

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 sympathy. 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.

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10 **ABSTRACT**

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

30 **Keywords:** dragonfly; Odonata; melanin; North America; species recognition; allopatry; wing;
31 sympathy; thermoregulation

32 INTRODUCTION

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

41 Pigmentation has also been linked with thermoregulation. For example, *Colias* butterflies living
42 at higher altitudes possess darker wings which enable greater absorption of solar energy ([Watt
43 1968](#)) and thermoregulation has been proposed as a selective pressure which drove the early
44 evolution of insect wings ([Kingsolver & Koehl 1985](#)). The dragonfly *Aeshna caerulea* (Ström)
45 holds its wings close to the ground in such a way as to create a pocket of warm air close to its
46 body, as well as undergoing temperature-mediated physiological colour change ([Sternberg 1996,
47 1997](#)). There has been some suggestion that the body colouration of coenagrionid damselflies
48 reflects the limits of their thermal tolerance ([Hilfert-Rüppell 1998](#)). Some equivocal evidence
49 exists for a negative relationship between temperature and the degree of wing pigmentation at a
50 population level in *Calopteryx* damselflies ([Outomuro & Ocharan 2011](#)), while broader
51 comparative analyses of the Calopterygidae show a strong association of pigmentation with
52 species at higher latitudes ([Svensson & Waller 2013](#)). An interspecific comparison of *C. virgo*
53 (darkly-pigmented) and *C. splendens* (less-pigmented) showed that *C. virgo* emerged earlier in
54 the year and maintained a higher body temperature at lower ambient temperatures ([Svensson &
55 Waller 2013](#)). Thus individuals inhabiting cooler regions or emerging during cooler parts of the
56 flight period may benefit from possessing melanin to assist with the absorption of solar radiation
57 to aid thermoregulation, resulting in a positive correlation between pigmentation and latitude (or
58 a negative correlation with temperature). However, field studies suggest that pigmented wings
59 are frequently cooler than the body temperature, suggesting that no heat transfer is occurring
60 ([Tsubaki et al. 2010](#)).

61 Pigmentation is also thought to play a role in species discrimination, influencing both
62 antagonistic conflict between males, and mate choice in both sexes. Two species-pairs of the

63 damselfly genus *Calopteryx*, one pair in Europe and one pair in North America, have received
64 particular attention with respect to interspecific interactions. *C. virgo* and *C. splendens* overlap
65 greatly in their distributions in Europe, with *C. virgo* extending slightly further south into the
66 Iberian peninsula and north into Scandinavia, while *C. splendens* is more common in eastern
67 Europe ([Dijkstra & Lewington 2006](#)). Reciprocal hybridisation has been documented in this pair
68 ([Tynkkynen et al. 2008](#)) and species isolation is based on both male and female mate
69 discrimination on wing pigmentation ([Svensson et al. 2007](#)). Interactions between males of the
70 two species tend to be dominated by *C. virgo*, and there is evidence that these interspecific
71 interactions may result from poor species recognition in *C. virgo* which mistake large-spotted *C.*
72 *splendens* for conspecifics ([Tynkkynen et al. 2004](#)). A decline in the size of the *C. splendens*
73 wing spot was found in association with greater *C. virgo* density ([Tynkkynen et al. 2004](#)) and
74 where the two species were sympatric ([Honkavaara et al. 2011](#)). Thus this species pair may
75 exhibit agonistic character displacement ([Grether et al. 2009](#)). This selection for smaller wing
76 spot size in territorial encounters may be traded-off against a selection for larger wing spot size
77 by female mate choice in *C. splendens* ([Siva-Jothy 1999](#)).

