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This thesis is based on the following publications, which are referred to in the text by their Roman numerals.

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- II Bauder J. A. S., Handschuh S., Metscher B. D., Krenn H. W. (2013) Functional morphology of the feeding apparatus and evolution of proboscis length in metalmark butterflies (Lepidoptera: Riodinidae). *Biological Journal of the Linnean Society* **110**: 291 - 304.
- III Bauder J. A. S., Warren A. D., Krenn H. W. (2014) Evolution of extreme proboscis lengths in Neotropical Hesperiiidae (Lepidoptera). *The Journal of Research on the Lepidoptera* **47**: 65 - 71.
- IV Bauder J. A. S., Warren A. D., Krenn H. W. (submitted) The role of long-proboscid Neotropical skipper butterflies (Lepidoptera: Hesperiiidae) in plant-pollinator networks. *Arthropod-Plant Interactions*.
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Contributions

The following table indicates the contributions of all authors to the respective publication.

	I	II	III	IV	V
experimental design	JB, HK	JB	JB	JB	JB
data collection	JB, NL	JB	JB	JB	JB
data analysis	JB	JB, BM	JB, AW	JB, AW	JB, LM, AW
graphical illustrations	JB	JB, SH	JB	JB	JB, LM
writing/editing	JB, HK	JB, HK	JB, HK	JB, HK	JB, HK, LM

Note: JB – Julia A. S. Bauder, SH – Stephan Handschuh, HK – Harald W. Krenn, NL – Nora R. Lieskonig, BM – Brian D. Metscher, LM – Linde Morawetz, AW – Andrew D. Warren.

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__1 GENERAL INTRODUCTION

Nectar feeding and its extreme adaptations

Nectar is commonly regarded as the world's most ubiquitous food source (Nicolson 2007), and birds, bats and insects have independently evolved adaptations which allow for its uptake (Pellmyr 2002; Muchhala & Thomson 2009; Johnson & Anderson 2010; Krenn 2010; Karolyi *et al.* 2012; Karolyi *et al.* 2013; Karolyi *et al.* 2014). Most conspicuous are elongations of the mouthparts which are often shaped as a proboscis in insects (Krenn *et al.* 2005). Nectar extracting mouthparts of insects have attained impressive extremes in euglossine bees (38.9 mm), certain tabanid and nemestrinid flies (85.8 mm), hawk moths (280 mm) and butterflies (49.9 mm) and often exceed twice the body length (Amsel 1938; Borrell 2005; Borrell & Krenn 2006; Kunte 2007; Pauw *et al.* 2009; Karolyi *et al.* 2012; Karolyi *et al.* 2014). These exaggerated morphologies evolved by natural selection and present examples for how adaptive departures from the usual isometric scaling relationships can represent a selective advantage in foraging (Kunte 2007). For example, nectar feeding butterflies feature disproportionately longer proboscides than non-nectar feeding butterflies, i.e., greater relative proboscis lengths (Kunte 2007).

In butterflies, the proboscis represents a combination of a microscopic sponge and a soda straw (Monaenkova *et al.* 2011). The two elongate galeae that compose the proboscis enclose the narrow tubular food canal (Krenn 2010). The apical region of the proboscis features small intake slits which allow for capillary fluid uptake into the food canal (Monaenkova *et al.* 2011). Subsequently, liquids are transported through the food tube inside the proboscis by the suction pump, which is located in the head and produces a pressure gradient (Eberhard & Krenn 2005; Davis & Hildebrand 2006).

Long proboscides in butterflies

In butterflies outside of the HesperIIDae, the proboscis normally measures about two thirds of the body length in European species (Paulus & Krenn 1996) and averages about 80 % in Neotropical butterflies (Kunte 2007). Extremely long proboscides were recorded for Neotropical *Eurybia*-butterflies (Riodinidae), measuring up to 49.9 mm (Kunte 2007) and for some Neotropical skipper butterflies (HesperIIDae), measuring

up to 45.8 mm. In some representatives of these families the proboscis may exceed twice the body length (Kunte 2007; **Chapters I, II, III, IV and V**). Although butterflies have taken centre stage in hundreds of taxonomic, ecological and anatomical studies and some butterfly species even became increasingly used as model organisms for studying evolution, behaviour or physiology, these two butterfly families have been largely left behind (DeVries *et al.* 1992; Warren *et al.* 2009). Hesperidae have received little attention from collectors because of their dull coloration and difficulties with species identification (Holloway *et al.* 2001; Warren *et al.* 2009), whereas Riodinidae are a diverse group of very tiny inconspicuous butterflies that combine all characteristics of a high tropical diversity with extreme rarity and were long treated as peculiar Neotropical members of Lycaenidae (DeVries *et al.* 1992; DeVries 1997). However, especially skipper butterflies represent a suitable study system for research on the evolution of long proboscides and phylogenetic differences of scaling relationships between proboscis length and body size (**Chapter III**) because they comprise a wide variety of both short- and long-proboscid species.

Costs and benefits of long proboscides

Despite the alleged advantage for nectar uptake from deep floral tubes (Nilsson 1988; May 1992), which cannot be used by insects with short proboscides (**Chapter IV**), only a few species of butterflies possess a proboscis that is longer than their body. Disadvantages of visiting deep flowers and/or of having a long proboscis can result in longer flower handling times (**Chapters I and V**), as has been recorded for hummingbirds, bumblebees, butterflies and some flies (Hainsworth 1973; Hainsworth & Wolf 1976; Inouye 1980; Harder 1983; Kunte 2007; Karolyi *et al.* 2013; **Chapter I**). Kunte (2007) reported a trade-off between proboscis length and food handling time in Neotropical butterflies. He concluded that long-proboscid butterflies harvest less nectar per time from the same flower than butterflies with normal sized proboscides. Because of this, he regarded the reduced foraging efficiency experienced by long-proboscid butterflies as a functional constraint for evolving extraordinarily long proboscides (Kunte 2007). However, flower handling time depends on the time required to enter and leave a flower (manipulation time) and the actual time needed to take up nectar (suction time) (Karolyi *et al.* 2013). In butterflies, manipulation time depends on uncoiling the proboscis spiral and finding an entrance into the flower as well as withdrawing and recoiling the proboscis. The suction time is determined by

the nectar intake rate, i.e., nectar volume flow per time unit. Therefore, increased flower handling times of long-proboscid butterflies could result from problems with flower manipulation, deceleration of nectar intake, or a combination of both. Alternatively, if both of these behavioural aspects were independent of proboscis length, prolonged flower handling times of long-proboscid butterflies would simply result from taking larger amounts of nectar from flowers than short-proboscid butterflies. In that case, prolonged flower handling times would represent an advantage and not a cost for evolving a long proboscis (**Chapter V**).

The rate of energy intake during influences foraging efficiency (Wolf *et al.* 1972; Heinrich 1975; Whitham 1977; May 1988) and reproductive fitness (Hainsworth *et al.* 1991). Rapid feeding should therefore be favoured by natural selection (Emlen 1966; Schoener 1971; Pyke *et al.* 1977). Nectar feeding through a tubular proboscis is subject to the physical laws of fluid dynamics, and both the morphological configuration of the feeding apparatus and nectar viscosity modify the rate of nectar intake (Kingsolver & Daniel 1979; Pivnick & McNeil 1985; Daniel *et al.* 1989; Kim *et al.* 2011; Lee *et al.* 2014). Biophysical models describe factors influencing the speed of fluid feeding and therefore help to understand the constraints regarding the evolution of extremely long proboscides (Kingsolver & Daniel 1979; Kingsolver & Daniel 1995; Lee *et al.* 2014). According to the law of Hagen-Poiseuille, the nectar intake rate of butterflies should increase linearly with increasing pressure difference produced by a suction pump and increase with the radius of the food canal to the exponent four. By contrast, it is expected to decline linearly with escalating proboscis length (Kingsolver & Daniel 1979; Daniel *et al.* 1989). Therefore on the one hand, insects could compensate for the negative influence of a long proboscis through changes in the radius of the food canal or the size of the suction pump (**Chapters II and V**), or otherwise bear this cost through a decreased intake rate (Borrell 2007). Compensating for the biophysically disadvantageous long proboscis by morphological and/or biometrical adaptations of the feeding apparatus could, on the other hand, evoke material costs for sustaining the functionality of the suctorial proboscis (**Chapter II**).

Evolution of exceptionally long insect mouthparts

Ever since Darwin's discovery of *Angraecum sesquipedale* in 1862, an orchid with an exceptionally long nectar spur ranging between 270 and 430 mm length (Wasserthal

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1997) and his prediction of the existence of a giant hawk moth pollinating this orchid (Darwin 1862), scientists all over the world have pondered over the selection mechanisms leading to the evolution of extremely long insect mouthparts (Nilsson 1988; Johnson & Steiner 1997; Wasserthal 1997; Nilsson 1998; Borrell 2005; Whittall & Hodges 2007; Johnson & Anderson 2010). Though the discovery of *Xanthopan morgani praedicta* in 1903 with a proboscis length of 220 mm verified Darwin's prediction, his coevolutionary model based on a mutual gain from the plant-pollinator interaction remains controversial (Wasserthal 1997; Wasserthal 1998; Nilsson 1998; Jermy 1999; Whittall & Hodges 2007). Darwin (1862) stated that plants with the longest nectar spurs gain a selective advantage because their reproductive organs optimally contact pollinators and therefore achieve the greatest reproduction, whereas pollinators with the longest proboscides have a selective advantage because they obtain the largest food reward. The most plausible alternative selection pathway is that floral tube length evolved when plants undergo evolutionary shifts between different pollinators with pre-existing proboscis lengths (Grant & Grant 1965; Johnson & Steiner 1997; Wasserthal 1997; Whittall & Hodges 2007). Selection mechanisms on proboscis length have been suggested by several authors and comprise (1) evolution of longer proboscides under directional selection from ambush predators such as spiders on flowers (Wasserthal 1997); (2) long proboscides pre-existed owing to selection on body size (Darwin 1862) and reflect an allometric relation with body size (Johnson & Anderson 2010); (3) interspecific competition among hawk moths for food rewards causing extreme proboscis lengths (Jermy 1999); (4) directional selection on proboscis length through energetic benefits (Pauw *et al.* 2009). Another aspect to the coevolutionary concept is that geographical variations in community structure can have effects on the strength and direction of selection (for a review see Johnson & Anderson 2010) resulting in geographically different outcomes of trait size in pollinators and plants. However, the most widely accepted coevolutionary scenario postulated by Darwin (1862) explaining the existence of long insect proboscides implies that long-proboscid flower visitors act as specialized pollinators of plants with deep floral tubes (**Chapter IV**). In fact, many studies confirmed that long-proboscid insects are generalistic foragers that exploit a great diversity of floral morphologies and use both deep and shallow flowers (Ranta & Lundberg 1980; Harder 1985; Nilsson *et al.* 1985; Nilsson *et al.* 1987; Haber & Frankie 1989; Corbet 2000; Goldblatt & Manning 2000; Agosta & Janzen 2005).

Although the majority of butterflies feed on floral nectar (Krenn 2010), the role of butterflies as effective pollinators remains doubtful in many examples (Wiklund *et al.* 1979; Wiklund 1981; Courtney *et al.* 1982). A mutualistic coevolutionary relationship between particular butterfly species and their preferred nectar host plants has only been demonstrated in two cases (Grant & Grant 1965; Gilbert 1972; Gilbert 1975; Levin & Berube 1972). In fact, some authors regard butterflies as opportunistic flower visitors that use resources as they become available during the season (Shreeve 1992; Tudor *et al.* 2004; Stefanescu & Traveset 2009). The effect of proboscis length on generalized or specialized flower use (**Chapter IV**) remains contradictory and to date has been investigated mainly in temperate butterfly communities (Porter *et al.* 1992; Corbet 2000; Tudor *et al.* 2004; Stefanescu & Traveset 2009; Tiple *et al.* 2009).

Long-proboscid butterflies: candidates for coevolution

Coevolution is most likely when interacting organisms have strong effects on each other's fitness (Janzen 1980), which is true for pollination mutualisms in which the insects that pollinate plants also depend on them as brood sites, i.e., figs and agaonid fig-wasps, as well as yuccas and *Tegiticula* moths (Cook & Rasplus 2003; Pellmyr 2003). Possible candidates for such tight coevolutionary mutualisms are butterflies belonging to the genus *Eurybia* (Riodinidae). The larvae of these butterflies feed exclusively on the flowers of several plant species belonging to Marantaceae (*Calathea* sp., *Ischnosiphon* sp. and *Maranta* sp.), Costaceae (*Costus* sp.) and Zingiberaceae (*Renealmia* sp.) (DeVries *et al.* 1992; DeVries 1997), and adults are seldom found away from thickets of *Calathea* or their other host plants (DeVries 1997). The nectar plants of adult *Eurybia*-butterflies are known to be the deep-tubed flowers of various species of *Calathea*, *Costus* and *Renealmia* which have flowers typically pollinated by hummingbirds or euglossine bees (DeVries 1997; Claßen-Bockhoff & Heller 2008). All of these observations raise the question, whether a mutualistic coevolutionary relationship exists between *Eurybia*-butterflies and their host plants? If adult long-proboscid *Eurybia*-butterflies contributed to the pollination of their larval host plants, thereby increasing the host plant's fitness, this interaction could be regarded as the third example for coevolution among butterflies (**Chapters I and V**).

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___2 AIMS AND OBJECTIVES

Chapter I

The extremely long-tongued Neotropical butterfly *Eurybia lycisca* (Riodinidae): Proboscis morphology and flower handling

In the first part of the thesis, I aimed to observe the behaviour of the long-proboscid butterfly species *Eurybia lycisca* when handling the flowers of *Calathea crotalifera* (Marantaceae) in their natural environment, a Costa Rican lowland rainforest, to find out, whether the adult butterflies act as pollinators of their larval host plants and can therefore be regarded as another example for a mutualistic coevolutionary insect-plant interaction. This study was the first to present a detailed morphological examination of the butterfly's extraordinary proboscis. Further, I described how the butterfly uses its proboscis to obtain nectar from its host plant and provided evidence for functional costs coming from long proboscides, i. e., long flower handling times.

During the copious amounts of observation time spent sitting in front of the *Calathea* flowers waiting for *Eurybia*-butterflies, I noticed that these butterflies were not the only ones that used these flowers as a nectar source. A number of different apparently long-proboscid skipper species also frequently visited *Calathea*-flowers. With this new information, I decided to dedicate part of my research to these HesperIIDae (**Chapters III, IV and V**).

Chapter II

Functional morphology of the feeding apparatus and evolution of proboscis length in metalmark butterflies (Lepidoptera: Riodinidae)

In the second part of the project, I quantified morphological adaptations of related short-proboscid and long-proboscid metalmark butterflies for comparing biometrical data of the whole feeding apparatus, including haemolymph pump muscles, suction pump muscles and various features of the proboscis. Based on these results, I estimated extra expenses and material costs of disproportionally long proboscides in Neotropical butterflies. In this way, I was able to discover morphological constraints on butterfly proboscis length that might limit its adaptability to flower tube length and thus could

2 Aims and Objectives

potentially end the proposed mutualistic coevolutionary arms race between flowers and their pollinators.

Chapter III

Evolution of extreme proboscis lengths in Neotropical HesperIIDae (Lepidoptera)

In this chapter, I analysed the scaling relationships between proboscis length and body size against a phylogenetic background, comparing the regression slopes of three HesperIIDae subfamilies. I wanted to know whether extreme proboscis lengths in skippers resulted from a proportional or disproportional increase with body size, and if these relationships differed between phylogenetic lineages. Having validated that scaling relationships characterized HesperIIDae subfamilies, I discussed the significance of scaling relationships on the evolution of ever longer mouthparts in butterflies compared to hawk moths.

Chapter IV

The ecological role of extremely long-proboscid Neotropical butterflies (Lepidoptera: HesperIIDae) in plant-pollinator networks

In this part of my PhD I observed skipper flower visitors of four different plants that differed in corolla length and analysed the effect of proboscis length on flower use. I tested the hypothesis that insects equipped with extremely long mouthparts specialize on flowers with deep nectar spurs. Alternatively, long-proboscid butterflies could use a variety of flowers regardless of nectar spur length in an opportunistic way: the longer the proboscis, the more flowers which can be visited. My observations pertained to the question whether long-proboscid butterflies can be regarded as efficient pollinators of their nectar host plants. As a result I was able to draw conclusions about the role of long-proboscid butterflies in plant-pollinator networks.

Chapter V

Functional constraints on the evolution of long butterfly proboscides: Lessons from Neotropical skippers (Lepidoptera: HesperIIDae)

In the final part of my work, I conducted feeding experiments to measure nectar intake rates of Neotropical skipper butterflies with varying proboscis lengths to rate functional costs of extremely long proboscides in butterflies. I evaluated the impact of proboscis length, body size and food canal area on intake rate. Finally, I discussed the effect of

intake rate and flower manipulation time on the foraging efficiency of long-proboscid butterflies and proposed an evolutionary scenario that explains why especially (large) skipper butterflies need long proboscides.

__3 MATERIAL AND METHODS

In an interdisciplinary approach carried out with many different butterfly species, (1) morphological analyses of the feeding apparatus of butterflies were completed with (2) functional analyses of standardized feeding experiments and (3) behavioural observations on flower choice under natural conditions in the field.

Morphology

The use of light microscopical techniques (**Chapters I, II, III and V**), X-ray microtomography (**Chapter II**) and scanning electron microscopy (**Chapter I**) delivered detailed qualitative and quantitative informations about the external and internal morphology of the feeding apparatus of long-proboscid butterflies. Especially the combination of optical sectioning, i. e. X-ray microtomography (**Chapter II**), and mechanical sectioning (**Chapters I, II and II**), i.e., serial semithin sectioning of resin-embedded body parts using a microtome, yielded detailed insights into the morphology of differently sized structures, ranging from whole butterfly heads to tiny proboscis sensilla. The MicroXTC X-ray microtomography system used in this study allowed for fields of view from 5 mm down to less than 500 µm (Metscher 2009), which rendered the discrimination of different tissues types by their specific gray values possible. Serial semithin sections were cut at a thickness of 1 µm and stained with Richardson-dye (Romeis 1989; Blumer *et al.* 2002; Pernstich *et al.* 2003), which colours various tissue types in different hues of blue, making the differentiation of structures beyond 500 µm possible. Quantification and visualisation of image stacks in AMIRA software yielded detailed 3D surface reconstructions of the suction pumps and haemolymph pumps inside the heads of butterflies and delivered quantitative data of muscle volumes in relation to head capsule volumes, making an interspecific comparison possible (**Chapter II**).

