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1 **Wing morphological responses to latitude and**
2 **colonisation in a range expanding butterfly**

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Abstract

Populations undergoing rapid climate-driven range expansion experience distinct selection regimes dominated both by increased dispersal at the leading edges and steep environmental gradients. Characterisation of traits associated with such expansions provides insight into the selection pressures and evolutionary constraints that shape demographic and evolutionary responses. Here we investigate patterns in three components of wing morphology (size, shape, colour) often linked to dispersal ability and thermoregulation, along latitudinal gradients of range expansion in the Speckled Wood butterfly (*Pararge aegeria*) in Britain (two regions of expansion in England and Scotland). We measured 774 males from 54 sites spanning 799km with a 10-year mean average temperature gradient of 4°C. A geometric morphometric method was used to investigate variation in size and shape of forewings and hindwings; colour, pattern, and contrast of the wings were examined using a measure of lightness (inverse degree of melanism). Overall, wing size increased with latitude by ~2% per 100km, consistent with Bergmann's rule. Forewings became more rounded and hindwings more elongated with history of colonisation, possibly reflecting selection for increased dispersal ability. Contrary to thermal melanism expectations, wing colour was lighter where larvae developed at cooler temperatures and unrelated to long-term temperature. Changes in wing spot pattern were also detected. High heterogeneity in variance among sites for all of the traits studied may reflect evolutionary time-lags and genetic drift due to colonisation of new habitats. Our study suggests that temperature-sensitive plastic responses for size and colour interact with selection for dispersal traits (wing size and shape). Whilst the plastic and evolutionary responses may in some cases act antagonistically, the rapid expansion of *P. aegeria* implies an overall reinforcing effect between these two mechanisms.

52

53 **Introduction**

54 A population may respond to climate change either by altering its phenotype to maintain local
55 fitness, or by shifting distribution and/or phenology to track its climatic envelope (Parmesan &
56 Yohe, 2003; Macgregor et al., 2019). During a range expansion populations on the range front
57 are subject to multiple selection pressures (Phillips, Brown & Shine, 2010). Any resulting
58 phenotypic changes are therefore created by evolutionary responses to both changing local
59 environments and the process of range expansion itself. Phenotype-environment optima under
60 equilibrium conditions, may be overridden or obscured by the expansion process as mal-adapted
61 genotypes can surf on the range front due to genetic drift (Burton & Travis, 2008). Moreover,
62 temperature-dependent reaction norms, and genetic correlations among traits, may help or hinder
63 adaptation to the new environment (Pujol et al., 2018). Phenotypic responses to climate change
64 have been reported for correlates of dispersal (Thomas et al., 2001; Hill, Griffiths & Thomas,
65 2011), body size (Daufresne, Lengfellner & Sommer, 2009), and colour lightness (linked to
66 thermal tolerance; Zeuss et al., 2014).

67

68 Contemporary evolution of dispersal traits is often closely linked to the process of range shifts
69 towards cooler climates (Parmesan, 2006; Hickling et al., 2006). Indeed, a common phenotypic
70 signature of range expansion, found in many species, is increased dispersal ability towards the
71 leading edge of a shifting range (Hughes, Hill & Dytham, 2003; Simmons & Thomas, 2004;
72 Phillips, Anderson & Schapire, 2006). This cline in dispersal ability is a product of *spatial*
73 *selection*, resulting from the combined effects of spatial sorting (assortative mating of dispersive
74 genotypes on the range edge) and density-dependent selection (Phillips, Brown & Shine, 2010).
75 Density-dependent selection is predicted to favour good dispersers colonising new habitat
76 patches, where they encounter much lower intraspecific competition, resulting in increased
77 fecundity and intrinsic growth rates at the range edge. Dispersal in insects, of which flight
78 performance is a key component, may be affected by several factors including morphological,
79 physiological, metabolic, and behavioural traits (Betts & Wootton, 1988; Berwaerts, Van Dyck
80 & Aerts, 2002; Niitepõld et al., 2009; Flockhart et al., 2017; Renault, 2020). Thorax size (highly
81 correlated to whole body size) is a widely accepted measure of dispersal ability in Lepidoptera as
82 it indicates flight muscle investment (Srygley & Chai, 1990; Hill, Thomas & Lewis, 1999;

83 Berwaerts, Van Dyck & Aerts, 2002). Wing size has been shown to be correlated with body size
84 measurements (e.g., thorax size, body length and dry mass) in several Lepidoptera species (Chai
85 & Srygley, 1990; Merckx & Van Dyck, 2006) and hence can be used as a proxy for overall size
86 and dispersal (Sekar, 2012). Wing shape has direct implications for aerodynamics during flight,
87 which affects the efficiency of flight strategies, flight ability and dispersal (Breuker, Brakefield
88 & Gibbs, 2007; Le Roy, Debat & Llaurens, 2019).

