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1 **Title:** The stable isotope ecology of mycalesine butterflies: implications for plant-insect co-
2 evolution

3

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21 insect co-evolution.

22

23 **Running headline:** Isotopic ecology of mycalesine butterflies

24

25

26 **Summary**

- 27 1. One of the most dramatic examples of biome shifts in the geological record is the rapid
28 replacement of C₃ vegetation by C₄ grasses in (sub-) tropical regions during the Late
29 Miocene-Pliocene. Climate-driven biome shifts of this magnitude are expected to have
30 a major impact on diversification and ecological speciation, especially in grazing taxa.
31 Mycalesine butterflies are excellent candidates to explore the evolutionary impact of
32 these C₃/C₄ shifts on insect grazer communities.
- 33 2. Mycalesine butterflies feed on grasses as larvae, have radiated spectacularly and occur
34 in almost all extant habitats across the Old World tropics. However, at present, we lack
35 a comprehensive understanding of the larval ecology of these butterflies and this
36 hampers investigations of co-evolutionary patterns among the geographically parallel
37 radiations of mycalesine butterflies and the remarkable evolutionary history of their
38 host plants.
- 39 3. By conducting several experiments under defined environmental conditions we
40 demonstrate that the feeding history of mycalesine larvae on C₃ and C₄ grasses can be
41 traced by analysing $\delta^{13}\text{C}$ in the organic material of the adult exoskeleton, while values
42 of $\delta^{18}\text{O}$ in the adult reflect atmospheric humidity during larval development.
- 43 4. To show the power of these isotopic proxies for ecological studies, we analysed the
44 isotopic composition of organic material obtained from adult butterflies sampled in two
45 extensive longitudinal surveys.
- 46 5. We observed strong associations among the larval ecology, habitat preferences of the
47 adult butterflies and patterns of seasonality, such that mycalesine species that inhabit
48 open environments are more opportunistic in their host plant choice but utilize C₃
49 grasses more frequently during the dry season. Crucially, the ability to process the less
50 palatable C₄ grasses appears to be phylogenetically clustered within mycalesine species,

51 suggesting that novel feeding adaptations may have evolved in response to the
52 ecological dominance of C₄ grasses in open savannah habitats.

53 **Introduction**

54 The origin of tropical savannahs is one of the most dramatic examples of climate-driven
55 biome shifts documented in the geological record (Edwards *et al.* 2010). Today's tropical
56 savannahs are dominated by grasses which utilize the C₄ photosynthetic pathway to fix
57 carbon, whereas only 3 to 8 million years ago these tropical environments were largely
58 dominated by C₃ vegetation (Osborne & Beerling 2006). A superior strategy for carbon
59 uptake and assimilation in warm climates and low atmospheric CO₂ concentrations
60 (Ehleringer, Cerling & Helliker 1997), in combination with different responses to climatic
61 extremes and wildfires (Edwards *et al.* 2010; Scheiter *et al.* 2012), led to the ecological
62 success of C₄ grasses in tropical environments (Ehleringer, Cerling & Helliker 1997). Such
63 major changes in biome characteristics would be expected to drive diversification across taxa.
64 For example, radiations in large terrestrial herbivores have been attributed to the expansion of
65 grasslands and changes in vegetation distribution, based on isotopic evidence about feeding
66 ecology from tooth enamel (MacFadden 2005; Kuerschner, Kvacek & Dilcher 2008).
67 However, surprisingly little is known about the impacts of these C₃/C₄ shifts on insect grazer
68 communities in grasslands.

69 Most herbivorous insects are specialized in their utilization of host plants and, in
70 response to this host specificity, plants often evolve chemical or physical defenses to reduce
71 herbivore-induced damage (Dres & Mallet 2002; Braby & Trueman 2006). This co-
72 evolutionary arms race is thought to play a crucial role in the evolution of key innovations
73 and drive diversification on each side of the plant–insect interaction (Ehrlich & Raven 1964;
74 Edger *et al.* 2015). In theory, C₄ photosynthesis should exert a strong influence on trophic
75 interactions with insect grazers via two key mechanisms. Firstly, C₄ grasses are expected to
76 have a higher physical resistance to herbivores because the anatomical changes required for
77 C₄ photosynthesis are associated with a higher density of leaf veins, fibre bundles and silica

78 cells (Wilson & Hattersley 1989; Bouchenak-Khelladi *et al.* 2009). Host plants with a high
79 physical resistance rapidly increase mandible wear in lepidopteran larvae, while high levels
80 of silica in grasses decrease nitrogen absorption, reducing feeding efficiency and insect
81 growth rates, respectively (Massey & Hartley 2006; Massey & Hartley 2009). Secondly,
82 because of the high efficiency of their carbon concentrating mechanism, C₄ leaves typically
83 have lower nutritional values (lower leaf protein and nitrogen) than C₃ foliage (Long 1999).
84 Therefore, generalist herbivores are predicted to prefer C₃ over C₄ leaves, despite possessing
85 the ability to process the latter, when both are available in the same habitat (see Caswell *et al.*
86 1973).

87 Here, we use data on butterflies of the subtribe Mycalesina (Nymphalidae: Satyrinae)
88 to examine how physiological and ecological differences between C₃ and C₄ grasses may
89 have impacted the evolutionary ecology of insect grazers. These tropical butterflies feed
90 mainly on grasses as larvae and, especially in the case of *Bicyclus anynana*, have become
91 important model organisms in ecological, evolutionary and developmental biology (van
92 Bergen *et al.* 2013; van den Heuvel *et al.* 2013; Mateus *et al.* 2014). Mycalesines have
93 radiated dramatically in Sub-Saharan Africa, Madagascar and Asia with over 300 extant
94 species (Kodandaramaiah *et al.* 2010; Aduse-Poku *et al.* 2015), and their evolutionary history
95 is expected to be closely tied to the rise to ecological dominance of C₄ grasses in savannah
96 habitats (Pena & Wahlberg 2008). In addition, mycalesine butterflies that inhabit C₄ grass-
97 dominated open habitats in East Africa and elsewhere in the Old World tropics, in contrast to
98 species found in the environmentally more stable forests, are faced with the challenge of
99 alternating seasons (Brakefield 2010). Many of these savannah species exhibit seasonal
100 polyphenism with alternative forms occurring in the wet and dry seasons (Windig *et al.* 1994;
101 Roskam & Brakefield 1999), such that polyphenism and adaptations to C₄ grass feeding are
102 expected to evolve in concert.

