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

Authors: Erik van Bergen\textsuperscript{1*}, Henry S. Barlow\textsuperscript{2}, Oskar Brattström\textsuperscript{1}, Howard Griffiths\textsuperscript{3}, Ullasa Kodandaramaiah\textsuperscript{1,4}, Colin P. Osborne\textsuperscript{5}, Paul M. Brakefield\textsuperscript{1}

Addresses:
\textsuperscript{1} Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, United Kingdom
\textsuperscript{2} P.O. Box 10139, 50704 Kuala Lumpur, Malaysia
\textsuperscript{3} Department of Plant Sciences, University of Cambridge, Cambridge, CB2 3EA, United Kingdom
\textsuperscript{4} School of Biology, Indian Institute of Science Education and Research Thiruvananthapuram, CET campus, Trivandrum 695016, India
\textsuperscript{5} Department of Animal and Plant Sciences, University of Sheffield, Sheffield, S10 2TN, United Kingdom

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

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Running headline: Isotopic ecology of mycalesine butterflies
Summary

1. One of the most dramatic examples of biome shifts in the geological record is the rapid replacement of C₃ vegetation by C₄ 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 C₃/C₄ 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 C₃ and C₄ grasses can be traced by analysing δ¹³C in the organic material of the adult exoskeleton, while values of δ¹⁸O 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 C₃ grasses more frequently during the dry season. Crucially, the ability to process the less palatable C₄ grasses appears to be phylogenetically clustered within mycalesine species,
suggesting that novel feeding adaptations may have evolved in response to the 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 C₄ photosynthetic pathway to fix carbon, whereas only 3 to 8 million years ago these tropical environments were largely dominated by C₃ vegetation (Osborne & Beerling 2006). A superior strategy for carbon uptake and assimilation in warm climates and low atmospheric CO₂ 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 C₄ 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 C₃/C₄ 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, C₄ photosynthesis should exert a strong influence on trophic interactions with insect grazers via two key mechanisms. Firstly, C₄ grasses are expected to have a higher physical resistance to herbivores because the anatomical changes required for C₄ photosynthesis are associated with a higher density of leaf veins, fibre bundles and silica.
cells (Wilson & Hattersley 1989; Bouchenak-Khelladi et al. 2009). Host plants with a high physical resistance rapidly increase mandible wear in lepidopteran larvae, while high levels of silica in grasses decrease nitrogen absorption, reducing feeding efficiency and insect growth rates, respectively (Massey & Hartley 2006; Massey & Hartley 2009). Secondly, because of the high efficiency of their carbon concentrating mechanism, C₄ leaves typically have lower nutritional values (lower leaf protein and nitrogen) than C₃ foliage (Long 1999). Therefore, generalist herbivores are predicted to prefer C₃ over C₄ leaves, despite possessing the ability to process the latter, when both are available in the same habitat (see Caswell et al. 1973).

Here, we use data on butterflies of the subtribe Mycalesina (Nymphalidae: Satyrinae) to examine how physiological and ecological differences between C₃ and C₄ grasses may have impacted the evolutionary ecology of insect grazers. These tropical butterflies feed mainly on grasses as larvae and, especially in the case of *Bicyclus anynana*, have become important model organisms in ecological, evolutionary and developmental biology (van Bergen et al. 2013; van den Heuvel et al. 2013; Mateus et al. 2014). Mycalesines have radiated dramatically in Sub-Saharan Africa, Madagascar and Asia with over 300 extant species (Kodandaramaiah et al. 2010; Aduse-Poku et al. 2015), and their evolutionary history is expected to be closely tied to the rise to ecological dominance of C₄ grasses in savannah habitats (Pena & Wahlberg 2008). In addition, mycalesine butterflies that inhabit C₄ grass-dominated open habitats in East Africa and elsewhere in the Old World tropics, in contrast to species found in the environmentally more stable forests, are faced with the challenge of alternating seasons (Brakefield 2010). Many of these savannah species exhibit seasonal polyphenism with alternative forms occurring in the wet and dry seasons (Windig et al. 1994; Roskam & Brakefield 1999), such that polyphenism and adaptations to C₄ grass feeding are expected to evolve in concert.
Our understanding of the larval ecology of this group is rudimentary in spite of many ecological studies. Natural host plant records for grass-feeding mycalesines are very limited and unreliable, as the larvae are generally cryptic and nocturnal feeders (Brakefield & Mazzotta 1995). This seriously hampers investigations on the evolution of feeding ecology in these butterflies. However, stable isotope analyses, which have become an important part of the ecologist’s toolbox over the last three decades, represent an unexploited opportunity to better understand larval feeding preferences and micro-climate conditions during development in mycalesine butterflies (West et al. 2006).