89
90 Morphological trends associated with poleward range expansion could be due to spatial selection
91 for dispersal ability, but could also reflect a genetic or plastic response to an environmental cline.
92 One such phenotype-environment relationship is an increase in body size with latitude
93 (Bergmann's rule; Bergmann, 1848). Originally described in mammals, this pattern (and its
94 converse) has been observed in ectotherms (Shelomi, 2012). Bergmann clines often suggest
95 genetic adaptation to different thermal environments but phenotypic plasticity also plays an
96 important role in producing body size clines (Atkinson & Sibly, 1997; Mousseau, 2006). The
97 temperature size rule, describes the plastic response of body size to developmental temperature
98 (smaller individuals at higher temperatures) in ectotherms (Atkinson, 1994). However, selection
99 for increased size at lower temperature is not supported across all species (Stillwell, Moya-lara &
100 Fox, 2008), and the relationship of size to temperature may evolve in different directions
101 between recently divergent populations that experience contrasting temperature regimes
102 (Kingsolver et al., 2007). Body size clines in ectotherms are further affected by season length
103 and voltinism, i.e. the number of generations per year, as growing time is positively correlated to
104 size (Chown & Gaston, 2010; Horne, Hirst & Atkinson, 2015; Zeuss, Brunzel & Brandl, 2017).
105 Above a certain latitude, shortening of season length reaches a point where the limited time
106 available for development, growth and foraging results in reduced overall body size with
107 latitude, i.e. a converse Bergmann cline (Blanckenhorn & Demont, 2004). The addition of a
108 second-generation can result in complex saw-tooth patterns of body size with season length
109 (Roff, 1980). Season length and voltinism may therefore explain why both the inverse and
110 classical Bergmann's rule have been documented in arthropods (Horne, Hirst & Atkinson, 2015),
111 suggesting that these patterns are not contradictory but part of a continuum (Blanckenhorn &
112 Demont, 2004).

113

114 Melanism is another trait often associated with adaptation to the thermal environment (thermal
115 melanism). For example, in ectotherms darker species frequently occur at higher latitudes or in
116 cooler climates (Zeuss et al., 2014; Heidrich et al., 2018). This observation is often explained by
117 the thermal melanism hypothesis, which states that darker individuals have an advantage in
118 cooler climates (Clusella Trullas, van Wyk & Spotila, 2007). In principle, all other things being
119 equal, ectotherms presenting a larger and/or darker surface area of melanised exocuticle should
120 show increased absorption of solar radiation compared to lighter individuals, thus reaching a
121 higher body temperature and at a faster rate. This could, in turn, allow activity at lower
122 temperatures, potentially enhancing mating opportunities (Clusella Trullas, van Wyk & Spotila,
123 2007) and dispersal (Mattila, 2015). It has been suggested that the basal part of the wing is the
124 most important for thermal regulation (Wasserthal, 1975), but other components of the wing
125 pattern may contribute to the thermal properties of wings (Brashears, Aiello & Seymoure, 2016).
126 Melanism also plays an important role in protection against UV radiation (Bishop et al., 2016;
127 Katoh, Tatsuta & Tsuji, 2018) and pathogens (Dubovskiy et al., 2013), which may lead to darker
128 individuals in warmer climates, opposing the trend predicted by a purely thermal explanation.
129 The degree of melanism may also be affected by selection on the colour pattern which has
130 important functions in mate choice (Jiggins et al., 2001; Kemp, 2007) and predation avoidance
131 (Bond & Kamil, 2002). Futhermore, seasonal polyphenism (the production of different
132 phenotypes in different seasonal generations) is widely documented in Lepidoptera, and
133 particularly prominent in multivoltine species (Kingsolver, 1995). This phenomenon is driven by
134 environmental cues (Roskam & Brakefield, 1999), often altering wing pattern, which can
135 potentially produce pattern differences across environmental gradients.

136

137 In this study, the Speckled Wood butterfly (*Pararge aegeria* (Linnaeus, 1758)), which has
138 undergone rapid range expansion in mainland Britain, was used to investigate phenotypic
139 changes in wing size, shape and melanism, with respect to colonisation history, latitude and
140 temperature. Flight morphological traits in *P. aegeria* differ across latitudinal clines in mainland
141 Europe, by habitat type (Vandewoestijne & van Dyck, 2010) and with mate location strategy
142 (Berwaerts, Van Dyck & Aerts, 2002). Bergmann's rule has been reported for the British
143 population, with larger individuals found further north (Dennis & Shreeve, 1989; Sibly, Winokur
144 & Smith, 1997), whereas the inverse pattern was seen in Sweden (Nylín & Svärd, 1991). An

145 increase in dispersal ability, using thorax size as an indicator, was found towards the expanding
146 range edge in the UK, with a potential evolutionary trade-off between reproduction and dispersal
147 (Hughes, Hill & Dytham, 2003). These studies of the British populations were limited to a
148 relatively small number of sites, when the distribution of *P. aegeria* in England and Scotland was
149 much less extensive. Detailed analysis of *P. aegeria* wing morphology, especially using
150 geometric morphometrics, across the range expansion in Britain is lacking. Dennis and Shreeve
151 (1989) report latitudinal variation in *P. aegeria* wing colour which is consistent with the
152 anecdotal perception of butterfly recorders that individuals from northerly populations tend to be
153 darker than those from southern populations. The cream spot pattern of *P. aegeria* has been
154 described to increase in lightness and size with latitude, possibly due to the interplay between
155 thermoregulatory requirements, mate choice and predator escape (Dennis & Shreeve, 1989).
156 However, to our knowledge, the qualitative perceptions of *P. aegeria* colour and pattern have not
157 been verified quantitatively.

