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

2   evolution

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

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Summary

1. One of the most dramatic examples of biome shifts in the geological record is the rapid replacement of C_3 vegetation by C_4 grasses in (sub-) tropical regions during the Late Miocene-Pliocene. Climate-driven biome shifts of this magnitude are expected to have a major impact on diversification and ecological speciation, especially in grazing taxa. Mycalesine butterflies are excellent candidates to explore the evolutionary impact of these C_3/C_4 shifts on insect grazer communities.

2. Mycalesine butterflies feed on grasses as larvae, have radiated spectacularly and occur in almost all extant habitats across the Old World tropics. However, at present, we lack a comprehensive understanding of the larval ecology of these butterflies and this hampers investigations of co-evolutionary patterns among the geographically parallel radiations of mycalesine butterflies and the remarkable evolutionary history of their host plants.

3. By conducting several experiments under defined environmental conditions we demonstrate that the feeding history of mycalesine larvae on C_3 and C_4 grasses can be traced by analysing $\delta^{13}C$ in the organic material of the adult exoskeleton, while values of $\delta^{18}O$ in the adult reflect atmospheric humidity during larval development.

4. To show the power of these isotopic proxies for ecological studies, we analysed the isotopic composition of organic material obtained from adult butterflies sampled in two extensive longitudinal surveys.

5. We observed strong associations among the larval ecology, habitat preferences of the adult butterflies and patterns of seasonality, such that mycalesine species that inhabit open environments are more opportunistic in their host plant choice but utilize C_3 grasses more frequently during the dry season. Crucially, the ability to process the less palatable C_4 grasses appears to be phylogenetically clustered within mycalesine species,

2

51   suggesting that novel feeding adaptations may have evolved in response to the

52   ecological dominance of C_4 grasses in open savannah habitats.

Introduction

The origin of tropical savannahs is one of the most dramatic examples of climate-driven biome shifts documented in the geological record (Edwards *et al.* 2010). Today's tropical savannahs are dominated by grasses which utilize the C_4 photosynthetic pathway to fix carbon, whereas only 3 to 8 million years ago these tropical environments were largely dominated by C_3 vegetation (Osborne & Beerling 2006). A superior strategy for carbon uptake and assimilation in warm climates and low atmospheric CO_2 concentrations (Ehleringer, Cerling & Helliker 1997), in combination with different responses to climatic extremes and wildfires (Edwards *et al.* 2010; Scheiter *et al.* 2012), led to the ecological success of C_4 grasses in tropical environments (Ehleringer, Cerling & Helliker 1997). Such major changes in biome characteristics would be expected to drive diversification across taxa. For example, radiations in large terrestrial herbivores have been attributed to the expansion of grasslands and changes in vegetation distribution, based on isotopic evidence about feeding ecology from tooth enamel (MacFadden 2005; Kuerschner, Kvacek & Dilcher 2008). However, surprisingly little is known about the impacts of these C_3/C_4 shifts on insect grazer communities in grasslands.

Most herbivorous insects are specialized in their utilization of host plants and, in response to this host specificity, plants often evolve chemical or physical defenses to reduce herbivore-induced damage (Dres & Mallet 2002; Braby & Trueman 2006). This co-evolutionary arms race is thought to play a crucial role in the evolution of key innovations and drive diversification on each side of the plant–insect interaction (Ehrlich & Raven 1964; Edger *et al.* 2015). In theory, C_4 photosynthesis should exert a strong influence on trophic interactions with insect grazers via two key mechanisms. Firstly, C_4 grasses are expected to have a higher physical resistance to herbivores because the anatomical changes required for C_4 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, C_4 leaves typically

83　have lower nutritional values (lower leaf protein and nitrogen) than C_3 foliage (Long 1999).