103 Our understanding of the larval ecology of this group is rudimentary in spite of many
104 ecological studies. Natural host plant records for grass-feeding mycalesines are very limited
105 and unreliable, as the larvae are generally cryptic and nocturnal feeders (Brakefield &
106 Mazzotta 1995). This seriously hampers investigations on the evolution of feeding ecology in
107 these butterflies. However, stable isotope analyses, which have become an important part of
108 the ecologist's toolbox over the last three decades, represent an unexploited opportunity to
109 better understand larval feeding preferences and micro-climate conditions during
110 development in mycalesine butterflies (West *et al.* 2006).

111 Isotopic data have, for instance, successfully been used to track migration patterns of
112 birds and insects (Wassenaar & Hobson 1998; Kelly *et al.* 2002; Brattström *et al.* 2010) and
113 to determine dietary preferences of fossil and modern animals (Ben-David, Flynn & Schell
114 1997; Cerling *et al.* 1997). These studies have shown that the feeding history of animals, with
115 respect to C₃ and C₄ grasses, can be traced by analysing the relative amount of carbon-
116 13 (¹³C) in the tissues of herbivores (Boutton, Cameron & Smith 1978; Fry, Joern & Parker
117 1978; Cerling *et al.* 1997; Fischer, O'Brien & Boggs 2004; Codron *et al.* 2012). Plants
118 discriminate against ¹³CO₂ during photosynthesis, but the C₄ pathway significantly lowers
119 this discrimination, leading to distinct non-overlapping differences in δ¹³C values between C₃
120 and C₄ plants (O'Leary 1988). These differences in ¹³C isotope ratios are transmitted to the
121 tissues of the herbivores that feed on the plant material (Cerling, Ehleringer & Harris 1998).
122 Furthermore, stable isotopes of oxygen, and in particular the ¹⁸O composition of the
123 haemolymph and organic material of the exoskeleton of terrestrial arthropods, can be used to
124 quantify the mean atmospheric conditions surrounding the animal before moulting (Ellwood
125 *et al.* 2011). Arthropods control the respiratory loss of water by opening and closing the
126 tracheal openings used for gas exchange. As water evaporates, diffusion and equilibration
127 fractionations favour the lighter isotope (¹⁶O) such that residual animal tissue water tends to

128 become enriched in the heavier isotope (^{18}O). Individuals that experience conditions of low
129 humidity, and therefore high rates of evaporation, prior to the final moult, show enriched
130 values of $\delta^{18}\text{O}$ in exoskeleton organic material.

131 The aims of this study were, firstly, to experimentally determine whether stable
132 isotopes of carbon and oxygen sampled from the exoskeleton of adult mycalesine butterflies
133 could provide key information about larval ecology. We used laboratory experiments to
134 elucidate the relationships between the environmental conditions during development and the
135 isotopic composition of the organic material obtained from the adult. Secondly, we analysed
136 the isotopic signatures of specimens from two extensive surveys of mycalesine butterflies,
137 one conducted in Africa and the other in Asia. Since these small herbivores are able to
138 complete development on a single or limited number of individual host plants and are
139 relatively immobile, we were able to investigate details of host plant-herbivore interactions in
140 the wild. This allowed us to explore whether the ecological and physiological differences
141 between C_3 and C_4 grasses are associated with the larval host plant and/or adult habitat
142 preferences of several mycalesine species.

143 The ability of mycalesines to process C_4 foliage is expected to be phylogenetically
144 clustered and associated with adult habitat preferences. Mycalesine species which remained
145 restricted to shaded forest understories, where the advantage of the C_4 pathway is normally
146 lost, are expected to be C_3 specialists. In contrast, species which successfully inhabit the more
147 open C_4 grass-dominated environments as adults are expected to have acquired novel feeding
148 adaptations and predicted to be more opportunistic and generalist in their larval host plant
149 choice. In addition, larvae which complete development in these open habitats are more
150 likely to respire at low levels of atmospheric humidity which should be reflected in the ^{18}O
151 composition of the adult exoskeleton.

152 In the C₄-dominated savannah habitats, host plant quality decreases rapidly during the
153 dry season as the environment gradually dries out. Seasonal patterns in host plant use are
154 predicted for generalists that inhabit these open habitats, with an increased preference for
155 high quality C₃ grasses during the dry season. For seasonal forms, we also predicted strong
156 associations between the values of $\delta^{18}\text{O}$ in organic material and the varying seasonal
157 environment in the open habitat. In contrast, the composition of oxygen stable isotopes is
158 likely to remain more constant in stable environments.

159

160 **Material and methods**

161 *Laboratory experiments*

162 We conducted four laboratory experiments under defined environmental conditions to
163 examine how isotopic signatures are established in mycalesine butterflies. The first two
164 experiments (A and B) investigated whether the feeding history of individual larvae on C₃
165 and C₄ grasses could be traced by analysing the $\delta^{13}\text{C}$ of adult leg tissue. They also explored
166 how a mixed larval diet of C₃ and C₄ plants affects the isotopic signature, and whether
167 different types of adult tissue share similar $\delta^{13}\text{C}$ values in our model species. The aim of the
168 next two experiments (C and D) was to examine the extent to which the relative humidity
169 (RH) of the environment affects ^{18}O composition in the organic material of mycalesine
170 butterflies. Individuals experiencing low RH are expected to lose water faster through
171 evaporation, which could lead to more enriched $\delta^{18}\text{O}$ values. The final experiment was
172 designed to identify the developmental period resulting in the evaporative signal found in
173 adult butterflies: is ^{18}O enrichment in the exoskeleton of adults the result of increased
174 evaporation rates in the host plants and transmitted to the tissue of the herbivorous insect
175 reflecting a signal of the atmospheric conditions during larval development, or is it
176 incorporated into the exoskeleton during the adult stage?

177 Experiments A and B were conducted with *Mycalesis mineus* (Linnaeus, 1758), an
178 Asian species which occurs throughout South and Southeast Asia from India to the Philippine
179 archipelago (Monastyrskii 2005). The founders of this population were collected near Khao
180 Chong Nature Reserve, Thailand in 2011. Experiments C and D used *Bicyclus anynana*
181 (Butler, 1879), an African species which occurs in seasonal habitats from Ethiopia to the
182 most northern provinces of South Africa (Larsen 1991). The stock population used for
183 experiments C and D was established in 2011 and indirectly originated from the Leiden
184 University laboratory stock population set up in 1988 from 80 gravid females collected near
185 Nkhata Bay in Malawi (Brakefield, Beldade & Zwaan 2009). Unless stated otherwise, all
186 larvae were reared on maize plants (*Zea mays*) in controlled environment chambers at 27°C,
187 70% RH, and L12:D12.

