Title page

Statement of authorship

Study was designed by EAJ, AJB, and ECT. Data were collected by EAJ, MPH, KW, AJB, and ECT. EAJ conducted analyses and wrote the first draft of the manuscript. All authors contributed substantially to revisions.

Data accessibility statement

Data and associated code has been provided as Supplementary Materials to be published alongside the manuscript.

Article title: Day-flying Lepidoptera larvae have a poorer ability to thermoregulate than adults

Running title: Thermoregulation across the life cycle

Keywords: Butterfly, life cycle, life stage, temperature, thermal ecology, thermoregulation

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Abstract

Changes to ambient temperatures under climate change may detrimentally impact small ectotherms that rely on their environment for thermoregulation, however there is currently a limited understanding of insect larval thermoregulation. As holometabolous insects, Lepidoptera differ in morphology, ecology, and behaviour across the life cycle, and so it is likely that adults and larvae differ in their capacity to thermoregulate. In this study we investigate the thermoregulatory capacity (buffering ability) of 14 species of day-flying Lepidoptera, whether this is influenced by body length or gregariousness, whether it differs between adult and larval life stages. We also investigated what thermoregulation mechanisms are used; microclimate selection (choosing locations with a particular temperature) or behavioural thermoregulation (controlling temperature through other means, such as basking). We found that Lepidoptera larvae differ in their buffering ability between species and body lengths, but gregariousness did not influence buffering ability. Larvae are worse at buffering themselves against changes in air temperature than adults. Therefore Lepidoptera may be more vulnerable to adverse temperature conditions during their larval life stage. Adults and larvae rely on different thermoregulatory mechanisms; adults primarily use behavioural thermoregulation, whereas larvae use microclimate selection. This implies that larvae are highly dependent on the area around their foodplant for effective thermoregulation. These findings have implications for the management of land and species, for example highlighting the importance of creating and preserving microclimates and vegetation complexity surrounding Lepidoptera foodplants for larval thermoregulation under future climate change.

Introduction

Climate change is a threat to species and ecosystems worldwide, with predicted impacts including rising average temperatures and an increasing frequency and intensity of extreme weather events (IPCC, 2014). These changes have wide-ranging impacts on many taxa, particularly small ectothermic organisms, such as insects, that rely on ambient conditions for thermoregulation (Elias 1991). The impacts of climate change on insects include changes in behaviour, development, synchronicity of ecological interactions, and survival (Bale *et al.* 2002).

Lepidoptera (butterflies and moths) are well-studied, diverse and widespread, have short generation times, and are ecologically sensitive to environmental change (Hill & Scheffers, 2021), in particular showing clear and detectable responses to temperature change (Roy & Sparks 2000). This makes them a valuable group for investigating the effects of temperature. Lepidoptera also have complex life cycles with specific habitat requirements at different stages (Kingsolver *et al.* 2011), however the majority of evidence on the effects of temperature comes from the adult life stage (Radchuk *et al.* 2013). As life stages can respond differently to temperature (Radchuk *et al.* 2013), there is an inherent risk in considering only the response of the adults, as this may not accurately reflect responses to temperature change across other life stages.

A previous study on adult British butterflies identified traits that influence their ability to thermoregulate (maintain a stable body temperature across a range of air temperatures; thermal buffering ability), with species with larger wings and those in the family Pieridae having the strongest buffering abilities, compared to smaller species and those in the family

Nymphalidae (Bladon *et al.* 2020). This pattern is also reflected in a community of tropical butterflies (Ashe-Jepson *et al.* 2023), which also identified colour as an important factor, whereby butterflies with dark wings had stronger thermal buffering abilities than pale butterflies. This implies that these traits (taxonomic family, size, and colour) could play a relatively consistent role in adult butterfly thermoregulation. A strong buffering ability could correspond to greater climate resilience, as individuals are able to elevate their body temperature in cold conditions, which would benefit larval development (Hong *et al.* 2014) and adult flight (Nève & Hall 2016), but lower their body temperature in hot conditions, which would prevent irreversible protein denaturation, unsustainable rises in metabolism, and other processes that would otherwise result in reduced survival and reproductive success (Heath *et al.* 1971; González-Tokman *et al.* 2020; Svensson *et al.* 2020). However it is also possible that a strong buffering ability may inhibit the evolution of tolerance to non-optimal temperatures (Ashe-Jepson *et al.* 2023), however to date this has not been investigated for Lepidoptera larvae.

Lepidopteran life stages differ in morphology, behaviour, and habitat use, which likely contributes to different thermal ecologies (MacLean *et al.* 2016). Day-flying adults can alter their body temperature using various mechanisms, including basking in the sun, using their wings to absorb solar radiation directly, or by reflecting solar radiation onto their body ("behavioural thermoregulation") (Watt 1968; Shanks *et al.* 2015). Adult butterflies can also increase or maintain their body temperature through flight metabolism (Mattila 2015). To cool down, adults can alter the circulation in their wings to radiate more heat (Tsai *et al.* 2020), or angle their wings parallel to the sun. These thermoregulation strategies allow adult butterflies to alter their body temperature independently from their immediate surrounding

area. Adults are also able to use flight to exploit microclimates over a wide area, choosing cooler microclimates to cool down and warmer microclimates to heat up ("microclimate selection") (Clench 1966). This strategy is dependent on the temperature conditions of the area surrounding the butterfly. Bladon *et al.* (2020) found inter-specific variation between these thermoregulatory strategies, with an overall tendency for adult butterflies to use behavioural thermoregulation over microclimate selection. In contrast, although Lepidoptera larvae can also bask to elevate their body temperature (Porter 1982; Karban 1998; Turlure *et al.* 2011), they lack wings and so have a smaller surface area for solar absorption, and lack the capacity to use these structures to radiate excess heat. They also have reduced mobility compared to adults, and so are only able to exploit microclimates within a restricted area. As a result, they may have reduced ability to maintain their body temperature within a tolerable range. It is also possible that butterflies differ in their thermal optima across the life cycle, for example for development as larvae and flight as adults. However, both life stages need to buffer their body temperature in order to achieve these temperatures. Previous studies have shown that Lepidoptera larvae are able to use these two strategies for thermoregulation, for example by using nearby microclimates to prevent overheating (Nice & Fordyce 2006), or behaviourally thermoregulating, such as basking in direct sunlight, to elevate their body temperature above ambient conditions (Joos *et al.* 1988). The ecological and morphological traits of the larval life stage are likely to also influence their ability to thermoregulate. For example, larvae change in size dramatically as they develop, unlike adults, and this is likely to alter the rate at which they gain and lose heat (Nielsen & Papaj 2015). Similarly, gregarious behaviour has been shown to alter the body temperatures of Lepidoptera larvae recorded in the field, with gregarious larvae showing higher and more stable body temperatures than solitary larvae (Bryant *et al.* 2000). However, these studies tend to be species-specific, and

may not reflect variation across a community. Additionally, little has been done to compare the use of these strategies across life stages.

Here, we investigate the thermal buffering ability (the ability to maintain body temperature across different air temperatures) and thermoregulatory mechanisms of 12 butterflies and two day-flying moths, comparing between larvae and adults. This builds upon our previous understanding of adult Lepidoptera thermoregulation (Bladon *et al.* 2020) by focusing on and comparing with the under-studied larval life stage.

Specifically, we address the following questions:

1. What is the range of species-specific thermal buffering ability across 14 day-flying Lepidoptera species as larvae, and is this influenced by family, size, colour, or gregarious behaviour?
2. Does thermal buffering ability differ between adults and larvae of the same species?
3. Do larvae differ in their use of microclimate selection or behavioural thermoregulation to control their body temperature across species?
4. Does use of microclimate selection and behavioural thermoregulation to control temperature differ between adults and larvae of the same species?

Methods

Study sites

Data on 12 species of day-flying Lepidoptera larvae (ten butterflies, two moths) were collected across four grassland nature reserves in Bedfordshire, UK: Totternhoe Knolls, Totternhoe Quarry, Pegsdon Hills, and Blows Downs (Figs S1, S2, Table 1). All sites are

heterogeneous calcareous grasslands that contain a mixture of open grassland and encroaching scrub, and support a diverse Lepidopteran community. Data on two species of generalist butterflies (*Pieris brassicae*, *Pieris rapae*), that visit the four reserves as adults but do not breed there in large numbers, were collected at an allotment in Girton, Cambridgeshire (Girton allotments, 52.235820, 0.084180). All surveys took place with the permission of and in collaboration with the Wildlife Trust of Bedfordshire, Cambridgeshire, and Northamptonshire, who own or manage all nature reserves surveyed, and with the permission of Girton Allotment Society for the allotment site. All data was collected in spring or summer months, and so ambient conditions reflect the natural conditions these species experience during development or reproduction but are unlikely to reflect the coldest temperatures these species experience. Data was also collected during the heatwave of 2022, when temperatures were high enough for adult butterflies to become inactive.

