

# Rating palatability of butterflies by measuring ant feeding behavior

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## KEY WORDS

Age, color, fruit-feeding, mimicry, Nymphalidae, sex, tropical

Entomologische Berichten 70 (2): 52-62

Certain bright colors of butterflies are often interpreted to signal that they are chemically defended, meaning that they are unpalatable or toxic to predators. We discuss several questions concerning predation on butterflies and how chemical defense might work. An important gap in our understanding is quantitative information about palatability on a community level. Here we present a novel method to quantify palatability used on 29 species of butterflies in a tropical forest in Uganda. Butterflies were mashed and offered as small drops to individual ants in Petri dishes. Ants tended to eat in shorter bouts from less palatable butterflies. The first results clarify one mimicry relationship as Batesian (one is unpalatable, the other is palatable), whereas another appeared more Müllerian (both are moderately unpalatable). Surprisingly, some brown (cryptically) colored butterflies also seemed to be chemically defended. Within one of the species we detected that individuals become more palatable as they age. We found no difference between the sexes in this species.

## Introduction

The vivid colors of many butterflies are often interpreted to signal to predators that they are unsuitable prey, generally as a result of their unpalatability or because they are poisonous (aposematism). Butterflies can become unpalatable by sequestering chemicals from their larval host-plant (i.e., Wiesen *et al.* 1994, Hesbacher *et al.* 1995, Trigo *et al.* 1996, Geuder *et al.* 1997, Schittko *et al.* 1999, Burghardt *et al.* 2001, Nishida 2002) or by synthesizing chemicals as adults (Engler-Chaouat & Gilbert 2007). The classic unpalatable butterfly advertises its unprofitability with contrasting colors – often black with white, yellow, or orange – on the dorsal and ventral side of the wings and may have dark spots near the base of the wings, or characteristic wing beat frequencies and flight patterns (Wourms & Wasserman 1985c, Srygley 2003, 2007). It typically flies slowly, apparently confident that it will be left alone by predators. These characteristics are thought to indicate that aposematic butterflies rely on their chemical defense for avoiding predation rather than on fast or erratic flight, crypsis, false heads, eyespots, or other predator evasion strategies.

Selection may act on the individual level when captured individuals are released alive (sometimes with minor damage) after the predator has detected the individual's unpalatability (Carpenter 1941, Vasconcellosneto & Lewinsohn 1984, Guilford & Cuthill 1991, DeVries 2002, 2003). Therefore, species that are not warningly colored may also be unpalatable.

However, while aposematic animals and their mimics provide stunning examples of the power of natural selection, theory of color pattern evolution is based on a number of hardly verified assumptions (Kassarov 2003, Gilbert 2005). Though unfortunate, it is not unusual to assume aposematic function (Guilford & Cuthill 1991) or lack of chemical defense without critical data. In addition, much remains to be learned about

predation pressures of wild butterflies, including the particular predators, the context of predation (i.e., in flight, at rest, when feeding, during copulation), and the proportion of butterfly mortality attributed to each of these predators.

## Who are the predators of butterflies?

Birds are often assumed to be an important selective force in butterfly color and flight pattern evolution (Carpenter 1935, 1944, Wourms & Wasserman 1985a, Mallet & Barton 1989, Langham 2006). Yet, butterflies are only occasionally found in the stomachs of birds (Collinge 1929 – more than 100,000 birds were killed and examined in the USA, India, Australia and the UK). Jantzen and Eisner (2008) argue that birds might simply write butterflies off as too elusive to catch (see also Kassarov 2003) and, because of their scales, too slippery to hold, and not nutritious enough to be worth the effort. However, although butterflies may be difficult to capture in flight (Larsen 1992b), they are easily captured while they are depositing eggs, mating, or feeding. Likewise, birds predate on hoverflies that are feeding on flowers by swooping down from a perch (Dlusskii 1984). In addition, some birds may specialize on butterflies, such as the Neotropical long billed anis (Burger & Gochfeld 2001) and jacamars (Pinheiro 2004).

Larsen (1992c) described temporary prey-specialization on migrant butterflies by blue cheeked bee-eaters in Africa where the birds only attacked butterflies that were crossing a river. Interestingly, in this case birds consumed butterflies on the wing, thus eliminating the handling time that is associated with their usual behavior of taking captured butterflies to a perch for inspection before ingestion (or rejection). Therefore, it seems that the single species migration over the river made such inspections unnecessary and rendered the butterflies more

**Table 1.** Host-plant and color pattern information on tested butterfly species. Palatability is expressed as the average number of seconds per feeding bout of the ants. Numbers below 100 (unpalatable) are green boxed, those above 200 are on red boxed (palatable).

**Tabel 1.** Voedselplant- en kleurpatrooninformatie van de getoetste vlindersoorten. De smaak van de vinder is uitgedrukt als de gemiddelde duur van mierenmaaltjes (in s). Wanneer dat minder was dan 100 ('vies') dan is de waarde groen omlijnd, en als mieren gemiddeld langer dan 200 s van een druppel dronken ('lekker') is de waarde rood omlijnd.