188 For experiment A, larvae were either reared on wheat (*Triticum aestivum*) or maize,
189 which are C₃ and C₄ grass species, respectively. Leaf samples were collected throughout
190 larval development in order to compare the $\delta^{13}\text{C}$ values of the host plant (N=16) and leg
191 tissue of adult butterflies (N=24). The same protocol was followed for experiment B.
192 However, on the first day of the fifth instar, the larvae within each cohort were randomly
193 transferred to a cage with either the original host plant or that of the alternative
194 photosynthetic pathway, and allowed to complete development. After eclosion, butterflies
195 were frozen at -20°C before the adults had fed, and then stored in individual envelopes until
196 further processing. For experiment B, $\delta^{13}\text{C}$ values were obtained from leg, antenna and wing
197 tissue (N=40).

198 In experiment C, larvae were reared as described above and, on the first day after
199 eclosion, adults were randomly transferred to a climate room with either the original RH
200 (70%) or to a low humidity climate room (20% RH). Here, the adults were fed on moist
201 banana and samples were collected from both cohorts on a daily basis for 21 consecutive

202 days (N=97). For the final experiment (D), host plants were cultivated under high (90%;
203 HIGH) or low RH (50%; LOW). Larvae were also reared under HIGH or LOW conditions
204 and on either HIGH or LOW host plants. The adults of these four cohorts were randomly
205 transferred to either a HIGH or a LOW climate room, resulting in eight experimental cohorts
206 (N=80). Adults were fed on moist banana, and after 14 days frozen and stored individually.
207 Schematic representations of these four laboratory experiments are given in figure S1.

208

209 *Field material*

210 We analysed the isotopic signatures of specimens from two independent longitudinal samples
211 of mycalesine butterflies, one collected in a seasonal open habitat in Africa and the other in a
212 more stable secondary forest in Asia. For the first dataset, the butterflies were caught daily
213 between June 1995 and May 1998 at Zomba, Malawi (15°22'S, 35°19'E). Here, three traps
214 were baited with fermenting banana and placed on the edge of the evergreen forest covering
215 the slopes of Zomba Mountain. The habitat rapidly changes into open grasslands and sparse
216 woodland-savannah just below the trapping site (Brakefield & Reitsma 1991; Windig *et al.*
217 1994). The second survey was conducted between December 2011 and January 2013 at the
218 Genting Tea Estate, Pahang, western Malaysia (3°21'N, 101°47'E). Five traps were located
219 near or in advanced secondary forest and baited twice a week with fermenting banana
220 (Holloway *et al.* 2013). All trapped mycalesine butterflies were killed and stored in
221 envelopes. After transport to Europe the samples were refrigerated ($\pm 5^{\circ}\text{C}$), before sorting
222 and analysis in 2013. In Malaysia, daily climatic data (minimum and maximum temperature,
223 RH and rainfall) were collected during the trapping period. In Malawi, climatic data were
224 obtained from Chancellor College, about 2 km from the trapping site.

225 Three African mycalesine species were abundant in the Zomba material; *Bicyclus ena*
226 (Hewitson, 1877), *B. safitza* (Westwood, 1850) and *B. vansoni* Condamin, 1965. Adults of *B.*

227 *ena* are normally found in open savannah habitats, especially on rocky hillsides (Pringle
228 1994; Windig *et al.* 1994). *B. safitza* has a wider distributional and ecological range, but the
229 adults mainly inhabit open grasslands (Larsen 2005). In contrast, adults of *B. vansoni* are
230 restricted to shaded forest habitats and forest margins (Kielland 1990; Windig *et al.* 1994).
231 Specimens were randomly selected from the available material, aiming to include at least
232 three males and three females of each species from every month (total N=587). The Genting
233 material contained eight mycalesine species, representing four genera; *Telinga janardana*
234 (Moore, 1857), *Mycalesis intermedia* (Moore, 1892), *M. oroatis*, Hewitson, 1864, *M. orseis*,
235 Hewitson, 1864, *M. visala*, Moore, 1858, *Mydosama fusca* (C. & R. Felder, 1860), *M.*
236 *maianeas* (Hewitson, 1864), and *Culapa mnasicles* (Hewitson, 1864) (N=367).

237 In addition to sampling tissues for isotopic analyses, the ventral surface of one
238 hindwing of each individual was photographed using a Canon EOS 600D camera with a
239 macro lens. These images were analysed with the image processing package Fiji v1.45b
240 (Schindelin *et al.* 2012) and the following wing pattern elements measured to classify
241 individuals into seasonal forms: (i) the wing size; (ii) radius and area of the inner black disc
242 of the eyespot in the 2nd cell (the fifth eyespot); and (iii) radius and area of the yellow outer
243 ring of the eyespot in the 2nd cell (adjusted from Wijngaarden & Brakefield 2001; figure S2).

244

245 *Isotopic analyses*

246 To assay the relative amount of stable isotopes of carbon in our specimens, leg tissue was
247 placed into 8 x 5 mm tin capsules, sealed and loaded into an auto-sampler. The tissue within
248 the capsule was combusted at 600 °C with a pulse of Oxygen and the resultant CO₂ fed into a
249 Costech Elemental Analyser and analysed for ¹³C/¹²C with an in-line Thermo DELTA V
250 mass spectrometer. Helium was used as a carrier gas and the gaseous products were separated
251 by a packed gas chromatographic molecular sieve column at a temperature of 90°C and

252 passed into the mass spectrometer via a Thermo ConFlo IV interface. The mass spectrometer
253 software is programmed to compare the area under the peak of CO₂ and the ¹³C/¹²C isotope
254 ratio. For the analysis of oxygen isotopes, the samples were placed in silver capsules. These
255 samples were pyrolysed at 1200°C using a Thermo Finnigan TC/EA attached to a Thermo
256 Delta V mass spectrometer via a ConFlo 3. Reference standards from IAEA in Vienna were
257 run at intervals throughout the sequence and these values are used to calibrate to the
258 international standards of ¹³C/¹²C ($\delta^{13}\text{C}$ Vienna-PDB) and ¹⁸O/¹⁶O ($\delta^{18}\text{O}$ V-SMOW).
259 Precision of analyses is better than 0.1 per mille for ¹³C/¹²C and about 0.4 per mille for
260 ¹⁸O/¹⁶O. Both analyses were conducted at the Godwin Laboratory for Palaeoclimate
261 Research, Department of Earth Sciences, University of Cambridge.

