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

2 evolution

3

4 **Authors:** Erik van Bergen[1*], Henry S. Barlow[2], Oskar Brattström[1], Howard Griffiths[3],

5 Ullasa Kodandaramaiah[1,4], Colin P. Osborne[5], Paul M. Brakefield[1]

6

7 **Addresses:**

8 [1] Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ,

9 United Kingdom

10 [2] P.O. Box 10139, 50704 Kuala Lumpur, Malaysia

11 [3] Department of Plant Sciences, University of Cambridge, Cambridge, CB2 3EA, United

12 Kingdom

13 [4] School of Biology, Indian Institute of Science Education and Research

14 Thiruvananthapuram, CET campus, Trivandrum 695016, India

15 [5] Department of Animal and Plant Sciences, University of Sheffield, Sheffield, S10 2TN,

16 United Kingdom

17

18 [*] Author for correspondence (ev274@cam.ac.uk)

19

20 **Keywords:** stable isotopes, larval ecology, C_4 photosynthesis, mycalesine butterflies, plant–

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 C_3 vegetation by C_4 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 C_3/C_4 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 C_3 and C_4 grasses can be

41   traced by analysing $\delta^{13}C$ in the organic material of the adult exoskeleton, while values

42   of $\delta^{18}O$ 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 C_3

49   grasses more frequently during the dry season. Crucially, the ability to process the less

50   palatable C_4 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 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

4

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 δ^{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 C_4-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 C_3 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 C_3

165   and C_4 grasses could be traced by analysing the $\delta^{13}C$ of adult leg tissue. They also explored

166   how a mixed larval diet of C_3 and C_4 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°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 *maianes* (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 ^{o}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. maianes* and *C. mnasicles*. The ability

332 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 $\lambda = 1.08$, $P < 0.05$;

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

527 All authors read and approved the final version of the manuscript for publication. Data are

528 deposited in the Dryad repository (doi:XXXXXXXXX).

529   7. TABLES

530

531   Table 1: For experiment C, we carried out a multiple linear regression with the δ^{18}O values as

532   the dependent variable, and the adult RH treatment and adult age, (i.e. time spend in RH

533   treatment), as independent variables. The non-significant interaction between both

534   independent variables (in bold) was interpreted as an absence of change in δ^{18}O values over

535   time in both adult RH treatments.

dependent variable	fixed effects	t	P
δ^{18}O values	Age	0.11	0.916
	RH adult stage	1.28	0.205
	Age : RH adult stage	0.09	**0.930**

536

537 Table 2: Minimum adequate models of the effect of RH during each experimental stage on

538 the $\delta^{18}O$ values obtained from adult organic material in experiment D. The exoskeleton of

539 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

540

541   Table 3: In Zomba, the proportion of larvae that developed on C_3 rather than C_4 grasses, was

542   significantly higher in *B. vansoni*; the species that is predominantly found in (semi-) shaded

543   forests and forest margins, while the larvae of the two species that inhabit the open grasslands

544   (*B. ena* and *B. safitza*) largely completed development on C_4 grasses. In addition, we

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

546   of C_3 host plants during the dry season in all species (minimum adequate model presented in

547   table S2). The change in relative consumption between both seasonal forms (DSF/WSF) is

548   greater than 1 in all species, indicating an increased utilization of C_3 host plants in DSF

549   individuals.