Body temperature recordings

Larvae were located during focused searches of their foodplants at peak times of the year in the summers of 2020, 2021, and 2022, focusing on reserves which hosted high numbers of each species (Table 1). When a larva was found, its body temperature was recorded without handling using a thermocouple with a handheld indicator (Tecpel Thermometer 305B), by gently pressing the probe onto the larva's dorsal surface without damaging it ("body temperature"). The surface temperature of the plant the larva was found on ("surface temperature") was then recorded, followed by the air temperature at waist height in shade ("air temperature"), both using the same thermocouple. We also recorded body length (using callipers), and colour (on a 1-6 scale, where 1 is almost white and 6 is almost black, adapted from Bladon *et al.* 2020) for each individual. For the three species with gregarious instars

(*Aglais io, Aglais urticae, Pieris brassicae*), we recorded whether they were gregarious at the time of recording (binary variable), and limited temperature recordings to a maximum of 10 randomly selected individuals per larval web, to avoid individual large groups having an undue influence on our dataset.

Adult data were taken from a combination of published datasets from Bladon *et al.* (2020) and Hayes & Turner (2023) all collected using similar protocols, with the majority coming from the same four nature reserves in the following years: 2009, 2018, 2019, and 2022. Additional adult data (n = 50) were also previously collected from a similar chalk grassland nature reserve, Winterbourne Downs, near Salisbury, in 2018 (Bladon et al. 2020). In brief, butterflies were caught by hand in nets without chasing (which would artificially increase their body temperature). Once caught, butterflies were kept in shade and had their thoracic temperature recorded by pressing the thermocouple through the net against the thorax, without touching the butterfly, within 60 seconds of capture. Air temperature was then recorded at waist height in shade, using the same thermocouple. If the butterfly was first seen landed on a perch, a perch temperature was recorded using the same thermocouple. Butterflies were then released.

Table 1: List of 12 butterfly and two day-flying moth species sampled as larvae, with species traits, sample sizes, and dates and locations of surveys (BD = Blows Downs, PH = Pegsdon Hills, TK = Totternhoe Knolls, TQ = Totternhoe Quarry, GA = Girton allotments). Colour scores follow Bladon et al. 2020. Ordered alphabetically by family.

Family	Species	Body length (range) (cm)	Colour	Colour score	Gregarious instars	Adult sample size	Larva sample size	Sites surveyed	Dates of surveys

Erebidae	*Tyria jacobaeae*	0.76-2.60	Yellow-black	4	N	22	18	GA	Aug-21
Hesperiidae	*Erynnis tages*	0.20-0.81	Green	2	N	75	23	TQ	June-July, 2021 & 2022
Lycaenidae	*Cupido minimus*	0.15-0.90	Green/brown	3.5	N	134	43	TQ	July 2020, 2021, & 2022
Lycaenidae	*Polyommatus coridon*	0.45-1.29	Green-yellow	1.5	N	219	44	TQ	Jun-21
Pieridae	*Anthocharis cardamines*	0.61-2.75	Green	2	N	19	48	BD	Jun-21
Pieridae	*Gonepteryx rhamni*	0.43-3.12	Green	2	N	56	90	TK, TQ	Jun-22
Pieridae	*Pieris brassicae*	0.18-3.50	Green-black	4	Y	81	48	GA	August 2021 & 2022
Pieridae	*Pieris napi*	0.45-1.64	Green	2	N	139	10	BD	Aug-21
Pieridae	*Pieris rapae*	0.40-2.80	Green	2	N	203	33	GA	August 2021 & 2022
Nymphalidae	*Aglais io*	0.44-4.84	Black	6	Y	16	274	BD, PH, TK, TQ	June-July, 2021 & 2022
Nymphalidae	*Aglais urticae*	0.17-3.29	Green-black	4	Y	35	109	BD, PH, TK, TQ	June-July, 2021 & 2022
Nymphalidae	*Vanessa atalanta*	0.43-2.56	Black	6	N	10	24	BD, PH, TK, TQ	June-July, 2021 & 2022
Riodinidae	*Hamearis lucina*	0.44-1.76	Brown	5	N	43	26	TQ	June-July, 2021 & 2022
Zygaenidae	*Zygaena filipendulae*	1.40-2.44	Green-black	4	N	74	17	BD, PH, TK, TQ	Aug-21

Data analysis

All analysis took place in R version 3.6.1 (R Core Development Team, http://www.r-project.org). We only included species with a minimum of ten recordings of body temperature

206 across a minimum air temperature range of 10°C, to ensure that estimates of buffering ability
207 were accurate. Model assumptions were tested with the 'sjPlot' package (Lüdecke 2021).
208 Model assessment used the 'car' package (Fox & Weisberg 2019). Plots were produced using
209 the 'ggplot2' (Wickham 2016) and 'interactions' packages (Long 2019). To check whether
210 explanatory variables (species, body length, colour) were associated with each other before
211 fitting in models, one-way ANOVA tests were used between all pairs of explanatory variables.
212 Due to the strong association between species and colour ($R^2 = 0.99$, $p < 0.001$), colour was
213 excluded from further analysis (see Table S1).
214
215 *Species-specific buffering abilities*
216 To calculate the buffering ability of each species, we fitted simple linear regression models of
217 body temperature against air temperature for each species. The slope of this relationship was
218 used to estimate the ability of species to alter their body temperature in relation to air
219 temperature. To aid interpretation, slopes were subtracted from one, so that higher values
220 indicate a stronger ability to maintain a relatively stable body temperature across a wide
221 range of air temperatures.
222
223 *Larval traits and buffering ability*
224 To identify which model best suit the data, a phylogenetic generalised least square (PGLS) or
225 multivariate linear model, larval trait (body length) was tested for a phylogenetic signal across
226 species using the 'phytools' package (Revell 2012) and a phylogenetic tree from Wiemers *et*
227 *al.* (2020). Due to no significant phylogenetic signal ($p = 0.231$), a multivariate linear model
228 was used.
229

To investigate whether species' traits influenced buffering ability, we fitted a multivariate linear model with body temperature as the response variable, and air temperature, individual body length, and species as explanatory variables. We also included an interaction term between each trait and air temperature. Because of the high number of families represented by only a single species in our dataset (Erebidae, Hesperiidae, Riodinidae, Zygaenidae), only Nymphalidae (3 species) and Pieridae (5 species) could be used to assess whether family identity influenced buffering ability. For these two families, another multivariate linear model was fitted, with body temperature as the response variable, and air temperature, family, colour, individual body length and each two-way interaction with air temperature as explanatory variables. Model selection was conducted through backwards stepwise selection, to avoid suppressor effects, where non-significant terms were removed until a minimal model was achieved in which all remaining terms were significant. The retention of a two-way interaction between air temperature and a trait in the optimal model indicates that the trait is important in explaining thermal buffering ability.