Isotopic data have, for instance, successfully been used to track migration patterns of birds and insects (Wassenaar & Hobson 1998; Kelly et al. 2002; Brattström et al. 2010) and to determine dietary preferences of fossil and modern animals (Ben-David, Flynn & Schell 1997; Cerling et al. 1997). These studies have shown that the feeding history of animals, with respect to C₃ and C₄ grasses, can be traced by analysing the relative amount of carbon-13 (¹³C) in the tissues of herbivores (Boutton, Cameron & Smith 1978; Fry, Joern & Parker 1978; Cerling et al. 1997; Fischer, O’Brien & Boggs 2004; Codron et al. 2012). Plants discriminate against ¹³CO₂ during photosynthesis, but the C₄ pathway significantly lowers this discrimination, leading to distinct non-overlapping differences in δ¹³C values between C₃ and C₄ plants (O’Leary 1988). These differences in ¹³C isotope ratios are transmitted to the tissues of the herbivores that feed on the plant material (Cerling, Ehleringer & Harris 1998).

Furthermore, stable isotopes of oxygen, and in particular the ¹⁸O composition of the haemolymph and organic material of the exoskeleton of terrestrial arthropods, can be used to quantify the mean atmospheric conditions surrounding the animal before moulting (Ellwood et al. 2011). Arthropods control the respiratory loss of water by opening and closing the tracheal openings used for gas exchange. As water evaporates, diffusion and equilibration fractionations favour the lighter isotope (¹⁶O) such that residual animal tissue water tends to
become enriched in the heavier isotope \((^{18}\text{O})\). Individuals that experience conditions of low humidity, and therefore high rates of evaporation, prior to the final moult, show enriched values of \(\delta^{18}\text{O}\) in exoskeleton organic material.

The aims of this study were, firstly, to experimentally determine whether stable isotopes of carbon and oxygen sampled from the exoskeleton of adult mycalesine butterflies could provide key information about larval ecology. We used laboratory experiments to elucidate the relationships between the environmental conditions during development and the isotopic composition of the organic material obtained from the adult. Secondly, we analysed the isotopic signatures of specimens from two extensive surveys of mycalesine butterflies, one conducted in Africa and the other in Asia. Since these small herbivores are able to complete development on a single or limited number of individual host plants and are relatively immobile, we were able to investigate details of host plant-herbivore interactions in the wild. This allowed us to explore whether the ecological and physiological differences between \(\text{C}_3\) and \(\text{C}_4\) grasses are associated with the larval host plant and/or adult habitat preferences of several mycalesine species.

The ability of mycalesines to process \(\text{C}_4\) foliage is expected to be phylogenetically clustered and associated with adult habitat preferences. Mycalesine species which remained restricted to shaded forest understories, where the advantage of the \(\text{C}_4\) pathway is normally lost, are expected to be \(\text{C}_3\) specialists. In contrast, species which successfully inhabit the more open \(\text{C}_4\) grass-dominated environments as adults are expected to have acquired novel feeding adaptations and predicted to be more opportunistic and generalist in their larval host plant choice. In addition, larvae which complete development in these open habitats are more likely to respire at low levels of atmospheric humidity which should be reflected in the \(^{18}\text{O}\) composition of the adult exoskeleton.
In the C₄-dominated savannah habitats, host plant quality decreases rapidly during the
dry season as the environment gradually dries out. Seasonal patterns in host plant use are
predicted for generalists that inhabit these open habitats, with an increased preference for
high quality C₃ grasses during the dry season. For seasonal forms, we also predicted strong
associations between the values of δ¹⁸O in organic material and the varying seasonal
environment in the open habitat. In contrast, the composition of oxygen stable isotopes is
likely to remain more constant in stable environments.