Field work

The flower-visiting behaviour of butterflies and their ability of pollinating their nectar plants (**Chapters I and IV**) were observed in a Costa Rican lowland rainforest area at the Tropical Research Station La Gamba (Puntarenas, Costa Rica, 8°45'N, 83°10'W, 81 m above sea level) situated near the Esquinas rainforest of the Piedras Blancas

3 Material and Methods

National Park. Feeding experiments with butterflies (**Chapter V**) were video-recorded with a commercially available video camera. Video footage was analysed with Sony PMB software and Adobe Photoshop CS 4 Extended 11.0.2.

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4 PUBLICATIONS

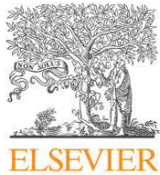
CHAPTER I

The extremely long-tongued Neotropical butterfly *Eurybia lycisca* (Riodiniinae): Proboscis morphology and flower handling.



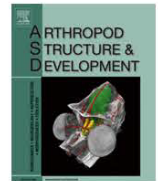
Eurybia lycisca resting on an inflorescence of *Calathea lutea*.

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The extremely long-tongued Neotropical butterfly *Eurybia lycisca* (Riodinidae): Proboscis morphology and flower handling

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ABSTRACT

Few species of true butterflies (Lepidoptera: Papilionoidea) have evolved a proboscis that greatly exceeds the length of the body. This study is the first to examine the morphology of an extremely long butterfly proboscis and to describe how it is used to obtain nectar from flowers with very deep corolla tubes. The proboscis of *Eurybia lycisca* (Riodinidae) is approximately twice as long as the body. It has a maximal length of 45.6 mm (mean length $36.5 \text{ mm} \pm 4.1 \text{ S.D.}$, $N = 20$) and is extremely thin, measuring only about 0.26 mm at its maximum diameter. The proboscis has a unique arrangement of short sensilla at the tip, and its musculature arrangement is derived. The flower handling times on the preferred nectar plant, *Calathea crotalifera* (Marantaceae), were exceptionally long (mean $54.5 \text{ sec} \pm 28.5 \text{ S.D.}$, $N = 26$). When feeding on the deep flowers remarkably few proboscis movements occur. The relationship between *Eurybia lycisca* and its preferred nectar plant and larval host plant, *Calathea crotalifera*, is not mutualistic since the butterfly exploits the flowers without contributing to their pollination. We hypothesize that the extraordinarily long proboscis of *Eurybia lycisca* is an adaptation for capitalizing on the pre-existing mutualistic interaction of the host plant with its pollinating long-tongued nectar feeding insects.

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1. Introduction

Elongate suctorial mouthparts have evolved convergently in various flower-visiting insects within the context of nectar feeding behavior (Krenn et al., 2005). Some insects have extremely long proboscides that greatly exceed body length. In a Neotropical hawk moth it reaches the remarkable length of 280 mm (Amsel, 1938). Darwin (1862) was the first to postulate that the development of greatly extended mouthparts takes place in a coevolutionary race that leads to a mutualistic specialization of nectar plants and their pollinating insects. Recent studies demonstrate that the long corollae of flowers exclude flower visiting insects that possess inappropriate mouthpart lengths, thus enhancing the chance of efficient pollination by insects with matching long proboscides (Anderson and Johnson, 2009; Anderson et al., 2008; Nilsson, 1988; Nilsson et al., 1985; Pauw et al., 2009). In return, these insects benefit from a greatly elongate proboscis by having more exclusive access to concealed nectar in deep corolla tubes (Haber and Frankie, 1989; Miller, 1997). Evolutionary scenarios postulate that the reciprocal relationships between flower depth and proboscis length in hawk moths (Lepidoptera: Sphingidae) result from mutualistic and coevolutionary adaptations (Alexandersson and Johnson,

2002; Johnson and Steiner, 1997; Nilsson, 1988; Nilsson et al., 1985). While previous studies largely focus on pollinator-mediated selection on flowers, specializations of the mouthparts have not been examined, except for proboscis length.

Compared with those of Hymenoptera and Diptera, the proboscis of Lepidoptera is a relatively simple organ. It is composed of two greatly elongate and coilable galeae, which together form the central food tube (reviewed by Kristensen, 2003). The morphology of the butterfly proboscis has been thoroughly studied along with its functional mechanisms and specializations to various food preferences (Eastham and Eassa, 1955; Knopp and Krenn, 2003; Krenn, 1990, 2010; Krenn and Mühlberger, 2002; Krenn et al., 2001).

In Papilionoidea, i.e. the true butterflies, the proboscis normally measures about two-thirds of the body length in species from Europe (Paulus and Krenn, 1996) and about 80% of body length in Neotropical butterflies (Kunte, 2007). Extremely long proboscides were recorded in the Neotropical genus *Eurybia* (Riodinidae), measuring up to 49.9 mm, equivalent to twice the body length (Kunte, 2007). While this is the greatest length recorded for a butterfly proboscis, neither the morphology nor the details of the flower-visiting behavior of any species of *Eurybia* have been examined to date. Species of *Eurybia* are known to frequently visit *Calathea* flowers to obtain nectar (DeVries, 1997). The aim of this study is to analyze the behavior of *Eurybia lycisca* (Westwood, 1851)

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while handling the flowers of *Calathea crotalifera* (Marantaceae), and to present the first morphological examination of the butterfly's extraordinary proboscis. Furthermore, the question is addressed whether the adult butterflies act as pollinators on the larval host plants (DeVries, 1997) and therefore could be regarded as an example of coevolutionary and mutualistic pollinator–plant interaction, as reported for senita moths (Fleming and Holland, 1998; Holland and Fleming, 2002) and yucca moths (Pellmyr, 2003; Pellmyr and Krenn, 2002).

2. Material & methods

The field part of the study was conducted at Tropical Research Station La Gamba (Puntarenas, Costa Rica; 8°45'N, 83°10'W; 81 m above sea level) near the Esquinas rainforest of the Piedras Blancas National Park from August to September 2009. Flower-handling behavior of *Eurybia lycisca* was recorded with a Sony V50 digital video camera. Handling time was defined as the amount of time the butterfly spent on the flower beginning with the uncoiling of the proboscis and ending when the proboscis was recoiled following nectar uptake or when the butterfly flew from the flower without visibly recoiling the proboscis. Proboscis length was measured in cold-immobilized individuals whose proboscis was partly uncoiled.

To investigate the morphology of the proboscis light microscopy, serial semithin sectioning and scanning electron microscopy were employed.

External features of the proboscis were examined in specimens fixed in 70% alcohol and then soaked in lactic acid for 5–7 days. Afterwards, the galeae were rinsed in distilled water and 30% ethanol. The galeae were then separated, embedded in polyvinyl-lactophenol on microscope slides and covered with a coverslip. Light microscopy was used to record the shape, number and length of sensilla and to investigate the surface structure of the galeae. Scanning electron microscopy was also used for external examination. Specimens were dehydrated in ethanol, air dried after treatment with hexamethyldisilazane (Bock, 1987), coated with gold and examined using a Fei XL30 (ESEM) scanning electron microscope.

Serial semithin sectioning was used for the light microscopic examination of the internal anatomy of the proboscis (procedure see Blumer et al., 2002). Specimens were fixed in 70% ethanol. The proboscis was separated from the head prior to embedding and additionally cut at the middle of its length and near the tip to ensure efficient impregnation of Agar Low Viscosity Resin under vacuum impregnation (procedure see Pernstich et al., 2006).

Drawings of the embedded parts of the proboscis were made using a stereomicroscope with the aid of a drawing tube. The drawings were used to determine the most suitable cutting plane to section the proboscis at the following intervals: 10–20%, 30–35%, 50–70% and 85–90% of overall proboscis length. Serial sections were cut on a microtome (Leica EM UC6) with a diamond knife (Histo Jumbo Diatome) at a thickness of 1 µm. A series of cross sections for a span of 100 µm was taken from the various proboscis regions. Sections were stained with a mixture of 1% azure II and 1% methylene blue in an aqueous 1% borax solution (confer Romeis, 1989) at 60–70 °C. Micrographs were taken with an Olympus CX41 microscope equipped with an Olympus E330 digital camera.

The length and inner diameter of the corolla from 20 flowers of *Calathea crotalifera* (Marantaceae) were measured with a digital caliper from different plant individuals growing in the garden of the Tropical Research Station (Table 1). Nectar amounts of unreleased flowers, which were previously covered over night with plastic bags, were measured using 10 µl syringes at 08:00 and 16:00. The superinflorescence of *Calathea crotalifera* consists of several bracts, each containing one to four flowers. The asymmetrical androgynous

Table 1

Morphometric comparison of *Eurybia lycisca* and its nectar plant, *Calathea crotalifera*.

[mm]	<i>Eurybia lycisca</i>		<i>Calathea crotalifera</i>
	Mean ± S.D. Min – max N		Mean ± S.D. Min – max N
Body length	18.5 ± 1.9 15.8–23.1 20	Flower length	39.5 ± 1.1 36.8–41.1 20
Proboscis length	36.5 ± 4.1 28.0–45.6 20	Corolla length	26.9 ± 1.0 24.7 ± 29.0 20
Proboscis diameter	— 0.24–0.26 —	Corolla diameter	0.7 ± 0.1 0.4–1.0 20

flower is composed of three choriseals and a synpetal corolla that consists of three petals. The androeceum is conjoined with the corolla and consists of one fertile petaloid stamen while other staminodia resemble petals. The superior gynoecium consists of three conjoined carpels to form a single style (Kennedy, 1983). Pollinating euglossine bees (Apidae) release a non-reversible pollen transfer mechanism (Claßen-Bockhoff and Heller, 2008a). It can be readily determined by visual examination of the flower whether its pollen release mechanism has been activated after visitation of an insect. This permitted us to postulate whether the respective visitor acts as a pollinator or not.

Statistical analyses were done using SPSS 16.0.

3. Results

3.1. Probing behavior on *Calathea crotalifera* flowers

The nectar-feeding behavior of *Eurybia lycisca* (Riodinidae) butterflies was observed on flowers of its preferred nectar plant, *Calathea crotalifera* (Marantaceae). The total handling time on single tubular flowers (mean corolla length 26.9 mm ± 1.0 S.D.; diameter 0.7 mm ± 0.1 S.D., *N* = 20) ranged between 23 sec and 151 sec (mean 54.5 sec ± 28.5 S.D., *N* = 26). Handling included proboscis uncoiling, searching for the corolla opening, uptake of nectar and proboscis withdrawal. After landing on a superinflorescence of *Calathea crotalifera*, the butterfly stepwise uncoils its proboscis. Full extension is achieved after 7–12 uncoiling steps which require a minimum of 2 sec and a maximum of 4 sec (*N* = 15). During uncoiling, the proboscis is turned steeply upward at the joint with the head. The butterfly stretches the legs to lift the body so that enough space is provided beneath to allow for the extension of the proboscis beyond the bend region in forwards and downwards direction (Fig. 1A). The uncoiled proboscis assumes a flexed position with the proximal region raised upward, a short bend region at about one third of the total length and the distal region in a vertical position. The butterfly uses the tip of the proboscis to locate the opening of the corolla before insertion. When the proboscis is inserted, the butterfly tilts forward enabling the tip of the proboscis to reach the bottom of the corolla tube. The inserted proboscis remains motionless for 9–130 sec (mean 35.1 sec ± 26.9 S.D., *N* = 30). During this time nectar is being taken up the proboscis. The mean nectar amount of unreleased flowers that can be taken up by butterflies ranges from 9.5 µl ± 2.9 S.D. (*N* = 20) in the morning to 20.6 µl ± 7.1 S.D. (*N* = 20) in the afternoon.

Eurybia lycisca can be ruled out as a pollinator of *Calathea crotalifera*, since examination of the flowers after visits revealed that they could not release the pollen trigger mechanism (Fig. 1B and C). In addition to *Eurybia lycisca*, only one species of hesperiid butterfly



Fig. 1. A. *Eurybia lycisca* probing with its extraordinary long proboscis for the corolla opening on a *Calathea crotalifera* flower. B. Untriggered flower in which the style (sty) holds back the hooded staminode (sta). C. Flower in which the pollen release mechanism has been triggered by an insect that is capable of pollinating the flower.

(*Calpodes ethlius*), two species of euglossine bee (*Euglossa* sp., *Exaerete smaragdina*) and the hummingbirds *Glaucis aene* and *Threnetes ruckeri* were observed to visit flowers of *Calathea crotalifera*. However, only euglossine bees were able to release the pollen trigger mechanism. Over a two day period, the number of flower visits to *Calathea crotalifera* was observed. 36.2–41.6% of flower visits were attributed to hummingbirds, 29.5–30.7% were *Eurybia lycisca*, 15.8–23.8% were euglossine bees and 10.5–11.9% were *Calpodes ethlius*.

3.2. Proboscis morphology

The length of the proboscis of *Eurybia lycisca* ranges between 28.0 mm and 45.6 mm (mean 36.5 mm $\pm$ 4.1 S.D., $N = 20$) (Table 1), which corresponds approximately to twice the body length. In resting position the proboscis forms at least 7 coils and is positioned between the labial palps beneath the head (Fig. 2A). In the uncoiled position, the region proximal to the bend region amounts to about 35% of the whole galeal length, while the region distal to the bend measures about 65% of the whole galeal length. The proboscis tapers continuously to the tip. Its diameter ranges from 0.26 mm in the bend region to 0.10 mm in the tip region in the male specimen and 0.24 mm–0.09 mm in the female specimen, respectively. The surface of the outer galeal wall bears continuous, vertical exocuticular ridges. The ridges have a corrugated texture that does not change from the proximal to the distal region of the proboscis. The tip region is characterized by slit openings between the otherwise tightly closed dorsal galeal linkage (Fig. 2B). The tip

region is 0.71 mm long, which corresponds to approximately 2.0% of the proboscis length.

Bristle-shaped sensilla trichodea occur along the whole length of the galea with a mean length of 83 $\mu\text{m} \pm 6.4$ S.D. ($N = 10$) in the basal region, 12.5 $\mu\text{m} \pm 7.4$ S.D. ($N = 10$) in the proximal region and 6.1 $\mu\text{m} \pm 1.0$ S.D. ($N = 20$) in the distal region. These sensilla become shorter from the basal to the distal region of the proboscis. Sensilla basiconica occur in the food canal and on the outside of the galea in a single row. Approximately 50 sensilla are present on the proboscis; with a mean density of 3.14 sensilla per mm. The apex of each galea is characterized by a cluster of 4–5 sensilla (Fig. 2B). The sensilla are composed of a short socket (mean length 3.4 $\mu\text{m} \pm 1.1$ S.D., $N = 12$) and an elongated sensory cone (mean length 10.1 $\mu\text{m} \pm 1.0$ S.D., $N = 12$).

The lumen of each galea contains a trachea and a nerve in the dorsal region of the cross-section, together with longitudinal musculature in the lateral and ventral regions (Fig. 2C). Lateral intrinsic galeal muscles occur throughout the whole length of each galea. The individual muscles follow a slightly oblique course; they originate on the dorso-lateral wall and extend obliquely to the ventro-lateral wall. Near the tip, the lateral intrinsic galeal muscles follow a nearly longitudinal course. A group of median intrinsic muscles is present only in the knee bend region. Their muscle strands run along the ventral galeal wall in a more or less longitudinal direction.

4. Discussion

Insect mouthparts that are considerably longer than the length of the body are known to occur in some specialized nectarivorous

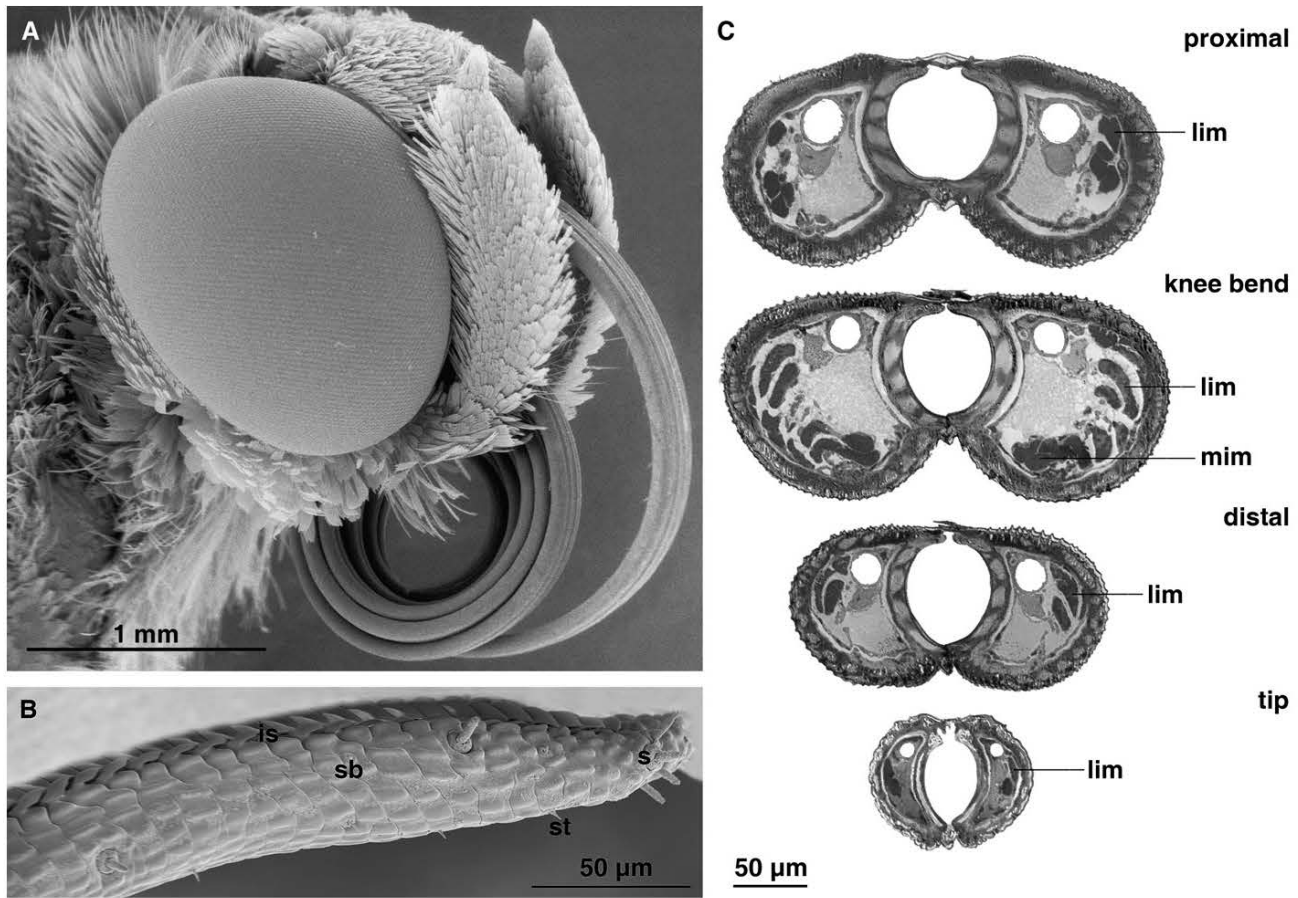


Fig. 2. A. Head of *Eurybia lycisca* (SEM) with coiled proboscis. B. Proboscis tip with four sensilla of uncertain type (s) on the apex of each galea (SEM): Intake slits (is) for imbibing nectar are located on the dorsal side of the tip region. Three different kinds of sensilla are distributed on the proboscis: sensilla of uncertain type (s) are restricted to the tip region sensilla basiconica (sb) and sensilla trichodea (st) can be found along the whole proboscis. C. Semithin sections at various points along the proboscis. Lateral intrinsic muscles (lim) occur throughout the length of the proboscis, median intrinsic muscles (mim) are present only in the knee bend.

insects such as hawk moths (Sphingidae), species of euglossine bees (Euglossini) and South African flies of Nemestrinidae and Tabanidae (Brachycera) (Krenn et al., 2005). Such extraordinarily long proboscides are rare in true butterflies, even in tropical species, and seem to be restricted to few representatives of Riodinidae and Hesperidae (Kunte, 2007). Here, we present the first study on the flower-handling behavior and the proboscis morphology of a riodinid butterfly from the genus *Eurybia*. Its proboscis is nearly twice the body length and is one of the longest among butterflies in terms of absolute length (Krenn, 2010).