Species	host-plant genus	family	palatability	abundance	sex	dorsal color pattern elements		discal
						main colors	sub-apical	
danaids	?	Asclepiadaceae+	38	rare	♂ / ♀	black & white	white (spots)	white spots
<i>Harma theobene</i>	<i>Lindackeria</i>	Achariaceae	66	common	♂	shades of grey	-	
					♀	yellow & brown		
<i>Cymothoe lurida</i>	?	Violaceae/	193	common	♂	ochre yellow		
		Achariaceae			♀	brown	white	
<i>Cymothoe herminia</i>	<i>Dasylepis</i>	Achariaceae	214	common	♂	creamy & brown	-	
					♀	black and white		
<i>Pseudacraea lucretia</i>	<i>Aningeria</i>	Sapotaceae	102	common	♂ / ♀	black & white	-	
<i>Euphaedra kakamegae</i>	<i>Aphania</i>	Sapindaceae	66	rare	♂	blue	white	
					♀	green	light blue	
<i>Euphaedra eusemoides</i>	<i>Uvariopsis</i>	Annonaceae	180	rare	♂ / ♀	orange & black	creamy	creamy
<i>Euphaedra alacris</i>	<i>Aphania</i>	Sapindaceae	194	common	♂ / ♀	orange & black	white	
<i>Euphaedra medon</i>	<i>Paullinia</i>	Sapindaceae	121	common	♂	metallic green	yellow	
					♀	brown	yellow	
<i>Euphaedra harpalyce</i>	<i>Blighia, Aphania,</i> <i>Pancovia</i>	Sapindaceae	124	common	♂ / ♀	brown	yellow	
<i>Euphaedra christyi</i>	?		318	rare	♂ / ♀	creamy & black	creamy	creamy
<i>Euphaedra uganda</i>	<i>Allophylus</i>	Sapindaceae	386	rare	♂ / ♀	blue	white	
<i>Euphaedra preussi</i>	?		212	rare	♂ / ♀	bluish green	white	
<i>Aterica galene</i>	<i>Pancovia +</i>	Sapindaceae	175	common	♂ / ♀	black & white	white spots	white spots
<i>Bebearia sophus</i>	<i>Pancovia, Chrysophyllum</i>	Sapotaceae	43	rare	♂ / ♀	brown	yellow (diffuse)	
<i>Charaxes fulvescens</i>	<i>Allophylus</i>	Sapindaceae	210	common	♂ / ♀	orange and white	-	
<i>Charaxes numenes</i>	<i>Hypocratia, Aphania,</i>	Hypocratiaceae/	113	rare	♂	blue	-	
		Sapindaceae			♀	brown		white
<i>Charaxes bipunctatus</i>	<i>Blighia, Aphania +</i>	Sapindaceae	199	rare	♂	blue	-	
					♀	brown		white
<i>Charaxes candiope</i>	<i>Croton</i>	Euphorbiaceae	261	rare	♂ / ♀	orange & brown	-	
<i>Charaxes brutus</i>	<i>Trichilia</i>	Meliaceae	43	rare	♂ / ♀	black & white	-	
<i>Charaxes cynthia</i>	?	Fabaceae	295	rare	♂ / ♀	black and red	-	
<i>Charaxes pleione</i>	<i>Acacia</i>	Fabaceae	256	rare	♂ / ♀	orange	-	
<i>Charaxes pollux</i>	<i>Bersama</i>	Meliantaceae	72	common	♂ / ♀	orange & black	-	
<i>Bicyclus sebetus</i>	?	Poaceae	126	rare	♂ / ♀	brown		violet
<i>Bicyclus graueri</i>	?	Poaceae	231	common	♂ / ♀	brown		violet
<i>Gnophodes chelys</i>	<i>Setaria</i>	Poaceae	250	common	♂ / ♀	brown	white	
							yellow	
<i>Melanitis leda</i>	<i>Setaria</i>	Poaceae	147	rare	♂ / ♀	brown	orange & black	
<i>Kallimoides rumia</i>	?		158	common	♂	brown	yellow	violet
					♀	brown	white	
<i>Hypolimnas monteironis</i>		Urticaceae	84	rare	♂ / ♀	blue, black & white	-	white

profitable prey. The importance of other types of predators has hardly been studied but records exist for dragonflies, chameleons, lizards, spiders, hornets, scorpions, centipedes, assassin bugs, ambush bugs, praying mantids, robber flies, beetles, ants, fish, frogs, toads, snakes, shrews, bats, monkeys, mice and sundew plants taking butterflies (Emmel 1976, Hayes 1981, Larsen 1981, 1992a, 2007, Vasconcellosneto & Lewinsohn 1984, DeVries 1987, Owen 1993, Alonsomejia & Marquez 1994, Wiklund 2005, Vlieger & Brakefield 2007, Wiklund *et al.* 2008, Molleman in press).

### How does chemical defense work in practice?

Information regarding the mechanisms or strategies that unpalatable butterflies use to avoid or survive predation attempts (i.e., beak mark tasting, emetic properties, handling time of separating palatable from unpalatable body parts) is also generally lacking (Wourms & Wasserman 1985b, Kassarov 1999). Furthermore, questions remain regarding the effectiveness of various defenses, the relative costs and trade-offs of investing in those defenses, and changes in the effectiveness of different strategies as butterflies age (Alonso-Mejia & Brower 1994).

## What do color patterns tell us?

Color patterns are difficult to interpret because the type of predator (e.g., bird, lizard, dragonfly), background, distance, angle, and light characteristics (e.g., intensity, wave-length, color pattern, polarization) can all be important factors, and color patterns can also be used for within-species communication and thermoregulation (Ruxton *et al.* 2004a, Vanewright & Boppré 1993).

While most research on mimicry has focused on precise mimicry, many butterfly species do not seem to neatly fit into this framework: they have bright colors at least on the upper side, are fast and maneuverable flyers, or their mimicry relationships are unclear. This is particularly true in the African tropics. Such butterflies may only in part rely on aposematism, or their colors may signal to predators that they are difficult to capture (Swynnertonian mimicry; Swynnerton 1926, van Someren & Jackson 1959, Larsen 1992b, Ruxton *et al.* 2004b). Therefore, more insight could be gained from an integrative view on defenses and life history. Most butterflies probably use a combination of defenses (Edmunds 1974, Ruxton *et al.* 2004a); for example, a butterfly may have cryptic undersides, upper sides with warning colors, and high flight speed (Srygley 1990, 2004, Pinheiro 2003, Tullberg *et al.* 2005, Vallin *et al.* 2006). As in marine worms (Kicklighter & Hay 2007), butterfly species may not invest in all defenses, as illustrated by the slow flight of very poisonous butterflies (Srygley 1990, Marden & Chai 1991). Individuals can also adopt different strategies at different stages in their life cycle (Wiklund & Sillentullberg 1985). The relative contribution of defensive strategies to survival in the wild has not been systematically studied in butterflies or other insects.