Species	Seasonal form	C_3	C_4	RC	DSF/WSF
B. ena	DSF	11	101	0.11	
	WSF	2	61	0.03	3.32
B. safitza	DSF	10	113	0.09	
	WSF	4	91	0.04	2.01
B. vansoni	DSF	69	19	3.63	
	WSF	78	27	2.89	1.26

550

551   Table 4: Minimum adequate models of the effects of species, sex and seasonal form on the

552     $\delta^{18}O$ values obtained from the field material in Genting and Zomba. The $\delta^{18}O$ values that

553   were measured for animals collected in the stable secondary forest in Genting were neither

554   correlated to the different species nor to the sex of the individuals. In contrast, in the seasonal

555   habitat in Zomba, the values of $\delta^{18}O$ of the different species and their seasonal forms were

556   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**

557

558   8. FIGURES

559

560   Figure 1: The left hand figure represents the data collected for experiment A. δ^{13}C in adult leg

561   tissue does not significantly differ from the isotopic signatures of the plant material. The δ^{13}C

562   values obtained from leg, antenna and wing tissue in experiment B are represented in the

563   right hand figure. For the first four instars, larvae were either reared on plants of wheat

564   (circles) or maize (squares). On the first day of the fifth instar, the larvae within each cohort

565   were randomly transferred to a cage with either the original host plants (solid lines) or host

566   plants of the alternative photosynthetic pathway (swapped: dashed lines), and allowed to

567   complete development. Filled symbols indicate individuals which completed development on

568   wheat while the blank symbols represent specimens which were feeding on maize during the

569   fifth instar. The isotopic signatures of adult tissue mainly reflect the isotopic composition of

570   the host plant which was consumed by the larvae during the final phase of development,

571   when most larval growth occurs and development of adult tissues begins. Error bars represent

572   95% confidence intervals.

573   Figure 2: The lower part of this graph is a schematic representation of temperature, relative

574   humidity and daily rainfall through the fluctuating dry and wet seasons in Zomba, Malawi

575   (left) and the stable secondary forest in Genting, Malaysia (right). The small red dots are

576   daily mean temperature measurements in Celsius while the red dashed line reflects the

577   seasonal fluctuations in temperature. The small blue dots are daily measurements of RH in

578   percent at 2 pm and the blue dashed line represents the seasonal fluctuations in relative

579   humidity. Purple bars represent the daily rainfall in mm. Temperature and rainfall are

580   associated with the left hand axis, relative humidity with the right hand axis. For Zomba, the

581   background colours provide a simplified representation of the dry season (yellow) and wet

582   season (green), while the dashed vertical grey lines divide the seasons into early and late. The

583    $\delta^{18}O$ values obtained from the exoskeleton of the specimens are represented above the

584   climatic data. $\delta^{18}O$ data have been corrected with five weeks to account for the time lag

585   between catching date and the climatic conditions during development. For Zomba, the red

586   circles are WSF individuals and black circles DSF.

587   Figure 3: The $\delta^{13}C$ values for all specimens collected in the stable environment in Genting

588   (N=367). Individuals with $\delta^{13}C$ values less than -25‰ were classified as C_3 feeders and

589   values above as C_4 feeders. Pie charts represent the proportion of C_3 feeders in green and C_4

590   feeders in yellow. Phylogenetic relationships were inferred from the work of Aduse-Poku et

591   al. (2015). In addition, here we refer to *Telinga janardana* (Moore, 1857), which is the novel

592   circumscription of the genus based on a taxonomic revision of the *Heteropsis* clade (see

593   Aduse-Poku *et al.* in press).

594   9. REFERENCES

595   Aduse-Poku, K., Brattström, O., Kodandaramaiah, U., Lees, D.C., Brakefield, P.M. &
596   Wahlberg, N. (2015) Systematics and historical biogeography of the Old World
597   butterfly subtribe Mycalesina (Lepidoptera: Nymphalidae: Satyrinae). *Bmc*
598   *Evolutionary Biology,* **15**.

599   Aduse-Poku, K., Lees, D.C., Brattström, O., Kodandaramaiah, U., Wahlberg, N. &
600   Brakefield, P.M. (in press) Molecular phylogeny and generic-level taxonomy of the
601   widespread palaeotropical *'Heteropsis* clade' (Nymphalidae: Satyrinae: Mycalesina).
602   *Systematic Entomology.*

603   Ben-David, M., Flynn, R.W. & Schell, D.M. (1997) Annual and seasonal changes in diets of
604   martens: Evidence from stable isotope analysis. *Oecologia,* **111,** 280-291.