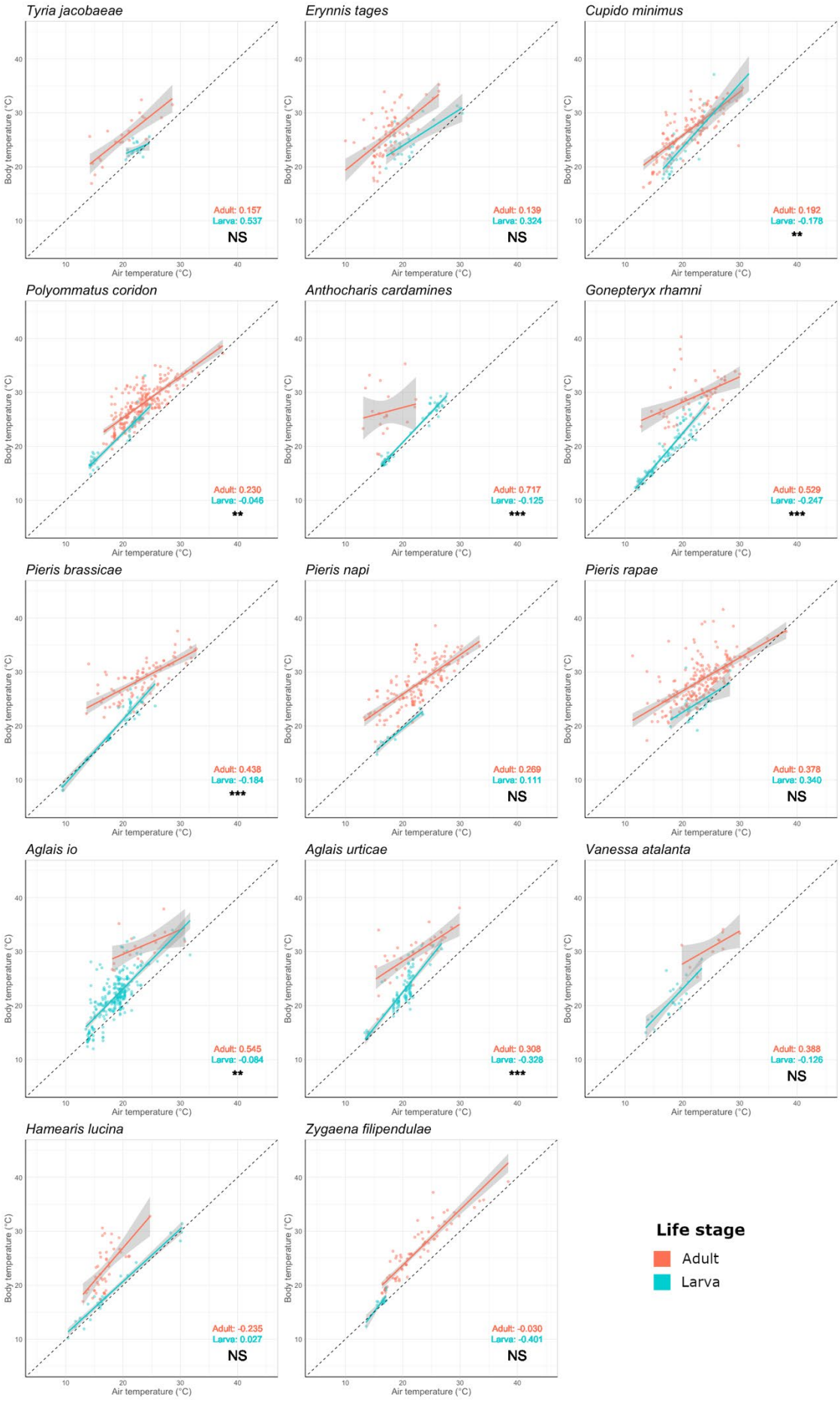
Gregarious species

To test whether gregarious versus solitary behaviour affects buffering ability, and to separate this from effects of body length, two species with gregarious instars (*A. urticae, P. brassicae*) were paired with similar but non-gregarious species within the same family (*A. urticae* with *V. atalanta, P. brassicae* with *P. rapae*) and tested individually. Although *A. io* is also gregarious, it was excluded from this analysis as there wasn't a suitable paired non-gregarious species available with similar traits known to influence thermal buffering abilities in butterflies (such as size, colour and family identity) (Bladon *et al.* 2020; Ashe-Jepson *et al.* 2023). We calculated a new size category variable for each pair, where the size of the largest

254 gregarious larva was used to define size categories ('large' for larvae above this length, 'small'
255 for larvae equal to or below this length). We then ran a multivariate linear regression per
256 species pair, with body temperature as the response variable, and air temperature, species,
257 and size category as explanatory variables, along with all two-way and the three-way
258 interaction terms. Model selection was conducted through backwards stepwise selection, as
259 described above. The retention of the three-way interaction would imply that the relationship
260 between size and buffering ability differs between gregarious and non-gregarious species,
261 indicating that gregariousness rather than size is influencing buffering ability.
262
263 *Comparing adult and larval thermal buffering abilities*
264 To compare the buffering ability of larvae and adults of the same species, adult body
265 temperature and air temperature data were taken from Bladon et al. (2020) and Hayes &
266 Turner (2023), and combined with larval data of the same species. Analysis was restricted to
267 14 species with more than ten body temperature records in each life stage to ensure that
268 estimates were accurate.
269
270 To test whether buffering ability differs between life stages, a multivariate linear regression
271 was run across all 14 species, with body temperature as the response variable, and air
272 temperature, species, and life stage as explanatory variables, with each two-way interaction
273 between air temperature and the other explanatory variables. Model selection was
274 conducted through backwards stepwise elimination, as described above. To test for species-
275 specific differences in buffering ability between life stages, individual multivariate linear
276 regressions were run per species, with body temperature as the response variable, and air

277 temperature and life stage as explanatory variables, with a two-way interaction between
278 explanatory variables.
279
280 *Thermoregulatory strategies: microclimate selection or behavioural thermoregulation*
281 To quantify the reliance on different thermoregulatory strategies across species, we
282 calculated two values following Bladon *et al.* (2020). Firstly, we calculated the difference
283 between the temperature of the surface on which the larva was found, and ambient air
284 temperature (hereafter 'microclimate selection'). Averaged across individuals, this value
285 describes the capacity of a species to select microclimates that differ in temperature from
286 ambient conditions. A positive value would indicate that the microclimate the larva occupied
287 was warmer than ambient conditions, whereas a negative value would indicate the larva
288 occupied a microclimate that was cooler than ambient conditions.
289
290 Secondly, we calculated the difference between larval body temperature and the
291 temperature of the surface where they were found (hereafter 'behavioural
292 thermoregulation'). This value describes the capacity of a species to alter their body
293 temperature relative to their chosen microclimate, through behaviours such as basking. A
294 positive value would indicate that the larva was warmer than the microclimate it occupied,
295 whereas a negative value would indicate that the larva was cooler.
296
297 To test how microclimate selection and behavioural thermoregulation differed across varying
298 ambient conditions in larvae, and whether this capacity was influenced by species identity,
299 body length, or colour, we fitted two multivariate linear models, with microclimate selection
300 or behavioural thermoregulation as response variables, and air temperature, species, and

301 body length as explanatory variables. Interaction terms were included between all
302 explanatory variables and air temperature. Model selection was conducted through
303 backwards stepwise elimination, as previously described.
304
305 To test how microclimate selection and behavioural thermoregulation differed between life
306 stages, we fitted two multivariate linear models, with microclimate selection or behavioural
307 thermoregulation as response variables, and air temperature, species, and life stage as
308 explanatory variables. Interaction terms were included between all explanatory variables and
309 air temperature. We restricted the data to eight species with a minimum of ten surface
310 temperature records in each life stage (*C. minimus, P. coridon, G. rhamni, P. napi, P. brassicae,*
311 *P. rapae, A. urticae, H. lucina*). Model selection was conducted through backwards stepwise
312 elimination, as previously described.
313
314 **Results**
315 In total, 1933 butterflies were recorded, 1126 of which were at the adult life stage, and 807
316 of which were at the larval life stage.
317
318 *Thermal buffering abilities of larvae*
319 Buffering abilities differed by taxonomy and morphology, but not ecology. Buffering abilities
320 for Lepidopteran larvae varied across species, and ranged from -0.401 (*Z. filipendulae*) to
321 0.537 (*T. jacobaeae*) (Fig 1).
322