## How can palatability be quantified?

To gain more insight into the various butterfly defensive strategies, methods must be developed to quantify and measure defenses. There are several studies demonstrating unpalatability of butterflies that use the learning curves of corvid birds, known for their intelligence (Brower *et al.* 1970, Pough & Brower 1977, Prudic *et al.* 2002) or chickens (Forsman & Appelqvist 1998, Aronsson & Gamberale-Stille 2008) (recent data show that they rely heavily on olfaction), but few studies include likely predators of butterflies in the wild (Pinheiro 1996, Langham 2006). In some studies butterfly extracts were injected into locusts or into exposed hearts of vertebrates to measure cardioactivity (e.g., Marsh *et al.* 1977). Probably the most effective experiment was carried out by Torben Larsen (1983) who personally chewed butterflies and then tasted them for one minute, something we do not recommend with butterflies that may have eaten civet dung or carrion, and this method may be risky in general (Pringle 2003, Hossler 2009).

The chemicals responsible for unpalatability are sometimes identified and the concentration of these chemicals is measured to characterize palatability (e.g., Cardoso 1997). These studies have found extensive within-species variation related to age, larval host-plant and adult diet. In addition, there are many scattered observations on predation on butterflies in the wild (Carpenter & Hale 1941, Larsen 1991, 1992c, Alonsomejia & Marquez 1994, Burger & Gochfeld 2001) and in captivity (Wourms & Wasserman 1985a, Brower 1988, Larsen 1992a, 2007). Such data can give information on palatability but do not allow researchers to fully distinguish aposematism from mimicry or other defense mechanisms (Carpenter & Hale 1941). When data on palatability are lacking, it is often inferred from mimetic relationships and phylogeny. For example, any danaid, ithomeine, acraid and arctiid is assumed to be unpalatable, because most

representatives of those groups that have been tested were shown to be unpalatable, they use toxic host-plants, or seem to be models in mimicry relationships.

## Using ants – Rationale for our method for quantifying palatability

In a tropical forest, the defenses of an organism need to be effective against a wide variety of predators for it to survive. Therefore, we conjecture that species that depend heavily on unpalatability need to be unpalatable to a wide range of predators, and as a result we expect congruence between predator species in their responses to butterfly chemical defenses. Here, we aim to quantify palatability using an adaptation of a method developed by Caroline Müller and colleagues for testing the effectiveness of hemolymph excretions of sawfly larvae to repel predatory ants (Müller *et al.* 2002). This approach is also akin to the cutting up of marine worms to assay their palatability to fish and crustaceans (Kicklighter *et al.* 2003, Kicklighter & Hay 2007), and Eisner *et al.* (2008) used wolf spiders and coccinellid beetles to measure the defensive potency of lycidic acid. Food preferences of ants have also been studied in the context of ant ecology (Blüthgen & Fiedler 2004) and caterpillar defenses (Coley *et al.* 2006), where intact caterpillars were exposed to ants.

Although ants attack and kill captive butterflies (in cages or in live traps), have been seen disassembling them in the wild (Hayes 1981), and can be important predators of nocturnal moths (Tammaru *et al.* 2001), we have no evidence that they are important butterfly predators in nature. However, in tropical forests ants are active during the night and could well attack resting butterflies. We assume here that actual/other predators of butterflies would rank the palatability similarly, as was demonstrated for bird-meat preferences of hornets, cats and humans (Cott 1947). We acknowledge that invertebrate predators often accept moderately chemically defended insects that are rejected by vertebrates (Vasconcelosneto & Lewinsohn 1984).

Although experiments using intact live butterflies and the actual predators remain preferable, we believe that assays with ants have several important advantages: 1) data can be collected quickly and at low cost; 2) there are no problems with obtaining permission to use ants (in contrast to insectivorous vertebrates); 3) palatability is tested in the absence of the confounding issue of the butterfly's visual appearance, therefore the required naivety in studies using vertebrates is not an issue; 4) concentration series can be used; 5) different body parts can be tested separately; 6) within-species variation in palatability can be examined; 7) animals from different parts of the world can be tested with the same assay, especially if frozen specimens are used (note that some butterfly species quickly lose their unpalatability when dead, Marsh *et al.* 1977); 8) a relatively small number of butterflies (or other insects) is needed for the tests; and 9) in many cases, it is virtually impossible to know the key predators of insects in their natural habitats.

## Material and Methods

### Study site

This study was conducted from June 2007 to August 2008 at Makerere University Biological Field Station in Kibale Forest National Park, Western Uganda. The field station borders selectively logged moist evergreen forest at an altitude of around 1500 m (figure II-A,B).