605   Blomberg, S.P., Garland, T. & Ives, A.R. (2003) Testing for phylogenetic signal in
606   comparative data: Behavioral traits are more labile. *Evolution,* **57,** 717-745.

607   Bouchenak-Khelladi, Y., Verboom, G.A., Hodkinson, T.R., Salamin, N., Francois, O., Ni
608   Chonghaile, G. & Savolainen, V. (2009) The origins and diversification of C-4
609   grasses and savanna-adapted ungulates. *Global Change Biology,* **15,** 2397-2417.

610   Boutton, T.W., Cameron, G.N. & Smith, B.N. (1978) Insect Herbivory on C-3 and C-4
611   Grasses. *Oecologia,* **36,** 21-32.

612   Braby, M.F. & Trueman, J.W.H. (2006) Evolution of larval host plant associations and
613   adaptive radiation in pierid butterflies. *Journal of Evolutionary Biology,* **19,** 1677-
614   1690.

615   Bradley, R.S. (2015) *Paleoclimatology: Reconstructing Climates of the Quaternary,* Third
616   Edition edn. Academic Press, Oxford, United Kingdom.

617   Brakefield, P.M. (2010) Radiations of Mycalesine Butterflies and Opening Up Their
618   Exploration of Morphospace. *American Naturalist,* **176,** S77-S87.

619   Brakefield, P.M., Beldade, P. & Zwaan, B.J. (2009) The African butterfly *Bicyclus anynana*:
620   a model for evolutionary genetics and evolutionary developemental biology.
621   *Emerging model organisms: a laboratory manual* (eds R. Behringer, A. Johnson & R.
622   Krumlauf), pp. 291-329. CSHL Press, Cold Spring Harbor.

623 Brakefield, P.M. & Mazzotta, V. (1995) Matching Field and Laboratory Environments -
624   Effects of Neglecting Daily Temperature-Variation on Insect Reaction Norms.
625   *Journal of Evolutionary Biology,* **8,** 559-573.

626 Brakefield, P.M., Pijpe, J. & Zwaan, B.J. (2007) Developmental plasticity and acclimation
627   both contribute to adaptive responses to alternating seasons of plenty and of stress in
628   *Bicyclus* butterflies. *Journal of Biosciences,* **32,** 465-475.

629 Brakefield, P.M. & Reitsma, N. (1991) Phenotypic Plasticity, Seasonal Climate and the
630   Population Biology of *Bicyclus* Butterflies (Satyridae) in Malawi. *Ecological*
631   *Entomology,* **16,** 291-303.

632 Brattström, O., Bensch, S., Wassenaar, L.I., Hobson, K.A. & Åkesson, S. (2010)
633   Understanding the migration ecology of European red admirals *Vanessa atalanta*
634   using stable hydrogen isotopes. *Ecography,* **33,** 720-729.

635 Caswell, H., Reed, F., Stephens.Sn & Werner, P.A. (1973) Photosynthetic pathways and
636   selective herbivory - hypothesis. *American Naturalist,* **107,** 465-480.

637 Cerling, T.E., Ehleringer, J.R. & Harris, J.M. (1998) Carbon dioxide starvation, the
638   development of C-4 ecosystems, and mammalian evolution. *Philosophical*
639   *Transactions of the Royal Society of London Series B-Biological Sciences,* **353,** 159-
640   170.

641 Cerling, T.E. & Harris, J.M. (1999) Carbon isotope fractionation between diet and bioapatite
642   in ungulate mammals and implications for ecological and paleoecological studies.
643   *Oecologia,* **120,** 347-363.

644 Cerling, T.E., Harris, J.M., MacFadden, B.J., Leakey, M.G., Quade, J., Eisenmann, V. &
645   Ehleringer, J.R. (1997) Global vegetation change through the Miocene/Pliocene
646   boundary. *Nature,* **389,** 153-158.