Material and methods

Laboratory experiments

We conducted four laboratory experiments under defined environmental conditions to
examine how isotopic signatures are established in mycalesine butterflies. The first two
experiments (A and B) investigated whether the feeding history of individual larvae on C₃
and C₄ grasses could be traced by analysing the δ¹³C of adult leg tissue. They also explored
how a mixed larval diet of C₃ and C₄ plants affects the isotopic signature, and whether
different types of adult tissue share similar δ¹³C values in our model species. The aim of the
next two experiments (C and D) was to examine the extent to which the relative humidity
(RH) of the environment affects ¹⁸O composition in the organic material of mycalesine
butterflies. Individuals experiencing low RH are expected to lose water faster through
evaporation, which could lead to more enriched δ¹⁸O values. The final experiment was
designed to identify the developmental period resulting in the evaporative signal found in
adult butterflies: is ¹⁸O enrichment in the exoskeleton of adults the result of increased
evaporation rates in the host plants and transmitted to the tissue of the herbivorous insect
reflecting a signal of the atmospheric conditions during larval development, or is it
incorporated into the exoskeleton during the adult stage?
Experiments A and B were conducted with *Mycalesis mineus* (Linnaeus, 1758), an Asian species which occurs throughout South and Southeast Asia from India to the Philippine archipelago (Monastyrskii 2005). The founders of this population were collected near Khao Chong Nature Reserve, Thailand in 2011. Experiments C and D used *Bicyclus anynana* (Butler, 1879), an African species which occurs in seasonal habitats from Ethiopia to the most northern provinces of South Africa (Larsen 1991). The stock population used for experiments C and D was established in 2011 and indirectly originated from the Leiden University laboratory stock population set up in 1988 from 80 gravid females collected near Nkhata Bay in Malawi (Brakefield, Beldade & Zwaan 2009). Unless stated otherwise, all larvae were reared on maize plants (*Zea mays*) in controlled environment chambers at 27ºC, 70% RH, and L12:D12.

For experiment A, larvae were either reared on wheat (*Triticum aestivum*) or maize, which are C$_3$ and C$_4$ grass species, respectively. Leaf samples were collected throughout larval development in order to compare the δ$^{13}$C values of the host plant (N=16) and leg tissue of adult butterflies (N=24). The same protocol was followed for experiment B. However, on the first day of the fifth instar, the larvae within each cohort were randomly transferred to a cage with either the original host plant or that of the alternative photosynthetic pathway, and allowed to complete development. After eclosion, butterflies were frozen at -20ºC before the adults had fed, and then stored in individual envelopes until further processing. For experiment B, δ$^{13}$C values were obtained from leg, antenna and wing tissue (N=40).

In experiment C, larvae were reared as described above and, on the first day after eclosion, adults were randomly transferred to a climate room with either the original RH (70%) or to a low humidity climate room (20% RH). Here, the adults were fed on moist banana and samples were collected from both cohorts on a daily basis for 21 consecutive
days (N=97). For the final experiment (D), host plants were cultivated under high (90%; HIGH) or low RH (50%; LOW). Larvae were also reared under HIGH or LOW conditions and on either HIGH or LOW host plants. The adults of these four cohorts were randomly transferred to either a HIGH or a LOW climate room, resulting in eight experimental cohorts (N=80). Adults were fed on moist banana, and after 14 days frozen and stored individually. Schematic representations of these four laboratory experiments are given in figure S1.

Field material

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

Three African mycalesine species were abundant in the Zomba material; *Bicyclus ena* (Hewitson, 1877), *B. safitza* (Westwood, 1850) and *B. vansonii* Condamin, 1965. Adults of *B.
ena are normally found in open savannah habitats, especially on rocky hillsides (Pringle 1994; Windig et al. 1994). *B. safitza* has a wider distributional and ecological range, but the adults mainly inhabit open grasslands (Larsen 2005). In contrast, adults of *B. vansoni* are restricted to shaded forest habitats and forest margins (Kielland 1990; Windig et al. 1994).

Specimens were randomly selected from the available material, aiming to include at least three males and three females of each species from every month (total N=587). The Genting material contained eight mycalesine species, representing four genera; *Telinga janardana* (Moore, 1857), *Mycalesis intermedia* (Moore, 1892), *M. oroatis*, Hewitson, 1864, *M. orseis*, Hewitson, 1864, *M. visala*, Moore, 1858, *Mydosama fusca* (C. & R. Felder, 1860), *M. maianes* (Hewitson, 1864), and *Culapa mnasicles* (Hewitson, 1864) (N=367).