158

159 *Pararge aegeria* in Britain provides an opportunity to examine the interplay and influence of
160 demographic factors (range expansion) and environmental factors (latitude and temperature, both
161 during development and in the recent past) in shaping morphological traits in a rapidly
162 expanding population. We investigated phenotypic changes in wing size and shape (linked to
163 dispersal ability and body size), as well as colour and pattern (potentially influencing
164 thermoregulation) across the expanded range of *P. aegeria* in mainland Britain. In common with
165 similar studies on wild-caught individuals that lack experimental and/or genetic data, our sample
166 does not allow strong inferences about the relative importance of phenotypic plasticity vs.
167 genotypic differences in determining the observed patterns in morphological variation. The
168 relative effect of demographic and environmental factors on morphology were evaluated through
169 collection of samples along the axes of range expansion, specifically to capture a wide range of
170 local population ages and colonisation histories (from core sites known to have been
171 continuously occupied at least since 1965 to leading edge sites colonised in 2015), as well as
172 over large gradients in latitude (covering a distance of 799km), mean 10-year annual temperature
173 (4°C) and mean temperature during development (6°C). We hypothesised that: 1) wing size is
174 larger in more recently colonised populations, as predicted by spatial selection; 2) wing size
175 increases with latitude, following Bergmann's and the temperature-size rules; 3) wing size is

176 smaller in populations with more generations; 4) wing shape changes to a more dispersive form
177 with colonisation history; 5) melanism increases with latitude and decreasing temperatures, in
178 accordance with the thermal melanism hypothesis; and 6) the cream spot wing pattern becomes
179 lighter and larger with latitude, as described by Dennis & Shreeve (1989).
180

181

182 **Materials & Methods**

183 **Study species and sample collection**

184 *Pararge aegeria* is a multivoltine species (capable of completing multiple generations in a year)
185 which occurs throughout Europe to western Asia. *Pararge aegeria* can follow three different
186 developmental pathways – pupal diapause, larval diapause or direct development – that may
187 differentially affect morphology (Van Dyck & Wiklund, 2002). Within the UK, the range of *P.*
188 *aegeria* has changed dynamically in the past 200 years. At the end of the 19th century there was a
189 contraction of populations to south-west England, Wales and a refuge population in western
190 Scotland, assumed to be in response to a change in climate (Emmet & Heath, 1990; Warren et
191 al., 2001). Since the 1970s, the distribution of *P. aegeria* began to expand northwards from
192 south-west England and Wales, argued to be mainly driven by increasing temperatures, but also
193 other factors such as habitat preference. A secondary range expansion from the refuge population
194 in western Scotland has recolonised much of central and northern Scotland. Increased habitat
195 fragmentation of woodland habitats resulted in a greater than expected lag in the rate of range
196 expansion compared to the climatic envelope (Hill, Thomas & Huntley, 1999). *P. aegeria* is now
197 widespread across the UK.

198

199 A total of 774 male *P. aegeria* were hand netted across 54 sites (10-20 males/site) during the
200 summers of 2016-2018 in the UK (Fig 1; Table S1). In order to capture the pattern of range
201 expansion, the site locations were chosen at a 10km grid scale aimed, firstly, at covering the
202 whole of the geographic range and, secondly, to include the full range of number of years since
203 *P. aegeria* was first recorded at 10km grid resolution. Specimens collected in 2018 were frozen
204 on or the day after collection using a liquid nitrogen dry shipper and subsequently transferred to -
205 80°C freezer for storage. Samples collected in 2016/17 were kept alive in a cool box for two days
206 until transfer to a -80°C freezer. Permission for sampling at sites was obtained from landowners,
207 including, but not limited to: Natural England, National Trust, Forestry Commission (England
208 and Scotland), Woodland Trust, Yorkshire Wildlife Trust, Norfolk Wildlife Trust, local councils
209 and site rangers through correspondence and verbal communication.

210

211 **Photography**

212 Wings were carefully removed from the body at their point of attachment using fine scissors. The
213 photographic setup consisted of Nikon D80 with Micro-Nikkor 40mm lens attached to a camera
214 stand, and two stand-alone speedlight (YN560IV) flashes (ISO 160, aperture $f/25$, exposure time
215 of $1/80$ seconds and compensation level +5.0). Size and shape analyses were carried out on *jpeg*
216 files, but for colour analysis, to retain more information, images were converted from RAW
217 Nikon images (NEF) to portable network graphics (png) using the programme XnConvert v.1.82.
218 Photographs were calibrated by the R package Patternize (Van Belleghem et al., 2018), using the
219 ColorGauge Micro Target (Image Science Associates) to account for any changes in lighting
220 (Fig.2A). Wings were graded into four wear categories, both for physical damage and scale
221 damage (1= no damage, 4= significant/total damage). The sum of these two factors, assumed to
222 be loosely correlated with butterfly age, was used as a factor in the analyses, or as a filter to
223 remove significantly damaged and old butterflies. The left wings were selected as a priority for
224 analysis, but if significant damage was present the right side was used instead.

225

226 **Environmental and demographic variables**

227 We focused on four demographic or environmental variables: 1) number of years since first
228 record or colonisation (years colonised); 2) latitude; 3) mean temperature during development;
229 and 4) 10-year mean annual temperature.

230

231 To determine if developmental temperature accounts for any differences in morphology,
232 latitudinal variation in emergence peaks had to be taken into account, due to *P. aegeria* being a
233 multivoltine species. The specimens in this study were mainly collected late May - early August,
234 which generally corresponds to the start of the second generation. However, more southerly
235 populations emerge earlier in the summer and undergo more generations than those further north.
236 Therefore, the emergence patterns of each 10km grid sampled, based on 10-year abundance data
237 from the Butterflies for the New Millennium (BNM) recording scheme (2007-2017), was
238 investigated using generalised additive models, in the R package mgcv (Wood, 2004). This
239 allowed identification of the number of generations at each grid, and the month of the second-
240 generation peak. To assess the effect of temperature during embryo, larva and pupa development
241 on morphological traits, we used the average temperature of the local second-generation peak

242 month and the preceding two months, in the year of collection (e.g., for a site with a second
243 generation peak in June, the mean temperature during development was calculated as the mean
244 of April- June in the collection year).