84　Therefore, generalist herbivores are predicted to prefer C_3 over C_4 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 C_3 and C_4 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 C_4 grasses in savannah

96　habitats (Pena & Wahlberg 2008). In addition, mycalesine butterflies that inhabit C_4 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 C_4 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 C_3 and C_4 grasses, can be traced by analysing the relative amount of carbon-

116   13 (^{13}C) 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 $^{13}CO_2$ during photosynthesis, but the C_4 pathway significantly lowers

119   this discrimination, leading to distinct non-overlapping differences in $\delta^{13}C$ values between C_3

120   and C_4 plants (O'Leary 1988). These differences in ^{13}C 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 ^{18}O 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 (^{16}O) 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 $\delta^{18}O$ 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 $\delta^{18}O$ 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 $\delta^{13}C$ 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 $\delta^{13}C$ 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 $\delta^{18}O$ 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 27ºC,

187   70% RH, and L12:D12.

188   For experiment A, larvae were either reared on wheat (*Triticum aestivum*) or maize,

189   which are C_3 and C_4 grass species, respectively. Leaf samples were collected throughout

190   larval development in order to compare the $\delta^{13}C$ 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, $\delta^{13}C$ 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 (± 5℃), 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 $^{\circ}$C with a pulse of Oxygen and the resultant CO_2 fed into a

249   Costech Elemental Analyser and analysed for $^{13}C/^{12}C$ 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 CO_2 and the $^{13}C/^{12}C$ isotope

254   ratio. For the analysis of oxygen isotopes, the samples were placed in silver capsules. These

255   samples were pyrolysed at $1200^{o}C$ 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 $^{13}C/^{12}C$ ($\delta^{13}C$ Vienna-PDB) and $^{18}O/^{16}O$ ($\delta^{18}O$ V-SMOW).

259   Precision of analyses is better than 0.1 per mille for $^{13}C/^{12}C$ and about 0.4 per mille for

260      $^{18}O/^{16}O$. 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 $\delta^{18}O$ 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 $\delta^{18}O$ 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 $\delta^{18}O$ 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 $\delta^{13}C$ values per species

276   falling within the typical ranges for C_3 or C_4 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 C_3 signatures ($N_{C3} : N_{C3}+N_{C4}$) 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   $\delta^{18}O$ 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 $\delta^{13}C$ in adult leg tissue does not significantly differ from the

295   isotopic signature of the larval host plant (C_3: t = -1.25, df = 12.49, P = 0.24 and C_4: 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 $\delta^{13}C$ 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 $\delta^{13}C$ values of individuals that

300   were swapped from the C_3 to the C_4 host plant at this final stage of larval development were

301 more similar to the isotopic signatures of individuals that were reared on C_4 plants during all

302 larval stages, and *vice versa* (table S1).

303   Experiment C showed that $\delta^{18}O$ 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 $\delta^{18}O$ 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 $\delta^{18}O$ 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 $\delta^{13}C$ values,

318 representing the isotopic composition of C_3 and C_4 grasses (individuals with $\delta^{13}C$ values less

319 than -25‰ were classified as C_3 feeders; figure S3). At Zomba, the proportion of larvae that

320 developed on C_3 grasses was significantly higher in *B. vansoni*, the species where adults are

321 predominantly found in (semi-)shaded forest habitats and forest margins (*B. vansoni:B. ena*

322 comparison, $X^2 = 187.16$, df =1, P < 0.01; *B. vansoni:B. safitza* comparison, $X^2 = 216.50$, df

323 =1, P < 0.01). The ratios of C_3:C_4 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 C_3 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 $\delta^{13}C$ 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 C_4 grasses throughout the year. We found no evidence for C_4 larval feeding in any

331   of the remaining four species; *M. oroatis*, *M. fusca, M. maianeas* and *C. mnasicles*. The

332   ability to use C_4 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 λ = 1.08, P < 0.05;

334   figure 3).

335

336   *Stable isotopes of oxygen in field material*

337   The $\delta^{18}O$ 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 $\delta^{18}O$ between *B. safitza* and *B. vansoni* (Tukey's HSD test, P = 0.54).