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

**Isotopic analyses**

To assay the relative amount of stable isotopes of carbon in our specimens, leg tissue was placed into 8 x 5 mm tin capsules, sealed and loaded into an auto-sampler. The tissue within the capsule was combusted at 600 °C with a pulse of Oxygen and the resultant CO$_2$ fed into a Costech Elemental Analyser and analysed for $^{13}$C/$^{12}$C with an in-line Thermo DELTA V mass spectrometer. Helium was used as a carrier gas and the gaseous products were separated by a packed gas chromatographic molecular sieve column at a temperature of 90°C and
passed into the mass spectrometer via a Thermo Conflo IV interface. The mass spectrometer software is programmed to compare the area under the peak of CO$_2$ and the $^{13}$C/$^{12}$C isotope ratio. For the analysis of oxygen isotopes, the samples were placed in silver capsules. These samples were pyrolysed at 1200°C using a Thermo Finnigan TC/EA attached to a Thermo Delta V mass spectrometer via a ConFlo 3. Reference standards from IAEA in Vienna were run at intervals throughout the sequence and these values are used to calibrate to the international standards of $^{13}$C/$^{12}$C ($\delta^{13}$C Vienna-PDB) and $^{18}$O/$^{16}$O ($\delta^{18}$O V-SMOW).

Precision of analyses is better than 0.1 per mille for $^{13}$C/$^{12}$C and about 0.4 per mille for $^{18}$O/$^{16}$O. Both analyses were conducted at the Godwin Laboratory for Palaeoclimate Research, Department of Earth Sciences, University of Cambridge.

Statistical analyses

All statistical analyses were performed with the R Statistical Package v 3.1.2 (R Development Core Team 2014). We used Student's t-tests to analyse the data from experiment A, and for experiment B we conducted two-way ANOVAs. For experiment C we carried out a multiple linear regression with the $\delta^{18}$O values as the dependent variable, and the adult RH treatment and adult age (i.e. time spent in RH treatment) as independent variables. A significant interaction between both independent variables can be interpreted as a differential change in $\delta^{18}$O values over time in both adult RH treatments. For experiment D, a three-way ANOVA was used to analyse the effect of RH during each experimental stage on the $\delta^{18}$O values obtained from adult organic material, initially fitting full models with each developmental stage, and their interactions, as fixed factors and removing non-significant terms successively. Dietary preferences among the three species from Zomba were investigated by using Chi-squared tests to compare the number of $\delta^{13}$C values per species falling within the typical ranges for C$_3$ or C$_4$ plants. We used a generalised linear model
(GLM) to test for seasonal patterns in host plant use. The number of individuals in each category was used as dependent variable with a Poisson distribution. The species, seasonal form and photosynthetic pathway of the host plant and their interactions were used as fixed factors. All interaction terms were initially included and backward elimination by Akaike Information Criterion (AIC) was used to find the minimum adequate model. For the Genting material we focused on the two most commonly used methods to estimate the phylogenetic signal: Blomberg’s K (Blomberg, Garland & Ives 2003) and Pagel’s $\lambda$ (Pagel 1999). Both K and $\lambda$ were estimated for the proportion of C$_3$ signatures ($N_{C3}$ : $N_{C3}+N_{C4}$) in each species using the function phylosig in the R package phytools (Revell 2012). Finally, two and three-way ANOVAs were used to analyse the effects of the species, sex and seasonal form on the $\delta^{18}$O values obtained from the field material. Again, this was done by initially fitting full models with all fixed factors and interactions, and then removing non-significant terms successively. This procedure was followed by a post hoc Tukey HSD test to identify differences between groups.

Results

Laboratory experiments

Experiment A revealed that $\delta^{13}$C in adult leg tissue does not significantly differ from the isotopic signature of the larval host plant (C$_3$: t = -1.25, df = 12.49, P = 0.24 and C$_4$: t = -1.47, df = 11.89, P = 0.17; figure 1a). The results from experiment B indicated that the different adult tissues of leg, antennae and wing material have very similar $\delta^{13}$C values (figure 1b). In addition, all analysed tissues largely reflect the isotopic composition of the nutrients obtained during the final phase of development, the fifth instar, as the $\delta^{13}$C values of individuals that were swapped from the C$_3$ to the C$_4$ host plant at this final stage of larval development were
more similar to the isotopic signatures of individuals that were reared on C4 plants during all larval stages, and *vice versa* (table S1).