245
246 To investigate potential morphological responses to multi-generational selection related to
247 geographic variation in temperature, we used 10-year mean annual temperature (T_{10}), at 1km
248 resolution, from the year prior to collection (e.g., for a butterfly sample collected in 2018, T_{10}
249 was calculated from 2007-2017). Ten years was chosen as a suitable timescale for phenotypic
250 response to selection, even for more recently colonised populations, whilst reducing the effect of
251 any large fluctuations in annual temperature. Temperature (monthly mean at a 1km resolution)
252 data for 2007-2017 were obtained from the Met Office HadUK-Grid UKCP18 dataset, available
253 through CEDA Archives. 2018 data were provided directly by the UK Met Office.

254
255 The pattern of range expansion was described by the number of years since the first record of *P.*
256 *aegeria* at each 10km grid (referred to as years colonised), using distribution data from the BNM
257 from 1965 onwards. The reliability of this type of data as an accurate reflection of changes in
258 species distribution relies on recorder effort and geographic coverage of records across the UK.
259 Previous studies that have accounted for recorder effort reveal that the expansion of *P. aegeria* is
260 a true occurrence and not due to changes in recorder effort (Parmesan et al., 1999). To assess the
261 reliability of the assumed pattern of expansion we applied an approach used in Macgregor et al.
262 (2019). For each grid, the percentage of regional species richness recorded was calculated (using
263 data for 58 butterfly species provided by BNM, where regional species richness was the total
264 number of species recorded in the 100 nearest neighbouring grids) in each year between 1965-
265 2014. Grids were considered well-recorded in a given year if 10% or more of regional species
266 richness was recorded. We then used this recording level to determine the level of confidence for
267 the year each 10km grid was colonised by *P. aegeria*. Grids in which *P. aegeria* was recorded
268 prior to 1975 were considered to be part of the core range of the species. From the remaining
269 grids, we considered that we had high confidence of the colonisation year for grids which had
270 been well-recorded in at least three years prior to the first record of *P. aegeria* (i.e. probable
271 absence, followed by presence). We considered that we had low confidence of the colonisation
272 year for grids in which the first record of *P. aegeria* coincided with or preceded the onset of

273 good recording (i.e. *P. aegeria* first recorded in or before the third well-recorded year), since it
274 was unclear whether such records represented true colonisation or simply the discovery of pre-
275 existing populations. The reported pattern of range expansion is evident even when core and high
276 confidence grids are considered in isolation (Fig.1), showing that it is not an artifact of increased
277 or changing patterns of recorder effort. Furthermore, the majority of sites sampled are from
278 10km grids that are categorised as core or high confidence sites, supporting our use of ‘years
279 colonised’ as an accurate metric for the sites studied.

280

281 **Wing landmarking and morphometrics**

282 To allow for comparison of geometric morphometry and pattern across individuals, 14 and 11
283 landmarks were digitised on the forewing and hindwing, respectively, using tpsUtil version 1.78
284 (Rohlf, 2019) and tpsDig2 version 2.31 (Rohlf, 2017). All landmarks were placed on vein-vein
285 or vein-wing margin intersections and provided adequate coverage of the overall shape and
286 internal structure of the wings (Fig.2). Landmarks were superimposed using generalised
287 Procrustes analysis performed using the R package geomorph (Adams & Otárola-Castillo, 2013)
288 and within MorphoJ version 1.07a (Klingenberg, 2011). This method standardises specimens to a
289 common coordinate system through controlling size, orientation and position to align
290 corresponding landmarks as closely as possible (Rohlf & Slice, 1990). Centroid size (CS) was
291 also calculated from the landmarks (square root of the sum of squared distances between each
292 landmark and the wing centroid), and was used as a measure of wing size and to account for
293 allometry in the analysis of wing shape.

294

295 *Size analysis*

296 Linear mixed effect models (LMM) fitted by restricted maximum likelihood (lme4 v.1.1-21
297 package; Bates et al., 2014), with *bobyqa* optimisation, was used to assess the effect of the four
298 environmental factors on wing size, quantified as CS. Random effects included were: 1) site
299 (10km grid) nested within regional expansion (i.e., south-west England or western Scotland), to
300 account for variation amongst sites and expansions, and 2) number of Julian days before or after
301 the peak of the second generation at a 10km scale that the sample was collected on (referred to as
302 the standardised collection date). Correlation of explanatory variables was investigated in R with

303 scatterplots and the Pearson correlation coefficient. The effect of voltinism on wing size was
304 tested using a two-sample t-test.

305
306 Significance testing for LMMs is not straightforward, as the denominator for degrees of freedom
307 is difficult to obtain for models with multiple levels (Baayen, Davidson & Bates, 2008);
308 therefore the package lme4 does not produce p-values (Bates et al., 2015). Instead, the t-value
309 from the LMM indicates the strength of the effect and some authors suggest a t-value of
310 magnitude over 1.96 can be considered significant, following the *t-as-z* approach (Luke, 2017).
311 Therefore, traditional p-values are not presented for LMMs in this study, and a t-value with a
312 magnitude of 1.96 or above is considered significant.