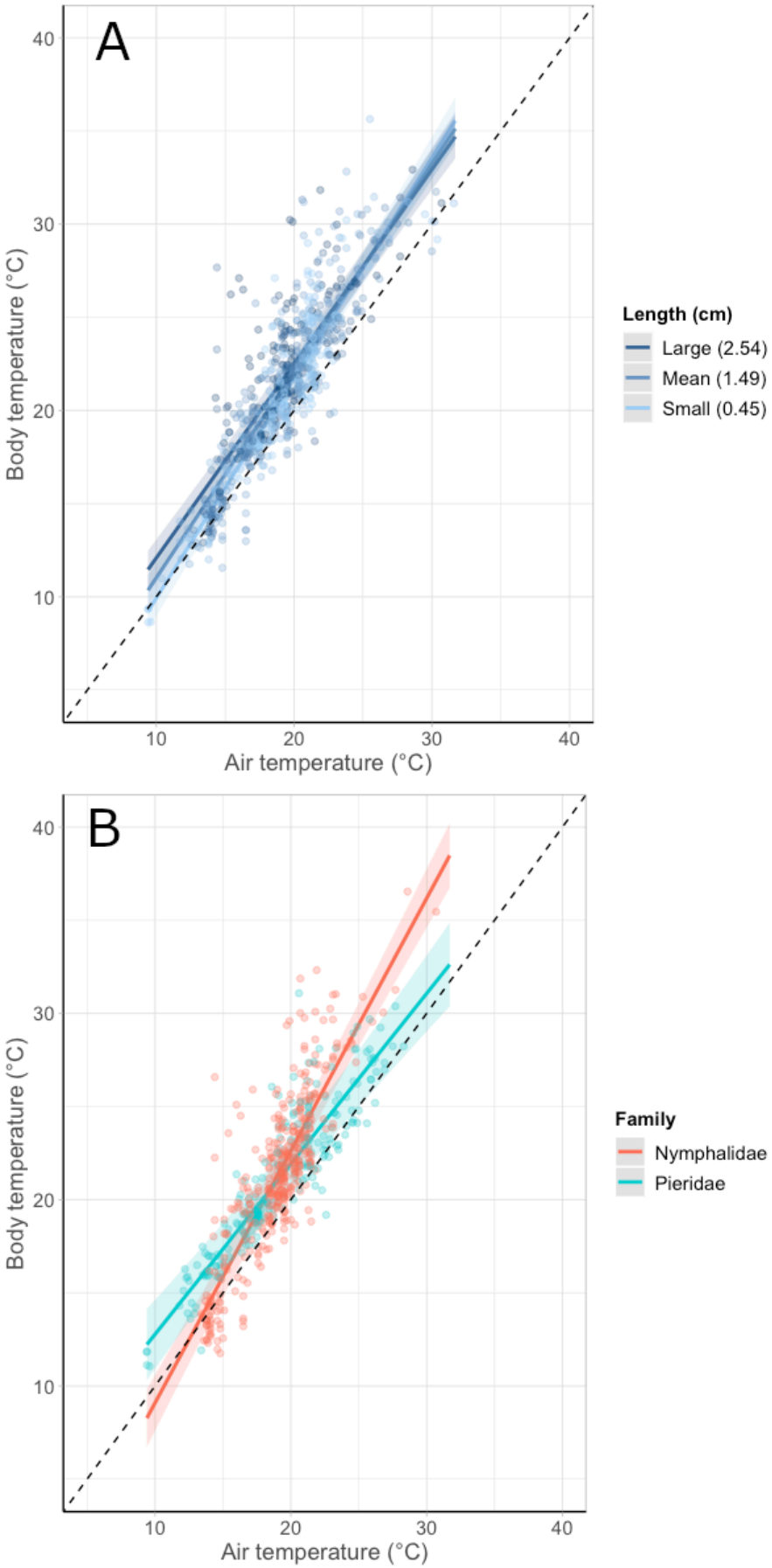

Tyria jacobaeae
Adult: 0.157
Larva: 0.537
NS
Erynnis tages
Adult: 0.139
Larva: 0.324
NS
Cupido minimus
Adult: 0.192
Larva: -0.178
**
Polyommatus coridon
Adult: 0.230
Larva: -0.046
**
Anthocharis cardamines
Adult: 0.717
Larva: -0.125
***
Gonepteryx rhamni
Adult: 0.529
Larva: -0.247
***
Pieris brassicae
Adult: 0.438
Larva: -0.184
***
Pieris napi
Adult: 0.269
Larva: 0.111
NS
Pieris rapae
Adult: 0.378
Larva: 0.340
NS
Aglais io
Adult: 0.545
Larva: -0.084
**
Aglais urticae
Adult: 0.308
Larva: -0.328
***
Vanessa atalanta
Adult: 0.388
Larva: -0.126
NS
Hamearis lucina
Adult: -0.235
Larva: 0.027
NS
Zygaena filipendulae
Adult: -0.030
Larva: -0.401
NS
Body temperature (°C)
Air temperature (°C)
Life stage
Adult
Larva

323

Figure 1: The relationship between air temperature (°C) and body temperature (°C) for 14 species of day-flying British Lepidoptera, split by life stage (adult and larva). Points show individuals. Coloured lines show the linear regression between air and body temperature, limited to the temperature ranges recorded (red = adult, blue = larva). Shaded areas show 95% confidence intervals. Dashed lines show a 1:1 relationship to aid visual comparison between species. Axes are standardised between plots. Panels are ordered alphabetically by family. Buffering ability estimates for each life stage are shown on each plot (calculated as one minus the regression slope, so that a large value indicates a strong thermal buffering ability, see Methods). Significant differences between regression slopes are denoted with NS = non-significant, * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.001$. Reported p-values testing the interaction term of life stage in each model.

Buffering ability differed significantly between species ($F = 5.18$, d.f. = 13, $p < 0.001$) and large larvae had stronger buffering abilities than small larvae ($F = 12.51$, d.f. = 1, $p < 0.001$) (Fig 2A, Table S2). Pieridae larvae had a stronger buffering ability than Nymphalidae larvae ($\chi^2 = 10.51$, d.f. = 1, $p = 0.001$) (Fig. 2B, Table S3).

The effect of body length on buffering ability did not differ between gregarious and non-gregarious species pairs (*P. brassicae* and *P. rapae*: $\chi^2 = 0.52$, d.f. = 1, $p = 0.696$. *A. urticae* and *V. atalanta*: $\chi^2 = 5.70$, d.f. = 1, $p = 0.210$, Table S4). Between *P. brassicae* and *P. rapae*, there was a significant difference in the effect of size on buffering ability ($F = 16.28$, d.f. = 1, $p < 0.001$), however this relationship did not differ between species ($F = 0.15$, d.f. = 1, $p = 0.696$). Between *A. urticae* and *V. atalanta*, though the buffering ability differed between species ($F = 0.78$, d.f. = 1, $p = 0.047$), this was not related to size ($F = 1.59$, d.f. = 1, $p = 0.210$).