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

88 Previous studies on patterns of pigmentation in *Calopteryx* damselflies have focused on its role in
89 specific processes such as antagonistic interactions, sexual signalling and immunology. Studies
90 of geographical variation in pigmentation have been conducted, but these were restricted to the
91 small proportion of the range in the southern UK for *Calopteryx splendens/virgo* ([10 populations,](#)
92 [Honkavaara et al. 2011](#)) and the northeast corner of the range of *C. maculata/aequabilis* ([26](#)
93 [populations, Waage 1979](#)). The broadest study of *Calopteryx maculata/aequabilis* was still

94 focused on the zone of sympatry between the two species, with few populations sampled far
95 beyond that zone ([15 populations, Mullen & Andrés 2007](#)). This study provides a
96 comprehensive description of variation in pigmentation which permits the comparison of
97 competing hypotheses at a continental scale using 907 *C. maculata* males from 34 sites across the
98 entire range. With these data, two potential hypotheses for geographical variation in the wing
99 pigmentation of *C. maculata* males are tested: (i) pigmentation is positively related to latitude (or
100 negatively related to temperature) as predicted by a thermoregulatory mechanism, and (ii)
101 pigmentation varies with range overlap with *C. aequabilis* as would be expected from character
102 displacement. Under the first hypothesis, it would be expected that wing pigmentation would
103 increase with latitude such that individuals experiencing colder average temperatures exhibited
104 more heat-absorbing pigment in their wings. Under the second hypothesis, we would expect a
105 discontinuity in the latitudinal pattern at the edge of the zone of sympatry. Given the generally
106 dark colour of *C. maculata* wings, this shift might take the form of a lightening of *C. maculata*
107 wings to provide greater difference compared to the black wing tip of *C. aequabilis*.

108 METHODS

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

124 Due to differences between individuals in the area of wing obscured by the thorax and the
125 accuracy of dissections, all wing images were modified to omit the arculus and all regions before
126 the first cross-veins. Wing pigmentation was measured as the average grayscale value of the
127 wing. Grayscale varies between 0 (black) and 255 (white), hence greater values correspond to
128 lighter, more transparent wings. This value was calculated for each pixel on the image, with an
129 average of 503,647 pixels (± 1202 SE) on the fore wing and 496,122 pixels (± 1260) on the hind
130 wing. All measurements were carried out in ImageJ ([Rasband 1997-2007](#)). During
131 measurements, any damage to wings was noted and those measurements which could not be
132 accurately quantified were excluded. This resulted in the exclusion of 140 fore wing and 116
133 hind wing pigmentation measurements (see Table S1 for sample sizes).

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

144 Response variables, predictors and model residuals were tested for normality using Shapiro-Wilk
145 tests. Transformations were applied where those assumptions were not met. General linear
146 models weighted by the square root of the sample size were constructed in R ([R Development](#)
147 [Core Team 2013](#)) with fore wing and hind wing pigmentation as response variables. It is unclear
148 (i) how atmospheric temperature relates to the temperature experienced by aquatic larvae and (ii)
149 what aspect of temperature variation might influence odonate pigmentation. As a result, latitude
150 was used as a general proxy for temperature variation. Furthermore, after transformation for
151 normality, latitude and annual temperature were very highly correlated ($r=0.860$, $p<0.001$).
152 Latitude, ordinal date on which specimens were collected, and sympatry with *C. aequabilis* were
153 included as predictors in the GLMs. An interaction between latitude and sympatry was also
154 included to evaluate the effect of range overlap on latitudinal trends. Where specimens were

155 collected across multiple dates, the average of those dates was taken for the sample. Subsets of
156 data corresponding to different regions of the range (i.e. whole range, allopatric range and
157 sympatric range) were analysed using Pearson correlations to investigate whether latitude-
158 pigmentation patterns varied. Similarity between specimens from different geographical
159 locations may be as much a function of their proximity as of any underlying patterns. This
160 should be corrected for using spatial autoregressive models, but only if the residuals of the
161 uncorrected models are spatially autocorrelated ([Diniz-Filho et al. 2003](#)). The residuals of all
162 models were tested for spatial autocorrelation using SAM v3.0 ([Rangel et al. 2010](#)).

163 Supplementary data on the degree of wing pigmentation in *C. maculata* females and *C.*
164 *aequabilis* females are available from a previous study and provide a useful comparison ([Waage](#)
165 [1979](#)). Mean transparency of the dark regions of female *C. maculata* and *C. aequabilis* wings,
166 and the proportion of the wing that was pigmented in *C. aequabilis* males were extracted from
167 Waage ([1979](#)), see Figure 3B-D. Data extraction was carried out using the GetData Graph
168 Digitizer v2.24 ([Fedorov 2008](#)). Waage used a densitometer to quantify relative transparency
169 between samples and so his absolute measurements are not directly comparable to the present
170 study. However, the data still permit a qualitatively comparison of variation between the species.
171 All data for the *C. maculata* males used in this study are available as electronic supplementary
172 information (Table S1 for site summaries, Table S2 for individual measurements).

