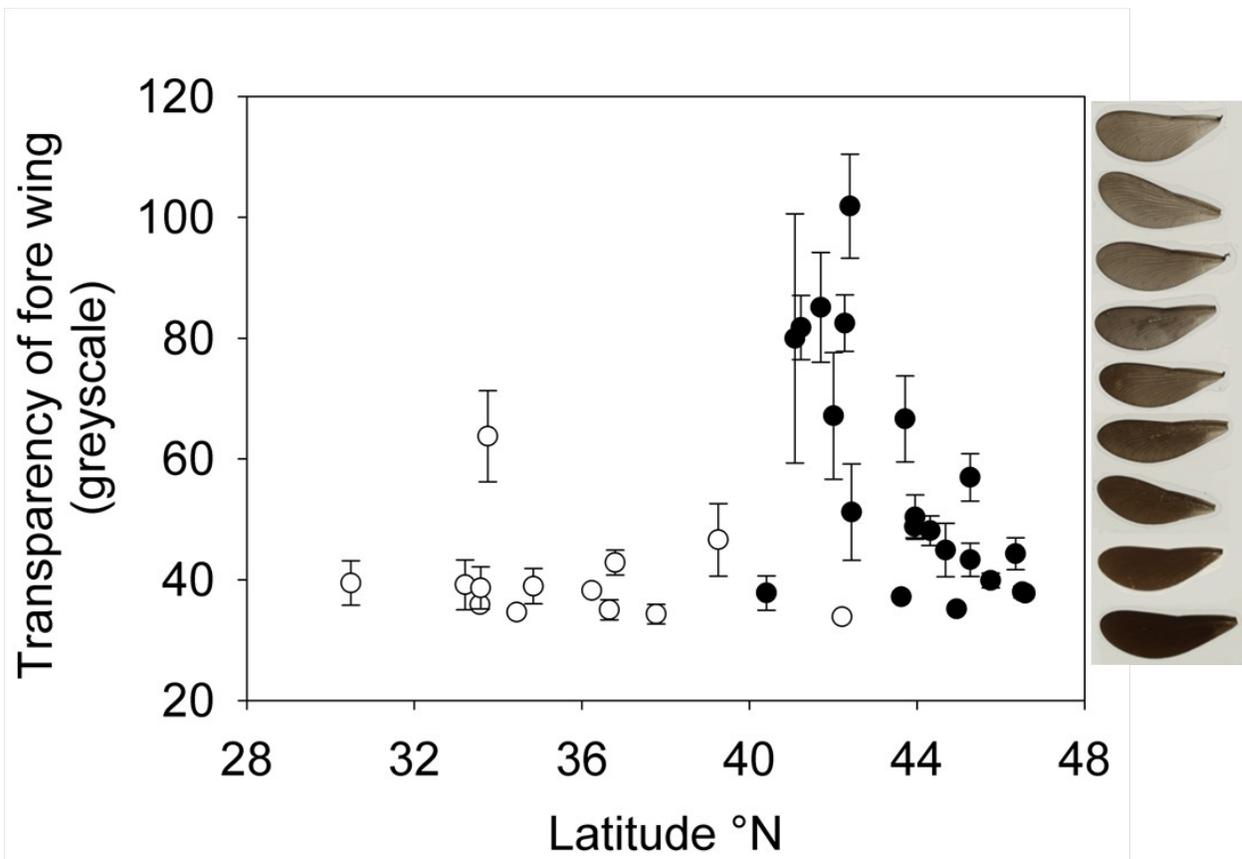
	Fore wing pigmentation		Hind wing pigmentation	
	F	p	F	p
Ordinal date	0.067	0.799	0.009	0.924
Latitude	1.104	0.302	2.613	0.117
Sympatry	19.423	<0.001	19.748	<0.001
Latitude*sympatry	4.203	0.049	4.338	0.046
R^2_{adj}		0.387		0.408