We found *Eurybia lycisca* to have an exceptionally long handling time on the particularly deep and thin corolla tubes of *Calathea*, which were known to be among the preferred nectar sources (DeVries, 1997). Analysis of the proboscis movements indicated that the phases of uncoiling, searching and insertion were slower compared to movements in butterflies with average-sized proboscides (Krenn, 1990, 2008). Kunte (2007) found that handling times were up to three times longer in butterflies with a relatively long proboscis. He concluded that they have a reduced foraging efficiency compared to butterflies with average proboscis lengths.

Eurybia lycisca butterflies remain on a single flower of *Calathea* for about 1 min during which time the proboscis is frequently motionless. Generally, butterflies perform a characteristic series of proboscis movements during flower probing, in which the distal region of the proboscis is repeatedly withdrawn and inserted into the flower (Krenn, 1990, 2008). These poking movements are nearly

entirely absent in visits of *Eurybia lycisca* on *Calathea crotalifera* flowers. Probably, the poking movements, which serve to search for nectar inside tubular corollae (Krenn, 1998), are not necessary in the narrow corollae of *Calathea crotalifera* since these flowers are frequented by few visitors and provide liberal amounts of nectar.

The deep corolla tube of *Calathea crotalifera* (mean length = 26.9 mm) restricts access of general flower visiting butterflies having a mean proboscis length of about 15.9 mm, calculated from data of 89 Neotropical butterfly species (Kunte, 2007). In comparison to *Lantana* flowers, which are visited by a great number of various butterfly species (Kunte, 2007), the flowers of *Calathea* species have a small spectrum of visiting butterflies, euglossine bees, hummingbirds and sugar birds (Claßen-Bockhoff and Heller, 2008b and present study). Only two species of butterflies, both with extraordinarily long mouthparts, were recorded during our study. It can be argued that the functional costs and the reduced foraging efficiency due to the extended flower handling time (Kunte, 2007) is balanced by a rich amount of nectar, which is available only to these few insects possessing a proboscis of matching length.

In contrast to that of most butterflies, the proboscis of *Eurybia lycisca* is equipped with only a small number of short sensilla. The sensory function of the various proboscis sensilla was described for the nymphalid butterfly, *Vanessa cardui* (Krenn, 1998). The bristle-shaped sensilla on the proboscis are interpreted to be mechanoreceptors which are responsible for detecting the width of the

flower's opening. The short blunt-tipped sensilla basiconica function as chemoreceptors (Inoue et al., 2009). The sensilla styloconica are interpreted to be contact chemo-mechanoreceptors, and are characterized by pores on the sensory cone and the presence of a tubular body (Altner and Altner, 1986; Krenn and Penz, 1998; Nagnan-Le Meillour et al., 2000). They are crucial for the detection of nectar and for assessing the position of the proboscis tip once inside a flower (Krenn, 1998). Two kinds of sensilla are present at the proboscis tip in *Eurybia lycisca*, i.e. very short bristle-shaped sensilla and short basiconica-like sensilla. Future work should concentrate on TEM examination of the tip sensilla to provide information on their ultrastructural composition and on their likely functional role. Meanwhile, the question remains open as to whether the tip sensilla are particularly short sensilla styloconica or sensilla basiconica. In the latter case, the sensilla styloconica would be entirely absent. Short or reduced sensilla styloconica were reported on the proboscides of some Papilionidae, Danainae and Ithomiinae (Paulus and Krenn, 1996; Petr and Stewart, 2004). The short size of sensilla on the proboscis of *Eurybia lycisca* can be interpreted to be an adaptation to the small diameter of the corolla of the preferred nectar plant, e.g. *Calathea crotalifera*. Presumably, long sensilla at the tip would hinder entrance into small lumina of a corolla, as observed in *Morpho peleides* butterflies where the long sensilla styloconica of the tip prevent access to flowers (Knopp and Krenn, 2003). Because flower morphology restricts most competing nectar feeding insects with a medium sized proboscis, there is a high probability that those with exceptionally long mouthparts will find greater amounts of nectar and the common sensillum equipment of the tip would be superfluous for nectar detection in simple and tubular shaped flowers. The morphology of the proboscis of *Eurybia lycisca* is seen as a modification of the proboscis morphology of the nectar feeding butterfly guild that is characterized by a rather short tip region with sparsely arranged short sensilla, in comparison to fruit, sap or dung-feeding butterflies which have a relatively long tip region with numerous and long sensilla styloconica (Krenn et al., 2001).

In principle, the proboscis anatomy of *Eurybia lycisca* resembles that of other butterflies. However, the proboscis contains remarkably few intrinsic galeal muscles. Nearly all species of butterflies possess two series of intrinsic galeal muscles in the proboscis lumen (Krenn and Muhlberger, 2002) which are responsible for coiling the proboscis (Krenn, 1990, 2000; Wannenmacher and Wasserthal, 2003). In *Eurybia lycisca* the two series of intrinsic muscles are found only in the short bend region at about one third of the proboscis length. Such a muscle arrangement was erroneously described for *Pieris brassicae* (Eastham and Eassa, 1955) and could not be verified in any Lepidoptera species in additional studies (Krenn and Kristensen, 2004; Krenn and Muhlberger, 2002) until now. The specific muscle arrangement is not common to other representatives of Riodinidae, as far as known, since the European species *Hamearis lucina* has a rather short proboscis which is equipped with lateral and medial intrinsic muscles throughout the proboscis. A Neotropical representative of genus *Nymphidium* has only lateral intrinsic musculature throughout the whole proboscis (Krenn and Muhlberger, 2002).

In the garden of the Tropical Research Station La Gamba, the most abundant flower visitors of *Calathea crotalifera* were hummingbirds and *Eurybia lycisca*. Furthermore, several euglossine bees regularly utilize this plant as a nectar source. Pollination is possible in all members of Marantaceae when the insect touches a trigger-like appendage of the hooded staminode which holds the style under tension. The style springs forward and scrapes off the foreign pollen, if present on the pollinator, and places its own pollen onto the pollinator's mouthparts (Pischtschan and Claßen-Bockhoff, 2008). The flower can be inspected after visitation to

determine whether the trigger mechanism was released and thus if the visitor served as a potential pollinator. Euglossine bees were found to be the pollinators of *Calathea crotalifera* since they were able to release the trigger mechanism of the staminode–style complex, corroborating previous results (Claßen-Bockhoff and Heller, 2008a). All other flower visitors failed to trigger the pollen transfer mechanism including *Eurybia lycisca*. Future work should confirm our hypothesis that releasing the trigger is unlikely due to the thinness of the butterfly's proboscis and the lack of energetic body movements of the butterfly when feeding, in contrast to the vigorous behavior of the euglossine bees. We therefore regard *Eurybia lycisca* as a nectar thief and not as a potential pollinator of *Calathea crotalifera*. Furthermore, *Eurybia lycisca* larvae predominantly feed on *Calathea* floral tissue (DeVries, 1997). Therefore we conclude that a two-fold antagonistic relationship exists between *Eurybia lycisca* and *Calathea crotalifera* that can be regarded as a parasitic interaction.

In flower-visiting insects with extraordinarily long mouthparts (e.g. hawk moths) the fit between nectar spur length and pollinator proboscis length has been regarded as a result of mutualistic coevolution, i.e., the matching traits evolved due to their functional interaction. The underlying evolutionary pathways were recently discussed (Whittall and Hodges, 2007). Either the nectar spurs and the proboscis length of a pollinator evolved in a one-to-one coevolutionary interaction, as proposed for species of Sphingidae and Nemestrinidae (Alexandersson and Johnson, 2002; Anderson and Johnson, 2009; Nilsson, 1987, 1988; Pauw et al., 2009) or the shift hypothesis applies, i.e. a one-sided selection favored the evolution of a particular long nectar spur to match a rather fixed proboscis length of pollinating insects (Whittall and Hodges, 2007). Neither of those evolutionary scenarios applies to the relationship between *Eurybia lycisca* and *Calathea crotalifera*. Since *Eurybia lycisca* is not the pollinator of its preferred nectar plant, *Calathea crotalifera*, which is the main larval host plant, the antagonistic nature of the animal–plant relationship is in no doubt. We argue that the enormous length of the proboscis in *Eurybia lycisca* evolved as an adaptation for stealing nectar from long and thin flowers, which are subject to selection from the pollinator's proboscis morphology and, therefore, probably have a fixed corolla length. In this case, the proboscis length of the nectar thief is subject to selection by the flower length. We conclude that the extraordinary proboscis length of *Eurybia lycisca* is an adaptation for gaining access to the nectar resources of flowers which are pollinated by other long-tongued visiting insects. Obviously, the extra costs of an extremely long proboscis and extended handling times are balanced by the high amounts of nectar in the flowers, which limit access to few flower visitors.

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CHAPTER II

Functional morphology of the feeding apparatus and evolution of proboscis length in metalmark butterflies (Lepidoptera: Riodinidae).



Sarota gyas sitting on a banana leaf.

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Functional morphology of the feeding apparatus and evolution of proboscis length in metalmark butterflies (Lepidoptera: Riodinidae)

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An assessment of the anatomical costs of extremely long proboscis mouthparts can contribute to the understanding of the evolution of form and function in the context of insect feeding behaviour. An integrative analysis of expenses relating to an exceptionally long proboscis in butterflies includes all organs involved in fluid feeding, such as the proboscis plus its musculature, sensilla, and food canal, as well as organs for proboscis movements and the suction pump for fluid uptake. In the present study, we report a morphometric comparison of derived long-tongued (proboscis approximately twice as long as the body) and short-tongued Riodinidae (proboscis half as long as the body), which reveals the non-linear scaling relationships of an extremely long proboscis. We found no elongation of the tip region, low numbers of proboscis sensilla, short sensilla styloconica, and no increase of galeal musculature in relation to galeal volume, but a larger food canal, as well as larger head musculature in relation to the head capsule. The results indicate the relatively low extra expense on the proboscis musculature and sensilla equipment but significant anatomical costs, such as reinforced haemolymph and suction pump musculature, as well as thick cuticular proboscis walls, which are functionally related to feeding performance in species possessing an extremely long proboscis. © 2013 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2013, **110**, 291–304.

ADDITIONAL KEYWORDS: flower-visitor – haemolymph pump – insect – mouthparts – sensilla – suction pump.

INTRODUCTION

Feeding in adult butterflies is of direct importance to individual fitness because the intake of carbohydrates and amino acids contributes to increased longevity, body weight maintenance, and lifelong fecundity in females (Murphy, Launer & Ehrlich, 1983; Hill & Pierce, 1989; Fischer, O'Brien & Boggs, 2004; Bauernfeind & Fischer, 2005). Additionally, a case-study on *Euphydryas editha* has shown that

egg distribution is constrained by the proximity of nectar plants (Murphy, 1983). Optimal foraging theory predicts that insects are bound to maximize their rate of food intake (Pyke, Pulliam & Charnov, 1977). Therefore, the performance of the feeding apparatus should be subject to a high selective pressure and allow these insects to feed even tiny amounts of nectar from variously shaped flowers in an efficient way (Krenn, Plant & Szucsich, 2005; Borrell & Krenn, 2006; Krenn, 2010).

Nectar foraging in insects comprises searching for a flower, the discovery of nectar within the flower, and the uptake of nectar (Boggs, 1988). Searching for a

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suitable flower requires locomotion organs and adequate sensory organs (i.e. colour vision and olfactory receptors on the antennae and mouthparts). The feeding apparatus is primarily responsible for flower handling, which includes the discovery and uptake of nectar. The feeding apparatus of butterflies comprises the tubular proboscis, a basal haemolymph pump for uncoiling the proboscis, and a suction pump located in the butterfly's head. The activity of the suction pump produces a pressure gradient to take up fluid through the food canal of the proboscis, which functions like a drinking straw (Krenn, 2010). Recent work on *Danaus plexippus* showed that the proboscis tip additionally functions like a nanosponge using capillary forces for initial fluid uptake (Monaenkova *et al.*, 2012). The production of a pressure gradient and resulting fluid transport from the tip of the proboscis through the food canal into the oesophagus imposes physical constraints such that fluid flow rate decreases as proboscis length increases (Kingsolver & Daniel, 1979). Evolutionary scenarios have been proposed that relate proboscis length of some Lepidoptera and Diptera with host flower morphology (Nilsson *et al.*, 1985; Nilsson, 1988; Johnson & Steiner, 1997; Alexandersson & Johnson, 2002; Anderson & Johnson, 2008, 2009; Pauw, Stofberg & Waterman, 2009). These well-studied model systems for insect–plant interactions demonstrate reciprocal adaptations and/or coevolutionary relationships between flower depth and insect proboscis length. However, with the exception of South African flies (Károlyi *et al.*, 2012), these studies focused mainly on the pollinator mediated selection on flowers. Apart from proboscis length, they do not consider other morphological adaptations of the mouthparts (e.g. haemolymph pump, additional musculature, and sensilla) or suction pumps.

Despite the putative advantage for nectar uptake from deep floral tubes, which cannot be used by insects with short proboscides, only a few species of butterflies possess a proboscis that is longer than the body. In the present study, we search for reasons for this phenomenon and pose the hypothesis that the haemolymph and nectar pumping organs of the feeding apparatus might cause constraints for the evolution of a particularly long proboscis. Thus, the functional morphology of proboscides that greatly exceed the body length is of interest in comparison to the morphology of short feeding organs. Neotropical representatives of the riodinid butterflies are suitable for comparing the whole feeding apparatus because the proboscis of representatives of the genus *Eurybia* may reach 49.9 mm, equivalent to double the body length (Kunte, 2007; Bauder, Lieskonig & Krenn, 2011), whereas, in other species of Neotropical Riodinidae, the proboscis is rather short. Despite

these differences in length, neither the micro-morphology of these mouthparts, nor the biomechanics of movements or of suction pumps have been studied so far. The purpose of the present study is to investigate exceptionally long proboscides and to estimate additional costs compared to short proboscides in Riodinidae. In this way, constraints on butterfly mouthpart length that might limit the adaptability to flower spur length should be discovered.

The coilable proboscis of Lepidoptera is composed of the two interlocked galeae of the maxillae. The basal parts of the maxillae connect the proboscis to the head and form a pair of pumping structures: the haemolymph pump. Uncoiling of the proboscis spiral results from the increase of an internal haemolymph pressure inside each galea as a result of the activity of the haemolymph pump. Coiling results from the elastic properties of the proboscis cuticle and the internal proboscis musculature (Bänziger, 1971; Krenn, 1990; Krenn, 2000; Wannenmacher & Wasserthal, 2003), which is arranged as a lateral and median series of muscles in the haemocoel of each galea (Eastham & Eassa, 1955; Krenn & Mühlberger, 2002; Krenn & Kristensen, 2004). During flower probing and nectar feeding the proboscis shows a distinct bend, located at 30–35% of the total proboscis length, which divides the proboscis into the proximal and distal regions (Eastham & Eassa, 1955; Bänziger, 1971; Krenn, 1990; Krenn & Mühlberger, 2002; Krenn *et al.*, 2005). The tip region at the apical end of the proboscis is characterized by rows of slits for fluid uptake (Paulus & Krenn, 1996; Krenn & Mühlberger, 2002). The morphology and functional mechanism of the proboscis of Lepidoptera has been reviewed by Krenn *et al.* (2005) and Krenn (2010).

Most previous studies are concerned with proboscis morphology and adaptations to various food sources in Lepidoptera (Büttiker, Krenn & Putterill, 1996; Krenn & Penz, 1998; Krenn, Zulka & Gatschnegg, 2001; Knopp & Krenn, 2003; Molleman *et al.*, 2005; Bauder *et al.*, 2011; Zaspel, Weller & Branham, 2011). In butterflies, variations of proboscis length and sensilla morphology were found to be candidates for adaptations to various feeding strategies and food sources (Krenn *et al.*, 2001). The sensory equipment of a proboscis of higher Lepidoptera includes mechanosensitive sensilla trichodea, chemoreceptive sensilla basiconica, and sensilla styloconica (Altner & Altner, 1986; Paulus & Krenn, 1996; Krenn, 1998). Sensilla styloconica are unique to the Eulepidoptera and evolved along with nectar feeding (Krenn & Kristensen, 2000). They are composed of a stylus and an apical sensory peg functioning as combined chemo-mechanoreceptors that are restricted exclusively to the tip region (Altner & Altner, 1986).