344   The $\delta^{18}O$ 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

Discussion

Our laboratory experiments indicate that dietary preferences for grasses with a C_3 or C_4 photosynthetic pathway can be traced accurately in mycalesine butterfly larvae by analysing the relative amount of ^{13}C deposited in the exoskeleton of adult butterflies. We have also shown that the values of $\delta^{18}O$ measured in adult organic material reflect atmospheric humidity during larval development. Together, these isotopic proxies provide key information about the larval feeding ecology and atmospheric environment of mycalesine butterflies in the wild, and enable three key inferences. First, species that inhabit open environments are more opportunistic in their larval host plant choice, whereas C_3 grass specialists were only found in the shaded habitat. Secondly, we observed seasonality in host plant utilization and larval respiration in the open savannah habitat, such that during the early dry season, larvae are more likely to use C_3 grasses and to experience higher rates of evaporation. Finally, our data suggest that the ability to process C_4 grasses is phylogenetically clustered within mycalesine species.

Proof of principle experiments

Animal organic tissue is enriched in $\delta^{13}C$, on average, by about 1‰ relative to the diet (Deniro & Epstein 1978), but enrichment in $\delta^{13}C$ can be as high as 14.1‰ in the tooth enamel of large mammals because of equilibrium fractionation associated with inorganic carbon deposition (Cerling *et al.* 1997; Cerling & Harris 1999). Our results indicate that the isotopic composition of leg tissue in mycalesine butterflies is not significantly enriched compared to the isotopic composition of the larval host plant (see also Fischer, O'Brien & Boggs 2004), suggesting that isotopic discrimination during digestion and assimilation is very limited in these species. In addition, we demonstrate that, in mycalesine butterflies, and probably, therefore, in other holometabolous insect herbivores, the isotopic signatures of adult tissue to

16

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 $\delta^{13}C$ 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 $\delta^{18}O$ 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 C_4 grasses in their

407 natural environment. In the material from Zomba, the C_3:C_4 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 C_4 grasses whereas individuals of *B. vansoni* from more shaded habitats mainly utilized C_3

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 $\delta^{13}C$ 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 $\delta^{18}O$ and $\delta^{13}C$

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 $\delta^{13}C$ values revealed clear differences in host plant preferences, with half of the

426 species frequently utilizing C_4 plants while the other species solely using C_3 grasses. This

427 indicates that there is no inherent barrier to forest species consuming C_4 grasses.

428 Furthermore, this observation conflicts with the prediction that generalists avoid C_4 leaves

429 when C_3 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 C_3 grasses more

450 frequently when the C_4 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 C_3 grass species increases during this period (Caswell *et al.* 1973). However,

453   the differences in $C_3:C_4$ 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 C_3

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 $\delta^{18}O$ 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 $\delta^{18}O$. 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 $\delta^{18}O$.

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, $\delta^{18}O$ 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 C_4. In contrast, species which acquired novel feeding adaptations

506   could colonize new, open ecological niches in the C_4-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

530

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 $\delta^{18}O$ 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 $\delta^{18}O$ values over

540 time in both adult RH treatments.

dependent variable	fixed effects	t	P
$\delta^{18}O$ 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 $\delta^{18}O$ 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
$\delta^{18}O$ 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 C_3 rather than C_4 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 C_4 grasses. In addition, we

550 observed a seasonal trend in host plant use towards an increased relative consumption (RC)

551 of C_3 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 C_3 host plants in DSF

554 individuals.

| Species	Seasonal form	C_3	C_4	RC	DSF/WSF
B. ena	DSF	11	101	0.11	3.32
	WSF	2	61	0.03	
B. safitza	DSF	10	113	0.09	2.01
	WSF	4	91	0.04	
B. vansoni	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   $\delta^{18}O$ values obtained from the field material in Genting and Zomba. The $\delta^{18}O$ 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 $\delta^{18}O$ of the different species and their seasonal forms were

561   significant different.