173 RESULTS

174 Note that throughout the results, wing transparency is used as the measure of colouration and this
175 is inversely related to the degree of pigmentation in the wing. A guide is shown in Figure 2 to
176 give examples of varying degrees of wing transparency. When individual data were averaged
177 across sites there was a highly significant correlation between hind and fore wing pigmentation
178 ($n=34$, $r=0.985$, $p<0.001$) and so only fore wing data are shown in Figures 1, 2 and 3A. The
179 results from GLMs demonstrate that sympatry with *C. aequabilis* and latitude influenced
180 variation in fore and hind wing pigmentation but that the effect of latitude was dependent upon
181 sympatry (Table 1). Residuals of these models were not spatially autocorrelated as revealed by
182 SAM and so no control for spatial autocorrelation was necessary. In the allopatric zone, male *C.*
183 *maculata* showed consistently dark wings (Figures 1 and 2). The exception was a single
184 population in Texas in which individuals had lighter wings. Where the range of *C. aequabilis*
185 overlaps the range of *C. maculata*, wings suddenly become lighter and then darken further north

186 in the sympatric zone. There is a significant correlation between latitude and mean population
187 pigmentation for populations of *C. maculata* when sympatric with *C. aequabilis* ($n=21$, $r=-0.743$,
188 $p<0.001$ – note that this negative correlation means increasing pigmentation with latitude) but not
189 for allopatric populations ($n=13$, $r=-0.280$, $p=0.332$) or populations as a whole ($n=34$, $r=0.150$,
190 $p=0.397$). The decline in pigmentation does not appear to result from a continuation of any
191 trends from the allopatric region but occurs suddenly.

192 The relative wing pigmentation between geographical locations that has previously been reported
193 for *C. maculata* females, *C. aequabilis* females, and *C. aequabilis* males ([Waage 1979](#)) appears to
194 follow the same geographic pattern as that found in the present study for *C. maculata* males
195 (Figure 3). In allopatri, wings tend to be more pigmented (lower transparency). At the southern
196 margin of the sympatric zone wings tend to be less pigmented (higher transparency) and
197 pigmentation increases towards the northern margin of the sympatric zone in all four groups.
198 Note that only qualitative comparisons are possible between the groups due to variation in the
199 methods used to obtain the data, but that these comparisons suggest a consistent pattern.

200 DISCUSSION

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

211 It has previously been stated that *Calopteryx maculata* "...has dark wings and shows little
212 geographic variation in the wing pattern" ([Mullen & Andrés 2007](#)) and that a "...sympatric shift in
213 wing pigmentation was exhibited by females of [C. maculata and C. aequabilis] but only by
214 males in C. aequabilis" ([Honkavaara et al. 2011](#)). This originated in a misinterpretation of the
215 work of Waage ([1979](#)) who focused only female pigmentation in *C. maculata* and did not

216 measure that in males, stating that "extent of the dark area in *C. maculata* males was not
217 measured as their wings are 95-100% pigmented" and "...wings of *C. maculata* are entirely dark
218 and vary only in size... among the populations sampled" ([Waage 1979, p106 and 108,](#)
219 [respectively](#)) but did not measure the intensity of pigmentation. However, from a broader
220 comparison of sites, the degree of variation is marked (see the comparison of wing pigmentation
221 levels in Figure 2) and varies depending on sympatry or allopatry with *C. aequabilis*. No
222 evidence exists for latitudinal patterns in pigmentation apart from a latitudinal decline in
223 pigmentation through the sympatric zone.