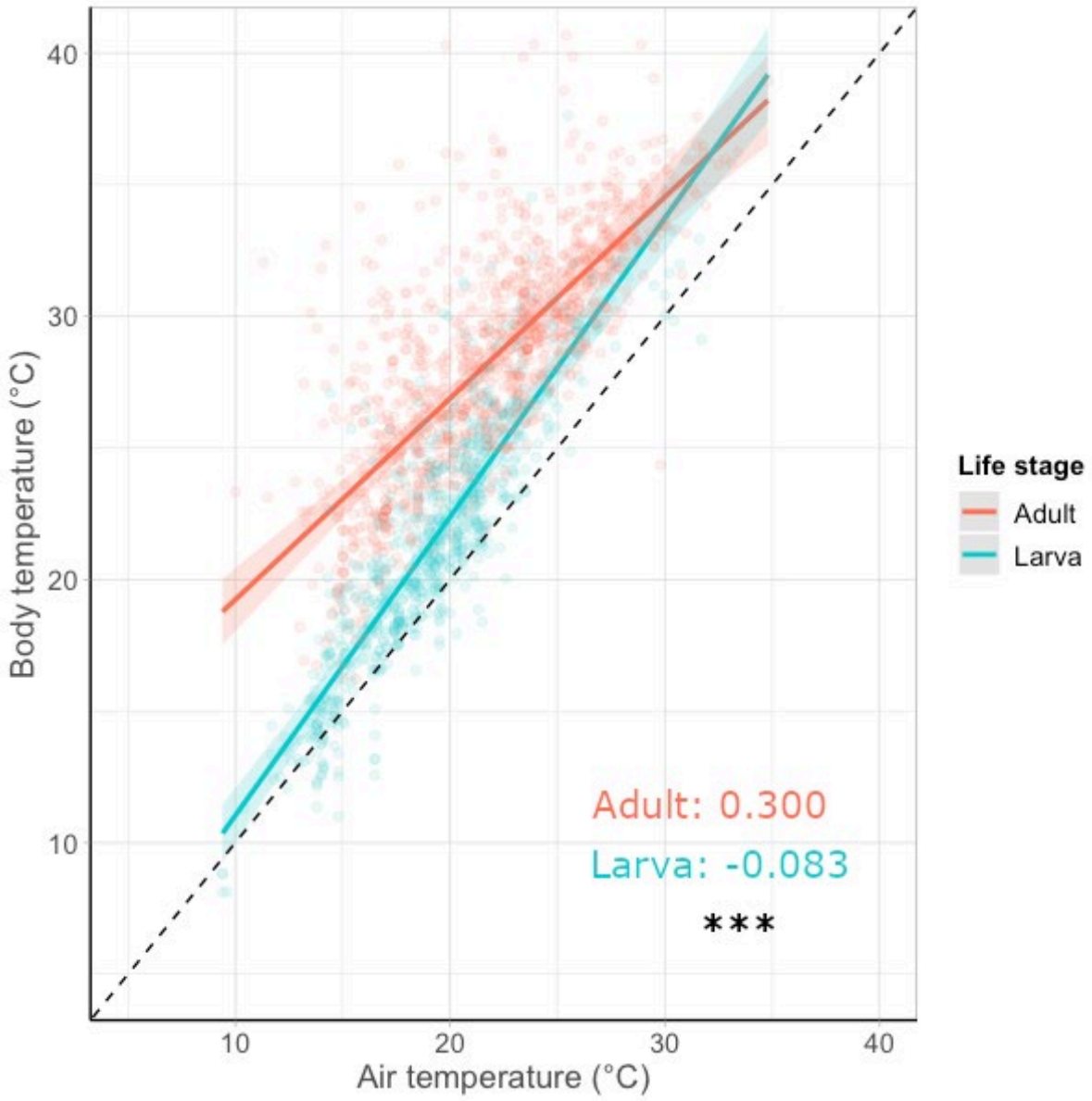

A
B
Body temperature (°C)
Air temperature (°C)
10
20
30
40
Length (cm)
Large (2.54)
Mean (1.49)
Small (0.45)
Family
Nymphalidae
Pieridae

348

Figure 2: The relationship between air temperature (°C) and body temperature (°C) for 14 species of day-flying British Lepidoptera as larvae, split by (A) body length (in cm, modelled as a continuous variable but split into three groups for plotting: large (2.54 cm), mean length (1.51 cm), and small (0.49 cm)), and (B) two families of British butterflies as larvae (Nymphalidae: 3 species, 387 individuals. Pieridae: 5 species, 229 individuals). Lines represent predicted values restricted to the range of temperatures observed. Points represent partial residuals (observed data points of individual butterflies with the effects of other variables accounted for). Shaded areas show 95% confidence intervals. Dashed lines show a 1:1 relationship to aid visual comparison between groups.

Thermal buffering abilities across life stages

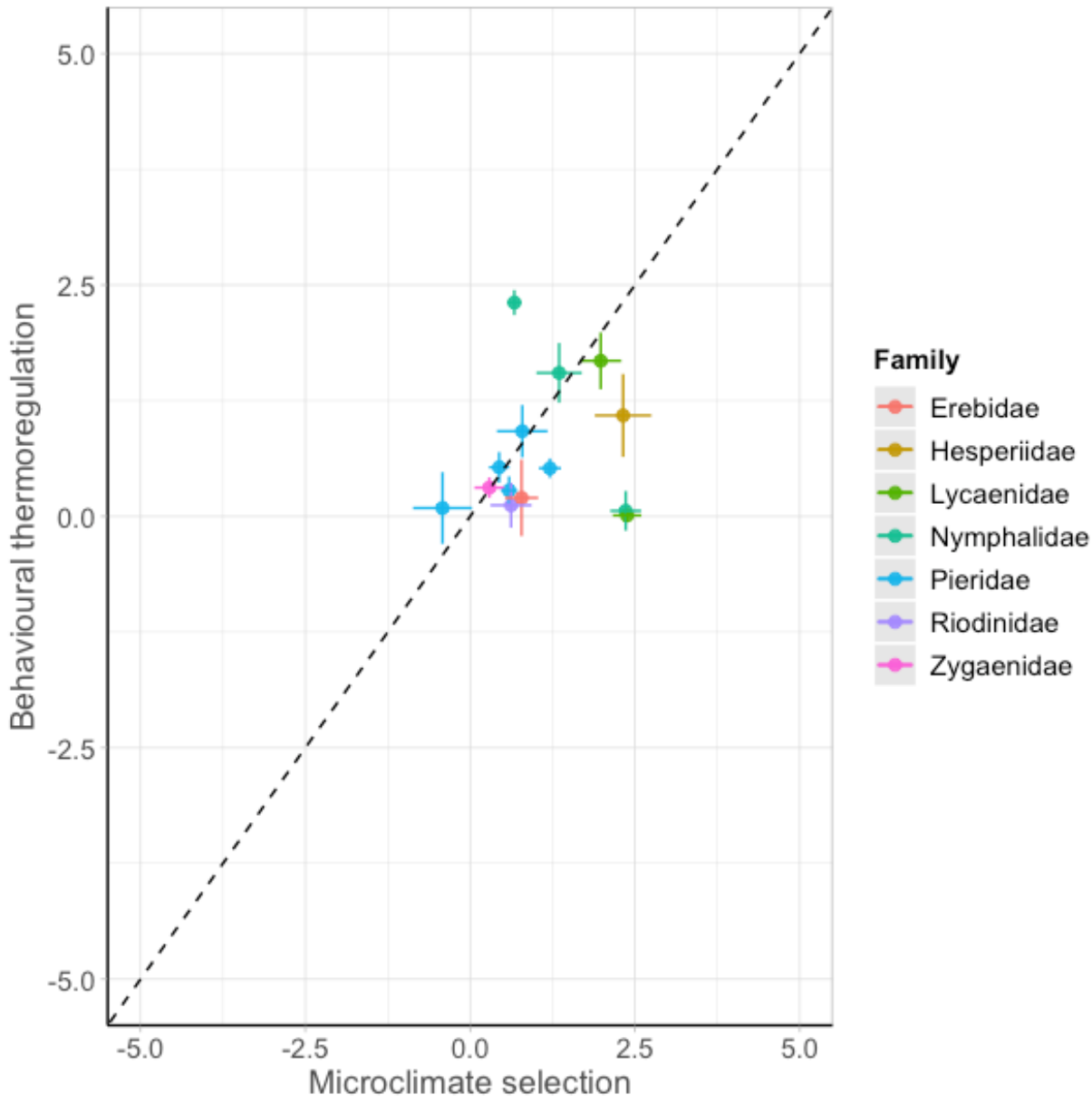
Across all species, buffering ability differed significantly between life stages, with larvae being poorer at buffering their body temperature against changes in air temperature than adults, particularly in their ability to warm up in cooler air temperatures ($F = 83.57$, d.f. = 1, $p < 0.001$) (Fig 1, 3).



365

366 Figure 3: The relationship between air temperature (°C) and body temperature (°C) for 14
367 species of day-flying British Lepidoptera, split by life stage. Points represent partial residuals
368 (observed data points of individual butterflies with the effects of the other variables
369 accounted for). Coloured lines show the linear regression between air and body temperature
370 per life stage, limited to the temperature ranges recorded. Shaded areas show 95%
371 confidence intervals. Dashed lines show a 1:1 relationship to aid visual comparison between
372 groups. Buffering ability estimates for each life stage are shown (calculated as one minus the
373 regression slope, so that a large value indicates a strong thermal buffering ability, see

374 Methods). Significant differences between regression slopes are denoted with NS = non-
375 significant, * = p < 0.05, ** = p < 0.005, *** = p < 0.001. Reported p-values testing the
376 interaction term of life stage in each model.
377
378 *Microclimate selection and behavioural thermoregulation*
379 The use of microclimate selection and behavioural thermoregulation varied widely across
380 species. Larval microclimate selection ranged from -0.42 (*P. napi*) to 2.38 (*P. coridon*), while
381 behavioural thermoregulation ranged from 0.01 (*P. coridon*) to 2.31 (*A. io*) (Fig 4).
382
383 The use of microclimate selection differed across air temperatures between species (F = 4.37,
384 d.f. = 13, p < 0.001) (Fig S3) and between larvae of different lengths (F = 6.78, d.f. = 1, p =
385 0.009) (Fig S4, Table S5). Small larvae occupied increasingly warm microclimates as air
386 temperature increased, whereas large larvae occupied relatively stable microclimates.
387
388 The use of behavioural thermoregulation differed across air temperatures between species
389 (F = 1.99, d.f. = 13, p = 0.019, Fig S5, Table S5), but did not differ between larvae of different
390 sizes (F = 3.04, d.f. = 1, p = 0.081).
391