262

263 *Statistical analyses*

264 All statistical analyses were performed with the R Statistical Package v 3.1.2 (R
265 Development Core Team 2014). We used Student's t-tests to analyse the data from
266 experiment A, and for experiment B we conducted two-way ANOVAs. For experiment C we
267 carried out a multiple linear regression with the $\delta^{18}\text{O}$ values as the dependent variable, and
268 the adult RH treatment and adult age (i.e. time spent in RH treatment) as independent
269 variables. A significant interaction between both independent variables can be interpreted as
270 a differential change in $\delta^{18}\text{O}$ values over time in both adult RH treatments. For experiment D,
271 a three-way ANOVA was used to analyse the effect of RH during each experimental stage on
272 the $\delta^{18}\text{O}$ values obtained from adult organic material, initially fitting full models with each
273 developmental stage, and their interactions, as fixed factors and removing non-significant
274 terms successively. Dietary preferences among the three species from Zomba were
275 investigated by using Chi-squared tests to compare the number of $\delta^{13}\text{C}$ values per species
276 falling within the typical ranges for C₃ or C₄ plants. We used a generalised linear model

277 (GLM) to test for seasonal patterns in host plant use. The number of individuals in each
278 category was used as dependent variable with a Poisson distribution. The species, seasonal
279 form and photosynthetic pathway of the host plant and their interactions were used as fixed
280 factors. All interaction terms were initially included and backward elimination by Akaike
281 Information Criterion (AIC) was used to find the minimum adequate model. For the Genting
282 material we focused on the two most commonly used methods to estimate the phylogenetic
283 signal: Blomberg's K (Blomberg, Garland & Ives 2003) and Pagel's λ (Pagel 1999). Both K
284 and λ were estimated for the proportion of C₃ signatures ($N_{C_3} : N_{C_3} + N_{C_4}$) in each species
285 using the function *phylosig* in the R package *phytools* (Revell 2012). Finally, two and three-
286 way ANOVAs were used to analyse the effects of the species, sex and seasonal form on the
287 $\delta^{18}\text{O}$ values obtained from the field material. Again, this was done by initially fitting full
288 models with all fixed factors and interactions, and then removing non-significant terms
289 successively. This procedure was followed by a *post hoc* Tukey HSD test to identify
290 differences between groups.

291

292 **Results**

293 *Laboratory experiments*

294 Experiment A revealed that $\delta^{13}\text{C}$ in adult leg tissue does not significantly differ from the
295 isotopic signature of the larval host plant (C₃: $t = -1.25$, $df = 12.49$, $P = 0.24$ and C₄: $t = -1.47$,
296 $df = 11.89$, $P = 0.17$; figure 1a). The results from experiment B indicated that the different
297 adult tissues of leg, antennae and wing material have very similar $\delta^{13}\text{C}$ values (figure 1b). In
298 addition, all analysed tissues largely reflect the isotopic composition of the nutrients obtained
299 during the final phase of development, the fifth instar, as the $\delta^{13}\text{C}$ values of individuals that
300 were swapped from the C₃ to the C₄ host plant at this final stage of larval development were

301 more similar to the isotopic signatures of individuals that were reared on C₄ plants during all
302 larval stages, and *vice versa* (table S1).

303 Experiment C showed that $\delta^{18}\text{O}$ in adult leg tissue is not affected by high rates of
304 local water evaporation during the adult stage. The interaction between the humidity
305 treatment and the age of the adult was non-significant ($P=0.93$) and the $\delta^{18}\text{O}$ values obtained
306 from adults that were transferred to the LOW (50% RH) humidity climate room remained
307 stable throughout the 21 day sampling period. Similar results were found for control
308 individuals which were kept under HIGH (90% RH) conditions for the same period (table 1).
309 Neither the RH conditions under which the host plants were cultivated, nor the RH
310 experienced during the adult phase, affected the $\delta^{18}\text{O}$ values in adult leg material in
311 experiment D. In contrast, the leg tissue of individuals reared under low RH and which had,
312 therefore, experienced increased rates of evaporation during the larval stage was significantly
313 enriched in ^{18}O (table 2).

314

315 *Stable isotopes of carbon in field material*

316 In both the open and seasonal habitat in Zomba, Malawi, as well as in the shaded secondary
317 forest in Genting, Malaysia, we found distinct bimodal distributions in $\delta^{13}\text{C}$ values,
318 representing the isotopic composition of C₃ and C₄ grasses (individuals with $\delta^{13}\text{C}$ values less
319 than -25‰ were classified as C₃ feeders; figure S3). At Zomba, the proportion of larvae that
320 developed on C₃ grasses was significantly higher in *B. vansonii*, the species where adults are
321 predominantly found in (semi-)shaded forest habitats and forest margins (*B. vansonii*:*B. ena*
322 comparison, $X^2 = 187.16$, $df = 1$, $P < 0.01$; *B. vansonii*:*B. safitza* comparison, $X^2 = 216.50$, df
323 $= 1$, $P < 0.01$). The ratios of C₃:C₄ feeding did not significantly differ between *B. ena* and *B.*
324 *safitza*, the two species where adults inhabit the open grasslands (*B. ena*:*B. safitza*
325 comparison, $X^2 = 0.04$, $df = 1$, $P = 0.86$). In addition, in all three species, we observed a trend

326 towards an increased use of C₃ host plants during the dry season, although this interaction
327 between seasonal form and host plant-use only approached significance ($z = 1.753$, $P = 0.08$;
328 table 3 and table S2). The $\delta^{13}\text{C}$ values of half of the eight mycalesines collected in Genting,
329 *T. janardana*, *M. intermedia*, *M. orseis* and *M. visala*, revealed that these species frequently
330 develop on C₄ grasses throughout the year. We found no evidence for C₄ larval feeding in any
331 of the remaining four species; *M. oroatis*, *M. fusca*, *M. maianeas* and *C. mnasicles*. The
332 ability to use C₄ grasses as the natural host was significantly correlated with the phylogenetic
333 relatedness of the Genting species (Blomberg's $K = 1.23$, $P < 0.05$; Pagels $\lambda = 1.08$, $P < 0.05$;
334 figure 3).