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Table 1. Numbers of individuals of 16 rioidinid species (collected at the Tropical Station La Gamba, Costa Rica, September–October 2010) in alphabetical order, which were measured regarding body length and proboscis length; given as minimum and maximum values where more than one individual per species was found

Species	N	Body length [mm]	Proboscis length [mm]
<i>Detritivora hermodora</i> (C. Felder & R. Felder, 1861)	1	9.0	5.9
<i>Emesis mandana</i> (Cramer, 1780)	1	15.0	9.7
<i>Eurybia elvina</i> Stichel, 1910	3	18.2–19.6	33.5–38.5
<i>Eurybia lycisca</i> Westwood, 1851	8	15.1–20.7	34.8–42.4
<i>Eurybia unxia</i> Godman & Salvin, 1885	8	14.0–19.1	26.2–32.0
<i>Euselasia aurantia</i> (A. Butler & H. Druce, 1872)	8	10.0–11.5	1.9–3.7
<i>Juditha molpe</i> (Hubner, [1808])	2	10.6–12.6	7.7–7.9
<i>Leucochimona lepida</i> (Godman & Salvin, 1885)	1	10.1	4.0
<i>Mesene phareus</i> (Cramer, 1777)	1	9.2	5.1
<i>Mesosemia asa</i> Hewitson, 1869	5	10.7–11.9	4.4–6.1
<i>Mesosemia zonalis</i> Godman & Salvin, 1885	1	12.5	5.2
<i>Napaea eucharila</i> (H. Bates, 1867)	1	14.4	6.6
<i>Nymphidium ascolia</i> Hewitson, [1853]	1	11.8	7.5
<i>Perophtalma lasus</i> Westwood, 1851	2	7.1–9.7	3.3–4.2
<i>Sarota chrysus</i> (Stoll, 1781)	1	9.9	3.1
<i>Sarota gyas</i> (Cramer, 1775)	6	6.2–8.0	2.6–3.3

The present study aimed to determine whether there are morphological and/or biometrical adaptations of the feeding apparatus of long-tongued Neotropical rioidinid butterflies in comparison to short-tongued, related species. The present study is the first to take into account that the haemolymph pump and the suction pump have to be considered in addition to the proboscis itself to understand the evolution of extremely long mouthparts. Based on morphometric analyses of all functional components of the feeding apparatus, the extra expenses and material costs of disproportionately long proboscides in butterflies can be estimated.

MATERIAL AND METHODS

FIELD WORK

The garden and surroundings of the Tropical Research Station La Gamba (Costa Rica, Puntarenas, 8°45'N, 83°10'W; 81 m above sea level) house a rich fauna of butterflies including 45 species of Rioidinidae, also known as metalmark butterflies (Krenn *et al.*, 2010). All studied individuals were collected with a hand net in September and October 2010. The butterflies were identified with reference to DeVries (1997). In total, 50 metalmarks belonging to 16 different species were sampled (Table 1). Live butterflies were immobilized by cooling to approximately 15 °C to measure body length with a digital caliper. Images of the manually uncoiled proboscides were taken with a digital camera (Olympus-μ-Tough-6010) to measure proboscis length using the software IMAGEJ (NIH). Afterwards, specimens were fixed in a mixture of

ethanol, acetic acid, and formaldehyde for 1–2 days; subsequently, they were transferred to 70% ethanol for storage.

CHOOSING SPECIES FOR MORPHOLOGICAL COMPARISONS

Relative proboscis length of all collected specimens was calculated by dividing proboscis length by body length. Representatives of 13 species have a relative short proboscis ranging between 0.2 and 0.7 times the body length. These species differ significantly from *Eurybia elvina*, *Eurybia lycisca*, and *Eurybia unxia*, which have a relative proboscis length ranging between 1.6 and 2.4 times the body length (Mann–Whitney *U*-test: $Z = -5.939$, $P = 0.0001$, $N = 50$; Fig. 1). Accordingly, all species possessing a proboscis that is shorter than the body are referred to as the short-tongued group, whereas the three species belonging to the genus *Eurybia* are termed the long-tongued group. For detailed anatomical analyses, all species of which we could collect at least three individuals were chosen in both groups (Table 1): *Euselasia aurantia*, *Mesosemia asa*, and *Sarota gyas* (short-tongued group), and *E. elvina*, *E. lycisca*, and *E. unxia* (long-tongued group). For each species, we analyzed two individuals, the third collected specimen served as a back-up in case of methodological problems. As far as possible, we chose males and females of each species to rule out sexual dimorphism of proboscis morphology, although we were not able to collect both sexes of *E. lycisca*, *E. elvina*, and *E. aurantia*. However, sexual dimorphisms relating to

proboscis morphology are unlikely because previous work on nymphalids showed that these do not exist even though puddling behaviour is performed by males (Molleman *et al.*, 2005).

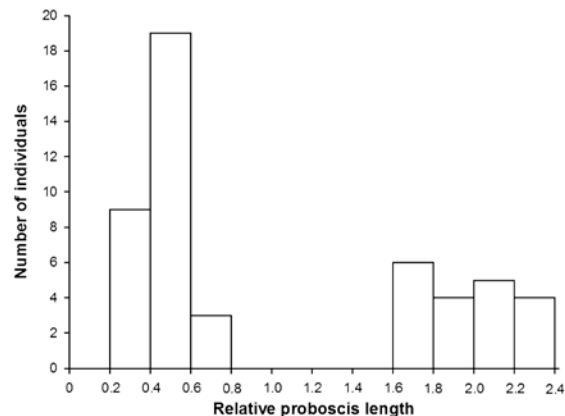


Figure 1. Frequency distribution of the ratio of proboscis length and body length in 50 collected Riodindae belonging to 16 species. Species with a proboscis that is shorter than the body (short-tongued group: relative proboscis length = 0.2–0.8) are distinct from *Eurybia* species (long-tongued group: relative proboscis length = 1.6–2.4) with a long proboscis (Mann-Whitney *U*-test: $Z = -5.939$, $P = 0.0001$, $N = 50$).

On each specimen, 15 biometrical characters were measured and categorized into characters of: (1) body size; (2) suction pump; (3) haemolymph pump; and (4) galea (Table 2).

Because long-tongued *Eurybia* species are larger than the three short-tongued species (Table 3), it was necessary to correct for differences in body size by comparing relative values (e.g. galeal cuticle volume in relation to the total galeal volume) for the comparisons between these groups. Statistical comparisons of all feeding apparatus characters in relation to head capsule volume, galeal length or galeal volume were made using SPSS, version 15.0 (SPSS Inc.). The Mann-Whitney *U*-test for non-parametric data was used, and the significance level was set at 0.05. Graphical figures were created with SIGMAPLOT, version 11.0 (Systat Software Inc.), CORELDRAW X5 (Corel Corp.), and PHOTOSHOP CS 4 (Adobe Systems).

NUMBER OF PROBOSCIS SENSILLA

The number and distribution of sensilla trichodea and styloconica along the proboscis were assessed by light microscopy. Because reliable counts of the very small sensilla basiconica can only be acquired from scanning electron microscopy samples, we chose to exclude these sensilla from our analysis. Proboscides were prepared by separating the two galeae, embedding in glycerine, and mounting on microscopic slides.

Table 2. Biometrical characters of three long-tongued species (*Eurybia elvina*, *Eurybia lycisca*, *Eurybia unxia*) and three short-tongued species (*Euselasia aurantia*, *Mesosemia asa*, *Sarota gyas*) were measured

Biometrical parameters	Scale unit	Method of measurement
Body size		
Body length	mm	Digital caliper
Head capsule volume	mm ³	μ-CT, 3D-reconstruction
Suction pump		
Volume of compressors	mm ³	μ-CT, 3D-reconstruction
Volume of dilators	mm ³	μ-CT, 3D-reconstruction
Haemolymph pump		
Number of external stipes muscles		μ-CT, 3D-reconstruction
Volume of external stipes muscles	mm ³	μ-CT, 3D-reconstruction
Volume of internal stipes muscles	mm ³	μ-CT, 3D-reconstruction
Galea		
Cuticula volume	mm ³	Serial semithin sectioning, 3D-reconstruction
Food canal volume	mm ³	Serial semithin sectioning, approximated to truncated cone
Galeal volume	mm ³	Serial semithin sectioning, 3D-reconstruction
Intragaleal muscle volume	mm ³	Serial semithin sectioning, 3D-reconstruction
Number of sensilla styloconica		Light microscopy
Number of sensilla trichodea		Light microscopy
Proboscis length	mm	Photographic images, IMAGEJ
Tip length	mm	Light microscopy

Two individuals per species were studied. CT, computed tomography.

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Table 3. Morphometry of the feeding apparatus of the long-tongued species (*Eurybia elvina*, *Eurybia lycisca*, *Eurybia unxia*) and the short-tongued species (*Euselasia aurantia*, *Mesosemia asa*, *Sarota gyas*)

	Long-tongued (N = 6)			Short-tongued (N = 6)			Mann–Whitney U-test	
	Median	Minimum	Maximum	Median	Minimum	Maximum	Z	P
Body size								
Body length (mm)	18.9	16.8	20.7	11.3	6.2	11.9		
Head capsule volume (mm ³)	1.6	1.2	2.0	0.8	0.2	0.9		
Suction pump								
Dilators (mm ³)	0.056	0.045	0.077	0.020	0.003	0.026		
Dilators (% of head capsule)	3.6	3.4	3.8	2.2	1.3	3.6	–2.242	0.026
Compressors (mm ³)	0.025	0.018	0.030	0.011	0.003	0.021		
Compressors (% of head capsule)	1.4	1.2	2.0	1.6	1.0	2.6	–0.641	0.589
Haemolymph pump								
External stipes muscles (mm ³)	0.115	0.091	0.151	0.007	0.003	0.014		
External stipes muscles (% of head capsule)	7.4	6.4	7.6	1.4	0.7	1.7	–2.882	0.002
Internal stipes muscles (mm ³)	0.015	0.010	0.020	0.001	0.001	0.002		
Internal stipes muscles (% of head capsule)	0.9	0.7	1.0	0.2	0.1	0.3	–2.882	0.002
Galea								
Galeal length (mm)	35.1	29.1	40.2	3.4	2.6	5.7		
Tip length (mm)	1.4	1.0	1.7	1.5	0.6	2.1		
Tip length (% of galeal length)	3.8	3.0	5.5	33.0	21.3	47.3	–2.887	0.002
Galea (mm ³)	0.189	0.136	0.233	0.022	0.020	0.043		
Cuticula (mm ³)	0.110	0.072	0.133	0.007	0.006	0.012		
Cuticula (% of galeal volume)	57.3	52.9	61.7	31.3	24.7	36.4	–2.882	0.002
Food canal (mm ³)	0.050	0.033	0.060	0.004	0.001	0.005		
Food canal (% of galeal volume)	24.7	24.2	30.3	14.6	1.5	23.4	–2.882	0.002
Internal galeal muscles (mm ³)	0.010	0.007	0.014	0.002	0.001	0.002		
Internal galeal muscles (% of galeal volume)	5.7	3.9	6.3	6.2	4.3	10.9	–0.801	0.485
Sensilla styloconica number	30.5	20.0	38.0	64.5	23.0	83.0		
Sensilla styloconica (per mm tip length)	22.2	11.8	38.0	40.7	38.3	48.0	–2.882	0.002
Sensilla trichodea number	294.0	192.0	373.0	71.0	55.0	91.0		
Sensilla trichodea (per mm galeal length)	8.8	5.3	9.8	21.0	11.6	27.1	–2.882	0.004

Volumetric parameters were compared using the Mann–Whitney U-test (N = 6 individuals per group).

Drawings of the galea and its sensilla equipment were made for a total of 12 specimens belonging to six species with the aid of a drawing tube attached to the microscope. Accordingly, counts of sensilla occurring along a single galea were made. Proboscis tip length was measured from the apical apex of the proboscis to the most proximal nectar intake slits and/or sensilla styloconica. Additionally, digital micrographs of the proboscis tip and its sensilla were made with an Olympus E-330 camera on an Olympus CX-41 microscope. Each proboscis tip was photographed in several focus planes. These images were imported into the software HELICONFOCUS, version 5.2 (HeliconSoft Ltd) and compiled into one completely focused image. After this treatment, the proboscides were again stored in 70% ethanol for further analyses.

SERIAL SEMITHIN SECTIONS

The proboscides were cut at the middle of their length and near the tip to ensure infiltration of Agar low viscosity resin (Agar Scientific) under vacuum after dehydration in an ascending ethanol series. Serial sections were cut on a microtome (Leica EM UC6) with a diamond knife (Histo Jumbo Diatome) at a thickness of 1 µm. Serial cross-sections of 200 sections per region for long-tongued species or 100 sections per region for short-tongued species were taken from the proximal region, the knee bend, three sub-regions of the distal region (at 25%, 50%, and 75% of its length), and the proboscis tip. Sections were stained with a mixture of 1% azure II and 1% methylene blue in an aqueous 1% borax solution (Romeis, 1989) at 60–70 °C.

Sections were digitally photographed with an Olympus E-330 camera on an Olympus CX-41 microscope. Microphotographs were enhanced in contrast and converted to grayscale using ADOBE PHOTOSHOP CS4 extended, version 11.0.2. After importing into the 3D reconstruction software AMIRA, version 5.3.3 (Zuse Institute), image stacks were aligned with the AlignSlices tool. Segmentation of the whole galea, its muscles and cuticle was performed manually using the brush tool. The MaterialStatistics tool was used to make volumetric calculations of the galea, as well as its muscles and cuticle, in each proboscis region (proximal: 0–30%, knee bend: 30–35%, distal: 35% to the beginning of the proboscis tip, and tip region, which is characterized by intake slits for nectar and the presence of sensilla styloconica). This procedure gave the volume of the galea, its muscles and cuticle for each proboscis region with spans of 200 μm in long-tongued species or spans of 100 μm in short-tongued riodinids. By multiplying the volume of the galea/muscles/cuticle per 1 μm proboscis length by the length of the respective proboscis region, and adding the volumes of each region, we can estimate the volume of a whole galea, its muscles and cuticle. Furthermore, we estimated the volume of the food canal by measuring the area of the food canal of the most proximal cross-section of the proboscis and of the most distal cross-section near the proboscis tip with IMAGEJ. The volume of the food canal was calculated by approximating it as a truncated cone.

MICRO-COMPUTED TOMOGRAPHY (CT)

Butterfly heads were prepared for X-ray microtomography by dehydration in an ascending ethanol series. Afterwards, the specimens were stained overnight in 1% elemental iodine dissolved in 100% ethanol. The samples were mounted in pipet tips filled with 100% ethanol (Metscher, 2009) and imaged using the Xradia MicroXCT-200 system with source settings of 40 kV and 200 μA and secondary optical magnification of either $\times 2$ or $\times 4$ depending on specimen size. Volume images were reconstructed with voxel sizes ranging from 3.6 to 7.3 μm . 3D reconstructions of the suction pump and the haemolymph pump were performed using AMIRA, version 5.3.3. Segmentation was done manually using the brush tool; volumetric calculations were made with the MaterialStatistics tool. The head capsule volume was reconstructed with compound eyes excluded because of a sexual dimorphism in eye size of some metalmark species (DeVries, 1997). The nomenclature of the suction pump musculature is given *sensu* Eberhard & Krenn (2005) and the nomenclature of the

haemolymph pump musculature is given *sensu* Krenn (2010).

RESULTS

BODY SIZE AND PROBOSCIS LENGTH

In the studied individuals of Riodinidae body length measured from 6.2 to 20.7 mm and proboscis length ranged between 1.9 and 42.4 mm ($N = 50$; Table 1). The comparison of the body length and proboscis length revealed two non-overlapping groups; one having a proboscis longer than the body and a second group possessing a proboscis that measures approximately half of the body length (Fig. 1).

With only a moderately larger body size, the long-tongued species of Riodinidae exhibit a ten-fold longer proboscis than the short-tongued group. The long-tongued and short-tongued species differ significantly in the proportions of the proboscis regions (Table 3). In relation to proboscis length, the representatives of the *Eurybia* species have a significantly shorter tip region than short-tongued species (Mann–Whitney *U*-test: $Z = -2.887$, $P = 0.002$, $N = 12$), because the absolute tip lengths are similar in all species (Table 3).

SENSILLA

The external surface of the proboscis is covered with various types of sensilla. Bristle-shaped sensilla trichodea are the most numerous (Table 3). Their numbers ranged from 192 to 373 in long-tongued metalmarks, whereas short-tongued metalmarks have lower absolute numbers of sensilla trichodea, ranging between 55 and 91. However, the long-tongued genus *Eurybia* is characterized by significantly lower sensilla trichodea numbers per mm proboscis length (Mann–Whitney *U*-test: $Z = -2.882$, $P = 0.004$, $N = 12$). All in all, short-tongued species have fewer sensilla trichodea than *Eurybia* species, although they are arranged more densely (Fig. 2A, B, C; Table 3).

Numbers of sensilla styloconica ranged from 20 to 38 in long-tongued species and from 23 to 83 in short-tongued species. Consequently, long-tongued species have a significantly lower number of sensilla styloconica per mm tip length than short-tongued species (Mann–Whitney *U*-test: $Z = -2.882$, $P = 0.002$, $N = 12$; Table 3). In the long-tongued *Eurybia* species, sensilla styloconica occur in relatively low numbers in the distal part of the distal region, as well as in the tip region. Four sensilla styloconica form a characteristic cluster at the proboscis tip (Fig. 2C). The stylus is very short and resembles a collared socket. The sensory peg of these sensilla exceeds the length of the stylus (Fig. 2C, G).

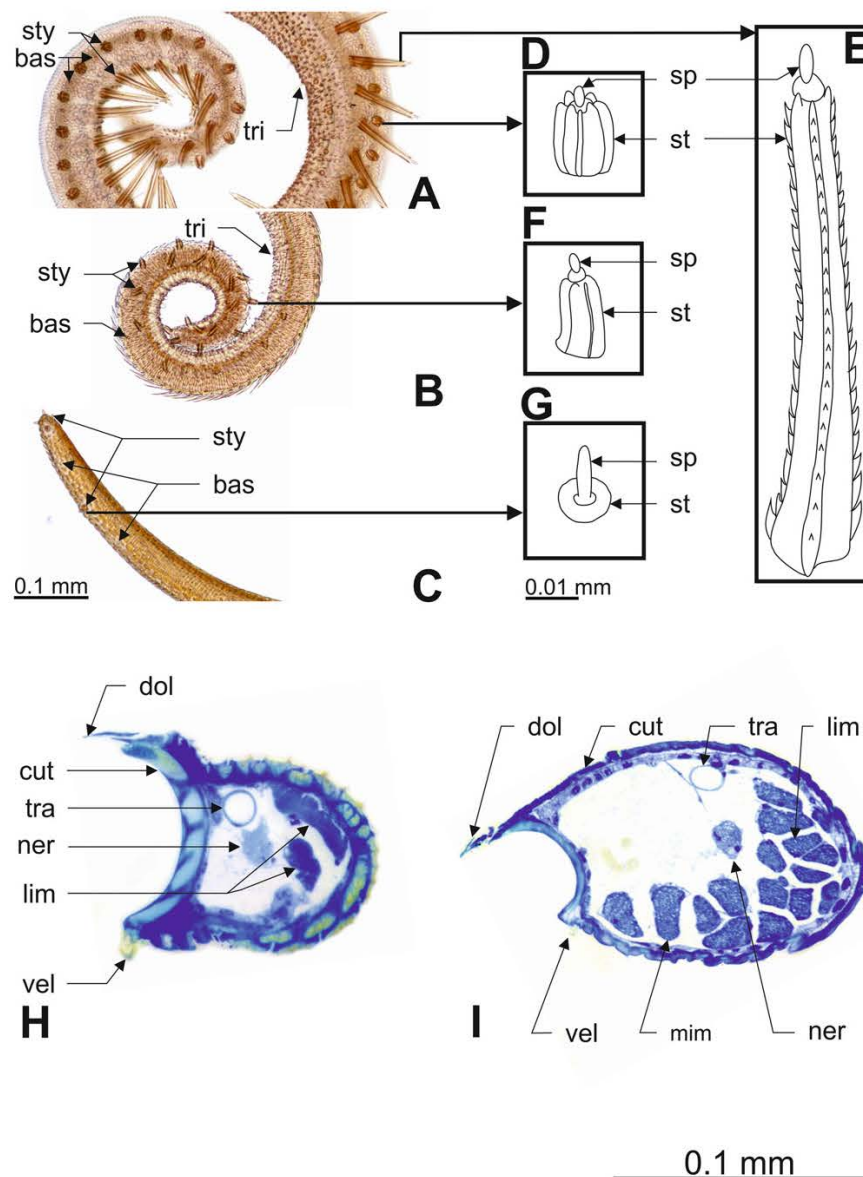


Figure 2. Proboscis morphology of long-tongued and short-tongued Riodinidae. A–C, proboscis tip equipped with various sensilla types (bas, sensilla basiconica; sty, sensilla styloconica; tri, sensilla trichodea): A, *Euselasia aurantia* (short-tongued). B, *Sarota gyas* (short-tongued). C, *Eurybia lycisca* (long-tongued). D, E, F, G, morphology of sensilla styloconica in these three species (sp, sensory peg; st, stylus). H, I, cross-sections of the distal proboscis of *Eurybia elvina* (long-tongued, H) and *Euselasia aurantia* (short-tongued, I) (cut, cuticula; dol, dorsal linkage; lim, lateral intrinsic muscles; mim, median intrinsic muscles; ner, nerve; tra, trachea; vel, ventral linkage).