| dependent variable	fixed effects	F	df	P
$\delta^{18}O$ values Genting	Species	0.44	7,358	0.876
	Sex	0.09	1,358	0.761
$\delta^{18}O$ 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
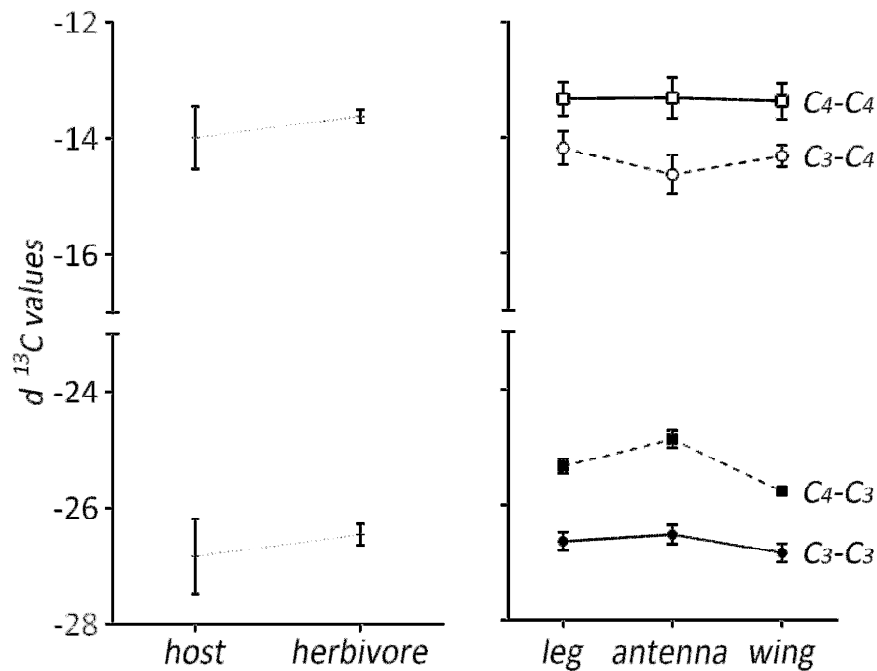
Figures

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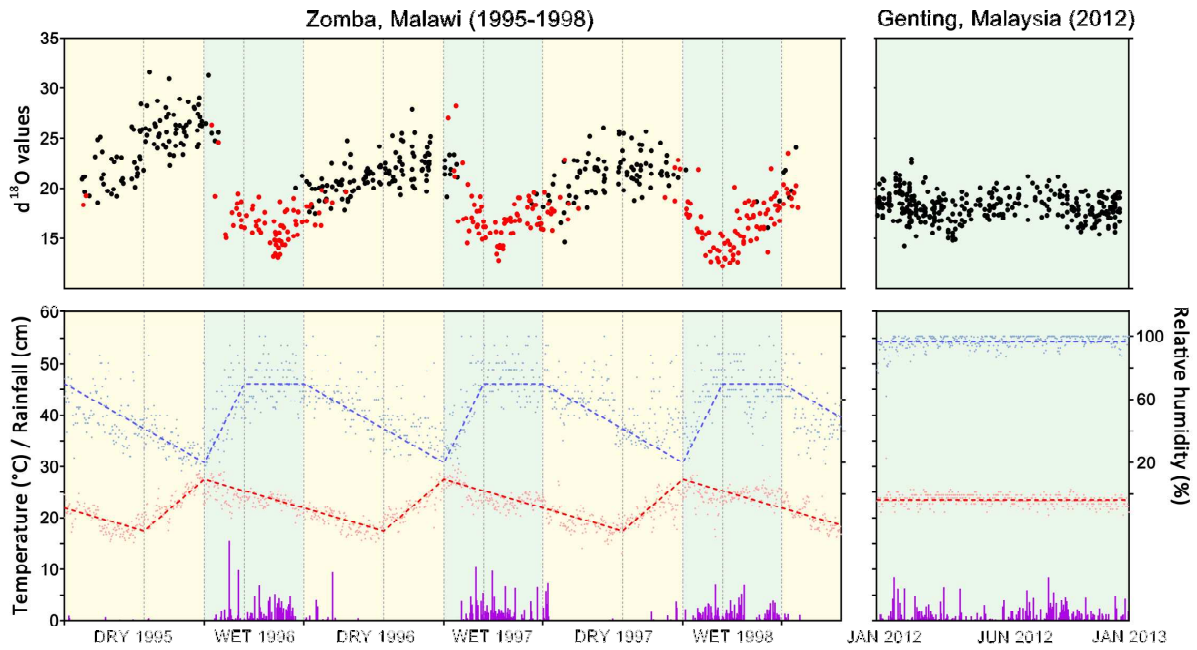
Figure 2: The lower part of this graph is a schematic representation of temperature, relative humidity and daily rainfall through the fluctuating dry and wet seasons in Zomba, Malawi (left) and the stable secondary forest in Genting, Malaysia (right). The small red dots are daily mean temperature measurements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