647 Codron, J., Codron, D., Sponheimer, M., Kirkman, K., Duffy, K.J., Raubenheimer, E.J.,
648   Melice, J.L., Grant, R., Clauss, M. & Lee-Thorp, J.A. (2012) Stable isotope series
649   from elephant ivory reveal lifetime histories of a true dietary generalist. *Proceedings*
650   *of the Royal Society B-Biological Sciences,* **279,** 2433-2441.

651 Deniro, M.J. & Epstein, S. (1978) Influence of Diet on Distribution of Carbon Isotopes in
652   Animals. *Geochimica Et Cosmochimica Acta,* **42,** 495-506.

653 Dres, M. & Mallet, J. (2002) Host races in plant-feeding insects and their importance in
654   sympatric speciation. *Philosophical Transactions of the Royal Society B-Biological*
655   *Sciences,* **357,** 471-492.

656 Edger, P.P., Heidel-Fischer, H.M., Bekaert, M., Rota, J., Glockner, G., Platts, A.E., Heckel,
657   D.G., Der, J.P., Wafula, E.K., Tang, M., Hofberger, J.A., Smithson, A., Hall, J.C.,
658   Blanchette, M., Bureau, T.E., Wright, S.I., dePamphilis, C.W., Eric Schranz, M.,
659   Barker, M.S., Conant, G.C., Wahlberg, N., Vogel, H., Pires, J.C. & Wheat, C.W.
660   (2015) The butterfly plant arms-race escalated by gene and genome duplications.
661   *Proceedings of the National Academy of Sciences of the United States of America,*
662   **112,** 8362-8366.

663 Edwards, E.J., Osborne, C.P., Stromberg, C.A.E., Smith, S.A., Bond, W.J., Christin, P.A.,
664   Cousins, A.B., Duvall, M.R., Fox, D.L., Freckleton, R.P., Ghannoum, O., Hartwell, J.,
665   Huang, Y.S., Janis, C.M., Keeley, J.E., Kellogg, E.A., Knapp, A.K., Leakey, A.D.B.,
666   Nelson, D.M., Saarela, J.M., Sage, R.F., Sala, O.E., Salamin, N., Still, C.J., Tipple, B.
667   & Consortium, C.G. (2010) The Origins of C(4) Grasslands: Integrating Evolutionary
668   and Ecosystem Science. *Science,* **328,** 587-591.

669 Ehleringer, J.R., Cerling, T.E. & Helliker, B.R. (1997) C-4 photosynthesis, atmospheric CO_2
670   and climate. *Oecologia,* **112,** 285-299.

671 Ehrlich, P.R. & Raven, P.H. (1964) Butterflies and plants - a study in coevolution. *Evolution,*
672   **18,** 586-608.

673 Ellwood, M.D.F., Northfield, R.G.W., Mejia-Chang, M. & Griffiths, H. (2011) On the vapour
674   trail of an atmospheric imprint in insects. *Biology Letters,* **7,** 601-604.

675 Fischer, K., O'Brien, D.M. & Boggs, C.L. (2004) Allocation of larval and adult resources to
676   reproduction in a fruit-feeding butterfly. *Functional Ecology,* **18,** 656-663.

677 Fry, B., Joern, A. & Parker, P.L. (1978) Grasshopper Food Web Analysis - Use of Carbon
678   Isotope Ratios to Examine Feeding Relationships among Terrestrial Herbivores.
679   *Ecology,* **59,** 498-506.

680 Heckathorn, S.A., McNaughton, S.J. & Coleman, J.S. (1999) C4 plants and herbivory. *C4*
681   *plant biology* (eds R.F. Sage & R.K. Monson), pp. 285–312. Academic Press, San
682   Diego, USA.