392

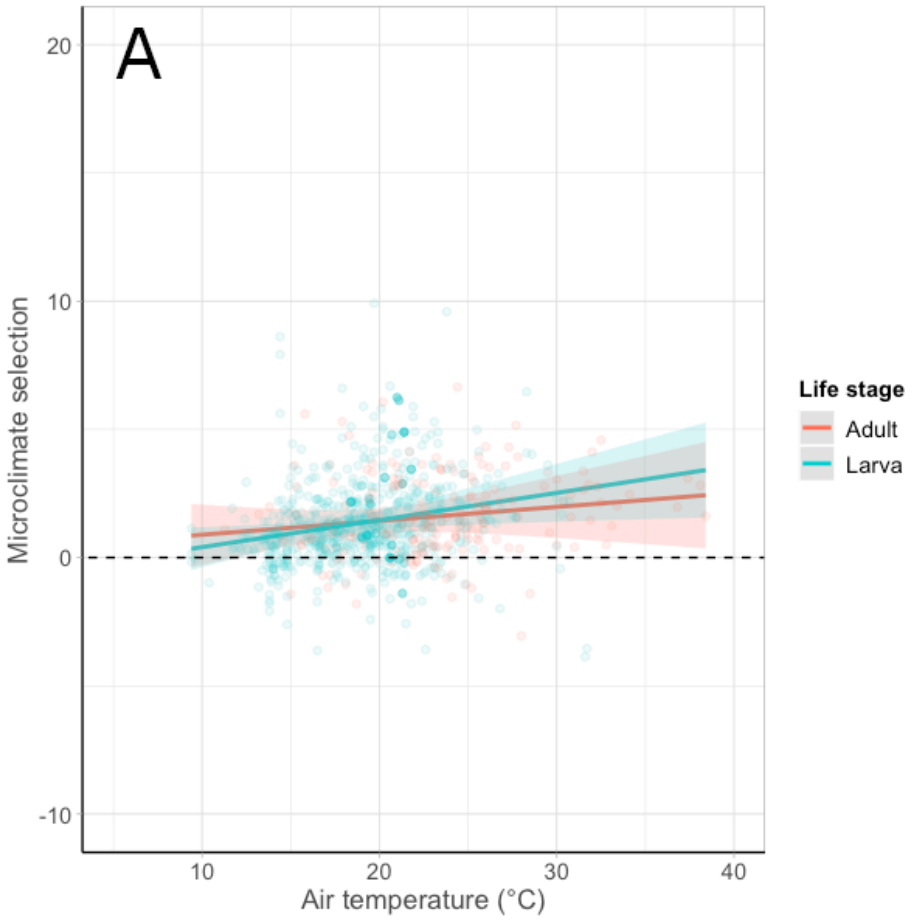
393 Figure 4: The relationship between microclimate selection (the difference between surface
394 temperature and air temperature) and behavioural thermoregulation (the difference
395 between body temperature and surface temperature) for 14 species of day-flying British
396 Lepidoptera, coloured by family (Erebidae: 1 species, Hesperiidae: 1 species, Lycaenidae: 2
397 species, Nymphalidae: 3 species, Pieridae: 5 species, Riodinidae: 1 species, Zygaenidae: 1
398 species). Points show mean values for each species, ± 1 standard error. Dashed lines show a
399 1:1 relationship to aid visual comparison between species.

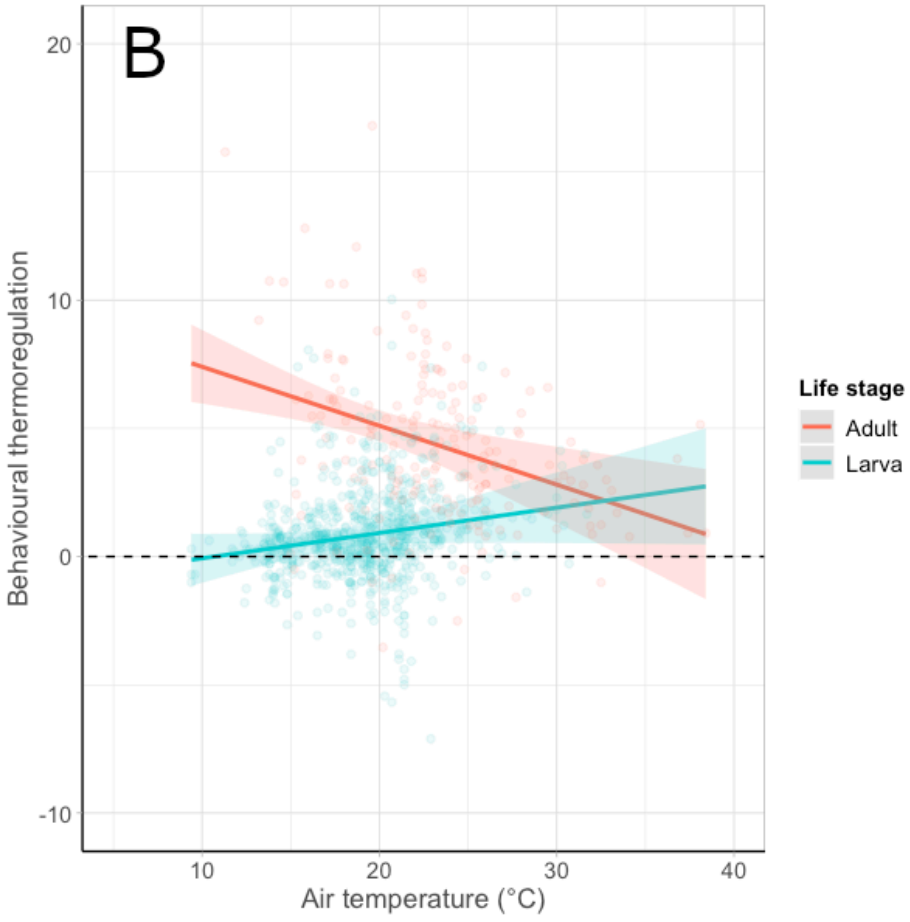
400

Microclimate selection and behavioural thermoregulation across life stages

The use of microclimate selection across air temperatures did not differ between life stages ($F = 1.65$, d.f. = 1, $p = 0.199$), but behavioural thermoregulation did ($F = 44.98$, d.f. = 1, $p < 0.001$) (Fig 5). At low air temperatures, adult butterflies were warmer than their chosen microclimate, and as the air temperature increased, the difference between their body temperature and microclimate temperature decreased. Contrastingly, the body temperature of larvae was similar to the microclimate they occupied at low air temperatures, and the difference between their body temperature and microclimate temperature increased as ambient conditions got warmer.

The use of these two strategies differed between life stages, whereby adult butterflies tended to rely more on behavioural thermoregulation, whereas larvae tended to rely on microclimate selection (Fig 6).


A
Microclimate selection
20
10
0
-10
10
20
30
40
Air temperature (°C)
Life stage
Adult
Larva


B
Behavioural thermoregulation
20
10
0
-10
10
20
30
40
Air temperature (°C)
Life stage
Adult
Larva

415

Figure 5: The relationship between (A) microclimate selection (the difference between microclimate temperature and ambient air temperature) and (B) behavioural thermoregulation (the difference between body temperature and microclimate temperature) across a range of air temperatures (°C) for 14 species of day-flying British Lepidoptera as adults and larvae. Points show data from individuals butterflies, and represent partial residuals (observed data points with the effects of the other variables accounted for). Lines represent predicted values restricted to the range of air temperatures observed. Shaded areas show 95% confidence intervals. The dashed horizontal line, indicating where no microclimate selection or behavioural thermoregulation is taking place, is marked to aid visual comparison between groups.