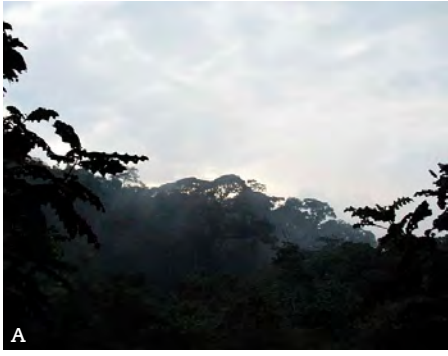




**Plate I** Photographs of butterflies: **A** *Euphaedra alacris*, **B** *E. eusemoides*, **C** *E. uganda*, **D** *E. kakamegae* female, **E** *E. kakamegae* male, **F** *E. kakamegae* caterpillars, **G** *E. medon* male, **H** *E. medon* female, **I** *E. harpalyce*, **J** *E. medon* (background) and *E. harpalyce* (foreground) undersides, **K** *Bebearia sophus* female, **L** *Aterica galena*, **M** *Cymothoe herminia*, **N** *C. lurida*, **O** *Harma theobene*, **P** *Pseudacraea lucretia*, **Q** *Charaxes numenes* female, **R** *Gnophodes chelys* female, **S** *Melanitis leda* upper side, **T** *Bicyclus sebetus*, **U** two *Kallimoides rumia* males, **V** *K. rumia* male upper side, **W** *Hypolimnas monteironis* male, **X** *E. christyi*. *Aterica galena* was photographed in Queen Elizabeth National Park (Uganda), *B. sophus* in the collection of the the African Butterfly Research Institute in Nairobi the others in Kibale National Park by Freerk Molleman.

**Plate I** Vlinderfoto's. *Aterica galena* is gefotografeerd in Queen Elizabeth National Park (Oeganda), *Bebearia sophus* in the ABRI-collectie in Nairobi, alle andere in Kibale National Park door Freerk Molleman.





**Plate II** Site and methods: **A** Misty morning in Kibale National Park Uganda, **B** View on the border of Kibale National Park, **C** Harriet and Chris in the forest, **D** performing the palatability experiment (left to right Bonny, Harriet, Chris, John, Freerk, Akoch), **E** Petri dish with droplets of sugar water and butterfly soup and ant. Photo D taken by Małgorzata E. Arlet, E by Melissa R. Whitaker, others by Freerk Molleman.

**Plate II** Locatie en methode: **A** Mistige morgen in Kibale National Park, **B** gezicht op de grens van het Nationale Park, **C** Harriet en Chris in het bos, **D** het experiment in volle gang (van links naar rechts Bonny, Harriet, Chris, John, Freerk, Akoch), **E** Petrischaaltje met een druppel suikerwater, een druppel vlindersoep en een mier. Foto D genomen door Małgorzata E. Arlet, E door Melissa R. Whitaker, de rest door Freerk Molleman.

## Study subjects

We conducted palatability tests using a wide variety of fruit-feeding butterflies that are often assumed to be palatable (Van Someren & Jackson 1959), and included a few danaiids as examples of putatively aposematic butterflies (table 1). Here we describe the coloration patterns and possible mimetic associations of the butterfly groups included in the palatability tests. For most species photographs are provided.

**Adoliadini** All *Euphaedra* species used are placed in different sub-genera and are not thought to be sister species (Hecq 1997). If we then consider the wide variety of color patterns within the genus *Euphaedra*, striking similarities are unlikely to be the result of a phylogenetic signal. Classic warning colors occur in the forest butterfly *Euphaedra alacris* Hecq (figure I-A), which faintly resembles the unpalatable open landscape butterfly *Danaus chrysippus* (Linnaeus), though these two species rarely co-occur. In addition, it resembles an uncommon diurnal arctiid moth with which it co-occurs. Other *Euphaedra* species have bright colors that are also within the classic aposematism color palette (*E. christyi* Sharpe, *E. eusemoides* Smith & Kirby (figure I-B)) and may be mimics of *Xanthospilopteryx* moths (T.B. Larsen, pers. comm.), whereas some are brightly colored but not within the classic aposematism color palette (*E. uganda* Aurivillius (figure I-C), *E. kakamegae* Carpenter, *E. medon* (Linnaeus) males (figure I-G)). *Euphaedra kakamegae* (figure I-D-F) is the only species with clearly warningly colored caterpillars in this data set (Molleman & Hecq 2005). *Euphaedra harpalyce* (Cramer) (figure I-I) and *E. medon* females both have brown upper sides with a yellow sub-apical band and are likely in some kind of mimicy relationship with each other. *Bebearia sophus* Fabricius (figure I-K) has some resemblance to *E. harpalyce* and *E. medon* (brownish with fragmented yellow sub-apical band). The undersides of all these species are more or less cryptic to human eyes when they are sitting on the forest floor, where they feed on fallen

fruits. *Aterica galena* Brown (figure I-L) is considered an imprecise mimic of the (likely) unpalatable *Amauris niavius* Linnaeus (Danainae).

**Limentidinae** *Cymothoe herminia* Smith (figure I-M), *C. lurida* Butler (figure I-N) and the closely related *Harma theobene* Doubleday (figure I-O) (Van Velzen *et al.* 2007) are moderately brightly colored and are thought to sequester unpalatable chemicals from their host-plants. *Pseudacraea lucretia* (Cramer) (figure I-P) is considered an *Amauris* mimic though with some pattern elements typical for acraids, and does not closely resemble any one particular species.