In the short-tongued species *E. aurantia* (Fig. 2A, D, E) and *M. asa*, two different kinds of sensilla styloconica occur on the proboscis tip: (1) short sensilla styloconica (Fig. 2D), distributed in a row on

the dorsal side of the galea, and (2) long sensilla styloconica (Fig. 2E). The long sensilla styloconica are arranged in a row on the ventral side near the tip, changing their position from ventral to lateral/dorsal

at the proximal end of the tip region where they form more or less one row of alternating short and long sensilla styloconica. In both sensilla styloconica types, the stylus is longer than the sensory peg but, in the long sensilla styloconica, this difference is extremely pronounced. The short sensilla styloconica are characterized by a cylindriform stylus with seven smooth ridges and a central sensory peg. The stylus of the long sensilla styloconica has an asymmetrical cross-section and consists of seven ridges, with at least three of them serrated, and bears a central sensory peg.

The sensilla styloconica of the short-tongued species *S. gyas* (Fig. 2B, F) are cylindriform, their stylus is longer than the sensory peg, has five smooth ridges, and the sensory peg is in a central position. These sensilla are distributed in two rows: one on the dorsal side and one on the lateral side of the galea. The row on the dorsal side terminates in the middle of the tip region; the row on the lateral side stretches across the whole proboscis tip.

MUSCLES AND CUTICLE OF THE GALEA

The long-tongued *Eurybia* species (Fig. 2H), as well as two of the short-tongued species, *M. asa* and *S. gyas*, are characterized by the occurrence of lateral intrinsic muscles throughout the whole galea and the presence of median intrinsic muscles only in the knee bend. In the short-tongued species *E. aurantia* (Fig. 2I), however, median intrinsic muscles occur in the knee bend as well as in the distal region.

Comparisons of the percentage of the intragaleal muscle volume per galeal volume and of the cuticular volume per galeal volume reveal that long-tongued species have a significantly thicker cuticle than short-tongued species (Mann–Whitney *U*-test: $Z = -2.882$, $P = 0.002$, $N = 12$), whereas the muscle volume is similar in all species (Table 3). Furthermore, long-tongued *Eurybia* species have a significantly larger food canal volume in relation to the galeal volume than the short-tongued species (Mann–Whitney *U*-test: $Z = -2.882$, $P = 0.002$, $N = 12$; Table 3).

MUSCLES OF THE HAEMOLYMPH PUMP

The haemolymph pump morphology correlates with proboscis length. In long-tongued *Eurybia* species, there are three stipes muscles (Fig. 3A), which are significantly larger in relation to the head capsule volume than in short-tongued species, which possess only two stipes muscles (Fig. 3B, Table 3).

The external stipes muscles attach to an apodeme on the flat part of the stipes. In long-tongued *Eurybia* species (Fig. 3A), two external stipes muscles can be found: the external stipes muscle 1 is paired and

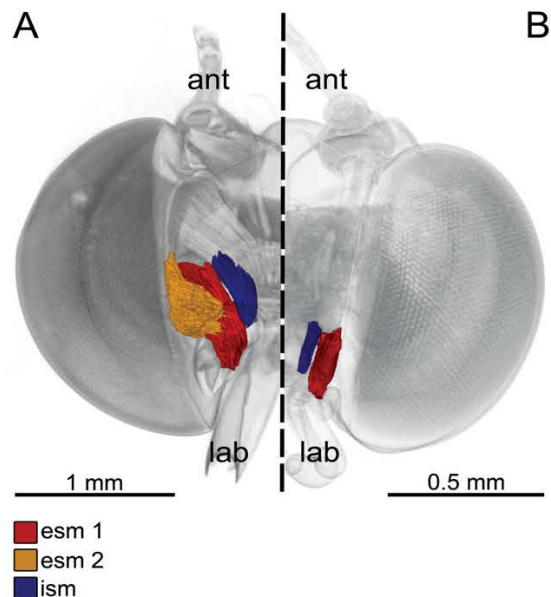


Figure 3. 3D reconstruction based on micro-computed tomography images of the stipes pump of long-tongued (A) and short-tongued (B) Riodindae, frontal view, proboscis removed. A, *Eurybia lycisca* (long-tongued): right half of the head with two external and one internal stipes muscles. External stipes muscle 1 (esm 1) originates on the gena and extends to a broad apodeme of the flat stipes part. External stipes muscle 2 (esm 2) originates on the tentorium and extends to the flat part of the stipes. The internal stipes muscle (ism) originates on the tentorium and extends to the stipes near the galeal base (ant, antenna; lab, labial palpus). B, *Sarota gyas* (short-tongued): left half of the head with one external and one internal stipes muscle. Esm 2 is missing in all short-tongued species.

originates on the gena at the lateral frontoclypeus, whereas the paired external stipes muscle 2 takes its origin on the anterior tentorium. In short-tongued species (Fig. 3B), there is only one external stipes muscle present (external stipes muscle 1), and the external stipes muscle 2 is missing. In all species, the internal stipes muscle originates on the posterior tentorium and extends to the posterior stipes part near the base of the galea.

MUSCLES OF THE SUCTION PUMP

The suction pump for nectar uptake is the largest muscular structure in the head and lies in the anterior half of the head (Fig. 4). The dorsal roof of the suction pump is covered with layers of transversal compressor muscles. Three dilator muscles attach on different sites of the suction pump: (1) The unpaired musculus frontoclypeo-cibarialis anterior originates at the

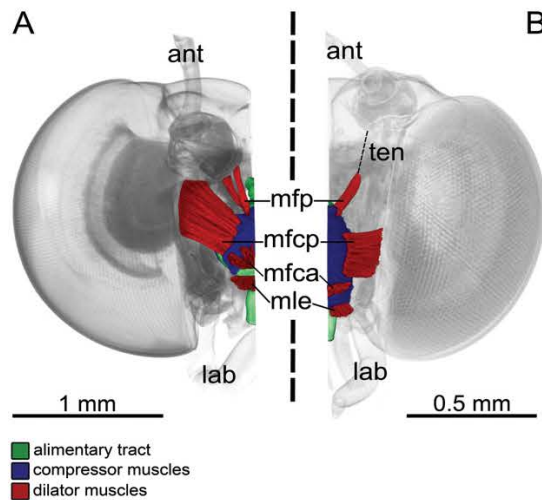


Figure 4. 3D reconstruction based on micro-CT images of the suction pump musculature of long-tongued (A) and short-tongued (B) Riodinidae, frontal view, proboscis removed. A, *Eurybia lycisca* (long-tongued): right half of the head with alimentary tract, compressor muscles and dilator muscles [musculus frontoclypeo-pharyngealis (mfp), musculus frontoclypeo-cibarialis posterior (mfcpc), musculus frontoclypeo-cibarialis anterior (mfca), musculus labro-epipharyngealis (mle)] of the suction pump. Compressors contract and reduce the suctorial cavity to swallow nectar into the oesophagus. Dilator muscles expand the suctorial cavity to produce a pressure gradient to take up nectar through the food canal of the straw-like proboscis (ant, antenna; lab, labial palpus). B, *Sarota gyas* (short-tongued): left half of the head, with alimentary tract, compressor muscles and dilator muscles of the suction pump. The dilator mfp is connected to its attachment site via a tendon (ten).

median frontoclypeus and extends to the anterior part of the dorsal wall of the suction pump. (2) The musculus frontoclypeo-cibarialis posterior is paired and originates on the lateral frontoclypeus and extends to the lateral dorsal wall of the suction pump. In *Eurybia* species, the origin of this muscle lies at the lateral/posterior frontoclypeus at the base of the antennae (Fig. 4A), whereas in the short-tongued species the origin is located at the lateral frontoclypeus (Fig. 4B). (3) The paired musculus frontoclypeo-pharyngealis originates on the lateral frontoclypeus near the base of the antennae and extends to the posterior part of the dorsal wall of the suction pump. This muscle is separated into two parts in the long-tongued species of genus *Eurybia* (Fig. 4A) but not in the short-tongued species (Fig. 4B). Another dilator muscle, the musculus labro-epipharyngealis attaches frontally at the median part of the labrum and extends to

the transition of the suction pump lumen into the proboscis food canal.

Comparison of the proportions of the dilator and compressor muscles in relation to the head capsule volume reveals significant differences concerning only the dilator muscles. The long-tongued *Eurybia* species have larger dilators than the short-tongued species (Table 3).

DISCUSSION

Insects present many examples of organs with disproportional size that evolved in context with sexual selection, such as horns in beetles (Emlen & Nijhout, 2000; Emlen, 2001). Similar extremely long organs that have been shaped by natural selection have received less attention, although there are examples of amazing lengths in probosces of flower-visiting insects (Borrell & Krenn, 2006). Because great size variations (an up to ten-fold difference in proboscis length) exist among related riodinid species, these mouthparts should be promising objects to study organ evolution and its limits from a quantitative point of view.

Because the *Eurybiini* nest within the subfamily Riodininae (Brower, 2008), it is likely that the extremely long proboscis of the representatives of the genus *Eurybia* is a derived feature that must have evolved in context with nectar feeding (Hall & Willmott, 2000).

The configuration of the feeding apparatus differs between the investigated long-tongued and short-tongued metalmarks regarding proboscis features and the size and composition of the haemolymph pump and suction pump. The proboscis of the representatives of *Eurybia* is unique in its extraordinary length, which may reach 49.9 mm (Kunte, 2007), the standing record in butterflies. The extremely long proboscis of *Eurybia* species comes from the elongation of the proximal and distal regions of the proboscis. The comparison of the proboscis proportions in long-tongued and short-tongued metalmarks revealed that the length of the tip region is the same in all species, even when the whole proboscis is ten times longer. The proboscis morphology of long-tongued *Eurybia* species is remarkable because of its low density of sensilla on the galeae, a large food canal and thick cuticular walls, as well as a short tip region equipped with relatively few and very short sensilla styloconica. The morphology of the associated inner structures indicates that the very long proboscis in metalmarks comes hand in hand with the presence of two, large external stipital muscles for uncoiling the very long and remarkably thin proboscis (Bauder *et al.*, 2011), as well as with reinforced dilator musculature of the

suction pump for creating the pressure drop required to suck up fluid through a proboscis twice the length of the body. The necessity of reinforcing the fluid pumps in the head and of developing a thick proboscis wall, as well as a large food canal in a thin proboscis, could therefore limit proboscis elongation in flower-visiting insects. The short proboscides of the metalmarks are characterized by a relatively high number of sensory sensilla, a small food canal, and thin cuticular walls, as well as a relatively long tip region covered with densely arranged and long sensilla styloconica. The morphology and arrangement of the sensilla styloconica predicts that these butterflies likely use other food sources than floral nectar since these features are similar to non-flower-visiting nymphalid butterflies (Krenn *et al.*, 2001; Molleman *et al.*, 2005).

Functional explanations for the sensilla densities and various morphologies in metalmarks come from other studies that analyzed flower-visiting behaviour and food preferences of Lepidoptera.

Sensilla trichodea function as mechanoreceptors and are assumed to provide information on the insertion depth of the proboscis (Krenn, 1990; Krenn, 1998). The sensilla styloconica of most butterflies are characterized by a long stylus that allows for the terminal sensory peg to function at a considerable distance from the surface of the galea in adult feeding (Paulus & Krenn, 1996; Krenn, 2010). These sensilla were found to be sensitive to solutions of sucrose and sodium chloride and to gather sensory information via chemical stimuli during feeding (Frings & Frings, 1956; Altner & Altner, 1986; Petr & Stewart, 2004). In addition, they are also assumed to provide tactile stimuli for detecting the opening of corolla tubes (Krenn, 1998). The morphology of these sensilla is highly variable in Lepidoptera and is known to be closely correlated with the feeding habits and preferred food sources of adult Lepidoptera (Paulus & Krenn, 1996; Krenn *et al.*, 2001; Knopp & Krenn, 2003; Petr & Stewart, 2004; Zaspel *et al.*, 2011).

Investigations on the flower-visiting behaviour of sphingid moths revealed that tactile information gathered by the proboscis on the corolla surface is needed to forage efficiently. Hawk moths decrease the time spent on individual flowers at the same time as increasing foraging efficiency (Goyret & Raguso, 2006). However, this may not apply for butterflies to such an extent. Because *Eurybia* butterflies do not hover in front of a flower like hawk moths but, instead, sit on the inflorescences and search for corolla entrances at a more leisurely pace, they do not expend high energetic costs (Bauder *et al.*, 2011). The present study suggests that there is no need for extra-investments concerning the sensory equipment of a very long proboscis. Alternatively, the prolonged

handling times at flowers of long-tongued butterflies (Kunte, 2007; Bauder *et al.*, 2011) could arise partly from a poor supply of tactile information as a result of the low number of tip sensilla and the consequently longer search times for the corolla opening.

The absence of prominent sensilla styloconica, which was detected for *Eurybia* species in the present study, has been found so far in only a few Eulepidoptera belonging to Danainae, Papilionidae, and Sphingidae (Paulus & Krenn, 1996; Petr & Stewart, 2004). This character state may be an adaptation to nectar feeding from deep floral tubes filled with nectar and may be viewed as an independent autapomorphy for each group (Petr & Stewart, 2004). A tip region equipped with short sensilla styloconica simply fits better even in narrow spurs and can be interpreted as a way of mitigating the material costs of the long proboscis but might restrict the food source to long-spurred flowers.

The sensilla styloconica morphology of the short-tongued metalmark species differs markedly from the long-tongued species and is similar to other, non-flower-visiting lycaenid and riodinid species. Because of this, it is likely that these species use food sources other than nectar, although DeVries (1997) reported that *Mesosemia asa* feeds on tiny flowers. Most non-flower-visiting butterflies possess conspicuously shaped long sensilla styloconica, which allow for feeding on evenly distributed fluids instead of fluid inside a floral tube (Paulus & Krenn, 1996; Petr & Stewart, 2004). The long styli of densely arranged sensilla styloconica form a brush-like structure to accumulate evenly distributed fluids near the food canal by capillary action in nymphalids (Krenn *et al.*, 2001).

The two differently sized types of sensilla styloconica occurring on the proboscis tip of *E. aurantia* and *M. asa* were also found in other lycaenid and riodinid species (Paulus & Krenn, 1996; Petr & Stewart, 2004). They use mainly food sources other than nectar (e.g. tree sap, honey dew from aphids) or exudates from insect galls and wet sand (Scott, 1974; Garling, 1984; Ebert & Rennwald, 1991; Stoffolano, 1995; Anthes, Fartmann & Hermann, 2008). Similar feeding preferences are also known from some metalmark species that feed on carrion and take up nutrients from puddles (Hall & Willmott, 2000).

The internal anatomy of the proboscis in the investigated species allows conclusions to be drawn about proboscis stiffness and recoiling. The muscles inside the galeae account for recoiling the proboscis into a tight spiral (Krenn, 2000). The longitudinal arrangement of these muscles differs between the investigated species: the short-tongued *E. aurantia* possesses both lateral and median muscles in the knee bend and in the distal region, whereas all the

other species investigated, regardless of proboscis length, have median intrinsic muscles only in the knee bend. Whether these groups of muscles carry out different functions is not yet known, although most butterflies possess both throughout the proboscis (Krenn & Mühlberger, 2002). Our study shows that the volume of intrinsic muscles per galeal volume is reduced in long-tongued *Eurybia* species. The proboscis of *Eurybia* species, which is ten times longer than those measured in the short-tongued group, contains approximately the same percentage of muscle volume per galeal volume. Although absolutely larger proboscides require absolutely more muscles, we conclude that galeal musculature constitutes no significant limit to proboscis length adaptation because galeal muscle volume is very small in comparison to the muscle volumes of the haemolymph and suction pump (Table 3). Therefore, no anatomical extra-investments in the galeal musculature appear to be necessary to move a very long proboscis. On the other hand, material costs of proboscis elongation affect the cuticular walls of the proboscis: the cuticle of long-tongued species is approximately two times thicker than the cuticle of short-tongued species. Krenn *et al.* (2001) suggested that long proboscides, which are suited for taking up nectar from narrow flower tubes, require a stiffer proboscis to retain their maneuverability.