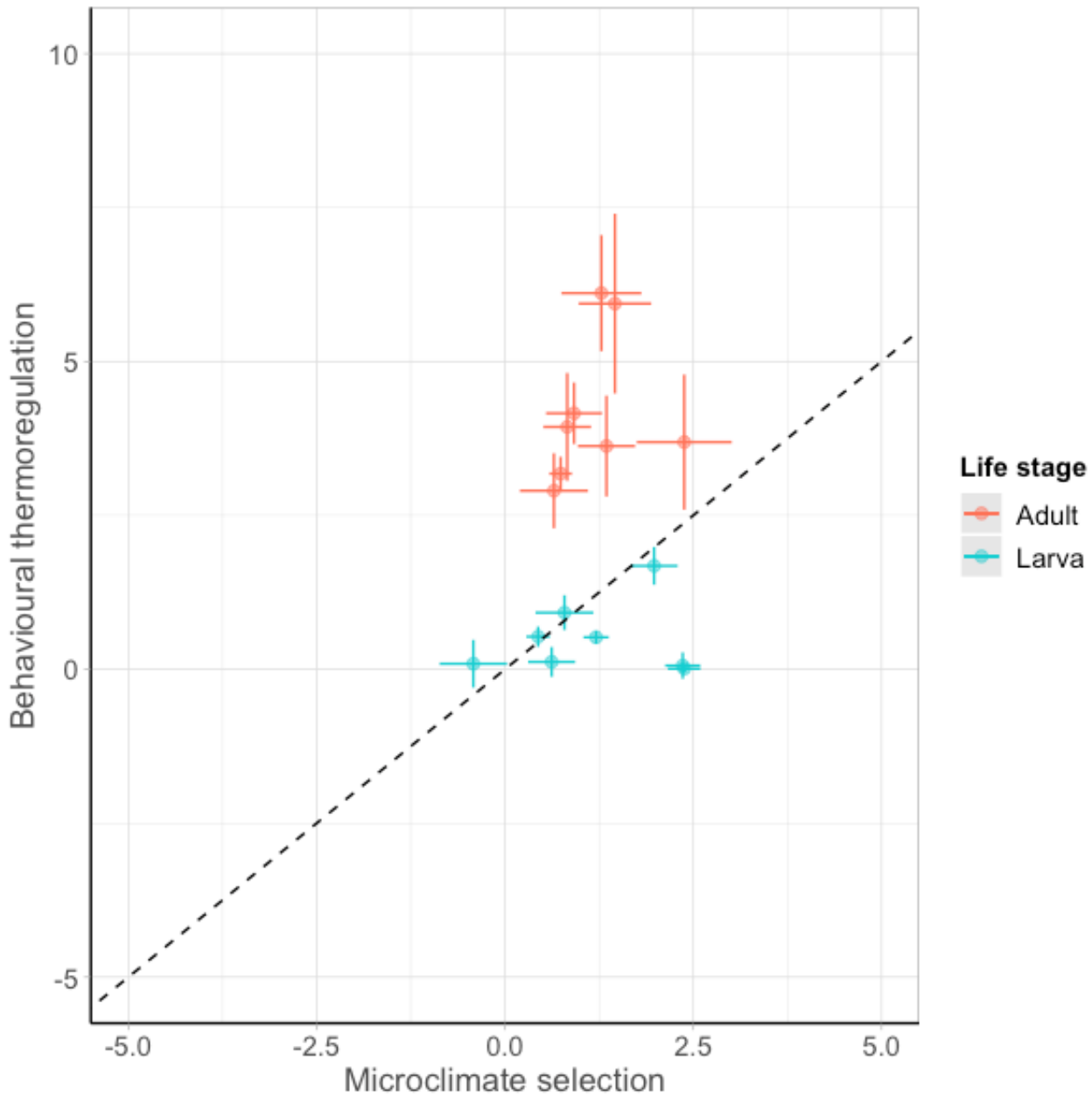


Figure 6: The relationship between microclimate selection (the difference between microclimate temperature and ambient air temperature) and behavioural thermoregulation (the difference between body temperature and microclimate temperature) for eight species of British butterflies as adults and larvae. Points show mean values per species, ± 1 standard error. The dashed line shows a 1:1 relationship, marked to aid visual comparison between groups.

436 **Discussion**

437 There was substantial variation in buffering ability between day-flying Lepidoptera species as
438 larvae. In particular, large larvae were better at buffering body temperature than small larvae,
439 but colour did not influence buffering ability. Of the two butterfly families tested, Pieridae
440 were better at buffering than Nymphalidae. We found some evidence that gregarious
441 caterpillars had lower buffering abilities than solitary caterpillars, but is it likely that this was
442 the result of their small body length rather than being gregarious. Therefore, gregariousness
443 is unlikely to be a trait that influences temperature control. We found that Lepidoptera have
444 a lower buffering ability as larvae than as adults, and that larvae tended to rely on
445 microclimate selection, whereas adults relied on behavioural thermoregulation. Finally, we
446 found that the size of larvae influenced microclimate choice under different temperatures,
447 with large larvae occupying relatively stable microclimates across air temperatures, but small
448 larvae occupying increasingly warm microclimates as ambient temperatures increased.
449
450 We found that large larvae were better at buffering air temperature than small larvae. This
451 pattern reflects results from a similar study, covering many of the same sites and species as
452 adults, which found that adult butterflies with longer wings tended to have stronger buffering
453 abilities than butterflies with short wings (Bladon *et al.* 2020). Larger larvae would have a
454 larger surface area for heat absorption and loss, both from radiation from the sun and
455 conduction from the surface they are in contact with. Owing to their larger volume, large
456 larvae would also experience more stable body temperatures, with fewer fluctuations, than
457 small larvae (Gilchrist 1990) (Kemp & Krockenberger 2004). This would allow large larvae
458 more time to seek alternative microclimates before their body temperature left tolerable
459 ranges. Large larvae are also likely to be able to travel further and faster than small larvae,

460 and should therefore be more able to locate suitable microclimates. This more stable body
461 temperature and greater surface area over which to gain or lose heat may explain the
462 stronger thermal buffering ability of large larvae. This finding means that extreme
463 temperatures may be particularly detrimental to larvae when they are small. As Lepidoptera
464 larvae tend to have high mortality at early instars (Zalucki & Kitching 1982; Zalucki *et al.* 2003),
465 our findings imply that the poor thermal buffering ability of small larvae may exacerbate this
466 trend.
467
468 Of the two butterfly families for which we had sufficient data, we found that Pieridae had
469 higher buffering ability than Nymphalidae. Interestingly, these two families represent the best
470 and worst thermal buffering abilities from the related study on adults (Bladon *et al.* 2020).
471 This implies that there is a consistent pattern in thermal buffering abilities, at least between
472 these two families, across life stages. This raises concern regarding future biodiversity loss
473 from Nymphalidae, the most speciose of the butterfly families (Hao *et al.* 2022). If poorer
474 buffering ability means that species experience temperatures above those they prefer or can
475 tolerate, this trend may mean this group is particularly vulnerable to increasing temperatures.
476 It is therefore possible that future climate change may disproportionately reduce Lepidoptera
477 diversity. The Nymphalidae larvae tested (*A. io, A. urticae, V. atalanta*) are dark in colour and
478 grow to relatively large sizes. *A. io* and *A. urticae* can live in relatively high densities, as they
479 are gregarious at low instars. However our findings do not indicate that these species traits
480 are contributing to the poor buffering ability of this family. Instead there may be other
481 phylogenetically conserved physiological traits that may have contributed to their poor
482 buffering ability, which merits further research.
483

Contrastingly, the evidence suggests that Pieridae have a strong ability to maintain their body temperature across a range of air temperatures, both as adults and larvae. This suggests they may be more resilient to changing ambient conditions, as they are less likely to experience non-optimal or damaging temperatures. This could be the result of differences in behaviour, physiology, or habitat choice between families. Pieridae larvae tend to be relatively pale (ranging from green to green-black) and hairless, and include economically important agricultural pest species of Brassicaceae crops (such as *P. rapae, P. brassicae*). As larvae, there is substantial variety in ecology and microhabitats occupied by pierids, and in this study pierids were located in areas with at least some structural complexity to exploit for thermoregulation. For example, although the *P. rapae* and *P. brassicae* larvae we sampled were located in a generally open allotment, they were most often found within the heart of their foodplants, sheltered in cool shady areas between leaves. *G. rhamni* were located on small to large trees (*Rhamnus cathartica*), these large foodplants could feasibly provide a variety of microclimates for the larvae to exploit for thermoregulation. It may be the availability of microclimates for thermoregulation surrounding the foodplants of this family that explain their strong thermal buffering ability. The exception to this is *A. cardamines,* which lives in an extremely exposed part of their foodplant, feeding on seedpods. This difference is evident when considering buffering ability and thermoregulatory mechanism; the species that are the strongest at buffering air temperature (*P. rapae, P. napi*) are the species that are also relying more on microclimate selection across air temperatures. Pierids being strong at buffering air temperature as both adults and larvae implies they may be resilient to temperature variation and climate change, with knock-on effects in cases where Pierid species are agricultural pests. For example, of all the species tested, *P. rapae* showed some of the strongest buffering abilities, both as adults and larvae. This species is native to