335

336 *Stable isotopes of oxygen in field material*

337 The $\delta^{18}\text{O}$ values collected throughout the sampling period in Genting did not differ among
338 the different species nor to the sex of the individuals (table 4). In contrast, in the seasonal
339 habitat in Zomba, the isotopic signatures were significantly higher in dry season form (DSF)
340 individuals compared to wet season form (WSF) specimens of the same species (table 4;
341 Tukey's HSD test, $P < 0.01$). In addition, *B. ena* was significantly more enriched in ^{18}O
342 compared to the other two species (Tukey's HSD tests, $P < 0.01$), while there was no
343 significant difference in $\delta^{18}\text{O}$ between *B. safitza* and *B. vansoni* (Tukey's HSD test, $P = 0.54$).
344 The $\delta^{18}\text{O}$ values of specimens collected during the early phase of the dry season were slightly
345 lower compared to the DSF individuals caught just before the following rainy period. In
346 addition, the WSF individuals that emerged immediately in the early wet season were more
347 enriched in ^{18}O compared to WSF specimens that had emerged in the middle of the wet
348 season (figure 2).

349

350

351 **Discussion**

352 Our laboratory experiments indicate that dietary preferences for grasses with a C₃ or C₄
353 photosynthetic pathway can be traced accurately in mycalesine butterfly larvae by analysing
354 the relative amount of ¹³C deposited in the exoskeleton of adult butterflies. We have also
355 shown that the values of δ¹⁸O measured in adult organic material reflect atmospheric
356 humidity during larval development. Together, these isotopic proxies provide key
357 information about the larval feeding ecology and atmospheric environment of mycalesine
358 butterflies in the wild, and enable three key inferences. First, species that inhabit open
359 environments are more opportunistic in their larval host plant choice, whereas C₃ grass
360 specialists were only found in the shaded habitat. Secondly, we observed seasonality in host
361 plant utilization and larval respiration in the open savannah habitat, such that during the early
362 dry season, larvae are more likely to use C₃ grasses and to experience higher rates of
363 evaporation. Finally, our data suggest that the ability to process C₄ grasses is phylogenetically
364 clustered within mycalesine species.

365

366 *Proof of principle experiments*

367 Animal organic tissue is enriched in δ¹³C, on average, by about 1‰ relative to the diet
368 (Deniro & Epstein 1978), but enrichment in δ¹³C can be as high as 14.1‰ in the tooth enamel
369 of large mammals because of equilibrium fractionation associated with inorganic carbon
370 deposition (Cerling *et al.* 1997; Cerling & Harris 1999). Our results indicate that the isotopic
371 composition of leg tissue in mycalesine butterflies is not significantly enriched compared to
372 the isotopic composition of the larval host plant (see also Fischer, O'Brien & Boggs 2004),
373 suggesting that isotopic discrimination during digestion and assimilation is very limited in
374 these species. In addition, we demonstrate that, in mycalesine butterflies, and probably,
375 therefore, in other holometabolous insect herbivores, the isotopic signatures of adult tissue to

376 a large extent reflect the isotopic composition of the host plant consumed by the larvae during
377 the final phase of development when most larval growth occurs and development of adult
378 tissues begins. The $\delta^{13}\text{C}$ values were comparable across tissues, although the isotopic
379 composition of antennal tissue showed a consistent deviation towards the isotopic of the host
380 plant utilized during the first four larval instars. This may indicate differences in the
381 allocation of nutrients acquired during the final larval instar amongst the analysed tissues, or
382 differences in the timing of the initiation of the development of these adult tissues during the
383 larval stage.

384 While ^{13}C has a proven track record of revealing dietary preferences, stable isotopes
385 of oxygen have rarely been used as a marker of abiotic conditions during development in
386 terrestrial insects and never been used in field studies. Using mycalesine butterflies under
387 defined experimental conditions, we show here that insect larvae respiring at low humidity
388 are significantly enriched in the ^{18}O composition of the organic material of the adult
389 exoskeleton. It has been demonstrated previously that the values of $\delta^{18}\text{O}$ of the haemolymph
390 continue to increase when adult insects are kept under low RH (Ellwood *et al.* 2011). Our
391 results indicate that this evaporative enrichment of adult haemolymph does not affect the
392 isotopic composition of the exoskeleton. A similar result was obtained for the increased
393 evaporation rates of the host plant during seedling establishment and growth (Helliker &
394 Ehleringer 2000). As a caveat we note, however, that the adults of laboratory experiments C
395 and D were only exposed to conditions of low humidity for a period of 21 and 14 days,
396 respectively. In the natural environment, DSF individuals have to cope with low humidity for
397 many months and our data do not allow us to exclude potential long-term effects of low
398 humidity on the isotopic composition of the exoskeleton. In general though, the ^{18}O signature
399 of the haemolymph of larvae is expected to be transferred to the organic material of the
400 exoskeleton of the adult around the time of the final moult, and our data indicate that the ^{18}O

401 composition of the chitin of terrestrial arthropods can be used as a direct indicator of
402 atmospheric humidity during larval development.

403

404 *Larval ecology*

405 In both the open habitat in Zomba, as well as in the shaded forest in Genting, the isotopic
406 signatures revealed that the larvae of some mycalesine species utilize C₄ grasses in their
407 natural environment. In the material from Zomba, the C₃:C₄ ratios of the different species
408 were strongly associated with the habitat preferences of the adult butterfly. Species that fly in
409 the more open grasslands, *B. ena* and *B. safitza*, were more likely to complete development
410 on C₄ grasses whereas individuals of *B. vansoni* from more shaded habitats mainly utilized C₃
411 host plants. None of these three species from a seasonal environment exclusively used host
412 plants from one of the two alternative photosynthetic pathways, suggesting that, within the
413 *Poaceae*, these species may indeed be opportunistic and generalist in their host plant choice.
414 Where the $\delta^{13}\text{C}$ values indicated that the larval host plant use of both open savannah species
415 was more similar, the oxygen signatures revealed that the *B. ena* larvae consistently respired
416 at lower RH during development, compared to *B. safitza* and *B. vansoni*, which completed
417 development under more humid atmospheric conditions. Adults of *B. safitza* normally inhabit
418 grasslands with scrub and open woodland and have a wider ecological range than those of *B.*
419 *ena*. The latter species is restricted to open savannah habitats and is likely to be more tolerant
420 of arid conditions during larval development. Overall, the combination of $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$
421 closely reflects the habitat preferences of adults.

422 In the shaded secondary forest in Genting where RH was constant and high
423 throughout the sampling period, the ^{18}O composition did not differ among species, indicating
424 that the atmospheric humidity experienced during development was similar for all species. In
425 contrast, the $\delta^{13}\text{C}$ values revealed clear differences in host plant preferences, with half of the

426 species frequently utilizing C₄ plants while the other species solely using C₃ grasses. This
427 indicates that there is no inherent barrier to forest species consuming C₄ grasses.
428 Furthermore, this observation conflicts with the prediction that generalists avoid C₄ leaves
429 when C₃ host plants are available (Caswell *et al.* 1973; see further below).