The haemolymph pump of *Eurybia* species features three muscles, whereas, in short-tongued species, one of the external stipes muscles is absent. During proboscis uncoiling, compression of the stipital tube is accomplished by the external stipes muscles pumping haemolymph from the head into the galeae (Krenn, 1990). The internal stipes muscle opens the stipital valve during coiling to allow back-flow of the haemolymph into the head (Bänziger, 1971; Krenn, 1990) and pulls back the galea base to lower the coiled proboscis (Wannenmacher & Wasserthal, 2003). Korzeev & Stekolnikov (2011) compared the musculature of the head between representatives of the superfamilies Cossioidea, Zygaenoidea, and Sesiioidea and found that the additional external stipes muscle first appeared in Zygaenidae, most probably from a splitting of the external stipes muscle 1. If the presence of three stipital muscles is a character configuration associated with proboscis length, we would expect representatives that possess a short proboscis to have a reduced stipital muscle set, whereas species with functional and long proboscides should have a complete set. So far, an additional external stipes muscle has been demonstrated in several members of Ditrysia, including many Papilionoidea (Schmitt, 1938; Eastham & Eassa, 1955; MacFarlane & Eaton, 1973; Wannenmacher & Wasserthal, 2003). Additional external stipes muscles are assumed to improve the mechanism of proboscis

uncoiling because it was detected in species possessing a long and movable proboscis and is absent in species belonging to Cossidae, Limacodidae, and Sesiidae with atrophic mouthparts (Korzeev & Stekolnikov, 2011). Comparative anatomy suggests that there are also butterfly species with a functional proboscis, such as members of Papilionidae, Nymphalidae, and Pieridae, which possess only one external stipes muscle, (Schmitt, 1938), as is the case in *E. aurantia*, *M. asa*, and *S. gyas* (Riodinidae). Consequently, the presence of a second external stipes muscle in the investigated long-tongued species cannot be interpreted as an exclusive adaptation necessary for uncoiling an extremely long proboscis. The larger size of the stipes muscles in relation to the head capsule of long-tongued *Eurybia* species is likely to improve proboscis uncoiling. Further studies should focus on the configuration of the haemolymph pump in other extremely long-tongued Lepidoptera, such as hawk moths and skippers, aiming to find a correlation between stipes musculature and proboscis length. Accordingly, we could determine whether the presence of three stipes muscles is a precondition for evolving a long proboscis as an adaptation to long nectar spurs.

The morphology of the suction pump is similar in all studied riodinid species and corresponds with other butterflies and moths (Eastham & Eassa, 1955; Srivastava & Bogawat, 1969; MacFarlane & Eaton, 1973; Miles & Booker, 1998; Eberhard & Krenn, 2005; Davis & Hildebrand, 2006). Differences between the studied species concern the number of dilator muscle bundles, as well as their volume. These muscles serve to raise the flexible roof of the suction pump when the opening to the food canal of the proboscis is opened. Fluid is transported into the chamber, and then either sphincter muscles or an oral valve close the opening into the proboscis, at the same time as the compressor muscles depress the lumen of the suction pump. In this way, fluid can be transported into the oesophagus (Schmitt, 1938; Eastham & Eassa, 1955; Srivastava & Bogawat, 1969; MacFarlane & Eaton, 1973; Miles & Booker, 1998; Eberhard & Krenn, 2005; Davis & Hildebrand, 2006). Because the proportion of dilator muscles in relation to the head capsule is larger in long-tongued riodinids and because the dilator muscle is divided into four bundles in *Eurybia* species in contrast to two bundles in the short-tongued riodinids, we hypothesize that a reinforcement of the suction pump evolved in context with a very long proboscis in *Eurybia*. This is consistent with the results showing that the suction pump of a fruit-piercing moth contain more muscle bundles compared to nectar feeding Lepidoptera and that this is an adaptation to drinking thick and viscous fruit sap (Srivastava & Bogawat, 1969).

Kunte (2007) noted that there is a trade-off between proboscis length and food handling time. He concluded that long-tongued butterflies harvest less nectar per time from the same flower than butterflies with normal sized proboscides. Because of this, he regarded the reduced foraging efficiency as a functional constraint for evolving disproportionately long proboscides. His presumption for this study was that, because of an allometric relationship between body size and cibarial muscle mass, a disproportionately long proboscis has a reduced nectar uptake rate resulting from a higher nectar flow resistance and therefore a greater handling time. Both Kingsolver & Daniel (1979), Pivnick & McNeil (1985) and Daniel, Kingsolver & Meyhöfer (1989) constructed mathematical models describing the mechanics and energetics of nectar feeding in butterflies, based on the Hagen-Poiseuille equation that describes the volume flow per time through a tube: the rate of volume intake is given by the pressure drop divided by the resistance to flow. This volumetric flow rate is inversely correlated with the length of the proboscis and directly correlated with the radius of the food canal, meaning that an increase in proboscis length leads to a decrease in the rate of intake and an increase of the food canal size leads to an increase of intake rate. In the present study, we show that, at least in extremely long-tongued *Eurybia* species, there are enlargements of the suction pump muscles, which probably create a higher pressure drop, and of the relative food canal size, which is larger than the food canal of short-tongued species. Especially an increase in the food canal radius is known to dramatically increase the rate of energy intake at all nectar concentrations, because nectar flow depends on the fourth power of the radius (Kingsolver & Daniel, 1979). Therefore, the enlargement of the food canal is a very efficient way for butterflies to compensate for lower flow velocities resulting from proboscis elongation but comes at the cost of cuticular investments. Also, a ten-fold increase in the cross-sectional area of the pump dilators leads to an approximately two-fold increase in energy intake rate at all nectar concentrations (Daniel *et al.*, 1989). Interestingly, Daniel *et al.* (1989) stated that: (1) to achieve the highest intake rates, the geometry of the entire feeding apparatus must be tuned, and (2) that isometric size increase of the feeding apparatus increases the energy intake rate at all nectar concentrations. In *Eurybia* species, we demonstrate a combination of these adaptations: (1) fine-tuning of dilator muscles and food canal size, and (2) scaling to a larger body size.

The present study has shown that anatomical investments in the suction pump dilators, the stipital muscles, and the proboscis walls can be considered

to maintain feeding performance in long-tongued riordinid butterflies. At the same time, these parts of the feeding apparatus can also limit further proboscis elongation because the space in a butterfly's head is finite. The present study provides novel information about the functional tuning of the feeding apparatus that may constrain organ evolution in an evolutionary arms race between plants and visiting nectar feeding insects.

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CHAPTER III

Evolution of extreme proboscis lengths in Neotropical Hesperiiidae (Lepidoptera).



Damas immaculata uncoiling its extremely long proboscis.

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Evolution of extreme proboscis lengths in Neotropical HesperIIDae (Lepidoptera)

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Abstract. Exaggerated morphologies have evolved in insects as adaptations to nectar feeding by natural selection. For example, the suctorial mouthparts of butterflies enable these insects to gain access to floral nectar concealed inside deep floral tubes. Proboscis length in Lepidoptera is known to scale with body size, but whether extreme absolute proboscis lengths of nectar feeding butterflies result from a proportional or disproportional increase with body size that differs between phylogenetic lineages remains unknown. We surveyed the range of variation that occurs in scaling relationships between proboscis length and body size against a phylogenetic background among Costa Rican HesperIIDae. We obtained a new record holder for the longest proboscis in butterflies and showed that extremely long proboscides evolved at least three times independently within Neotropical HesperIIDae. We conclude that the evolution of extremely long proboscides results from allometric scaling with body size, as demonstrated in hawk moths. We hypothesize that constraints on the evolution of increasingly long butterfly proboscides may come from (1) the underlying scaling relationships, i.e., relative proboscis length, combined with the butterfly's flight style and flower-visiting behaviour and/or (2) developmental constraints during the pupal phase. Lastly, we discuss why butterflies did not evolve similar scaling relationships as hawk moths.

Key words: Skippers, hawk moths, scaling relationship, allometry, flower-visiting behaviour, metamorphosis.

INTRODUCTION

Exaggerated morphologies in animals are mainly known from traits that evolved by sexual selection and competition for access to mates, such as the antlers of elk or the horns of beetles (Emlen, 2001). Typically, these extraordinary features vary intraspecifically, so that not all individuals of a species express the trait to the same extent, and trait size often, but not always, scales with body size (Emlen & Nijhout, 2000). The slopes of the scaling relationships between the

dimensions of each trait and variation in body size can vary from no slope (size-invariant trait expression), very steep slopes (traits become disproportionately larger with increasing body size) to negative slopes (traits become proportionately smaller with increasing body size; Emlen & Nijhout, 2000). Scaling relationships for morphological traits in insects have evolved and can be measured by comparing related taxa. This is because scaling relationships result from developmental processes that regulate the growth of body parts and these processes are influenced by the manner in which genotypes respond to environmental conditions during growth (for a review see Emlen & Nijhout, 2000).

Exaggerated morphologies in insects do not evolve by sexual selection alone, but also by natural selection. For example, the extremely elongate mouthparts of hawk moths, butterflies, nemestrinid flies or euglossine bees evolved as adaptations for gaining access to food resources, i.e., floral nectar concealed in deep corolla tubes (Darwin, 1862; Johnson & Steiner, 1997; Alexandersson & Johnson, 2002; Johnson *et al.*, 2002; Borrell, 2005; Pauw *et al.*, 2009; Krenn, 2010). These studies present examples of how

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adaptive departures from the usual proportional scaling relationships can represent a selective advantage in foraging (Kunte, 2007). Interspecific comparative studies on hawk moths and butterflies showed that proboscis length is correlated positively with body size (Agosta & Janzen, 2005; Corbet, 2000; Kunte, 2007), and that nectar feeding butterflies have disproportionately longer proboscides than non-nectar feeding butterflies (Kunte, 2007). Until now, there have been no studies on the differences between the scaling relationships of butterflies with extremely long and short proboscides in relation to their phylogenetic background.

Here, we surveyed the range of variation that occurs in scaling relationships between proboscis length and body size in Neotropical HesperIIDae butterflies. We tested whether extreme absolute proboscis lengths in skippers results from a proportional increase of proboscis length and body size or from a disproportional increase, i.e., greater relative proboscis lengths. To the end, the significance of scaling relationships on the evolution of ever longer mouthparts in butterflies is discussed.

MATERIAL AND METHODS

Study site and field work

Sampling of HesperIIDae was carried out in the garden and surroundings of the Tropical Station La Gamba (SW Costa Rica: Puntarenas Province, Piedras Blancas National Park, 8°45'N, 83°10'W; 81 m a.s.l.) in September-October 2010, September-October 2012 and January-February 2013. The Tropical Research Station is surrounded by a mosaic of habitats including primary forest, secondary forest and intensively used land (Weissenhofer *et al.*, 2008; Krenn *et al.*, 2010). Skippers were collected with a hand net and stored in 70 % ethanol. Classification of taxa follows the most recent phylogeny of HesperIIDae (Warren *et al.*, 2009).

Morphometrics

Body length and proboscis length was measured in representatives of 75 species belonging to three subfamilies of HesperIIDae (HesperIIDae: 41; Eudaminae: 17; Pyrginae: 17). The numbers of measurements for each species depended on its commonness and ease of capture, and ranged from 1 to 39. Mean body size, proboscis length and relative proboscis length (absolute proboscis length divided by body length) for each species are given in Table 1.

In the year 2010, body length and proboscis length of live specimens was measured. Skippers were cooled to approximately 20° C. Subsequently, body length of immobilized butterflies was measured with a digital caliper. The proboscis was uncoiled manually with the aid of a dissection needle, fixed with insect pins and photographed with an Olympus μ -Tough 6000 digital camera (Olympus, Tokyo, Japan). These photographs were imported to ImageJ (U.S. National Institutes of Health, Bethesda, USA) and measured with the aid of the segmented line tool.

In the years 2012 and 2013, body length and proboscis length of ethanol-preserved specimens was measured. Body length was measured by pinning the body of each specimen in a lateral position to a foam mat. After taking a micrograph of the body, the proboscis of each specimen was separated from the head at its base, uncoiled and fixed on a foam mat using insect pins. Micrographs of the body and the proboscis were taken using a Nikon SMZ 1500 stereomicroscope (Nikon, Tokyo, Japan) equipped with an Optocam-I digital camera (Nikon, Tokyo, Japan). Micrographs were imported to ImageJ and body length as well as proboscis length was measured with the aid of the segmented line tool.

Statistical analyses

We used analyses of covariance for testing if the scaling relationships between body size and proboscis length, i.e., relative proboscis length of HesperIIDae species, differs among the three subfamilies HesperIIDae, Eudaminae and Pyrginae. ANCOVA was used to test the assumption of homogeneity of slopes among these three groups. Analyses were conducted with untransformed data in the statistical package IBM SPSS Statistics 21.0 (IBM Corporation, New York, USA). Graphical illustrations were prepared using SigmaPlot 12.5 (Systat Software Incorporated, San Jose, California, USA) and CorelDRAW X6 (Corel Corporation, Munich, Germany).

RESULTS

Body size and proboscis length were measured for a total of 370 individuals of HesperIIDae belonging to 75 species and 50 genera. Mean proboscis length per species varied eightfold between 6.4 mm and 51.8 mm, whereas mean body length per species ranged from 9.0 mm to 30.4 mm, varying only threefold (Table 1). Mean relative proboscis length also varied considerably between 0.5 (i.e., proboscis is half as long as the body) and 2.4 (i.e., proboscis is more than twice as long as the body). The longest proboscis ever discovered

Table 1. Body length, absolute proboscis length and relative proboscis length, measured in 370 individual skippers representing 75 species and 50 genera. Note: Given are mean values ($\pm$ standard deviation), whenever more than one individual per species was measured.

Species	N	Body length [mm]	Proboscis length [mm]	Relative proboscis length
Eudaminae				
<i>Astraptes fulgerator</i> azul (Reakirt, [1867])	1	25.5	23.1	0.9
<i>Astraptes alardus</i> latia Evans, 1952	2	25.5 ($\pm$ 2.1)	23.8 ($\pm$ 0.4)	0.9 ($\pm$ 0.1)
<i>Astraptes anaphus</i> annetta Evans, 1952	1	23.9	19.5	0.8
<i>Astraptes brevicauda</i> (Plötz, 1886)	1	19.8	19.7	1.0
<i>Astraptes talus</i> (Cramer, 1777)	1	21.7	17.8	0.8
<i>Autochton longipennis</i> (Plötz, 1882)	9	17.3 ($\pm$ 1.3)	16.0 ($\pm$ 1.3)	0.9 ($\pm$ 0.05)
<i>Autochton zarex</i> (Hübner, 1818)	2	18.8 ($\pm$ 0.3)	16.3 ($\pm$ 1.5)	0.9 ($\pm$ 0.1)
<i>Bungalotis quadratum</i> quadratum (Sepp, [1845])	1	30.4	39.4	1.3
<i>Cogia calchas</i> (Herrich-Schäffer, 1869)	7	14.7 ($\pm$ 1.3)	11.8 ($\pm$ 1.2)	0.8 ($\pm$ 0.03)
<i>Drephalys heraclides</i> E. Bell, 1942	1	20.0	14.3	0.7
<i>Dyscophellus porcius porcius</i> (C. Felder & R. Felder, 1862)	1	24.9	25.5	1.0
<i>Spathilepia clonius</i> (Cramer, 1775)	9	19.1 ($\pm$ 2.0)	15.5 ($\pm$ 1.3)	0.8 ($\pm$ 0.04)
<i>Typhedanus undulatus</i> (Hewitson, 1867)	1	16.2	12.4	0.8
<i>Urbanus procne</i> (Plötz, 1881)	5	18.9 ($\pm$ 1.5)	15.6 ($\pm$ 0.8)	0.8 ($\pm$ 0.07)
<i>Urbanus simpliciis</i> (Stoll, 1790)	16	18.7 ($\pm$ 1.6)	16.3 ($\pm$ 0.7)	0.9 ($\pm$ 0.06)
<i>Urbanus tanna</i> Evans, 1952	9	20.4 ($\pm$ 1.3)	16.6 ($\pm$ 0.6)	0.8 ($\pm$ 0.03)
<i>Urbanus teleus</i> (Hübner, 1821)	13	18.3 ($\pm$ 1.5)	15.9 ($\pm$ 0.9)	0.9 ($\pm$ 0.04)
Pyrginae				
Pyrrhopygini				
<i>Mysoria ambigua</i> (Mabille & Boulet, 1908)	4	23.2 ($\pm$ 1.0)	15.3 ($\pm$ 0.6)	0.7 ($\pm$ 0.03)
<i>Pyrrhopyge phidias</i> evansi E. Belt, 1947	1	27.5	15.9	0.6
Celaenorrhini				
<i>Celaenorrhinus darius</i> Evans, 1952	1	21.1	29.8	1.4
<i>Celaenorrhinus monartus</i> (Plötz, 1884)	1	15.4	20.4	1.3
Erynnini				
<i>Chiomara mithrax</i> (Möschler, 1879)	1	15.4	10.6	0.7
<i>Ebrietas osyris</i> (Staudinger, 1876)	1	19.5	11.8	0.6
Pyrgini				
<i>Pyrgus orcus</i> (Stoll, 1780)	3	13.7 ($\pm$ 0.3)	8.1 ($\pm$ 0.1)	0.6 ($\pm$ 0.01)
<i>Xenophanes tryxus</i> (Stoll, 1780)	3	11.7 ($\pm$ 0.2)	8.5 ($\pm$ 1.7)	0.7 ($\pm$ 0.1)
Achlyodini				
<i>Achlyodes busirus</i> heros Ehrmann, 1909	1	19.6	13.3	0.7
<i>Milanion marciana</i> Godman & Salvin 1895	1	13.3	9.4	0.7
<i>Ouleus panna</i> EVANS, 1953	1	11.7	10.6	0.9
Carcharodini				
<i>Nisoniades ephora</i> (Herrich-Schäffer, 1870)	1	15.2	10.1	0.7
<i>Nisoniades godma</i> Evans, 1953	3	14.7 ($\pm$ 0.3)	10.5 ($\pm$ 0.3)	0.7 ($\pm$ 0.03)
<i>Nisoniades rubescens</i> (Möschler, 1877)	3	14.8 ($\pm$ 0.4)	10.0 ($\pm$ 0.7)	0.7 ($\pm$ 0.1)
<i>Noctuana stator</i> (Godman, 1899)	1	16.8	8.9	0.5
<i>Staphylus ascalaphus</i> (Staudinger, 1876)	1	10.9	8.4	0.8

Table 1. (Cont.)