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

Stable isotopes of carbon in field material

In both the open and seasonal habitat in Zomba, Malawi, as well as in the shaded secondary forest in Genting, Malaysia, we found distinct bimodal distributions in $\delta^{13}C$ values, representing the isotopic composition of C3 and C4 grasses (individuals with $\delta^{13}C$ values less than -25‰ were classified as C3 feeders; figure S3). At Zomba, the proportion of larvae that developed on C3 grasses was significantly higher in *B. vansoni*, the species where adults are predominantly found in (semi-)shaded forest habitats and forest margins (*B. vansoni*: *B. ena* comparison, $X^2 = 187.16$, df =1, $P < 0.01$; *B. vansoni*: *B. safitza* comparison, $X^2 = 216.50$, df =1, $P < 0.01$). The ratios of C3:C4 feeding did not significantly differ between *B. ena* and *B. safitza*, the two species where adults inhabit the open grasslands (*B. ena*: *B. safitza* comparison, $X^2 = 0.04$, df =1, $P = 0.86$). In addition, in all three species, we observed a trend
towards an increased use of C₃ host plants during the dry season, although this interaction between seasonal form and host plant-use only approached significance ($z = 1.753$, $P = 0.08$; table 3 and table S2). The $\delta^{13}$C values of half of the eight mycalesines collected in Genting, *T. janardana*, *M. intermedia*, *M. orseis* and *M. visala*, revealed that these species frequently develop on C₄ grasses throughout the year. We found no evidence for C₄ larval feeding in any of the remaining four species; *M. oroatis*, *M. fusca*, *M. maianes* and *C. mnasicles*. The ability to use C₄ grasses as the natural host was significantly correlated with the phylogenetic relatedness of the Genting species (Blomberg’s $K = 1.23$, $P < 0.05$; Pagels $\lambda = 1.08$, $P < 0.05$; figure 3).

**Stable isotopes of oxygen in field material**

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

Our laboratory experiments indicate that dietary preferences for grasses with a C\textsubscript{3} or C\textsubscript{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\textsubscript{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\textsubscript{3} grasses and to experience higher rates of evaporation. Finally, our data suggest that the ability to process C\textsubscript{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
a large extent reflect the isotopic composition of the host plant consumed by the larvae during the final phase of development when most larval growth occurs and development of adult tissues begins. The $\delta^{13}C$ values were comparable across tissues, although the isotopic composition of antennal tissue showed a consistent deviation towards the isotopic of the host plant utilized during the first four larval instars. This may indicate differences in the allocation of nutrients acquired during the final larval instar amongst the analysed tissues, or differences in the timing of the initiation of the development of these adult tissues during the larval stage.

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

Larval ecology

In both the open habitat in Zomba, as well as in the shaded forest in Genting, the isotopic signatures revealed that the larvae of some mycalesine species utilize C₄ grasses in their natural environment. In the material from Zomba, the C₃:C₄ ratios of the different species were strongly associated with the habitat preferences of the adult butterfly. Species that fly in the more open grasslands, *B. ena* and *B. safitza*, were more likely to complete development on C₄ grasses whereas individuals of *B. vansoni* from more shaded habitats mainly utilized C₃ host plants. None of these three species from a seasonal environment exclusively used host plants from one of the two alternative photosynthetic pathways, suggesting that, within the *Poaceae*, these species may indeed be opportunistic and generalist in their host plant choice.

Where the δ¹³C values indicated that the larval host plant use of both open savannah species was more similar, the oxygen signatures revealed that the *B. ena* larvae consistently respired at lower RH during development, compared to *B. safitza* and *B. vansoni*, which completed development under more humid atmospheric conditions. Adults of *B. safitza* normally inhabit grasslands with scrub and open woodland and have a wider ecological range than those of *B. ena*. The latter species is restricted to open savannah habitats and is likely to be more tolerant of arid conditions during larval development. Overall, the combination of δ¹⁸O and δ¹³C closely reflects the habitat preferences of adults.