224 From a previous study it has been shown that *C. aequabilis* reduces its pigmentation
225 progressively in populations that are located deeper inside the range of *C. maculata* ([wing](#)
226 [pigmentation is significantly correlated with latitude, see Table 1 in Waage 1979](#)) and it has been
227 assumed that this was to enhance species recognition where dark-winged *C. maculata* were
228 present. However, it appears that the northward increase in pigmentation within the sympatric
229 zone is also present in *C. maculata*, with qualitatively similar trends in increasing pigmentation
230 further north in both sexes of both *C. maculata* and *C. aequabilis*. This pattern is consistent with
231 the existence of convergent character displacement ([Grant 1972](#)) with male wing pigmentation
232 changing to match variations in female *C. aequabilis* pigmentation intensity and male *C.*
233 *aequabilis* wing spot size (see Figure 3 for a comparison with Waage's ([1979](#)) data). However, a
234 notable difference between the two trends is that the decline in pigmentation in *C. maculata*
235 males is found in both wings, while this is only true for the hind wing in *C. aequabilis* ([Waage](#)
236 [1979](#)). This difference may relate to variations between species in the "cross-displays" performed
237 by males to court females, which result in the more prominent display of hind wings in *C.*
238 *aequabilis* ([Waage 1973](#)).

239 It is important to note that the previous demonstration of character displacement involved
240 behavioural differences between species. The key observation was that *C. maculata* males
241 exhibit a greater ability to discriminate between conspecific and heterospecific females when in
242 sympatry than when in allopatry ([Waage 1975](#)). This can be taken as evidence of a cost of
243 confused mating by *C. maculata* which leads to a selective pressure acting on the reinforcement
244 of species identification. The present study provides a description of apparent convergence in a
245 character that is key to discriminating between species, which appears to render both sexes of
246 both species less discriminable. This observation runs counter to what would be expected given

247 this selection pressure. Furthermore, the pattern cannot be explained by correlations with latitude
248 or temperature, which might be predicted based on the fact that melanin can play a role in
249 thermoregulation at higher latitudes (leading to a positive correlation between latitude and
250 pigmentation), as the levels of pigmentation return to allopatric levels (dark pigmentation) at the
251 northern-most sites (Figures 1, 2 and 3).

252 The pattern of results effectively appear to rule out temperature and latitude as causal factors in
253 the broader trend, although these have been suggested to influence geographic variation in
254 odonate colour ([Hilfert-Rüppell 1998](#)) and are strongly associated with the occurrence of wing
255 pigmentation across the Calopterygidae as a group ([Svensson & Waller 2013](#)). It is worth noting
256 that this is a purely correlative study, albeit involving intensive sampling of populations across an
257 extensive geographical area. The relatively constant levels of wing transparency through the
258 allopatric zone offer no evidence of a latitudinal or temperature-driven cline in pigmentation,
259 although the results are consistent with such a cline (showing declining transparency further
260 north) within the zone of sympatry. Stream temperatures measured *in situ* would provide a far
261 closer approximation of the thermal environment within which the animals develop, and there is
262 the potential that *Calopteryx* sp. may specifically select water bodies with particular thermal
263 regimes ([as they do for a large number of other parameters, Siva-Jothy et al. 1995](#)). Hence, while
264 latitude and temperature are strongly correlated, small (e.g. catchment) scale variations in water
265 temperature may still be operating. *Calopteryx* can be maintained in laboratory conditions and so
266 a common garden experiment that examined the impacts of varying temperature on larvae from
267 multiple populations would provide a thorough and robust test of these competing hypotheses.
268 Another mechanism that has been implicated in driving changes in odonate colour is the
269 avoidance of harassment by conspecifics ([Van Gossum et al. 2001](#)) or heterospecifics ([Tynkkynen
270 et al. 2004](#)). Evidence for this playing a role in calopterygid damselflies is present in the
271 diversity of wing morphs exhibited by European species. Males of *Calopteryx exul* (Selys),
272 inhabiting north Africa, have no pigmentation on their wings and so resemble females.
273 *Calopteryx virgo virgo* has no pigmentation at the base or the tip of the wing and resembles
274 *Calopteryx splendens* found in eastern Europe. *Calopteryx splendens balcanica* females have
275 pigmented wings which resemble the male (androchrome). Wing pigmentation polymorphism is
276 also present in males of the calopterygid damselfly *Mnais costalis* (Selys), where clear-winged
277 males exhibit a "sneaker" mating strategy while orange-winged males are territorial ([Plaistow &
278 Tsubaki 2000](#)). Therefore, there is evidence from the Calopterygidae for male-mimicking

279 females, female-mimicking males and, possibly, heterospecific mimicry. Establishing which, if
280 any, of these explanations best fit the *C. maculata/C. aequabilis* system would require further
281 experiments along the same lines as those conducted previously ([Tynkkynen et al. 2004](#); [Waage](#)
282 [1979](#)).