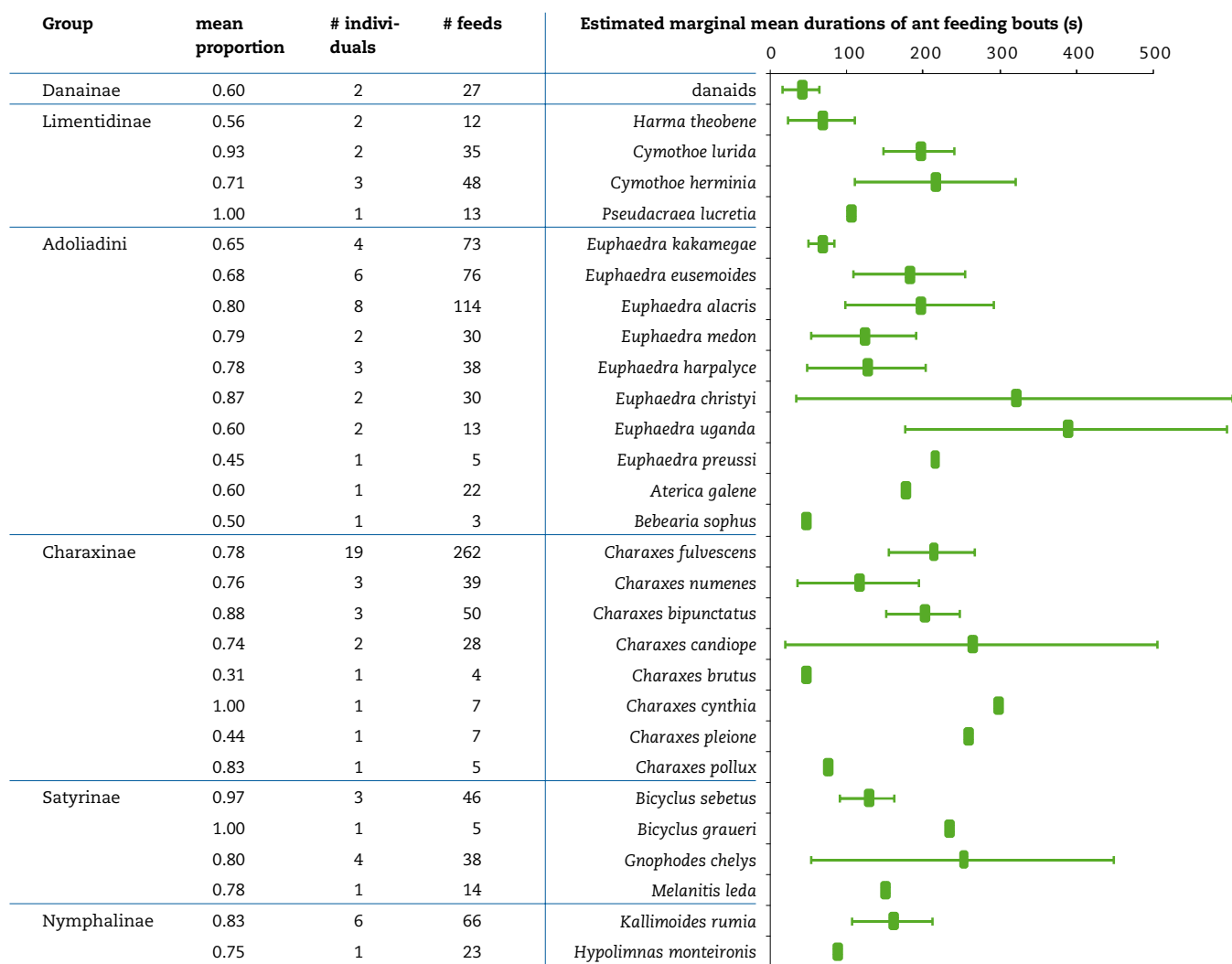
**Charaxinae** *Charaxes* typically have some bright and intricate coloration on the upper sides of the wings, with mostly cryptic undersides (figure I-Q). Some females faintly resemble the brown *Euphaedra* species.

**Satyrinae** All included satyrines (*Gnophodes*, *Melanitis*, *Bicyclus* (figure I-R-T)) are cryptically colored with minimal eyespots. The upper sides are brown with violet sub-apical (to discal) bands, or white or yellow sub-apical bands, and in *Melanitis leda* Linnaeus (figure I-S) there is an eyespot-like subapical pattern.

**Nymphalinae** *Kallimoides rumia* Doubleday is considered a leaf mimic and males can also be perceived as bird mimics, as the butterfly's tail resembles a bird's beak (figure I-T,V). *Hypolimnys monteironis* Druce (figure I-W) has bright blue on its upper sides, but undersides are more cryptic.

## Experimental methods

Butterflies were either reared from field-collected caterpillars or were collected from the field as adults using sweep nets (Danaiids) or baited traps (all others) (figure II-C). They were



1. Duration of individual feeding bouts of ants feeding on butterfly suspensions with information on subfamily, proportion of feedings on butterfly (compared to sugar solution) and sample sizes. For those species having at least two individuals tested, the Estimated Marginal Mean durations of feeding bouts are given with 95% confidence intervals at mean order number of feeding (2.47) and order quadratic (5.05). This means that averages are statistically corrected so that they are directly comparable among species. For species represented by a single individual, we reported only the mean duration.

1. Het aantal seconden dat een mier aan één stuk van een druppel vlindersoep drinkt met informatie betreffende de subfamilie van de vlinder, de fractie maaltjes van vlindersoep (vergeleken met suiker water) en het aantal observaties. Voor soorten waarbij meer dan één vlinder gebruikt is, hebben we het gemiddelde gecorrigeerd voor het volgnummer van elk maaltje van individuele mieren. Als maar één vlinder is gebruikt hebben we alleen het gemiddelde weergegeven.

killed and legs, wings and head were removed before weighing. Each specimen was then ground up with 3× its weight of boiled rainwater using a mortar and pestle. The resulting suspension was then placed in a vial, and a second solution was prepared in another vial that contained the same amount of water as was added to the butterfly suspension. A 10% sugar solution was added to both vials so that both solutions would have a 5% sugar concentration. One droplet of each solution (one pair per Petri dish) was then placed approximately 2 cm apart near the center of a Petri dish (droplets were named 'butterfly' and 'sugar'). Ants – *Myrmecaria natalensis* (Smith) – were collected by hand from nearby ant-trails and one ant was introduced into each Petri dish (figure II-E). Observations were made by teams of 2-5 researchers (figure II-D). Each person could observe up to six Petri dishes, each containing one pair of droplets and one ant, while one person recorded notes. We noted the start and end times for each ant feeding bout (in s), and which droplet was fed on for each feeding. Each trial lasted 30 min.