In the shaded secondary forest in Genting where RH was constant and high throughout the sampling period, the δ¹⁸O composition did not differ among species, indicating that the atmospheric humidity experienced during development was similar for all species. In contrast, the δ¹³C values revealed clear differences in host plant preferences, with half of the
species frequently utilizing C₄ plants while the other species solely using C₃ grasses. This indicates that there is no inherent barrier to forest species consuming C₄ grasses. Furthermore, this observation conflicts with the prediction that generalists avoid C₄ leaves when C₃ host plants are available (Caswell et al. 1973; see further below).

Seasonal variation at field sites

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

In the seasonal habitat in Zomba, we found a tendency to utilize C₃ grasses more frequently when the C₄ host plants potentially become less palatable during the early dry
season. This observation suggests that the preference of grass-feeding generalist herbivores for high quality $C_3$ grass species increases during this period (Caswell et al. 1973). However, the differences in $C_3:C_4$ ratios between seasonal forms may also reflect seasonal variation in the availability of the two types of host plants or indicate that there may be a longer window of opportunities for successful development and adult recruitment in forest margins, where $C_3$ grass species are expected to be more prevalent.

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

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

Phylogenetic signal

Of the three species of *Bicyclus* we sampled in the seasonal environment, adults of *B. vansoni*, in contrast to *B. ena* and *B. safitza*, are normally found in more shaded habitats. Interestingly, *B. vansoni* is not considered to be a true forest species as it is frequently found in the semi-shaded forests margins while most *Bicyclus* species, and especially the basal lineages of the genus, are less tolerant to habitat disturbance and only found in habitats with complete canopy cover. This may indicate that only those mycalesine species that frequently interact with C$_4$ grass-dominated open environments, or have done so in the past, are able to utilize the C$_4$ host plants that are available in their natural habitat. In this context, the results from the more stable environment in Genting are particularly interesting. Here, four out of the eight species solely utilized C$_3$ host plants during larval development while, evidently, C$_4$ grasses were available in their natural habitat. The ability to use C$_4$ grasses as the natural host...
is significantly correlated with the phylogenetic relatedness of the species, indicating that closely related species exhibit similar host plant preferences in this habitat. This is consistent with the hypothesis that those mycalesine species that have been restricted to shaded forest understories throughout their evolutionary history have not evolved adaptations to cope with the lower palatability of C_4. In contrast, species which acquired novel feeding adaptations could colonize new, open ecological niches in the C_4-dominated habitats which were comparatively free of competition from other herbivores, resulting in divergent selection and ultimately speciation (Heckathorn, McNaughton & Coleman 1999).

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

Acknowledgements

We are grateful to John Thompson and one anonymous reviewer for suggestions which greatly improved our manuscript, to John Wilson and Hok Kim Loong for collecting and providing the field material in Malawi and Malaysia, respectively, and Dave Osbaldeston, Pragya Singh and James Rolfe for practical assistance and technical support. EvB, HG, CPO and PMB conceived and designed the experiments; HSB, UK (Genting) and PMB (Zomba) organised and supervised the longitudinal sampling; EvB, UK and OB identified the specimens; EvB performed the experiments, analysed the data and wrote the manuscript. This
work was supported by the European Research Council grant EMARES (250325) to PMB.

All authors read and approved the final version of the manuscript for publication. Data are deposited in the Dryad repository (doi:XXXXXXXXXX).
Table 1: For experiment C, we carried out a multiple linear regression with the $\delta^{18}$O values as the dependent variable, and the adult RH treatment and adult age, (i.e. time spend in RH treatment), as independent variables. The non-significant interaction between both independent variables (in bold) was interpreted as an absence of change in $\delta^{18}$O values over time in both adult RH treatments.

<table>
<thead>
<tr>
<th>dependent variable</th>
<th>fixed effects</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\delta^{18}$O values</td>
<td>Age</td>
<td>0.11</td>
<td>0.916</td>
</tr>
<tr>
<td></td>
<td>RH adult stage</td>
<td>1.28</td>
<td>0.205</td>
</tr>
<tr>
<td></td>
<td>Age : RH adult stage</td>
<td>0.09</td>
<td>0.930</td>
</tr>
</tbody>
</table>
Table 2: Minimum adequate models of the effect of RH during each experimental stage on the δ^{18}O values obtained from adult organic material in experiment D. The exoskeleton of individuals that experienced low RH during the larval stage was significantly enriched in ^{18}O.