29

593
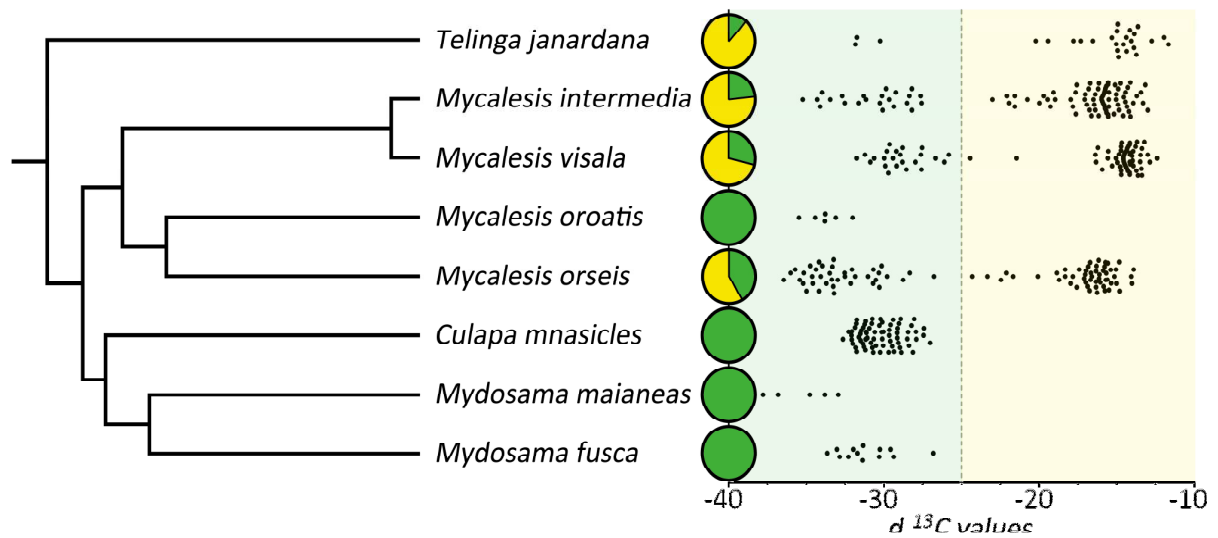
Figure 3: The δ^{13}C values for all specimens collected in the stable environment in Genting (N=367). Individuals with δ^{13}C values less than -25‰ were classified as C_3 feeders and values above as C_4 feeders. Pie charts represent the proportion of C_3 feeders in green and C_4 feeders in yellow. Phylogenetic relationships were inferred from the work of Aduse-Poku et al. (2015). In addition, here we refer to *Telinga janardana* (Moore, 1857), which is the novel circumscription of the genus based on a taxonomic revision of the *Heteropsis* clade (see Aduse-Poku *et al.* in press).

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