430

431 *Seasonal variation at field sites*

432 The climatic data were used to discover when seasonal shifts occurred during the period
433 when the specimens were collected. In the seasonal habitat in Zomba, Malawi, periods of
434 increased rainfall normally started around the beginning of November and extended into
435 April of the next year. Temperature starts to rise about two months before the first rains. The
436 RH increases rapidly during these rainy periods, peaks about six weeks after the onset of the
437 first rains and gradually decreases throughout the entire dry season. The dry season of 1995
438 was especially arid, with weekly means of RH as low as 21% immediately before the first
439 rains of that year. In contrast, in the secondary forest in Genting, Malaysia, the temperature
440 and RH remained constant and relatively high throughout the year (figure 2). Substantial
441 phenotypic variation was found for most species in both surveys. In Genting, the phenotypic
442 variability was not correlated to any of the measured abiotic factors. However, in the material
443 from Zomba, phenotypic variation was clearly associated with the seasonal climatic
444 fluctuations in all three species of *Bicyclus* (see also Windig *et al.* 1994). In each year, the
445 first wet season form (WSF) individuals appear soon after the onset of the first rains when the
446 temperature is high and humidity is increasing rapidly. In contrast, dry season form (DSF)
447 adults begin to emerge when the environment is gradually drying out and the temperature is
448 significantly lower (Brakefield, Pijpe & Zwaan 2007).

449 In the seasonal habitat in Zomba, we found a tendency to utilize C₃ grasses more
450 frequently when the C₄ host plants potentially become less palatable during the early dry

451 season. This observation suggests that the preference of grass-feeding generalist herbivores
452 for high quality C₃ grass species increases during this period (Caswell *et al.* 1973). However,
453 the differences in C₃:C₄ ratios between seasonal forms may also reflect seasonal variation in
454 the availability of the two types of host plants or indicate that there may be a longer window
455 of opportunities for successful development and adult recruitment in forest margins, where C₃
456 grass species are expected to be more prevalent.

457 Mycalesine species that developed in the stable and shaded habitat in Genting had
458 similar $\delta^{18}\text{O}$ values throughout the year, while we observed significant differences between
459 seasonal forms in the material collected in the open seasonal habitat in Zomba. Here, the ^{18}O
460 composition of DSF individuals was significantly enriched in all species. The butterflies
461 collected immediately after the onset of the first rains expressed WSF phenotypes as are
462 induced by high temperatures experienced during the late larval and early pupal stages (Kooi
463 & Brakefield 1999). Interestingly, these early WSF individuals also demonstrated an enriched
464 ^{18}O composition of the exoskeleton indicating that they had experienced conditions of low
465 RH, and therefore high rates of evaporation, during larval development. In the early wet
466 season, these individuals are likely to utilize the first vegetation that appears after a long
467 period of drought and develop while the environment is becoming increasingly more humid
468 (see figure 2). Later in the wet season, when the temperature is still relatively high and the
469 maximum levels of RH are reached, larvae develop into WSF individuals and have
470 comparatively low values of $\delta^{18}\text{O}$. The temperature drops further during the early months
471 following the final rains and DSF individuals begin to appear. The environment is then
472 gradually drying out as is reflected by enrichment in the ^{18}O composition of adult
473 exoskeletons. The temperature rises significantly during the final phase of the dry season
474 while the RH continues to drop. No recruitment occurs during this part of the season as the
475 grasses in the open savannah habitats dry out and disappear completely (see also Windig *et*

476 *al.* 1994). The butterflies collected in this period likely completed development in the early
477 dry season and survived until they could reproduce with the onset of the next rains. This is
478 confirmed by the absence of intermediate or WSF butterflies with extremely high values of
479 $\delta^{18}\text{O}$.

480 These results clearly indicate that the ratios of stable isotopes of carbon and oxygen
481 obtained from adult organic material can shed light on the larval ecology of insect herbivores
482 and contribute to our understanding of local tropical communities. To our knowledge, this is
483 the first study in which stable isotopes of oxygen have been used in an ecological context in
484 terrestrial arthropods. However, $\delta^{18}\text{O}$ tends to decrease with increasing latitude, altitude and
485 towards the continental interior due to environmental effects on the source water (Bradley
486 2015), which could make biological comparisons across ecological communities and
487 continental gradients challenging.

488

489 *Phylogenetic signal*

490 Of the three species of *Bicyclus* we sampled in the seasonal environment, adults of *B.*
491 *vansoni*, in contrast to *B. ena* and *B. safitza*, are normally found in more shaded habitats.
492 Interestingly, *B. vansoni* is not considered to be a true forest species as it is frequently found
493 in the semi-shaded forests margins while most *Bicyclus* species, and especially the basal
494 lineages of the genus, are less tolerant to habitat disturbance and only found in habitats with
495 complete canopy cover. This may indicate that only those mycalesine species that frequently
496 interact with C_4 grass-dominated open environments, or have done so in the past, are able to
497 utilize the C_4 host plants that are available in their natural habitat. In this context, the results
498 from the more stable environment in Genting are particularly interesting. Here, four out of the
499 eight species solely utilized C_3 host plants during larval development while, evidently, C_4
500 grasses were available in their natural habitat. The ability to use C_4 grasses as the natural host

501 is significantly correlated with the phylogenetic relatedness of the species, indicating that
502 closely related species exhibit similar host plant preferences in this habitat. This is consistent
503 with the hypothesis that those mycalesine species that have been restricted to shaded forest
504 understories throughout their evolutionary history have not evolved adaptations to cope with
505 the lower palatability of C₄. In contrast, species which acquired novel feeding adaptations
506 could colonize new, open ecological niches in the C₄-dominated habitats which were
507 comparatively free of competition from other herbivores, resulting in divergent selection and
508 ultimately speciation (Heckathorn, McNaughton & Coleman 1999).

509 The results of this small-scale comparative analysis of host plant use in mycalesine
510 butterflies are encouraging and emphasize the importance of a more detailed investigation of
511 co-evolutionary patterns between mycalesines and their natural host plants. The phylogenetic
512 relationships of about 200 species, across the entire Mycalesina subtribe, have been inferred
513 recently (Aduse-Poku *et al.* 2015). With a robust phylogenetic framework readily available
514 and the applicability of stable isotopes verified, it is now timely to investigate whether the
515 evolutionary history of mycalesine butterflies is closely tied to the evolutionary history of
516 their hosts and the colonisation of open habitats.