<table>
<thead>
<tr>
<th>dependent variable</th>
<th>fixed effects</th>
<th>F</th>
<th>df</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ^{18}O values</td>
<td>RH host plant</td>
<td>0.11</td>
<td>1,76</td>
<td>0.743</td>
</tr>
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<td></td>
<td>RH larval stage</td>
<td>75.76</td>
<td>1,76</td>
<td>&lt;0.001</td>
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<tr>
<td></td>
<td>RH adult stage</td>
<td>2.51</td>
<td>1,76</td>
<td>0.118</td>
</tr>
</tbody>
</table>
Table 3: In Zomba, the proportion of larvae that developed on C₃ rather than C₄ grasses, was significantly higher in *B. vansoni*; the species that is predominantly found in (semi-) shaded forests and forest margins, while the larvae of the two species that inhabit the open grasslands (*B. ena* and *B. safitza*) largely completed development on C₄ grasses. In addition, we observed a seasonal trend in host plant use towards an increased relative consumption (RC) of C₃ host plants during the dry season in all species (minimum adequate model presented in table S2). The change in relative consumption between both seasonal forms (DSF/WSF) is greater than 1 in all species, indicating an increased utilization of C₃ host plants in DSF individuals.

<table>
<thead>
<tr>
<th>Species</th>
<th>Seasonal form</th>
<th>C₃</th>
<th>C₄</th>
<th>RC</th>
<th>DSF/WSF</th>
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</thead>
<tbody>
<tr>
<td><em>B. ena</em></td>
<td>DSF</td>
<td>11</td>
<td>101</td>
<td>0.11</td>
<td>3.32</td>
</tr>
<tr>
<td></td>
<td>WSF</td>
<td>2</td>
<td>61</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td><em>B. safitza</em></td>
<td>DSF</td>
<td>10</td>
<td>113</td>
<td>0.09</td>
<td>2.01</td>
</tr>
<tr>
<td></td>
<td>WSF</td>
<td>4</td>
<td>91</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td><em>B. vansoni</em></td>
<td>DSF</td>
<td>69</td>
<td>19</td>
<td>3.63</td>
<td>1.26</td>
</tr>
<tr>
<td></td>
<td>WSF</td>
<td>78</td>
<td>27</td>
<td>2.89</td>
<td></td>
</tr>
</tbody>
</table>
Table 4: Minimum adequate models of the effects of species, sex and seasonal form on the δ¹⁸O values obtained from the field material in Genting and Zomba. The δ¹⁸O values that were measured for animals collected in the stable secondary forest in Genting were neither correlated to the different species nor to the sex of the individuals. In contrast, in the seasonal habitat in Zomba, the values of δ¹⁸O of the different species and their seasonal forms were significant different.

<table>
<thead>
<tr>
<th>dependent variable</th>
<th>fixed effects</th>
<th>F</th>
<th>df</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ¹⁸O values Genting</td>
<td>Species</td>
<td>0.44</td>
<td>7,358</td>
<td>0.876</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>0.09</td>
<td>1,358</td>
<td>0.761</td>
</tr>
<tr>
<td>δ¹⁸O values Zomba</td>
<td>Species</td>
<td>27.78</td>
<td>2,564</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>0.11</td>
<td>1,564</td>
<td>0.742</td>
</tr>
<tr>
<td></td>
<td>Seasonal form</td>
<td>481.29</td>
<td>1,564</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Figure 1: The left hand figure represents the data collected for experiment A. δ\(^{13}\)C in adult leg tissue does not significantly differ from the isotopic signatures of the plant material. The δ\(^{13}\)C values obtained from leg, antenna and wing tissue in experiment B are represented in the right hand figure. For the first four instars, larvae were either reared on plants of wheat (circles) or maize (squares). On the first day of the fifth instar, the larvae within each cohort were randomly transferred to a cage with either the original host plants (solid lines) or host plants of the alternative photosynthetic pathway (swapped: dashed lines), and allowed to complete development. Filled symbols indicate individuals which completed development on wheat while the blank symbols represent specimens which were feeding on maize during the fifth instar. The isotopic signatures of adult tissue mainly reflect the isotopic composition of the host plant which was consumed by the larvae during the final phase of development, when most larval growth occurs and development of adult tissues begins. Error bars represent 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.
Figure 3: The $\delta^{13}$C values for all specimens collected in the stable environment in Genting (N=367). Individuals with $\delta^{13}$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 *Teltinga 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).
9. REFERENCES


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