Species	N	Body length [mm]	Proboscis length [mm]	Relative proboscis length
<i>Staphylus caribea</i> (Williams & E. Bell, 1940)	4	11.2 (± 0.9)	8.0 (± 0.2)	0.7 (± 0.05)
Hesperiinae				
Clade 113				
<i>Lycas godart boisduvalii</i> (Ehrmann, 1909)	1	25.7	45.7	1.8
<i>Perichares adela</i> (Hewitson, 1867)	8	23.2 (± 1.5)	44.5 (± 4.9)	1.9 (± 0.1)
<i>Perichares lotus</i> (A. Butler, 1870)	1	22.8	48.3	2.1
<i>Pyrhopygopsis socrates orasus</i> (H. Druce, 1876)	1	26.1	34.4	1.3
Calpodini				
<i>Aroma henricus henricus</i> (Staudinger, 1876)	4	20.9 (± 1.6)	29.9 (± 1.8)	1.4 (± 0.04)
<i>Calpodes ethlius</i> (Stoll, 1782)	6	24.6 (± 2.5)	39.8 (± 3.9)	1.6 (± 0.04)
<i>Carystoides escalantei</i> H. Freeman, 1969	5	23.2 (± 1.1)	33.2 (± 1.5)	1.4 (± 0.09)
<i>Carystoides hondura</i> Evans, 1955	2	22.7 (± 1.4)	28.9 (± 0.3)	1.3 (± 0.1)
<i>Damas clavus</i> (Herrich-Schäffer, 1869)	20	23.4 (± 1.9)	49.5 (± 2.1)	2.1 (± 0.1)
<i>Damas immaculata</i> Nicolay, 1973	2	22.1 (± 2.0)	52.0 (± 1.0)	2.4 (± 0.2)
<i>Panoquina ocola ocola</i> (W. H. Edwards, 1863)	14	16.3 (± 0.9)	13.7 (± 0.5)	0.8 (± 0.05)
<i>Saliana esperi esperi</i> Evans, 1955	8	18.6 (± 1.0)	36.5 (± 2.5)	2.0 (± 0.2)
<i>Saliana longirostris</i> (Sepp, [1840])	1	26.4	42.7	1.6
<i>Saliana salius</i> (Cramer, 1775)	3	23.3 (± 0.6)	47.2 (± 5.7)	2.0 (± 0.2)
<i>Saliana severus</i> (Mabille, 1895)	1	29.7	51.8	1.8
<i>Saliana triangularis</i> (Kaye, 1914)	9	20.9 (± 1.5)	41.1 (± 2.1)	2.0 (± 0.1)
<i>Talides hispa</i> Evans, 1955	2	25.0 (± 1.5)	45.0 (± 0.7)	1.8 (± 0.1)
<i>Talides sergestus</i> (Cramer, 1775)	1	22.1	36.6	1.7
<i>Thracides phidon</i> (Cramer, 1779)	1	27.0	42.0	1.6
<i>Tromba xanthura</i> (Godman, 1901)	1	20.9	48.2	2.3
Anthoptini				
<i>Anthoptus epictetus</i> (Fabricius, 1793)	6	11.9 (± 0.8)	12.9 (± 0.4)	1.1 (± 0.08)
<i>Anthoptus insignis</i> (Plötz, 1882)	1	12.0	12.2	1.0
<i>Corticea lysias lysias</i> (Plötz, 1883)	7	12.4 (± 0.9)	12.6 (± 1.1)	1.0 (± 0.04)
Moncini				
<i>Apastus gracilis gracilis</i> (C. Felder & R. Felder, 1867)	6	9.0 (± 0.7)	6.4 (± 0.7)	0.7 (± 0.07)
<i>Arita arita</i> (Schaus, 1902)	1	18.8	27.4	1.5
<i>Callimormus radiola radiola</i> (Mabille, 1878)	6	9.9 (± 0.4)	9.0 (± 0.5)	0.9 (± 0.06)
<i>Cymaenes alumna</i> (A. Butler, 1877)	7	12.9 (± 0.9)	15.9 (± 0.9)	1.2 (± 0.09)
<i>Cymaenes tripunctus theogenis</i> (Capronnier, 1874)	1	16.9	20.3	1.2
<i>Flaccilla aecas</i> (Stoll, 1781)	1	15.1	20.0	1.3
<i>Lerema ancillaris</i> (A. Butler, 1877)	1	16.0	20.5	1.3
<i>Mnasilus allubita</i> (A. Butler, 1877)	3	11.2 (± 0.02)	12.8 (± 0.6)	1.1 (± 0.1)
<i>Mnasitheus chrysophrys</i> (Mabille, 1891)	1	10.1	9.3	0.9
<i>Morys geisa</i> (Möschler, 1879)	39	14.6 (± 1.2)	20.2 (± 1.4)	1.4 (± 0.09)
<i>Morys micethus</i> (Godman, 1900)	8	15.6 (± 0.9)	19.1 (± 1.2)	1.2 (± 0.07)
<i>Papias phaeomelas</i> (Hübner, [1831])	21	14.5 (± 1.3)	19.3 (± 4.0)	1.3 (± 0.2)
<i>Papias phainis</i> Godman, 1900	2	13.3 (± 0.6)	16.3 (± 0.2)	1.2 (± 0.1)
<i>Papias subcostulata</i> (Herrich-Schäffer, 1870)	29	17.3 (± 1.2)	24.8 (± 2.6)	1.4 (± 0.1)

Table 1. (Cont.)

Species	N	Body length [mm]	Proboscis length [mm]	Relative proboscis length
<i>Vehilius stictomenes illudens</i> (Mabille, 1891)	6	12.4 ($\pm$ 1.0)	13.1 ($\pm$ 0.9)	1.1 ($\pm$ 0.05)
<i>Vettius marcus</i> (Fabricius, 1787)	1	14.6	21.4	1.5
Hesperiini				
<i>Pompeius pompeius</i> (Latreille, [1824])	14	15.5 ($\pm$ 1.0)	14.5 ($\pm$ 0.8)	0.9 ($\pm$ 0.06)
<i>Quinta cannae</i> (Herrich-Schäffer, 1869)	7	18.8 ($\pm$ 1.2)	21.7 ($\pm$ 1.1)	1.2 ($\pm$ 0.06)

in butterflies thus far was in a specimen of *Damas immaculata* Nicolay, 1973 (Hesperiinae: Calpodini) and measured 52.7 mm. Several individuals had proboscides measuring more than 50 mm, such as specimens of *Damas clavus* (Herrich-Schäffer, 1869) (Hesperiinae: Calpodini), *Perichares adela* (Hewitson, 1867) (Hesperiinae: Clade 113), *Saliana salius* (Cramer, 1775) (Hesperiinae: Calpodini) and *Saliana severus* (Mabille, 1895) (Hesperiinae: Calpodini). The shortest proboscis measuring only 5.3 mm was found in a representative of the species *Apastus gracilis gracilis* (C. Felder & R. Felder, 1867) (Hesperiinae: Moncini).

Proboscis lengths of 75 species were categorized according to the quartiles of the data range as (1) short: $\leq$ 12.6 mm (first quartile), (2) medium: $>$ 12.7 to $\leq$ 17.8 mm (second quartile), (3) long: $>$ 17.9 to $\leq$ 29.9 mm (third quartile) and (4) extremely long: $>$ 30.0 mm (fourth quartile; see Figure 1). 70 % of the species representing the subfamily of Hesperiinae were characterized by long (12 out of 41 species) and extremely long (17 out of 41 species) proboscides. By contrast, most Pyrginae had short proboscides (12 out of 17 species). Within Eudaminae, medium sized proboscides were most abundant (9 out of 17). Extremely long proboscides occurred within Hesperiinae, but also in a single species of Eudaminae.

Within all three subfamilies, proboscis length increased with increasing body length (Hesperiinae: $F_{(1, 39)} = 184.3$, $p < 0.0001$; Eudaminae: $F_{(1, 15)} = 83.0$, $p < 0.0001$; Pyrginae: $F_{(1, 15)} = 7.3$, $p < 0.05$). The regression slopes of the three subfamilies differed significantly (Figure 2). For every 1 mm body length gain, proboscis length increased by 2.4 mm within Hesperiinae, by 1.5 mm within Eudaminae and by 0.7 mm within Pyrginae.

Hesperiinae had the steepest slope, indicating that these butterflies had disproportionately long proboscides, i.e., higher relative proboscis lengths. Within Hesperiinae, two groups (Calpodini and clade 113) had the highest relative proboscis lengths (mean = 1.8) and departed from the isometric scaling relationships of other Hesperiinae such as Moncini (mean = 1.2), Anthoptini (mean = 1.0) and Hesperiini (mean = 1.1).

DISCUSSION

Longest proboscis among butterflies found within Hesperiidae

Among insects, the world record holder concerning absolute proboscis length is *Amphimoea walkeri* (Boisduval [1875]) (Sphingidae). The proboscis of this Neotropical hawk moth measures up to 280 mm (Amsel, 1938). Among butterflies, the standing record regarding proboscis length has been held by the riordinid butterfly *Eurybia patrona* Staudinger, 1876. Its proboscis measures up to 49.9 mm (Kunte, 2007). In addition, exceptionally long proboscides were noted in at least four genera of Hesperiidae (Kunte, 2007). Here, we provide further evidence that Hesperiidae comprise many species with exceptionally long proboscides. Further, we now have a new record holder for absolute proboscis length in butterflies: *D. immaculata* with a proboscis length of up to 52.7 mm.

Evolution of extremely long proboscides

Mapped onto a cladogram (Warren *et al.*, 2009), we conclude that extremely long proboscides among Neotropical Hesperiidae presumably evolved at least three times independently (Figure 3), once within the subfamily Eudaminae and twice within groups of Hesperiinae: viz. Hesperiinae-Calpodini, and Hesperiinae-clade 113 (Table 1). Nearly all members of the tribe Calpodini analysed in this study were characterized by long or even extremely long proboscides, except *Panoquina ocola ocola* (W. H. Edwards, 1863), which had a medium-sized proboscis measuring only 13.7 mm on average. However, it is possible that other extremely long-proboscis species could also be found among Palaeotropical Hesperiidae. By contrast, extremely long proboscides in butterflies outside of the Hesperiidae are known to occur only within a single genus of Riordinidae, *Eurybia* (Kunte, 2007; Bauder *et al.*, 2011; Bauder *et al.*, 2013).

Our data showed that each of the three investigated skipper subfamilies Hesperinae, Eudaminae and Pyrginae featured a characteristic scaling relationship between body size and proboscis length, i.e., relative proboscis length. Hesperinae had the steepest slope, indicating that these butterflies had disproportionately long proboscides. Therefore, extreme absolute proboscis lengths in skipper butterflies are the result of allometry (slope of regression line: 2.4 for Hesperinae) and do not scale isometrically with body size (slope of regression line would be 1.0).

What prevents butterflies from evolving even longer mouthparts?

The evolution of extreme absolute proboscis lengths in skipper butterflies is closely linked to extreme relative proboscis lengths, since body size and absolute proboscis length scaled allometrically. In hawk moths, the extreme proboscis length of *Amphimoea walkeri*, 280 mm, corresponds to the fourfold of body length (Amsel, 1938), whereas our present data and those of former studies (Kunte, 2007; Bauder *et al.*, 2011; Bauder *et al.*, 2013) showed that relative proboscis length in butterflies never exceeds 2.5. These results indicate that proboscis length in hawk moths can exceed that of butterflies not only because hawk moths are larger, but also because of a steeper scaling relationship between body size and proboscis length. Two not mutually exclusive explanations for what keeps butterflies from evolving equally long mouthparts in relation to body size as hawk moths could be found in differences regarding the flower-visiting behavior and/or metamorphosis.

A crucial difference between butterflies and hawk moths regards their flower-visiting behavior: hawk moths typically hover over or in front of flowers during nectar uptake (Farina *et al.*, 1994), whereas nearly all butterflies need to sit on the flower to feed (Krenn, 2008), except for Troidini (Papilionidae). In butterflies, uncoiling a very long proboscis is limited by how far a butterfly can bend back its head and stretch its legs to allow for straightening of the proboscis spiral while sitting on the flower. None of these problems apply to hawk moths, which can modulate the space needed for uncoiling by hovering at an acceptable distance in front of or over the flower. Although absolute proboscis length determines access to nectar in flowers with deep tubes, relative proboscis length plays a crucial role during the uncoiling process and might constrain butterflies from evolving even longer mouthparts.

Further, developmental constraints could limit the evolution of proboscis length in butterflies since proboscis formation takes place in a developmental sheath on the ventral side of the pupa (Lowe *et al.*,

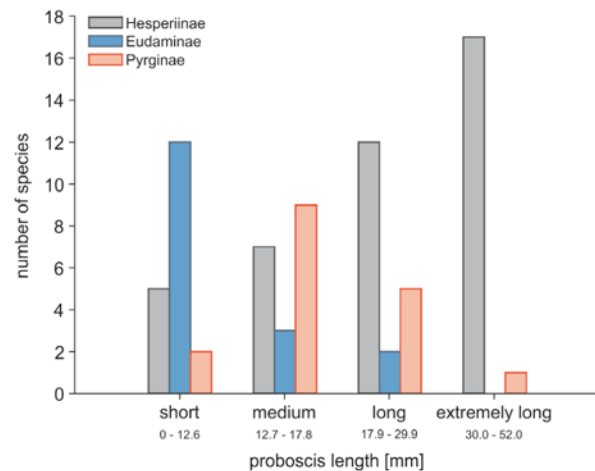


Figure 1. Categorization of proboscis lengths measured in 75 species representing three subfamilies of Hesperidae (Hesperinae, Eudaminae, Pyrginae) according to quartiles of data range: short: ≤ 12.6 mm; medium: 12.7 to 17.8 mm; long: 17.9 to 29.9 mm; and extremely long: 30.0 to 52.0 mm.

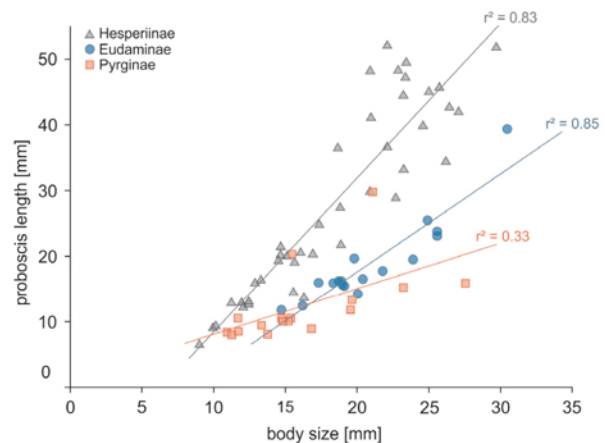


Figure 2. The allometric relationship between body size and proboscis length in Costa Rican Hesperidae butterflies. Hesperinae (N = 41 species) had significantly longer proboscides for a given body size compared to Eudaminae (N = 17 species) or Pyrginae (N = 17 species). Regression lines were fitted as: Hesperinae: $y = 2.4x - 15.1$; Eudaminae: $y = 1.5x - 12.3$; and Pyrginae: $y = 1 + 0.7x$. Scaling relationships differed significantly among the three subfamilies (ANCOVA, homogeneity of regression slopes, Hesperinae-Eudaminae: $p < 0.05$; Eudaminae-Pyrginae: $p < 0.05$; Hesperinae-Pyrginae: $p < 0.0001$).

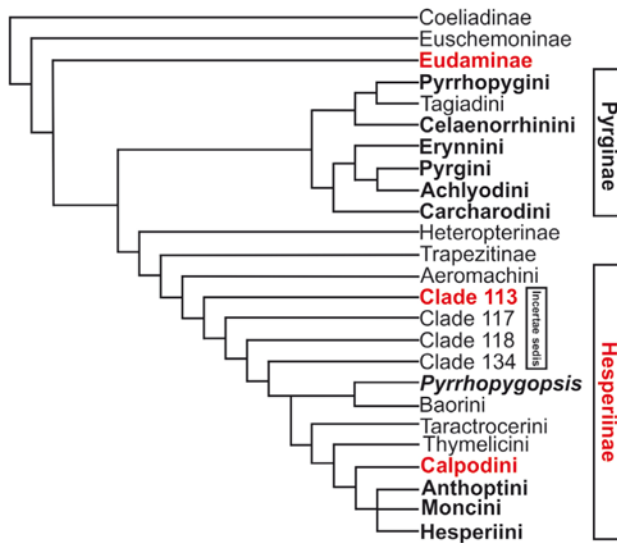


Figure 3. Simplified cladogram of the family Hesperidae (Warren *et al.*, 2009). Extremely long proboscides evolved at least three times independently within Neotropical Hesperidae in representatives of the subfamilies Eudaminae and two tribes of Hesperiinae. Note: Taxa printed in bold are represented in this study, taxa printed in red include species with extremely long proboscides that exceed 30 mm in length.

2013), where the galeae are straight and arranged parallel to each other. Since the developmental sheath contains the full length of the unfolded proboscis, this organ grows accordingly to accommodate the extreme length of the adult proboscis and may extend a full body length beyond the last abdominal segment (Figure 40A, p. 137: DeVries, 1997). Further elongation of this fragile and thin pupal organ might constrain proboscis length evolution in butterflies. By contrast, the pupae of long-proboscid hawk moths during metamorphosis develop a heavily sclerotized, hook-shaped external outgrowth that contains a loop of the developing proboscis that allows for the formation of a proboscis of much greater length (Patočka, 1993).

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CHAPTER IV

The role of long-proboscid Neotropical skipper butterflies (Lepidoptera: HesperIIDae) in plant-pollinator networks.



Eurybia unxia (left) and *Carystoides escalantei* (right) probing the flowers of *Calathea lutea* without releasing the trigger mechanism.

Bauder J. A. S., Warren A. D., Krenn H. W. (submitted) The role of long-proboscid Neotropical skipper butterflies (Lepidoptera: HesperIIDae) in plant-pollinator networks. *Arthropod-Plant Interactions*.

The ecological role of extremely long-proboscid Neotropical butterflies (Lepidoptera: Hesperiidae) in plant-pollinator networks.

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Summary

Extremely long proboscides of insect flower visitors have been regarded as an example of a coevolutionary arms race, assuming that these insects act as efficient pollinators for their nectar host plants. However, the effect of proboscis length on generalized or specialized flower use remains unclear and the efficiency of butterfly-pollination is ambiguous. Neotropical Hesperiidae feature a surprising variation of proboscis length, which makes them a suitable study system to elucidate the role of extremely long-proboscid insects in plant-pollinator networks. The results of this study show that skippers with longer proboscides visit plant species with deeper nectar spurs to take up food. Skippers equipped with extremely long proboscides neither include short-tubed flowers in their diet nor visit more plant species than those with shorter proboscides. Our observations indicate that extremely long-proboscid butterflies steal nectar from their preferred nectar host plants, *Calathea* sp., instead of contributing to pollination. Finally, we discuss the impact of nectar robbery by these butterflies on their nectar host plants and their legitimate pollinators, euglossine bees.

Introduction

Many scientists have pondered over the evolutionary processes that led to the development of particularly elongate proboscides in flower-visiting insects (Darwin 1862; Johnson 1997; Johnson and Anderson 2010; Muchhala and Thomson 2009; Nilsson 1988; Nilsson 1998; Pauw et al. 2009; Rodríguez-Gironés and Llandres 2008; Rodríguez-Gironés and Santamaría 2007; Wasserthal 1997; Wasserthal 1998; Whittall and Hodges 2007). The most widely accepted hypothesis for the evolution of extreme mouthpart lengths is that they coevolved with long nectar spurs of angiosperms. In this way, the plant partner secures its pollination and the insect partner gains exclusive access to plenty of nectar (Darwin 1862; Nilsson 1998).