517

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530

531 **Data accessibility**

532 Data deposited in the Dryad Digital Repository: doi:10.5061/dryad.n4m62 (van Bergen et al.,
533 2016).

534 **Tables**

535

536 Table 1: For experiment C, we carried out a multiple linear regression with the $\delta^{18}\text{O}$ values as
 537 the dependent variable, and the adult RH treatment and adult age, (i.e. time spend in RH
 538 treatment), as independent variables. The non-significant interaction between both
 539 independent variables (in bold) was interpreted as an absence of change in $\delta^{18}\text{O}$ values over
 540 time in both adult RH treatments.

dependent variable	fixed effects	t	P
$\delta^{18}\text{O}$ values	Age	0.11	0.916
	RH adult stage	1.28	0.205
	Age : RH adult stage	0.09	0.930

541

542 Table 2: Minimum adequate models of the effect of RH during each experimental stage on
 543 the $\delta^{18}\text{O}$ values obtained from adult organic material in experiment D. The exoskeleton of
 544 individuals that experienced low RH during the larval stage was significantly enriched in ^{18}O .

dependent variable	fixed effects	F	df	P
$\delta^{18}\text{O}$ values	RH host plant	0.11	1,76	0.743
	RH larval stage	75.76	1,76	<0.001
	RH adult stage	2.51	1,76	0.118

545

546 Table 3: In Zomba, the proportion of larvae that developed on C₃ rather than C₄ grasses, was
547 significantly higher in *B. vansoni*; the species that is predominantly found in (semi-) shaded
548 forests and forest margins, while the larvae of the two species that inhabit the open grasslands
549 (*B. ena* and *B. safitza*) largely completed development on C₄ grasses. In addition, we
550 observed a seasonal trend in host plant use towards an increased relative consumption (RC)
551 of C₃ host plants during the dry season in all species (minimum adequate model presented in
552 table S2). The change in relative consumption between both seasonal forms (DSF/WSF) is
553 greater than 1 in all species, indicating an increased utilization of C₃ host plants in DSF
554 individuals.

Species	Seasonal form	C₃	C₄	RC	DSF/WSF
<i>B. ena</i>	DSF	11	101	0.11	3.32
	WSF	2	61	0.03	
<i>B. safitza</i>	DSF	10	113	0.09	2.01
	WSF	4	91	0.04	
<i>B. vansoni</i>	DSF	69	19	3.63	1.26
	WSF	78	27	2.89	

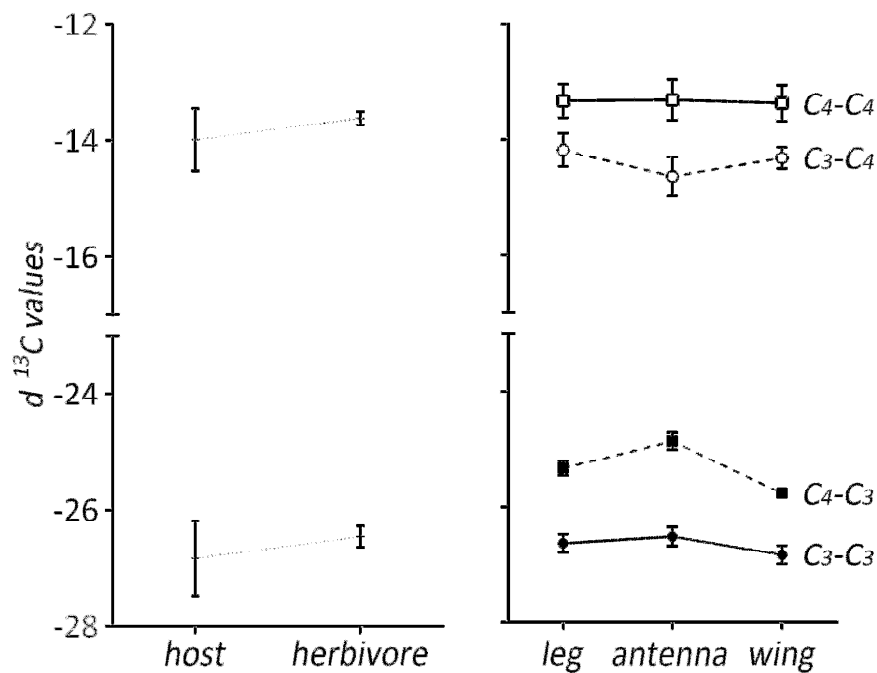
555

556 Table 4: Minimum adequate models of the effects of species, sex and seasonal form on the
 557 $\delta^{18}\text{O}$ values obtained from the field material in Genting and Zomba. The $\delta^{18}\text{O}$ values that
 558 were measured for animals collected in the stable secondary forest in Genting were neither
 559 correlated to the different species nor to the sex of the individuals. In contrast, in the seasonal
 560 habitat in Zomba, the values of $\delta^{18}\text{O}$ of the different species and their seasonal forms were
 561 significant different.

dependent variable	fixed effects	F	df	P
$\delta^{18}\text{O}$ values Genting	Species	0.44	7,358	0.876
	Sex	0.09	1,358	0.761
$\delta^{18}\text{O}$ values Zomba	Species	27.78	2,564	<0.001
	Sex	0.11	1,564	0.742
	Seasonal form	481.29	1,564	<0.001