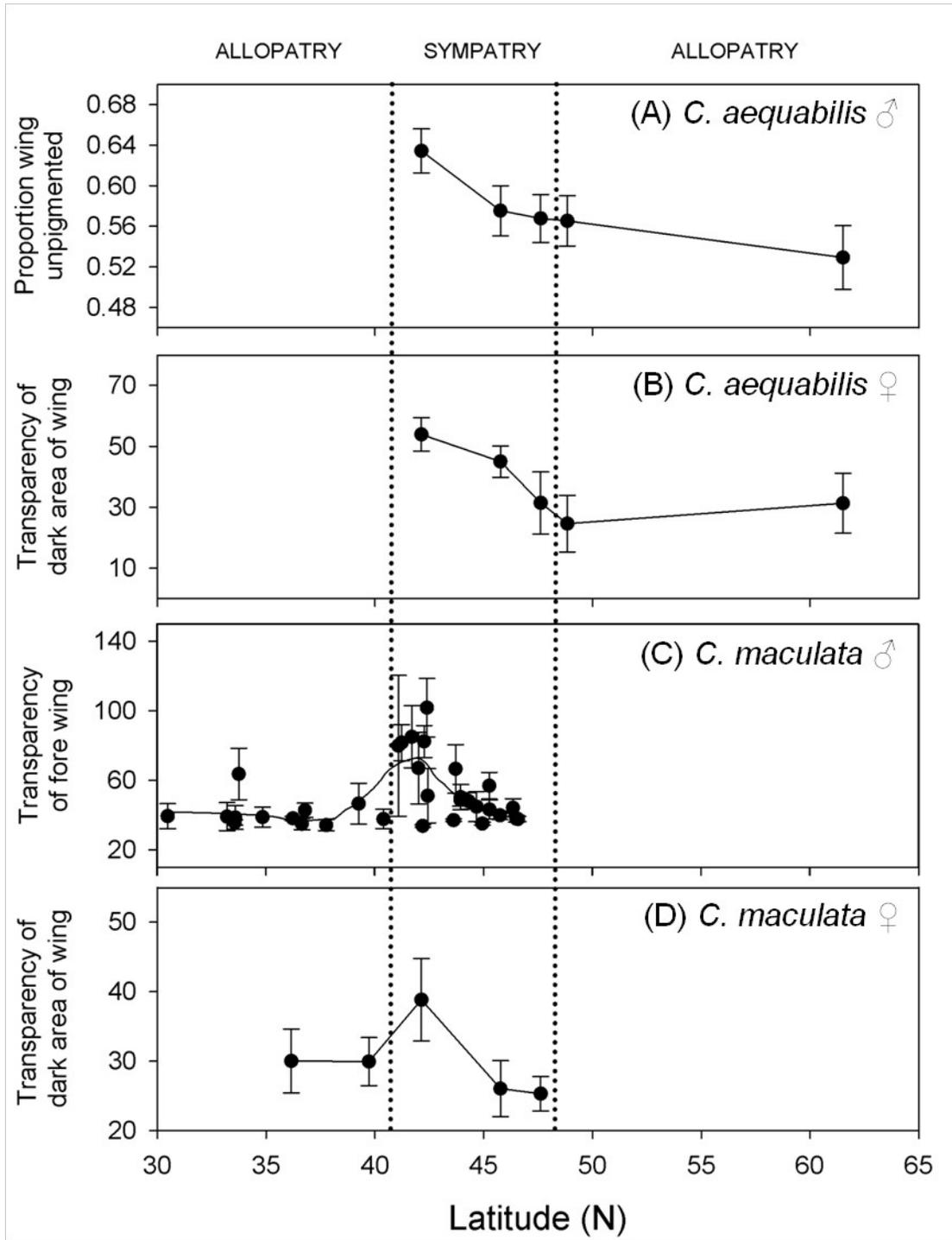
448 Figure 1 – Geographical distribution of *Calopteryx maculata* (light grey) and *Calopteryx*
 449 *aequabilis* (hashed region). Points mark the location of sampling sites for *C. maculata* and the
 450 size of the point is proportional to the greyscale value of the wing pigmentation intensity (larger
 451 symbol = lighter wings). See Figure 3 for illustrations of wing pigmentation variation.



452 Figure 2 – Diagram of *C. maculata* wing with the region omitted from the analysis (shaded in
453 grey). Arc=arculus.



454 Figure 3 – Latitudinal patterns of pigmentation in *C. maculata* males where populations are
455 allopatric (open circles) and sympatric (closed circles) with *C. aequabilis*. Error bars are 1SE.
456 Displayed on the right are examples of wings with pigmentation intensities corresponding to their
457 locations on the y-axis. See Table S1 for sample sizes and means, and Table S2 for raw data.



458 Figure 4 – Comparison of variation wing pigmentation between (A) *C. aequabilis* males, (B) *C.*
459 *aequabilis* females, (C) *C. maculata* males (present study), and (D) *C. maculata* females ([1979](#)).
460 All y-axes represent qualitatively the same trait: quantity of pigment in the wing, with low values
461 corresponding to darker wings and higher values to greater transparency. However, the measures
462 vary in the way in which they were collected: for *C. aequabilis* males it is the proportion of the
463 wing length that is pigmented, for *C. aequabilis* and *C. maculata* females it is the transparency of
464 the dark area of the wing (using a densitometer), and for *C. maculata* males it is the mean
465 greyscale value of a wing scan (see methods above). Line fitted through *C. maculata* male data is
466 a loess curve and other lines simply connect data points. All error bars are 95% confidence
467 intervals. Note in all cases that pigmentation is highest at the southern margin of the zone of
468 sympatry with subsequent declines further north.