313

314 *Shape analysis*

315 To investigate shape changes, independently of size, a multivariate regression of the Procrustes
316 coordinates against wing size (log CS) was carried out (10,000 permutations). This method
317 accounts for allometric patterns by producing a regression score that corresponds to the shape
318 variable with the greatest covariation to size. The residuals of this regression can therefore be
319 treated as a size-adjusted shape variable. This method has been used widely to account for
320 allometry in many morphometric studies (van Heteren et al., 2016; Curth, Fischer & Kupczik,
321 2017). An analysis of covariance (ANCOVA) was used to determine if allometry was
322 significantly different between forewings and hindwings.

323

324 The resulting size-adjusted variables were then used for a two-block partial least squares (2B-
325 PLS) method across all individuals (Rohlf & Corti, 2000). The 2B-PLS method aims to capture
326 the greatest amount of covariation between two blocks of variables (here size-adjusted shape as
327 block one, and environmental variables as block two) of equal weight. This method calculates a
328 RV coefficient, that can be interpreted as a multivariate generalization of the bivariate R^2 , and
329 used to determine the strength of the covariation between blocks (Klingenberg, 2009). A
330 permutation test (10,000 repetitions) was used to compare the observed association against the
331 null hypothesis of complete independence. Shape changes associated with PLS axes are shown
332 using wireframe diagrams against the mean (or consensus) wing shape. All 2B-PLS analyses

333 were carried out in MorphoJ v1.07a (Klingenberg, 2011) and plotted with ggplot2 (Wickham,
334 2016).

335

336 **Wing colour, pattern and contrast**

337 *Pararge aegeria* wings are brown with a principally cream spot pattern. Four complementary
338 measures of colour and pattern were investigated: 1) average degree of lightness across the basal
339 3rd and entire wing surfaces (dorsal and ventral surfaces on forewing and hindwing); 2) average
340 lightness within brown and cream areas (forewing dorsal only); 3) the relative area of brown and
341 cream (forewing dorsal only); and 4) the contrast between brown to cream areas (forewing dorsal
342 only).

343

344 Degree of lightness of the forewing and hindwing dorsal and ventral surfaces was investigated
345 using the mean grey value for the whole wing and the basal third of the wing, closest to the body
346 (basal colour). It has been suggested that wing basal colour is the most important for thermal
347 regulation (Wasserthal, 1975). An ImageJ (FiJi distribution) macro was created to select the
348 individual wings from the background, rotate them to the same orientation, separate wings into
349 thirds (perpendicular to the longest axis), convert RGB images to 8-bit grey, and calculate the
350 mean grey value for the whole wing and each wing section. The full 8-bit grey scale ranges from
351 0 (complete black) to 255 (complete white). Wings with scale damage of 4 were removed from
352 the analysis, leaving 709 forewings and 642 hindwings. The effect of the environmental
353 variables on mean grey values was analysed in a LMM, fitted by restricted maximum likelihood
354 and *nmk bw* optimisation (lme4 v.1.1-21 package; Bates et al., 2015). Site nested within regional
355 expansion (i.e., south-west England or western Scotland), and the standardised collection date
356 metric were included as random effects. Significance testing followed the method detailed for
357 size analysis.

358

359 To investigate variation in the brown and cream areas separately (dorsal forewing only as it has
360 the most discrete pattern), a macro script for ImageJ (FiJi distribution) was written to calculate
361 the mean grey value and area (number of pixels) for each (filtered for scale damage of 4). Linear
362 regression was used to assess the relationship of brown (or cream) area with latitude, and an
363 ANCOVA to determine if these relationships differed significantly from each other. The

364 relationship of brown to cream colours in the forewing was quantified by linear regression on the
365 residuals of each colour to latitude (to focus on the underlying relationship). Finally, the
366 difference between the cream and brown mean grey value was calculated to produce a simple
367 measure of average contrast between the dark and light areas of the wing. The effect of the
368 environmental variables on contrast between brown and cream was analysed in a LMM, fitted by
369 restricted maximum likelihood and *nmkpw* optimisation (*lme4* v.1.1-21 package; Bates et al.,
370 2015). The random effects included were the same as for the overall lightness analysis.
371

372

373 **Results**

374

375 **Wing morphometrics**

376 *Size*

377 Forewing and hindwing size increase significantly with latitude (t-values > 1.96) and in more
378 recently colonised populations (shown by negative relationship of size to an increase in number
379 of years colonised), consistent with Bergmann's rule and spatial selection. Forewing size is also
380 weakly associated with temperature during development (t= 2.64) but not in hindwings (t=1.65).
381 In general, each of the environmental factors (latitude, years colonised, 10-year temperature
382 average, and temperature during development) show a consistent effect (both in the strength and
383 direction) on forewing and hindwing size, although latitude seems to have a stronger effect on
384 hindwings compared to forewings (Table 1; Fig.3). T₁₀ (10-year mean annual temperature)
385 produced the lowest t-values (0.57, 0.58) across the environmental variables for both wings,
386 indicating no effect of recent past temperature on wing size. Correlation between explanatory
387 variables was considered acceptable for LMMs (Figure S1). The Pearson correlation coefficient
388 ranged between 0.14 (years colonised with temperature during development) to -0.74 (latitude
389 with mean annual temperature). Although latitude is often considered a proxy for annual
390 temperature, it also incorporates other environmental gradients that follow latitude, for example
391 day length and amount of sunlight etc. Therefore, it was decided to retain both T₁₀ and latitude in
392 the model.