683 Helliker, B.R. & Ehleringer, J.R. (2000) Establishing a grassland signature in veins: O-18 in
684   the leaf water of C-3 and C-4 grasses. *Proceedings of the National Academy of*
685   *Sciences of the United States of America,* **97,** 7894-7898.

686 Holloway, J.D., Barlow, H.S., Hok, K.L. & Chey, V.K. (2013) Sweet or Savoury? Adult
687   Feeding Preferences of Lepidoptera Attracted to Banana and Prawn Baits in the
688   Oriental Tropics. *Raffles Bulletin of Zoology***,** 71-90.

689 Kelly, J.F., Atudorei, V., Sharp, Z.D. & Finch, D.M. (2002) Insights into Wilson's Warbler
690   migration from analyses of hydrogen stable-isotope ratios. *Oecologia,* **130,** 216-+.

691 Kielland, J. (1990) *Butterflies of Tanzania.* Hill House Melbourne, Australia.

692 Kodandaramaiah, U., Lees, D.C., Muller, C.J., Torres, E., Karanth, K.P. & Wahlberg, N.
693   (2010) Phylogenetics and biogeography of a spectacular Old World radiation of
694   butterflies: the subtribe Mycalesina (Lepidoptera: Nymphalidae: Satyrini). *Bmc*
695   *Evolutionary Biology,* **10**.

696 Kooi, R.E. & Brakefield, P.M. (1999) The critical period for wing pattern induction in the
697   polyphenic tropical butterfly *Bicyclus anynana* (Satyrinae). *Journal of Insect*
698   *Physiology,* **45,** 201-212.

699 Kuerschner, W.M., Kvacek, Z. & Dilcher, D.L. (2008) The impact of Miocene atmospheric
700   carbon dioxide fluctuations on climate and the evolution of terrestrial ecosystems.
701   *Proceedings of the National Academy of Sciences of the United States of America,*
702   **105,** 449-453.

703 Larsen, T.B. (1991) *The butterflies of Kenya and their natural history.* Oxford University
704   Press, Oxford.

705 Larsen, T.B. (2005) *Butterflies of West Africa.* Apollo Books, Stenstrup, Denmark.

706 Long, S.P. (1999) Environmental responses. *The Biology of C4 Photosynthesis* (eds R.F. Sage
707   & R.K. Monson), pp. 209–243. Academic Press, San Diego, CA, USA.

708 MacFadden, B. (2005) Terrestrial Mammalian Herbivore Response to Declining Levels of
709   Atmospheric CO2 During the Cenozoic: Evidence from North American Fossil
710   Horses (Family Equidae). *A History of Atmospheric CO2 and its Effects on Plants,*
711   *Animals, and Ecosytems* (ed. C.T. Ehleringer JR, Dearing MD), pp. 273–292.
712   Springer, Berlin.

713   Massey, F.P. & Hartley, S.E. (2006) Experimental demonstration of the antiherbivore effects
714   of silica in grasses: impacts on foliage digestibility and vole growth rates.
715   *Proceedings of the Royal Society B-Biological Sciences,* **273,** 2299-2304.

716   Massey, F.P. & Hartley, S.E. (2009) Physical defences wear you down: progressive and
717   irreversible impacts of silica on insect herbivores. *Journal of Animal Ecology,* **78,**
718   281-291.

719   Mateus, A.R.A., Marques-Pita, M., Oostra, V., Lafuente, E., Brakefield, P.M., Zwaan, B.J. &
720   Beldade, P. (2014) Adaptive developmental plasticity: Compartmentalized responses
721   to environmental cues and to corresponding internal signals provide phenotypic
722   flexibility. *Bmc Biology,* **12**.

723   Monastyrskii, A.L. (2005) *Butterflies of Vietnam: Nymphalidae: Satyrinae*. Dolphin Media,
724   Hanoi.

725   O'Leary, M.H. (1988) Carbon Isotopes in Photosynthesis. *Bioscience,* **38,** 328-336.

726   Osborne, C.P. & Beerling, D.J. (2006) Nature's green revolution: the remarkable evolutionary
727   rise of C-4 plants. *Philosophical Transactions of the Royal Society B-Biological*
728   *Sciences,* **361,** 173-194.