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

296 The importance of thermoregulatory behaviour has been noted in a number of Odonata ([for a](#)
297 [review see Hassall & Thompson 2008](#)), and a number of adaptations are present. There has been
298 a suggestion that variations in body pigmentation with latitude in *Orthetrum cancellatum* (L)
299 contribute to enhanced absorption of solar energy ([Hilfert-Rüppell 1998](#)). Similarly, a melanic
300 form of *Sympetrum striolatum* (Charpentier) found only at the northern range margin where such
301 pigmentation could assist in thermoregulation was previously described as a separate species,
302 *Sympetrum nigrescens*, before molecular studies demonstrated that the two were synonymous
303 ([Pilgrim & Von Dohlen 2007](#)). The only other study of range-wide variation in wing
304 pigmentation in a damselfly (*Calopteryx splendens*) also demonstrated no consistent patterns with
305 latitude ([Sadeghi et al. 2009](#)), and this has been attributed to different wing morphs being
306 genetically distinct gene pools with intermediates resulting from hybridisation ([Sadeghi et al.](#)
307 [2010](#)). This study similarly shows no unequivocal evidence of a latitudinal cline, despite the
308 wide climatic range over which the species occurs. However, the increased pigmentation at

309 higher latitudes within the sympatric zone is consistent with a potential role in thermoregulation
310 within this region.

311 It has been demonstrated that the extent of pigmentation in male wings is an honest indicator of
312 immune function in a number of calopterygid damselflies ([Hetaerina americana](#), [Contreras-](#)
313 [Garduño et al. 2006](#); [Calopteryx splendens](#), [Rantala et al. 2000](#); [Calopteryx splendens](#)
314 [xanthostoma](#), [Siva-Jothy 2000](#)). As such, variation in pigmentation may be under indirect
315 selective pressures acting on immune function. Parasites increase in diversity and abundance
316 closer to the equator ([Poulin & Morand 2000](#)) and there is some evidence that virulence follows a
317 similar pattern ([Møller et al. 2009](#)). This selection may result in greater immune function at
318 lower latitudes ([e.g. Ardia 2007](#)). Thus we may expect to observe greater pigmentation at lower
319 latitudes where immune challenge is at its highest. However, damselflies emerging later in the
320 season exhibit greater immune responses ([Yourth et al. 2002](#)) which are associated with higher
321 temperatures ([Robb & Forbes 2005](#)). The melanotic encapsulation involved in the immune
322 response follows the same biochemical pathways as those involved in melanisation of the cuticle
323 ([Marmaras et al. 1996](#)). Thus, a negative correlation between pigmentation and latitude (or a
324 positive correlation with temperature) is predicted by both parasite-mediated selection and plastic
325 responses to temperature, but there is no evidence of this pattern in the present study.

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

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Table 1(on next page)

General linear models describing variation in wing pigmentation in *Calopteryx maculata*.

Table 1 – Parameter estimates from general linear models (weighted by square root of sample size) describing variation in wing pigmentation in *Calopteryx maculata* males. DF=1 for all parameters.

	Fore wing pigmentation			Hind wing pigmentation		
	Parameter	t	p	Parameter	t	p
Ordinal date	-0.0017	-0.298	0.768	-0.0015	-0.367	0.717
Latitude	-0.3068	-0.994	0.329	-0.0944	-0.428	0.672
Sympatry	2.8438	5.097	<0.001	2.2590	5.591	<0.001
Latitude*sympatry	-0.5589	-1.574	0.126	-0.5280	-2.067	0.048
R ² adj			0.547			0.581

Figure 1

Map of sites with transparency of wings

Figure 1 – Geographical distribution of *Calopteryx maculata* (light grey) and *Calopteryx aequabilis* (hashed region). Points mark the location of sampling sites for *C. maculata* and the size of the point is proportional to the grayscale value of the wing pigmentation intensity (larger symbol = lighter wings) for areas of sympatry (filled symbols) and allopatry (open symbols). See Figure 3 for illustrations of wing pigmentation variation.