393

394 The populations varied between two and three generations per year across the 10km grid
395 sampled, and size of forewings (mean CS of 2.60 (2 generations) and 2.55 (3 generations)) and
396 hindwings (mean CS of 2.39 (2 generations) and 2.33 (3 generations)) are significantly smaller
397 (1.9% and 2.5% smaller in forewings and hindwings, respectively) in populations with three
398 generations (t-test: t=4.96, df=661.36, p<0.001 for forewing; t=4.45, df=598.26, p<0.001 for
399 hindwing).

400

401 *Shape*

402 Shape is significantly associated with size (log CS) both for forewing and hindwing, reflecting
403 shape allometry ($p < 0.0001$; Fig. 4A). Allometry is less pronounced in the forewing, where the
404 regression explained 1.25% of shape variance compared to 4.14% in the hindwing (ANCOVA of
405 regressions, $F = 21.53$, $df = 1$, $p < 0.001$; slope of 1.31 ± 0.14 and 2.25 ± 0.14 respectively; Fig. 4A and
406 4B). Shape changes for forewings and hindwings relative to size and the consensus shape are
407 shown in Figure 4C-F, respectively. Overall, larger wings have increased width and roundness
408 compared to smaller individuals. The shape difference between small and large forewings is
409 most noticeable for landmark 7, which moves further away from the consensus shape with
410 increasing size, and for landmark 1, which is shifted inwards, producing relatively broader and
411 shorter wings. In the hindwings, landmarks 5 and 11 are more separated from each other in large
412 hindwings, resulting in relatively wider wings compared to smaller hindwings.

413

414 The 2B-PLS analysis focused on covariation between the size-adjusted shape (block 1) and
415 environmental variables (block 2). Overall, the permutation test showed a significant covariation
416 between the two blocks both for the forewing ($p = 0.006$) and hindwing ($p = 0.0001$), supporting
417 non-independence of the two blocks. The overall strength of association between the two blocks
418 (as explained by the RV coefficient) is weak, at 0.012 and 0.025, respectively. The first PLS axis
419 (PLS1) explained 73% and 91% for forewing and hindwing, respectively, and was principally
420 loaded by years colonised and latitude (for both wings), but showed a weak correlation (0.20 and
421 0.22 respectively; Table 2; Fig. 5A and B). This suggests that, out of the environmental factors
422 studied, the range expansion process has the largest effect on shape. T_{10} was always loaded on
423 the fourth PLS axis, explaining the least variation across both wings, implying a minimal effect
424 of recent past temperature on wing shape.

425

426 Shape changes associated with PLS1 (associated most strongly with years colonised; Fig. 5C-F)
427 and the second PLS axis (PLS2; mainly latitude; not shown) indicate a general tendency towards
428 longer, narrower forewings (i.e., with increasing numbers of years colonised). Within PLS1 this
429 increase mainly occurs between the base or shoulder of the wing (landmark 1) to the apex
430 (landmarks 12-14), while the distance between landmark 1 and 7 decreases (Fig. 5C and 5D). In
431 comparison, the hindwing PLS1 (and PLS2) becomes more rounded. For PLS1, the increased

432 roundness seems to be due to the majority of landmarks at the tail edge of the wing being more
433 separated from one another (Fig. 5E and 5F).

434

435 **Colour, pattern and contrast**

436 The average lightness (average grey value) of whole wing surfaces, whether dorsal or ventral,
437 has a complex relationship with latitude, with periodic fluctuations of lightness that are
438 consistent in the forewing and hindwing (Fig. 6). The relationship of average lightness with the
439 demographic and environmental variables are similar across the basal third of the wing (Table 3)
440 and whole wing (Table S2). The lightness of ventral surfaces, both of forewings and hindwings,
441 becomes significantly darker with increasing temperature during development, but significantly
442 lighter with increasing latitude (Table 3; greyness value of 0 is black and 255 is white). The
443 direction of effects is consistent among all four wing surfaces, although the trend is not
444 significant for dorsal surfaces. There is no detectable effect of T_{10} and years colonised on any
445 wing surfaces. These effects account for variation due to population (England or Scotland) and
446 sampling date.

447 The relationship between latitude and mean grey value of the dorsal forewing depends
448 significantly on the colour considered (ANCOVA, $F=27.69$, $df=1$, $p<0.001$). Both brown and
449 cream become significantly lighter with latitude ($p<0.001$), but the cream area becomes lighter
450 faster (i.e., further north) than the brown area (slopes of 3.38 ± 0.25 vs 1.49 ± 0.26 greyscale units
451 per degree of latitude, respectively). These slopes reflect a strong positive correlation between
452 the lightness of brown and cream areas, which is independent of the latitudinal trend ($R^2= 0.68$;
453 $p<0.001$; Fig. 7). However, the relative proportion of the dorsal forewing surface that is brown
454 increases significantly with latitude ($R^2=0.09$, $df=702$, $p<0.001$). The analysis of contrast
455 (difference between cream and brown mean grey values) shows that the level of contrast
456 increases significantly with latitude (est.= 1.26 ± 0.50 , $df=46.37$, $t= 2.54$; Fig. 8), even when
457 accounting for population (England or Scotland) variation and collection date relative to the site-
458 specific emergence peak.