508 Eurasia, but has become an invasive pest of wild and cultivated Brassicaceae around the world
509 (Ryan *et al.* 2019). This ability to maintain their body temperature within tolerable ranges
510 across the life cycle may partly explain their successful invasion across regions.
511
512 However, there is a possibility that strong thermal buffering ability may inhibit adaptation to
513 tolerate higher temperatures, as individuals would rarely be exposed to selection to evolve
514 mechanisms to cope with non-optimal temperatures (Muñoz 2022). For example, a recent
515 study on tropical adult butterflies has shown that species with a weaker buffering ability also
516 had lower thermal tolerance (Ashe-Jepson *et al.* 2023). This theory, dubbed the 'Bogert effect'
517 (Huey *et al.* 2003), may mean that species with strong buffering abilities may initially be less
518 impacted by changing temperatures. However, should microclimates be lost or an extreme
519 weather event were to occur whereby temperatures rise or fall outside of tolerable ranges
520 (such as during a heatwave), species with strong buffering abilities could have a reduced
521 thermal tolerance compared to species with weak buffering abilities, and may be
522 disproportionately affected. This implies that most species may be vulnerable to climate
523 change, however further study is needed to determine whether thermal buffering ability
524 interacts with thermal tolerance in Lepidoptera larvae (Ashe-Jepson *et al.* 2023).
525
526 Aspects of ecology or evolutionary history may explain some of the species buffering abilities
527 we detected. For example, predation is a strong selective pressure on Lepidopteran larvae
528 which can alter the morphology and behaviour of species (Sugiura 2020). Species with
529 reduced predation pressure may be able to thermoregulate more freely. Of the species
530 tested, *T. jacobaeae* is the only aposematically coloured larva, and is chemically defended
531 from predation (McLellan *et al.* 2021). Because of this, larvae of this species tends to be active

– feeding or basking openly – during the day (Dempster 1982). These traits may explain the strong buffering ability of this species. Strong selective pressure to maintain body temperatures within narrow ranges may also contribute to the evolution of stronger buffering abilities. *T. jacobaeae* may again be an example of this, whereby in peak abundance years, their foodplant (*Senecio jacobaea*) can be defoliated, and the fastest developing larvae are the most likely to survive (Dempster 1971). As development rate is correlated positively with temperature in insects (Ratte 1984), individuals that are able to maintain high body temperatures, especially at low air temperatures and without overheating, would be at a selective advantage. Another factor that may influence the buffering ability of a species is their evolutionary history. Larvae may have evolved within a system that not require them to buffer air temperature, such as under forest canopies (De Frenne *et al.* 2019), which themselves buffer ambient conditions. Outside of those habitats, their buffering ability may be weak. *Z. filipendulae* may be an exampleof this, as it is largely distributed in coastal areas in the UK, (Gutiérrez *et al.* 2001), which tend to have warmer and more stable temperatures than inland (Met Office 2013). Therefore, this species may be adapted to a coastal climate, perhaps resulting in weak selection for buffering ability in this species.

We found larval day-flying Lepidoptera are worse at buffering their body temperature than adults. As air temperatures increase, most adult Lepidoptera can slow their rate of heat gain, and at high air temperatures, even lower their body temperature below ambient conditions (Bladon *et al.* 2020; Ashe-Jepson *et al.* 2023). Adult Lepidoptera are particularly good at raising their body temperature at low air temperatures; adults were almost 10°C warmer than ambient conditions in cool air temperatures. In contrast, larvae are generally thermo-conformers at low air temperatures, and gain heat rapidly as ambient temperatures increase.

This means that Lepidoptera at the larval life stage are likely to be particularly vulnerable to both hot and cold ambient conditions. As temperature is influential for larval development rate, the inability to elevate their body temperature in cold conditions could prolong the time spent as vulnerable larvae, increase the risk of exposure to predators and parasites, and therefore result in higher larval mortality (Benrey & Denno 1997). In hot ambient conditions, larvae show a limited ability to slow the rate at which they gain heat, placing them at risk of their body temperatures reaching damaging and even lethal levels. The difference between adult and larvae is likely explained by differences in morphology and thermoregulation strategies between life stages. Firstly, adult Lepidoptera have morphological traits that improve their thermal buffering ability compared to larvae, particularly their wings, which they can use to absorb solar radiation or reflect it onto the body to warm up (Watt 1968; Shanks *et al.* 2015), or use to radiate heat into the environment to cool down (Tsai *et al.* 2020). The stronger buffering ability of adults compared to larvae means that species' responses to a changing climate may be driven by the larval life stage, for which there is limited available data to make accurate predictions. Most studies investigate the adult life stage only (Radchuk *et al.* 2013), and relatively little is known about the other life stages (Kingsolver *et al.* 2011). This means we may be unprepared to enact effective conservation strategies to protect Lepidoptera larvae from the effects of temperature change. In this study we utilised an existing adult dataset to complement a new larval dataset to provide a more holistic understanding of Lepidoptera responses to temperature change, while also providing the next steps in research on larvae. This study demonstrates the value of focusing on the larval life stage, and comparing across life stages. A shift in research focus is needed to continue to address this research gap.

580 The differences in buffering ability across life stages may also be partly explained by the
581 alternative thermoregulatory strategies the life stages depend on. We found that adults relied
582 more on behavioural thermoregulation, whereas larvae relied more on microclimate
583 selection. In addition, we found little evidence of change of strategy across air temperatures
584 for larvae, whereas adults rely less on behavioural thermoregulation and marginally more on
585 microclimate selection under increasing air temperatures. This could be because the wings of
586 adult Lepidoptera enable more effective heat transfer for behavioural thermoregulation. In
587 addition, flight also enables adults to access microclimates over a wider area than that
588 available to larvae (Clench 1966). We found limited evidence that adults were relying on
589 microclimate selection for thermoregulation, suggesting that behavioural thermoregulation
590 is the more effective strategy for adults. In contrast, larvae showed less variation in their
591 strategies, and generally depended on microclimate selection over behavioural
592 thermoregulation. This means that larval thermoregulation is restricted and dependent on
593 their local environment, generally in the immediate area around their foodplant. This
594 highlights the importance of maintaining vegetation complexity surrounding Lepidoptera
595 foodplants, to provide larvae with opportunities to thermoregulate in adverse ambient
596 conditions. The reliance on microclimate selection as a strategy for thermoregulation may
597 explain the poor thermal buffering ability of larvae compared to adults.
598
599 There are caveats to this study that may have impacted our results and must be considered.
600 In particular, the data presented comes from only 14 species, and may not be representative
601 of other communities. We call for more studies focussing on the larval life stage of
602 Lepidoptera to investigate the generality of our findings. In addition, our larval data were
603 collected within a single brood. For species with multiple broods per year, data were collected

on the largest brood at peak abundance, to maximise the sample size achieved. As different generations within a year experience different thermal conditions, this may have influenced the thermal buffering abilities we recorded. Similarly, as all data collected were from wild-caught individuals and across multiple years, it is possible that the thermal conditions experienced throughout ontogeny and between years differed, influencing the thermal buffering abilities detected. However, as we used the same sites each year, and collected data at roughly the same time of year, we expect this effect to be limited.

Conclusions

We have identified that species-specific thermal buffering ability and thermoregulation strategies differ across the life cycle of 14 species of day-flying Lepidoptera. We found that larvae may be more vulnerable to temperature variation than adults, especially when small, young, or in the family Nymphalidae. We also found that Lepidoptera larvae rely on microclimate selection for thermoregulation, over behavioural thermoregulation, whereas the opposite is true for adults. This implies that larvae are dependent on their local environment for thermoregulation, and so require microclimates in the immediate area around their foodplant to thermoregulate effectively. These findings can be used to inform the management of land and species, for example to preserve microclimates and vegetation complexity surrounding Lepidoptera foodplants for larval thermoregulation.

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634

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