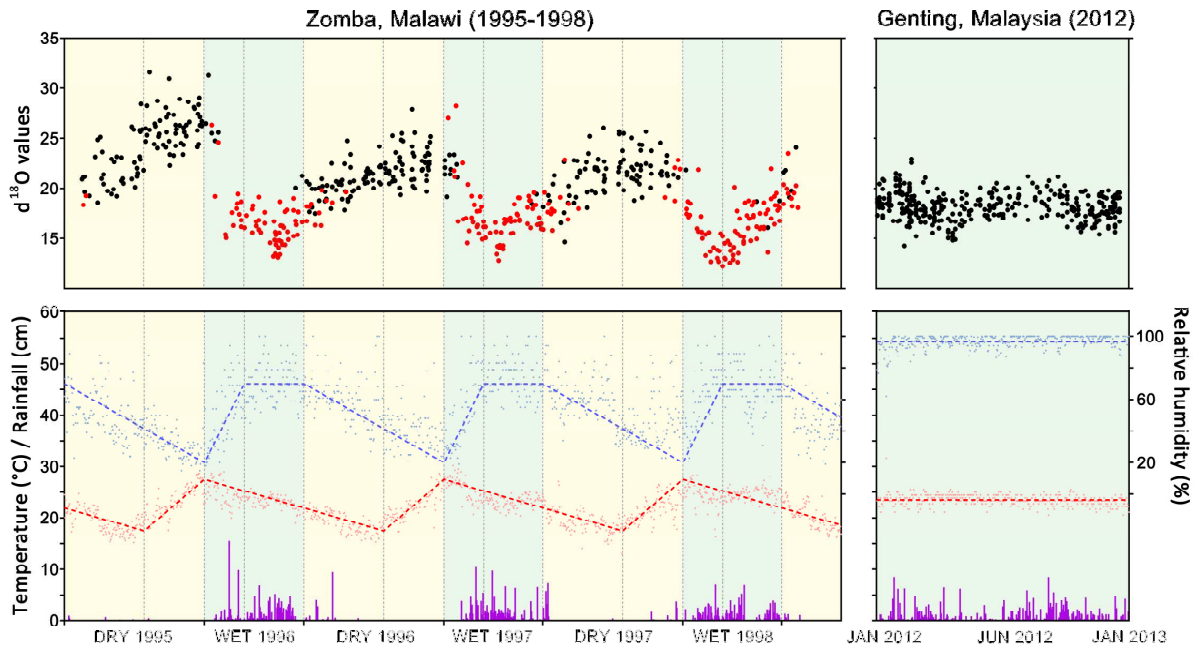
562

563 **Figures**



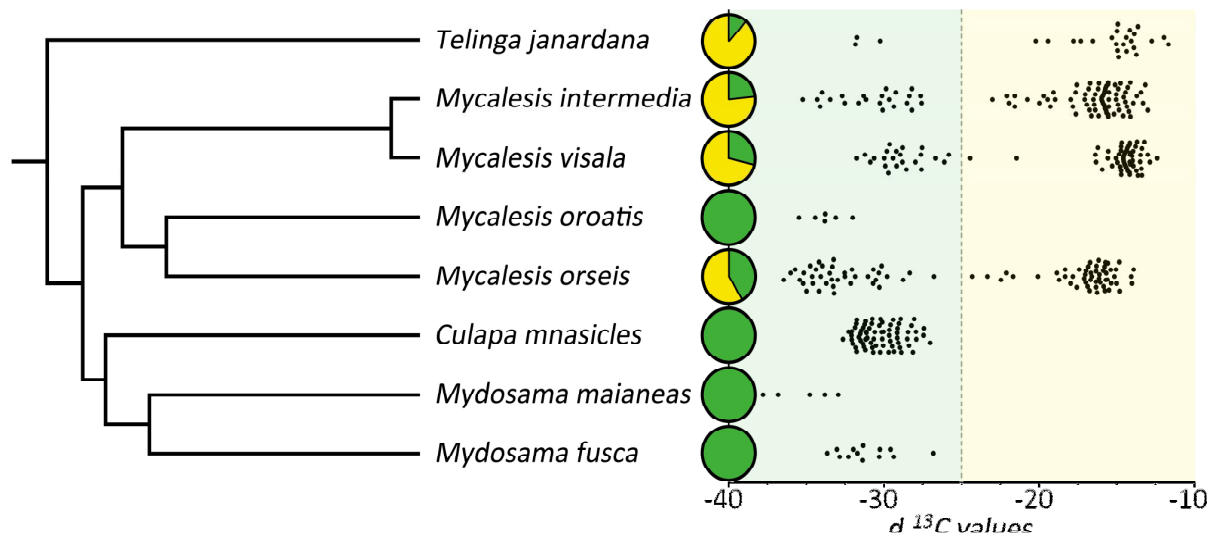
564

565 Figure 1: The left hand figure represents the data collected for experiment A. $\delta^{13}C$ in adult leg
 566 tissue does not significantly differ from the isotopic signatures of the plant material. The $\delta^{13}C$
 567 values obtained from leg, antenna and wing tissue in experiment B are represented in the
 568 right hand figure. For the first four instars, larvae were either reared on plants of wheat
 569 (circles) or maize (squares). On the first day of the fifth instar, the larvae within each cohort
 570 were randomly transferred to a cage with either the original host plants (solid lines) or host
 571 plants of the alternative photosynthetic pathway (swapped: dashed lines), and allowed to
 572 complete development. Filled symbols indicate individuals which completed development on
 573 wheat while the blank symbols represent specimens which were feeding on maize during the
 574 fifth instar. The isotopic signatures of adult tissue mainly reflect the isotopic composition of
 575 the host plant which was consumed by the larvae during the final phase of development,
 576 when most larval growth occurs and development of adult tissues begins. Error bars represent
 577 95% confidence intervals.



578

579 Figure 2: The lower part of this graph is a schematic representation of temperature, relative
 580 humidity and daily rainfall through the fluctuating dry and wet seasons in Zomba, Malawi
 581 (left) and the stable secondary forest in Genting, Malaysia (right). The small red dots are
 582 daily mean temperature measurements in Celsius while the red dashed line reflects the
 583 seasonal fluctuations in temperature. The small blue dots are daily measurements of RH in
 584 percent at 2 pm and the blue dashed line represents the seasonal fluctuations in relative
 585 humidity. Purple bars represent the daily rainfall in mm. Temperature and rainfall are
 586 associated with the left hand axis, relative humidity with the right hand axis. For Zomba, the
 587 background colours provide a simplified representation of the dry season (yellow) and wet
 588 season (green), while the dashed vertical grey lines divide the seasons into early and late. The
 589 $\delta^{18}\text{O}$ values obtained from the exoskeleton of the specimens are represented above the
 590 climatic data. $\delta^{18}\text{O}$ data have been corrected with five weeks to account for the time lag
 591 between catching date and the climatic conditions during development. For Zomba, the red
 592 circles are WSF individuals and black circles DSF.



593

594 Figure 3: The $\delta^{13}\text{C}$ values for all specimens collected in the stable environment in Genting
 595 (N=367). Individuals with $\delta^{13}\text{C}$ values less than -25‰ were classified as C₃ feeders and
 596 values above as C₄ feeders. Pie charts represent the proportion of C₃ feeders in green and C₄
 597 feeders in yellow. Phylogenetic relationships were inferred from the work of Aduse-Poku et
 598 al. (2015). In addition, here we refer to *Telinga janardana* (Moore, 1857), which is the novel
 599 circumscription of the genus based on a taxonomic revision of the *Heteropsis* clade (see
 600 Aduse-Poku *et al.* in press).

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