459

460

461 **Discussion**

462 This study documents detailed wing morphological variation (size, shape and colour) in the
463 Speckled Wood butterfly, *P. aegeria*, across two recently expanded populations in mainland
464 Britain, suggesting differing responses to environmental and demographic factors. The size of *P.*
465 *aegeria* increases with latitude, consistent with Bergmann's rule, and during the range expansion
466 process, with more recently colonised populations being larger than core populations. Shape
467 changes, independent of size, are most strongly associated with colonisation history. Forewing
468 shape becomes more rounded, whereas hindwing shape becomes longer, in more recently
469 colonised populations and with latitude. The distribution of average lightness (opposite of
470 melanism) is more strongly associated with temperature during development than it is to latitude,
471 and runs contrary to the traditional thermal melanism hypothesis. Furthermore, the area of brown
472 relative to cream increases with latitude, but not enough to overcome the general lightening in
473 both areas. Finally, the contrast between brown and cream areas increases with latitude,
474 accounting for the human perception that individuals become darker further north. Overall, this
475 study sheds light on the interaction of temperature-sensitive plastic traits and selection during a
476 mainly climate-driven range expansion.

477

478 During range expansion, sections of a population experience different and new environmental
479 conditions that may result in local adaptation, be it through genetic changes or phenotypic
480 plasticity. Our analyses are of wild-caught individuals who experienced different environments
481 during development, making it impossible to separate plastic from genetic effects. Previous
482 broad sense heritability estimates in a *P. aegeria* population from southern Sweden ($h^2 = 0.38-$
483 0.45) indicate high potential for evolutionary responses in comparable morphological traits,
484 including wing size and colour pattern (Van Dyck & Matthysen, 1998). Furthermore, a positive
485 correlation between thorax investment and wing shape, which was associated with acceleration
486 performance during take-off in males, had a heritability of 0.15 (Berwaerts, Matthysen & Dyck,
487 2008). This heritability estimate is specific to acceleration performance, and so caution should be
488 taken if relating this to general dispersal ability. Thus, although we cannot quantify the effect of
489 developmental environment on our phenotypic distributions, there is likely to be a degree of
490 heritable genetic variance in all of our measured traits.

491

492 **Size and shape variation**

493 Forewing and hindwing size in *P. aegeria* increases with colonisation and latitude. Larger
494 individuals were found in more recently colonised populations, following the expectation under
495 spatial selection if larger wings are associated with dispersal, for which there is some evidence
496 (Sekar, 2012; Flockhart et al., 2017). Our findings support the conclusion of Hughes et al.
497 (2003), which were based on thorax size across a much more limited number of sites and
498 geographic range of *P. aegeria*. On the reasonable assumption that wing size is directly
499 correlated to body size in this species, as in other Lepidoptera (Chai & Srygley, 1990), increased
500 wing size with latitude follows Bergmann's rule, in agreement with previous studies (Dennis &
501 Shreeve, 1989; Sibly, Winokur & Smith, 1997). Temperature during development shows a
502 positive relationship with forewing size, which runs counter to the prediction of the temperature
503 size rule (Atkinson & Sibly, 1997), but is consistent with experimental results (C. Macgregor,
504 unpub.). Within this underlying temperature-size relationship, there is also an additional effect of
505 season length and development time, as reflected by the observed reduction in size with number
506 of generations. A limitation of using ambient temperature as a measure of temperature during
507 development is that the micro-climate that individuals experience can be significantly modified
508 by behaviour and other environmental factors, such as humidity and sunlight. Under fast
509 demographic change, as in the range expansion of *P. aegeria*, the process of spatial selection
510 may override selection pressures from environmental gradients. For example, in the Scottish
511 expansion, wing size has responded more strongly to selection for dispersal than to the
512 environmental gradients (all recently colonised site are noticeably larger than the core
513 populations, irrespective of latitude and temperature).

514

515 The shape of forewings and hindwings, independent of size, were found to change during the
516 range expansion process and with increasing latitude. Forewings are more rounded and
517 hindwings are narrower in more recently colonised populations. Spatial selection during a range
518 expansion often results in increased dispersal ability towards the leading edge (Phillips, Brown &
519 Shine, 2010). The finding that the number of years colonised has the strongest loading to shape
520 in both the forewings and hindwings suggests these shapes are more dispersive in this species.
521 However, interpreting the functional consequences of fine-scale wing shape variation for

522 different dimensions of flight performance (e.g., dispersal efficiency, acceleration,
523 manoeuvrability) and tying shape changes to adaptive evolution is a complex and currently an
524 unresolved problem (Le Roy, Debat & Llaurens, 2019). In fact, large, long and more pointed
525 forewings are often considered the most dispersive form as this is associated with (less
526 metabolically costly) gliding flight and migratory species, e.g. the Monarch butterfly (*Danaus*
527 *plexippus*; Altizer & Davis, 2010; Flockhart et al., 2017). However, this assumption may not
528 hold true for all species. For example, in the Glanville Fritillary (*Melitaea cinxia*), dispersive
529 females have more rounded wings (Breuker, Brakefield & Gibbs, 2007), whilst another study
530 found little or no evidence linking wing morphology to dispersal ability (Hanski et al., 2002).

531
532 In *P. aegeria*, the female is thought to be the more dispersive sex (Shreeve, 1986) and, in
533 general, is larger with more rounded wings compared to males (Pellegroms, Van Dongen & Van
534 Dyck, 2009). Mate location strategy in male *P. aegeria* varies between two behaviours, perching
535 or patrolling, requiring different flight dynamics (Shreeve, 1984). Perchers require high
536 acceleration to defend a territorial sunlit patch and intercept passing females, whereas patrollers
537 require attributes for more sustained flight as they move from one spot to another in search of a
538 female. The differing flight requirements of these behaviours is reflected in their thorax size,
539 wing morphology and colour (Van Dyck, Matthysen & Dhondt, 1997; Van Dyck & Matthysen,
540 1998; Berwaerts, Van Dyck & Aerts, 2002). An increase in forewing roundness was also found
541 in *P. aegeria* males across a large latitudinal (700km) gradient from France to Netherlands,
542 where a decrease in aspect ratio (AR; calculated as $4 \times \text{forewing length}^2 / \text{forewing area}$), was
543 found further north (Vandewoestijne & Van Dyck, 2011). The AR is widely used as a predictor
544 of flight performance but does not account for allometry, as in the geometric morphometric
545 approach used in this study. Therefore, comparisons between studies based on AR and this one
546 should be considered carefully. Nonetheless, Vandewoestijne and Van Dyck's finding supports
547 the pattern seen here, suggesting a common trend in wing shape with increasing latitude in this
548 species.