729   Pagel, M. (1999) Inferring the historical patterns of biological evolution. *Nature,* **401,** 877-
730   884.

731   Pena, C. & Wahlberg, N. (2008) Prehistorical climate change increased diversification of a
732   group of butterflies. *Biology Letters,* **4,** 274-278.

733   Pringle, E.L.L. (1994) *Pennington's Butterflies of Southern Africa*. Struik Winchester, Cape
734   Town.

735   R Development Core Team (2014) R: A language and environment for statistical computing.
736   R Foundation for Statistical Computing, Vienna, Austria. URL: http://www.R-
737   project.org.

738   Revell, L.J. (2012) phytools: an R package for phylogenetic comparative biology (and other
739   things). *Methods in Ecology and Evolution,* **3,** 217-223.

740   Roskam, J.C. & Brakefield, P.M. (1999) Seasonal polyphenism in *Bicyclus* (Lepidoptera :
741   Satyridae) butterflies: different climates need different cues. *Biological Journal of the*
742   *Linnean Society,* **66,** 345-356.

743   Scheiter, S., Higgins, S.I., Osborne, C.P., Bradshaw, C., Lunt, D., Ripley, B.S., Taylor, L.L.
744   & Beerling, D.J. (2012) Fire and fire-adapted vegetation promoted C4 expansion in
745   the late Miocene. *New Phytologist,* **195,** 653-666.

746   Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T.,
747   Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.Y., White, D.J.,
748   Hartenstein, V., Eliceiri, K., Tomancak, P. & Cardona, A. (2012) Fiji: an open-source
749   platform for biological-image analysis. *Nature Methods,* **9,** 676-682.

750   van Bergen, E., Brakefield, P.M., Heuskin, S., Zwaan, B.J. & Nieberding, C.M. (2013) The
751   scent of inbreeding: a male sex pheromone betrays inbred males. *Proceedings of the*
752   *Royal Society B-Biological Sciences,* **280**.

753   van den Heuvel, J., Saastamoinen, M., Brakefield, P.M., Kirkwood, T.B.L., Zwaan, B.J. &
754   Shanley, D.P. (2013) The Predictive Adaptive Response: Modeling the Life-History
755   Evolution of the Butterfly *Bicyclus anynana* in Seasonal Environments. *American*
756   *Naturalist,* **181,** E28-E42.

757   Wassenaar, L.I. & Hobson, K.A. (1998) Natal origins of migratory monarch butterflies at
758   wintering colonies in Mexico: New isotopic evidence. *Proceedings of the National*
759   *Academy of Sciences of the United States of America,* **95,** 15436-15439.

760   West, J.B., Bowen, G.J., Cerling, T.E. & Ehleringer, J.R. (2006) Stable isotopes as one of
761   nature's ecological recorders. *Trends in Ecology & Evolution,* **21,** 408-414.

762   Wijngaarden, P.J. & Brakefield, P.M. (2001) Lack of response to artificial selection on the
763   slope of reaction norms for seasonal polyphenism in the butterfly *Bicyclus anynana.*
764   *Heredity,* **87,** 410-420.

765   Wilson, J.R. & Hattersley, P.W. (1989) Anatomical characteristics and digestibility of leafes
766   op Panicum and other grass genera with C-3 and different types of C-4 photosynthetic
767   pathway. *Australian Journal of Agricultural Research,* **40,** 125-136.

768   Windig, J.J., Brakefield, P.M., Reitsma, N. & Wilson, J.G.M. (1994) Seasonal polyphenism
769   in the wild - survey of wing patterns in 5 species of *Bicyclus* butterflies in Malawi.
770   *Ecological Entomology,* **19,** 285-298.