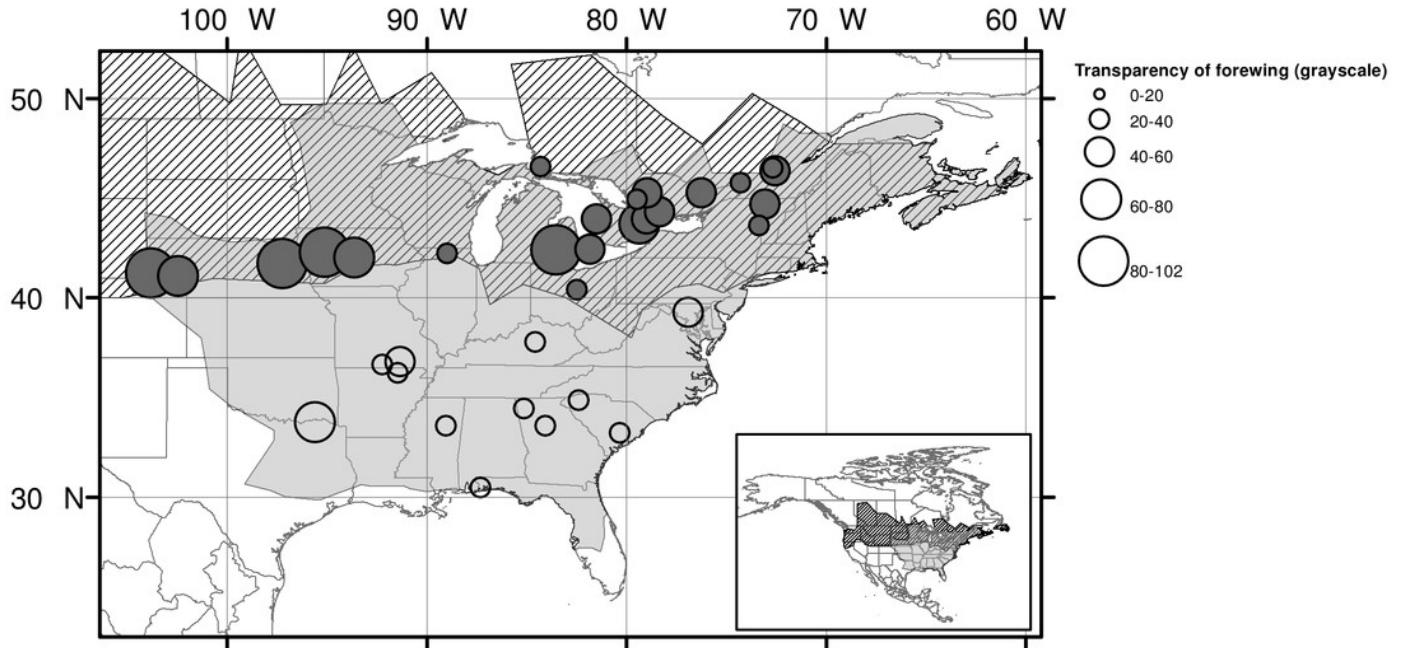


Figure 2

Variation in wing pigmentation in *Calopteryx maculata*

Figure 2 – Latitudinal patterns of pigmentation in *C. maculata* males where populations are allopatric (open circles) and sympatric (closed circles) with *C. aequabilis*. Error bars are 1SE. The y-axis is arranged to show increasing levels of pigment from bottom to top, but note that the measurement was grayscale where higher values correspond to lighter colours. Displayed on the right are examples of wings with pigmentation intensities corresponding to their locations on the y-axis. See Table S1 for sample sizes and means, and Table S2 for raw data.