## Results

Data were obtained from 87 butterflies. Sample sizes per species were usually small, with few exceptions (figure 1). All species were fed on by ants and the proportion of feeding bouts on butterfly solution vs. sugar solution was typically between 0.5 and 1, demonstrating that the butterfly solution was generally preferred over the sugar solution (figure 1). The proportion of feeding bouts on butterfly vs. sugar solution provides only one value per ant and is dependent on which droplet is encountered first, so future experiments might better omit the sugar solution droplet. Upon first contacting the butterfly solution droplet, ants typically ran away to the edge of the Petri dish and groomed themselves, but later returned to the droplet to feed. This 'expression of aversion' was noted in particular for ants tasting *Bicyclus graueri* (Rebel), and *H. monteironis*, but we did not record it systematically. We found significant differences in the average length of feeding bouts among the butterfly species (Box 1, figure 1).

Feeding bouts were shortest for danaid specimens. Within

## Box 1

## Statistical analysis

For each specimen tested, the proportion of ant feeding bouts on butterfly solution was calculated. Subsequently, we focused on the feeding bout durations on 'butterfly'. Q-Q plots were used to determine that the feeding-bout durations were approximately gamma distributed (estimated shape parameter 0.503, scale parameter 0.003). Subsequently, we used Generalized Estimating Equations (gamma dependent variable) with an exchangeable working correlation matrix to estimate the effects of species and order of feeding bout for individual ants. For the species with the largest sample size, *Charaxes fulvescens*, we also explored effects of sex and age (freshly emerged vs. collected from the forest). Visual examination of a plot of consecutive feeding-bout durations of individual ants showed that first feedings tend to be shorter than subsequent feedings, and after seven feedings the feeding-bouts tend to become shorter. The fourth feeding bout seemed to be on average the longest, therefore, we included the order of each feeding bout and a quadratic term defined as  $(\text{order} - 4)^2$  as covariates in our analysis. At least two individuals per species need to be tested in order to estimate a 'species' effect, however, the number of feeding-

bouts for an individual butterfly is allowed to be just one with the GEE-method.

Effect estimates (B) with standard errors along with Type III test model results of Generalized Estimating Equations are given in the table below. The Estimated Marginal Mean durations of feeding bouts for 18 species represented in figure 1 are not derived from the GEE-method but differ only slightly from these results.

	B ± S.E.	Wald $\chi^2$	df	P
Intercept	4.943 ± 0.188	2392.48	1	<0.001
Species		108.96	17	<0.001
Order	0.093 ± 0.024	14.82	1	<0.001
Order-quadratic	-0.022 ± 0.007	10.38	1	0.001

We found no differences between the sexes in *C. fulvescens* (GEE-method: Sex P=0.3, Order P<0.001, Order-quadratic P=0.003). Older *C. fulvescens* appeared to be more palatable than freshly emerged individuals (GEE-method: Age P<0.001, Order p<0.001, Order-quadratic p=0.002).

the other sub-families tested, some species were fed on shorter than others. The most convincingly unpalatable species was *E. kakamegae*, and *H. theobene* was also fed on for relatively short time periods. Species that are putatively cryptic, Satyrinae and *Kallimoides* spp., tended to appear rather unpalatable to the ants. Older *Charaxes fulvescens* Aurivillius appeared to be more palatable than freshly emerged individuals, but there were no differences between the sexes in this species.

## Discussion

### The methods

These preliminary data indicate that the duration of ant feeding bouts can be used to assess butterfly palatability. The results can be used to generate hypotheses on palatability that can be further tested with other animals, preferably realistic predators. However, with very few observed attacks on these butterflies in the wild, we can only guess that predators are insectivorous birds, lizards, and chameleons, and perhaps large praying mantids, spiders and dragonflies. One form of unpalatability that was not investigated in this study is the concentration of repellent chemicals in scales on wings and body, which may allow predators to detect unpalatability before ingestion and allow butterflies to survive predation attempts (Vasconcelosneto & Lewinsohn 1984). Therefore, it is impossible to fully assess the role of chemical defense in these species because all appendages were removed, and therefore only scales of the body were in the test solution. While realizing the need for further tests, we interpret shorter feeding bouts as indicative of low palatability.

The results for *C. fulvescens* show that butterfly palatability can change with age, which corroborates previous results from monarch butterflies (Alonso-Mejia & Brower 1994). Therefore, care is needed when interpreting results from freshly emerged (young) specimens as well as field-caught (older) butterflies. We did not find differences in palatability between the sexes

in *C. fulvescens*, but such differences may be important in other species (Pough & Brower 1977).

### Which species seem unpalatable and could this have been predicted?

Our results support the claim that danaids are particularly unpalatable. However, there are also significant differences among the other species. Our data suggest that *H. theobene* and *E. kakamegae* are unpalatable. For *H. theobene* this could be predicted from its weak flight and moderately colorful upper sides of wings. Butterflies in this group are also thought to sequester chemicals from their host plants (Van Velzen *et al.* 2007). However, this species does not appear to be in any close mimicry association. Unpalatability of *E. kakamegae* could be predicted from the warningly colored gregarious caterpillars. *Euphaedra kakamegae* females could then be the mimetic model for *E. uganda* (the females are difficult to distinguish even though they are placed in different subgenera; Hecq 1997) on which ants typically fed for long periods of time. *Euphaedra kakamegae* males could be mimetic models for *E. preussi* and possibly *E. medon* males that all have metallic green upper sides with (slightly differently shaped) light sub-apical bands, but are easily distinguishable from the putatively cryptic undersides.