549
550 The majority of studies focus solely on forewing shape. Although butterflies are mostly
551 antermotoric (require, and predominantly use, forewings for flight), hindwings increase linear
552 and turning acceleration, so are particularly important for aerial agility and predator evasion

553 (Jantzen & Eisner, 2008). The distinct roles of wings in flight, coupled with our finding that
554 shape changes are different between forewings and hindwings, suggest that evolutionary factors
555 may differentially affect forewing and hindwing shape. This study highlights the need to include
556 both wings in future studies.

557

558 **Colour and pattern variation**

559 *Pararge aegeria* has been previously described as becoming darker further north in Britain
560 (Dennis & Shreeve, 1989). However, using quantitative measurements and a more spatially
561 extensive sample, our results do not follow the expectation from the thermal melanism
562 hypothesis (i.e., decreasing lightness with latitude). Lightness fluctuates with latitude producing
563 a wave-like pattern that is consistent across both wings. The basal area becomes lighter with
564 latitude, whereas average lightness decreases with temperature during development (darker with
565 increasing temperature). Increased lightness at cooler temperatures during development could
566 support an energetic trade-off between overall growth and melanin production, which is costly to
567 synthesise (Talloe, Van Dyck & Lens, 2004). The production of melanin may be subject to
568 selection pressures unrelated to thermoregulation (True, 2003). The level of melanism has
569 implications for disease resistance (Wilson et al., 2001; Dubovskiy et al., 2013) UV protection
570 (Bishop et al., 2016; Katoh, Tatsuta & Tsuji, 2018), predation (Bond & Kamil, 2002) and sexual
571 selection (Jiggins et al., 2001; Kemp, 2007). Our samples are of male individuals and so changes
572 in colour due to selection for thermoregulatory properties (increased melanism) are likely to be
573 constrained by sexual selection (Tuomaala, Kaitala & Rutowski, 2012). Behavioural traits such
574 as posture during basking, which was not assessed in this study, also effect thermal regulation
575 (Kingsolver, 1985; Berwaerts et al., 2001). In *P. aegeria*, wing colour is associated with
576 alternative mate-location strategies, with perchers being lighter in colour than patrollers (Van
577 Dyck & Matthysen, 1998). The sample used here is likely biased towards perchers due to the
578 increased likelihood of spotting and netting perching individuals. Furthermore, previous studies
579 that find decreased lightness with latitude also include thorax colour which was not possible in
580 this study (Zeuss et al., 2014).

581

582 The perceived darkening of *P. aegeria* at higher latitudes (as reported by recorders and the
583 authors of this study) is probably due to the relative changes of brown and cream areas on the

584 forewing. The area of brown colour increases significantly with latitude, which may also have
585 thermal regulatory consequences. The strong correlation between the lightness of brown and
586 cream areas is indicative of an underlying genetic and developmental mechanism controlling the
587 ‘background’ production of melanin across the whole wing surface that is also sensitive to
588 environmental cues during the larval or pupal stages. Nevertheless, the cream colour increases in
589 lightness significantly faster than the brown colour, resulting in an overall increase in contrast
590 between the brown and cream patches. Few studies have looked at the effect of pattern on
591 thermoregulation, but the wing band pattern in Banded Peacock (*Anartia fatima*) has been shown
592 to slow the rate of heating but not the overall thoracic temperature equilibrium (Brashears, Aiello
593 & Seymoure, 2016). The consequences of the specific traits detailed here on thermal properties
594 of *P. aegeria* wings have not been studied to date, and so conclusions relating these findings to
595 the thermal regulation should be made with care.

596

597 **Conclusion**

598 We have shown that the rapid expansion of *P. aegeria* across a temperature gradient in a
599 spatially fragmented landscape is associated with shifts in morphological traits that are
600 differentially affected by the environmental and demographic factors studied. Wing size and
601 shape are most strongly linked to latitude, following Bergmann’s rule, and colonisation,
602 consistent with selection on dispersal. The spatial distribution of average lightness is only weakly
603 related to latitude and more associated with a plastic response to temperature during
604 development, which on the surface would appear to run contrary to the thermal melanism
605 hypothesis. Interpretation of the patterns observed here must take account of the dynamic nature
606 of this recent and ongoing range expansion. Populations are likely to be changing phenotypically
607 through adaptation to local environmental conditions and secondary immigration. Genetic drift
608 associated with colonisation, and evolutionary time-lags, may also account for some of the high
609 variance in phenotype-environment associations. The non-equilibrium state of many of the local
610 populations sampled, and an overriding role of selection for traits linked directly to range
611 expansion, may explain some of the weak phenotype-environment associations observed. The
612 planned application of genetic markers to this sample will help disentangle the roles of
613 developmental plasticity, selection, genetic drift and gene flow on these morphological traits.

614

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626

627

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