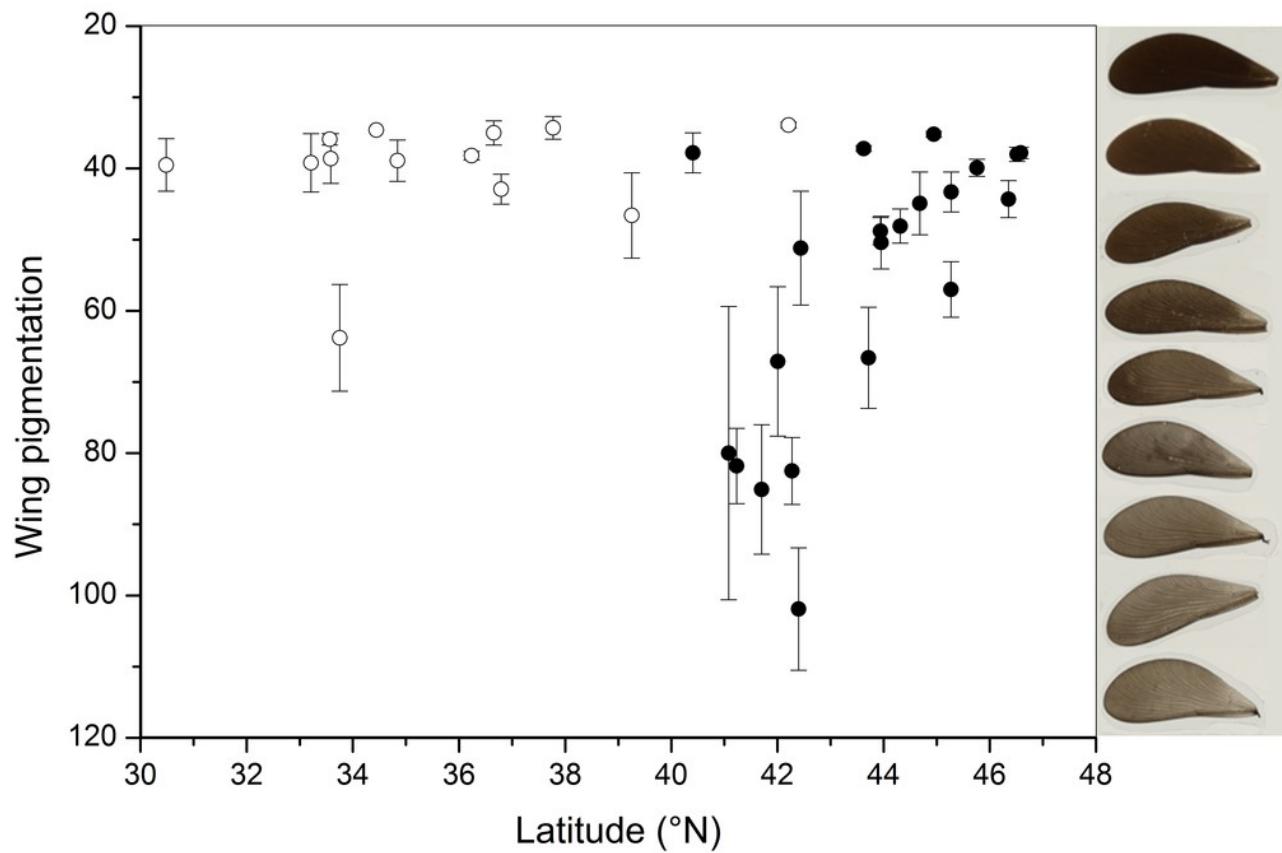


Figure 3

Comparison of wing pigmentation variation between *Calopteryx maculata* and *C. aequabilis*

Figure 3 – Comparison of variation wing pigmentation between (A) *C. maculata* males (from the present study), (B) *C. aequabilis* females (from Waage, 1979), (C) *C. maculata* males (from Waage, 1979), and (D) *C. maculata* females (from Waage, 1979). All y-axes represent qualitatively the same trait: pigmentation of the wing, with low values (top of y-axes) corresponding to darker wings and higher values (bottom of y-axes) to greater transparency. However, the measures vary in the way in which they were collected: for *C. aequabilis* males it is the proportion of the wing length that is unpigmented, for *C. aequabilis* and *C. maculata* females it is the transparency of the dark area of the wing (using a densitometer), and for *C. maculata* males it is the mean grayscale value of a wing scan (see methods above). All error bars are standard errors. Note in all cases that pigmentation is highest at the southern margin of the zone of sympatry with subsequent declines further north. t