Aposematism has been shown before for one *Euphaedra* species, *E. cyarissa* (Cramer) (Larsen 2007). This species is an especially weak flyer and occurs in more exposed habitats compared to the majority of *Euphaedra* species which typically occur in the understory of forests. Aposematism could also be expected for *E. eusemoides* and *E. christyi* on the basis of their color patterns and weak flight (and for *E. eusemoides* also based on the gregarious caterpillars with moderate warning colors), *E. alacris* (warning colors), and *E. kakamegae* (gregarious caterpillars with warning colors), but we only found a strong indication of unpalatability for *E. kakamegae*. Therefore, Batesian mimicry of the aforementioned arctiid moths may be at play in *E. alacris*, *E. eusemoides* and *E. christyi*. Another case of mimicry



within our sample of *Euphaedra* species is *E. harpalyce* and the females of *E. medon* (further discussed below with cryptic species). Both species appeared moderately unpalatable and their mimicry relationship could then be classified as Müllerian. This idea contrasts with the Swynnertonean mimicry, as has been proposed for this species pair in which the large *E. harpalyce* is the model for the (supposedly easier to capture) *E. medon* females (Van Someren & Jackson 1959). However, these explanations may not be mutually exclusive and should be tested further.

Within the Limentidinae we noted that the slow-flying *H. theobene* tends to be less palatable than the more agile *Cymotrhoe* species, corroborating that animals may focus on investing in either chemical or escape defense (Kicklighter & Hay 2007). The warningly colored *P. lucretia* (viewed as imprecise acraid or *Amauris* mimic) may be aposematic on its own as was suggested by Larsen (2007).

Among the studied Charaxinae, our data show that *C. numenes* (figure I-Q) may be moderately unpalatable. Unpalatability of a *Charaxes* species has not been reported before. Other possible unpalatable *Charaxes* species are *C. pollux* and *C. brutus* (figure 1, but note the small sample size). The latter breeds on *Trichilia rubescens* Oliver (Meliaceae), a plant that Ugandan chimpanzees may use as a medicine against malaria (Krief et al. 2004), while other species of *Trichilia* are used by humans as a medicine or insecticide elsewhere (Eldeen et al. 2005, Lehman et al. 2007, Adeniyi et al. 2008). Both of these *Charaxes* species seem (at least locally) restricted to one host-plant species, which is an unusual condition in this genus and may be related to sequestration of host-plant chemicals for defense.

Regarding (unsystematic) behavioral observations, ants seemed to respond particularly negatively to *H. monteironis*. Unpalatability of *Hypolimnas* has been suggested before: 1) *H. bolinia* contains cardio-active substances but is not distasteful to several bird species (Marsh et al. 1977) and 2) a chameleon avoided *H. misippus*, both female (a near perfect mimic of *D. chrysippus*) and male (looking completely different) (Larsen 1992a).

### Crypsis and chemical defense – could it be?

One surprising result from the data was that cryptic species, including the satyrines and *K. rumia*, appeared to be rather unpalatable. This suggests that cryptic species may employ some chemical protection from predators during flight, when crypsis cannot be expected to be effective. Another indication that cryptic Lepidoptera can be unpalatable is that geometrid moths (generally cryptic adults, but occasionally caterpillars are also warningly colored) were the least acceptable moths to birds in Venezuela (Collins & Watson 1983). It would be interesting to know whether the color brown (with yellow sub-apical bands?) is viewed as a warning color by visually hunting predators. If so, this may be related to the evolution of mimicry in brown butterflies, most notably the *E. medon*-*E. harpalyce* group, which both appeared to be moderately unpalatable, and possibly including some *Bebearia* and *Euriphene* species. It may also apply to the brown females of *C. lurida*, *Gnophodes chelys* Fabricius females

(figure I-R), *Melanitis ansorgei* Rothschildt, and some *Charaxes* species that have white sub-apical or discal (table 1). In addition, there could be more precise mimicry within *B. graueri*, *B. sebetus* and *K. rumia* which all have a similar wing-shape, two parallel lines on the ventral side of the wings, and violet sub-apical bands (table 1). However, convergent evolution and a phylogenetic signal (for the two *Bicyclus* species) are possible alternative explanations for these similarities.

### How are host plants related to chemical defense?

Our results demonstrate that host-plant chemistry is a poor predictor of palatability of butterflies. *Aphania senegalensis* (Juss. ex Poir.) Radlk. (Sapindaceae) is used by *E. kakamegae*, *E. alacris*, *E. harpalyce* and *C. numenes* (table 1), but only *E. kakamegae* was clearly unpalatable, whereas *E. harpalyce* and *E. alacris* seemed at most slightly unpalatable and *C. numenes* was non-significantly less palatable than some of the other *Charaxes* included (figure 1). That host-plant chemistry is a poor predictor of insect palatability was demonstrated before by Müller and colleagues: sawfly larvae sequestered host-plant glucosinolates from species of Brassicaceae while two species of pierid butterflies did not (Müller et al. 2003, 2001). Beck and Fiedler (2009) suggested that on average, vines provide butterflies with more defensive chemicals than other growth forms. This is corroborated in our data: *E. medon*, *C. numenes* (the vine *Hypocratia plumbea* Vieillot appears to be the main host plant for *C. numenes* in Kibale National Park) and *H. monteironis* breed on vines and seem to be moderately unpalatable.

### Conclusions

These analyses provide evidence that, 1) palatability assays on whole individuals or body parts using ants can be a useful tool in the study of insect ecology and evolution, and 2) we can expect results that challenge existing assumptions on defenses and mimicry relationships.

### Acknowledgements

We thank Boniface Balyeganira, Harriet Kesiime, Christopher Aliganyira, Francis Katuramu Kanywanii, John Koojo, Francis Akoch Edigu, Małgorzata E. Arlet and Mary Alum, for their invaluable assistance in the field and in the laboratory. We thank Torben Larsen for sending us important literature, Phil Ward for identifying our ant samples (to genus at least, species level taxonomy of these ants is under revision at the moment), and Elizabeth Long, Philip DeVries, Konrad Fiedler, Annette Aiello, and Torben Larsen and anonymous Smithsonian Tropical Research Institute staff members for insightful discussion and critical comments. Thanks to Janny Tuinenga en Gerard Molleman for their help with the Dutch summary. We are grateful to Mark Grote for statistical advice. This study was conducted with kind permission of the Uganda Wildlife Authority (UWA) and the Ugandan National Council for Science and Technology (UNCST). The funding was provided by the National Institute on Aging (PO1 AG022500-01 and PO1 AG608761-10 to JRC).

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Accepted: 13 February 2010

## Samenvatting

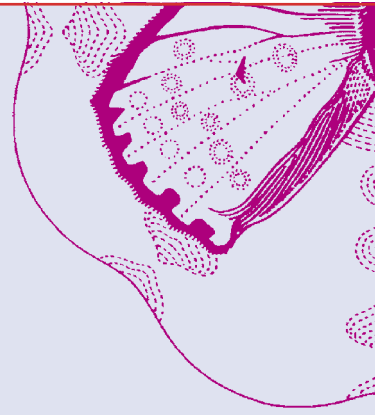
### De eetbaarheid van vlinders vergeleken met behulp van mieren

Vaak wordt gedacht dat de kleurpatronen van vlinders gelden als waarschuwing aan de predatoren dat een vlinder onsmakelijk of zelfs giftig is. De predator doet in dat geval een slechte ervaring op met vlinders die een bepaald kleurpatroon hebben, en laat vervolgens vlinders met dat patroon met rust. Toch is er nog veel onduidelijk. Zo is het bijvoorbeeld niet bekend welke predatoren bepaalde soorten doden, onder welke omstandigheden, en hoe die predatoren de kleuren zien en de smaak beoordelen. Het hier besproken onderzoek had als doel om een methode te ontwikkelen om de smaak van vlinders te kunnen vergelijken. Omdat vlinders, zeker in een tropisch bos, zich tegen een groot aantal potentiële predatoren moeten verdedigen, is te verwachten dat chemische verdediging (onsmakelijkheid en giftigheid) werkt tegen verschillende predatoren. We hebben gekozen voor mieren als predatoren, en geven negen voordelen van het werken met deze insecten. Vlinders werden opgekweekt van rupsen of verzameld als adult in Kibale National Park, een tropisch bos in het westen van Oeganda. De gebruikte soorten zijn algemene, (middel)grote vlinders die als adult fruit eten. Met suikerwater werd er van de vlinders een papje gemaakt en een druppel daarvan werd naast een druppel water met dezelfde concentratie suiker aangeboden aan individuele mieren. Vervolgens hebben we gemeten hoelang elke mier aan één stuk van de pap dronk. Op deze manier hebben we de smaak van 87 individuen van in totaal 28 vlindersoorten gemeten. Van verwanten van de monarchvlinder die bekend staan om hun onsmakelijkheid, werd steeds maar kort gegeten. Van vlinders waarvan niet bekend was of ze smakelijk waren of niet, werd over het algemeen langer gegeten. We hebben gevonden dat *Euphaedra kakamegae* (met zwart-wit geringde rupsen) niet smakelijk is. De als adult sterk op de vrouwelijke *E. kakamegae* gelijkende *E. uganda* lijkt smakelijk te zijn. De mannetjes van *E. kakamegae* lijken van boven (dorsaal) weer enigszins op die van *E. medon* en *E. preussi*. Het lijkt er dus op dat verschillende vlindersoorten profiteren van de





aanwezigheid van de onsmakelijke *E. kakamegae* door middel van Batesiaanse mimicry. De *E. medon* vrouwtjes en de *E. harpalyce* zijn van boven nauwelijks te onderscheiden en zijn beide matig onsmakelijk volgens de mieren en dit duidt dus op Mülleriaanse mimicry. Sommige soorten met waarschuwingkleuren bleken niet erg onsmakelijk te zijn, en mogelijk imiteren deze vlinders onsmakelijke of giftige motten. Het was ook opvallend dat sommige vlinders met een schutkleur onsmakelijk leken te zijn en we kunnen ons dus voorstellen dat 'bruin met een gele streep bij de punt van de voorvleugel' ook een signaal is aan predatoren dat de vlinder niet smakelijk is. Veel van de mogelijke signaalfuncties en mimicryrelaties die op deze manier aan het licht kwamen waren nog niet eerder zo bekeken. Binnen één soort (*Charaxes fulvescens*, waarvan we de meeste gegevens verzameld hebben) vonden we geen verschil tussen de sexen, maar pas uit de pop gekropen vlinders bleken minder smakelijk te zijn dan oudere vlinders die in het bos gevangen waren. We stellen dat meten hoelang mieren van een insect eten in een door ons geprepareerd papje een handige methode is om insectensoorten en verschillende groepen binnen soorten met elkaar te kunnen vergelijken. Daar zouden weleens nog meer interessante en verrassende resultaten uit kunnen komen.



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\*voor meer informatie en mogelijkheden om in Oeganda aan vlinders te werken. Dit voorjaar komt ook een veldgids uit van FM.