Although the majority of butterflies feed on floral nectar (Krenn 2010), their role as effective pollinators remains doubtful in many examples (Courtney et al. 1982; Wiklund 1981; Wiklund et al. 1979). A mutualistic coevolutionary relationship between particular butterfly species and their preferred nectar host plants has only been demonstrated in two cases (Gilbert 1972; Gilbert 1975; Grant and Grant 1965; Levin and Berube 1972). In fact, some authors regard butterflies as opportunistic flower visitors that use resources as they become available during the season (Shreeve 1992; Stefanescu and Traveset 2009; Tudor et al. 2004). The effect of proboscis length on generalized or specialized flower use remains contradictory and to date has been investigated mainly in temperate butterfly communities (Corbet 2000; Porter et al. 1992; Stefanescu and Traveset 2009; Tiple et al. 2009; Tudor et al. 2004).

Here, we investigated Neotropical skipper butterflies (Hesperiidae) with a wide range of proboscis lengths to test the hypothesis that insects equipped with extremely long mouthparts specialize on flowers with deep nectar spurs. Alternatively, the hypothesis could be forwarded that extremely long-proboscid butterflies use a wide variety of flowers regardless of nectar spur length in an opportunistic way: the longer the proboscis, the more flowers which can be visited. Finally, we raise the question whether extremely long-proboscid butterflies act as pollinators of their nectar plants and thus constitute another example of a coevolved pollination mutualism, as in extremely long-proboscid hawk moths and orchids.

Material and methods

Flower use and species sampling

We chose four flowering plant species (Verbenaceae: *Lantana camara*, *Stachytarpheta frantzii*; Marantaceae: *Calathea crotalifera*, *Calathea lutea*) growing in the garden and surroundings of the Tropical Station La Gamba (Costa Rica, Puntarenas, Piedras Blancas National Park, 8°45'N, 83°10'W; 81 m a.s.l.) for recording skipper visitation (Figure 1 A-B) during an overall observation time of approximately 240 hours (September – October 2012, January – February 2013). Skippers were collected with a hand net after they landed on flowers and subsequently uncoiled the proboscis. Specimens were stored in 70 % ethanol. Classification of taxa follows the recent phylogeny of Hesperiidae (Warren et al. 2009).

Measuring proboscis length

Proboscis length of ethanol-preserved specimens was measured. The proboscis of each specimen was separated from the head at its base, uncoiled and fixed on a foam mat using insect pins. Micrographs of the proboscis were taken using a Nikon SMZ 1500 stereomicroscope (Nikon, Tokyo, Japan) equipped with an Optocam-I digital camera (Nikon, Tokyo, Japan). Micrographs were imported to ImageJ (U.S. National Institutes of Health, Bethesda, USA) and proboscis length was measured with the aid of the segmented line tool.

Floral biology and corolla length

The small flowers of *L. camara* (Verbenaceae) are mostly yellow or orange in colour, changing to red or scarlet with age, and form a slightly curved corolla tube (Figure 2). They are arranged in hemispheric inflorescences, up to 3 cm wide, that can be used by butterflies as a landing platform (Woodson et al. 1973).

The flowers of *S. frantzii* are larger than those of *L. camara* and are coloured purple (Figure 2). The corolla is fused to a slender cylindrical tube which is semi-immersed in the rachis of spikes. The flowers are arranged in terminal inflorescences (Woodson et al. 1973).

The yellow, tubed flowers of *C. crotalifera* and *C. lutea* (Marantaceae, Figure 2) feature a unique pollination mechanism, which can be easily observed by the naked eye (see Bauder et al. 2011, p. 124, Figure 1B-C). Pollination occurs only when the insect touches a trigger-like appendage of the hooded staminode, which holds the style under

tension. The style then springs forward, scrapes off any pollen away from the insect and simultaneously places its pollen onto the flower visitor (Pischtschan and Claßen-Bockhoff 2008). Since the style movement is easily visible and flowers can be inspected after visitation, we were able to determine whether skippers released the trigger and thus potentially act as efficient pollinators.

To measure corolla lengths, flowers were collected from different plant individuals at several locations in the garden of the Tropical Station. Freshly picked flowers were used for estimating corolla length of *L. camara* and *S. frantzii*. Prior to measuring, the slightly curved corolla of each flower was straightened with the aid of a dissection needle. The length of the corolla was measured from the petals to the beginning of the ovaries using a digital caliper. Measurements of ethanol-preserved flowers of *C. crotalifera* and *C. lutea* were performed by A. Ruppel in the course of a diploma thesis (Ruppel 2013). Flowers were photographed with a Nikon D3100 SLR digital camera (Nikon, Tokyo, Japan) equipped with an AF-S DX 18-55 VR objective (Nikon, Tokyo, Japan) and corolla length was measured from the petals to the ovaries (Ruppel 2013) with the software UTHSCSA ImageTool 3.0 (University of Texas, San Antonio, Texas, U.S.A.). All plant species differed significantly in corolla length ($X^2_{(3)} = 121.4$, $p < 0.0001$; Post-Hoc Tests: $p < 0.05$; Bonferroni-corrected). *C. lutea* had the deepest nectar spurs measuring 31.4 ± 2.5 mm ($N = 95$), those of *C. crotalifera* were 25.0 ± 1.4 mm deep ($N = 43$). Nectar spurs of *S. frantzii* measured 16.1 ± 1.3 mm ($N = 11$). *L. camara* had the shortest nectar spurs (10.4 ± 0.9 mm, $N = 11$).

Statistics

Statistics were calculated with the statistical package IBM SPSS Statistics 21.0 (IBM Corporation, New York, USA). Analyses were done with untransformed data using a Kruskal-Wallis ANOVA. For Post-hoc tests Mann-Whitney U-Tests (Bonferroni corrected) were used. Graphical illustrations were done with SigmaPlot 12.5 (Systat Software Incorporated, San Jose, California, U.S.A.), CorelDRAW X6 (Corel Corporation, Munich, Germany) and Adobe Photoshop CS4 Extended 11.0.2 (Adobe Systems Incorporated, San Jose, California, USA).

Results

A total of 148 individuals of skippers belonging to 45 species and 30 genera were found to visit the flowers of *L. camara*, *S. frantzii*, *C. crotalifera* and *C. lutea* during the period of observation (Table 1). Whereas both *L. camara* and *S. frantzii* received frequent visits from other butterflies, e. g. Pieridae, Nymphalidae, Papilionidae and Lycaenidae, the two species of *Calathea* were visited exclusively by Hesperidae and butterflies of the genus *Eurybia* (Riodiniae), confirming the results of a previous study (Bauder et al. 2011).

Proboscis lengths of the skippers differed significantly according to the nectar host plants utilized ($X^2_{(3)} = 96.8$, $p < 0.0001$; Figure 3 A). Visitors of *L. camara*, the flower with the shortest corolla length, had significantly shorter proboscides than the visitors of the other three investigated nectar host plant species (Post-Hoc Tests: $p < 0.05$; Bonferroni-corrected). Visitors of *S. frantzii* were also significantly different from other flower visitors concerning their proboscis length, and had longer proboscides than the visitors of *L. camara* (Post-Hoc Tests: $p < 0.05$; Bonferroni-corrected). Furthermore, the flower visitors of two *Calathea* species had longer proboscides than visitors of *L. camara* and *S. frantzii* (Post-Hoc Tests: $p < 0.05$; Bonferroni-corrected). However, proboscis lengths of skippers that visited *C. crotalifera* and *C. lutea* were similar (Post-Hoc Test: $p > 0.05$; Bonferroni-corrected), although the corolla depths of these two plant species differed significantly from each other. Generally, skipper butterflies with extremely long proboscides, i.e. longer than 30 mm, visited flowers with deep nectar spurs and skippers with shorter proboscides used flowers with shorter nectar spurs. Our data show that skippers with extremely long proboscides refrained from visiting short-tubed flowers, since the number of interactions with flowers of different nectar host plant species did not increase with increasing proboscis length (Figure 3B). Instead, the interaction pattern is compartmentalized, indicating that skipper flower visitors with shorter proboscides are separated from skippers with longer proboscides with respect to their preferred flowers, each using different set of flowering plants as their source of nectar.

Discussion

Food resource partitioning is held to be a driving force for the coexistence of animals (Hespenheide 1973; Inouye 1980; Ranta and Lundberg 1980; Schoener 1974) and is often estimated using morphological traits, such as size differences between animals or differences in mouthparts in relation to the size of food particles. With respect to nectar-drinking flower visitors, such as butterflies and moths, this refers to differences in mouthpart length. According to this hypothesis of competition avoidance, extremely long-proboscid skippers should specialize in visiting flowers that correspond to their mouthpart lengths and avoid short corolla flowers. Alternatively, since butterflies are regarded as generalist flower visitors (Corbet 2000; Nilsson 1988; Nilsson et al. 1985), the number of plant species available to them could be a function of their proboscis length (Agosta and Janzen 2005). Thus species with long proboscides could potentially utilize short flowers in addition to long flowers, and it would be expected that the number of flowering species visited would be greater than with short-proboscid butterflies.

Our data support the hypothesis of competition avoidance, since the extremely long-proboscid skippers generally did not visit flowers with short nectar spurs, such as *L. camara* or *S. frantzii*, but preferred the deep corolla flowers of the two *Calathea* species. Furthermore, extremely long-proboscid skippers did not discriminate between the flowers of *C. crotalifera* and *C. lutea*, probably because these flowers look alike regarding their floral morphology and colour and grow in the same habitats (Ruppel 2013).

Further, our observations showed that the deep corollae of *Calathea* flowers received visits from fewer skipper species (and no visits from other butterflies except for extremely long-proboscid butterflies from the genus *Eurybia*) than the short corollae of *L. camara* and *S. frantzii*. Therefore, extremely long-proboscid Lepidoptera might be released from the level of competition experienced by short-proboscid Lepidoptera (Agosta and Janzen 2005) and may choose deep flowers, which cannot be used by short-proboscid butterflies. The same was confirmed earlier for British butterfly assemblages (Porter et al. 1992; Corbet 2000). Extremely long-proboscid skippers, such as the calpodines (Hesperiinae), are known to live in shady, forested habitats (Warren et al. 2009). *Calathea* plants are normally found in deep shade of tree trunks (Ruppel, unpublished), and thus probably have similar habitat requirements as extremely long-proboscid skippers. Because of this, we hypothesize that extremely

long-proboscid skippers make the best of their situation by using nectar plants flowering in their proximity and harvesting plenty of nectar out of deep tubes.

Many studies have demonstrated a close match between plant and pollinator morphology, such as correlations between proboscis length of pollinators and corolla depth of flowers on the scale of species-specific interactions, that have served as supposed examples of coevolution (Alexandersson and Johnson 2002; Grant and Grant 1965; Grant and Grant 1983; Harder 1985; Johnson and Steiner 1997; Nilsson 1988; Nilsson 1998; Nilsson et al. 1985). Although our results showed that the proboscis lengths of skipper butterflies differed significantly between the visited flower species, we did not observe a close match between extremely long proboscides and deep tubes: Extremely long-proboscid skipper butterflies that used *C. crotalifera* and *C. lutea* as nectar source, had a mean proboscis length of 43.4 mm, exceeding the corolla length of *C. crotalifera* by 18.4 mm and *C. lutea* by 12 mm. Similarly, the proboscides of *E. lycisca* butterflies in Costa Rica that fed on the nectar of *C. crotalifera* and *C. lutea* plants measured 36.5 mm on average (Bauder et al. 2011), exceeding the corolla depth of *C. crotalifera* and *C. lutea* by 11.5 mm or 5.1 mm, respectively. Since the butterflies' proboscides are longer than the floral tube, they are not forced to plunge their head into the corolla entrance and successful pollination is very unlikely. Apart from that, previous analyses on the flower-visiting behaviour of extremely long-proboscid butterflies (Bauder et al. 2011) as well as observations in the course of this study demonstrate that neither skipper nor metalmark butterflies were able to release the trigger mechanism of *Calathea*-flowers in contrast to their legitimate pollinators, euglossine bees (Borrell 2005; Janzen 1971). Euglossine bees feature long proboscides ranging between 13.8 and 38.9 mm length (Borrell 2005). However, these bees are generalistic flower visitors, because the number of visited plant species increases with proboscis length (Borrell 2005). These results raise the question, why these long-tubed flowers have specialized on generalistic bees (Borrell 2005) and how nectar-thieving butterflies fit into this network. Schemske (1981) and Borrell (2005) hypothesized that many steady-state understorey plants, such as *Calathea* or *Costus*, are unable to support the energetic needs of an entire pollinator population because of their low density, the small size of some species and few flowers blooming simultaneously (Janzen 1971). They argued that increasing nectar production would be energetically costly, but by morphologically excluding short-proboscid insects as potential nectar consumers, these plants can still

provide plenty of nectar to individual euglossine bees, which learn to include these plants in the same nectar traplines daily (Borrell 2005; Janzen 1971). If that is true, the long nectar spurs of *Calathea* plants could have evolved not via directional selection exerted by a specialized pollinator (Darwin 1862), but by competition among sympatric flowers for inclusion on a pollinator's foraging route (Garrison and Gass 1999). Nectar thieving butterflies would ransack *Calathea* nectar stores, leaving less nectar for pollinating Euglossini. The average nectar amount that an extremely long-proboscid skipper butterfly could take up during a flower visit is roughly estimated at 7.1 μl (Bauder, data will be published elsewhere; amount of ingested nectar was calculated by multiplying the average nectar intake rate of skippers that visited *Calathea*-flowers with the average suction time on *Calathea crotalifera* flowers). Given that the average nectar amount of a flower belonging to the genus *Calathea* measures about 14.4 μl (Ruppel 2013), a single skipper butterfly could empty half of a flower's nectar reserves in a single visit. If extremely long-proboscid butterflies were abundant visitors of *Calathea* and had a serious impact on the nectar availability for the legitimate pollinators of *Calathea* plants, pollinator sharing (Macior 1971) would be adaptive for two sympatric *Calathea* species, such as *C. lutea* and *C. crotalifera*. Plant species with restricted distributions and low densities increase the effective density of food rewards to pollinators and pollination probabilities by converging in floral characters, termed floral mimicry (Macior 1971). Since the flowers of *C. lutea* and *C. crotalifera* are both coloured yellow and share similar morphologies, further studies focussing on their local distribution, flowering phenology, nectar secretion patterns and interspecific hybridization (Schemske 1981) could elucidate whether these similarities arise from pollinator sharing or phylogenetic relationship. The pollinator sharing - hypothesis is especially tantalizing keeping in mind that these plants not only suffer from nectar robbery by extremely long-proboscid skipper and metalmark butterflies, but also from flower predation exerted by the larvae of *Eurybia* butterflies, which feed on *Calathea* flowers and not on foliage (DeVries 1997, Horvitz et al. 1987).

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Table 1. Proboscis length and flowers visited of 148 specimens representing 45 species and 30 genera of Hesperidae. *Note:* Mean values $\pm$ standard deviation of proboscis lengths are given, whenever more than 1 individual per species was measured. When two plant species were visited, the number of observed flower visits is given in parentheses.

Species of Hesperidae	N	Proboscis length [mm]	Flowers visited
Eudaminae			
<i>Astraptes alardus latia</i> EVANS, 1952	1	23.5	<i>C. lutea</i>
<i>Astraptes anaphus anetta</i> EVANS, 1952	1	19.5	<i>S. frantzii</i>
<i>Autochton longipennis</i> (PLÖTZ, 1882)	3	17.3 $\pm$ 1.2	<i>S. frantzii</i>
<i>Autochton zarex</i> (HÜBNER, 1818)	2	16.3 $\pm$ 1.5	<i>S. frantzii</i>
<i>Bungalotis quadratum quadratum</i> (SEPP, [1845])	1	39.4	<i>C. lutea</i>
<i>Cogia calchas</i> (HERRICH-SCHÄFFER, 1869)	3	12.3 $\pm$ 0.6	<i>L. camara</i> (N = 2) <i>S. frantzii</i> (N = 1)
<i>Spathilepia clonius</i> (CRAMER, 1775)	2	16.8 $\pm$ 0.2	<i>S. frantzii</i>
<i>Typhedanus undulatus</i> (HEWITSON, 1867)	1	12.4	<i>L. camara</i>
<i>Urbanus procne</i> (PLÖTZ, 1881)	3	15.9 $\pm$ 0.1	<i>S. frantzii</i> (N = 2) <i>L. camara</i> (N = 1)
<i>Urbanus simplicius</i> (STOLL, 1790)	8	16.5 $\pm$ 0.7	<i>L. camara</i> (N = 6) <i>S. frantzii</i> (N = 2)
<i>Urbanus tanna</i> EVANS, 1952	7	16.7 $\pm$ 0.3	<i>S. frantzii</i> (N = 6) <i>L. camara</i> (N = 1)
<i>Urbanus teleus</i> (HÜBNER, 1821)	4	16.3 $\pm$ 0.6	<i>L. camara</i> (N = 3) <i>S. frantzii</i> (N = 1)
Pyrginae			
Pyrrhopygini			
<i>Mysoria ambigua</i> (MABILLE & BOULLET, 1908)	4	15.3 $\pm$ 0.6	<i>S. frantzii</i> (N = 3) <i>L. camara</i> (N = 1)
Celaenorrhini			
<i>Celaenorrhinus darius</i> EVANS, 1952	1	29.8	<i>S. frantzii</i>
Carcharodini			
<i>Nisoniades godma</i> EVANS, 1953	1	10.7	<i>L. camara</i>

Table 1 continued.

Species of HesperIIDae	N	Proboscis length [mm]	Flowers visited
HesperIIDae			
Clade 113			
<i>Lycas godart boisduvalii</i> (EHRMANN, 1909)	1	45.7	<i>C. lutea</i>
<i>Perichares adela</i> (HEWITSON, 1867)	8	44.5 ± 4.9	<i>C. lutea</i> (N = 7) <i>C. crotalifera</i> (N = 1)
<i>Perichares lotus</i> (A. BUTLER, 1870)	1	48.3	<i>C. lutea</i>
<i>Pyrrhopygopsis socrates orasus</i> (H. DRUCE, 1876)	1	34.4	<i>C. lutea</i>
Calpodini			
<i>Aroma henricus henricus</i> (STAUDINGER, 1876)	2	30.6 ± 2.0	<i>C. crotalifera</i>
<i>Calpodes ethlius</i> (STOLL, 1782)	4	42.2 ± 1.5	<i>C. lutea</i> (N = 3) <i>C. crotalifera</i> (N = 1)
<i>Carystoides escalantei</i> H. FREEMAN, 1969	5	33.2 ± 1.5	<i>C. lutea</i>
<i>Carystoides hondura</i> EVANS, 1955	2	28.9 ± 0.3	<i>C. lutea</i> (N = 1) <i>C. crotalifera</i> (N = 1) <i>C. lutea</i> (N = 8)
<i>Damas clavus</i> (HERRICH-SCHÄFFER, 1869)	13	49.5 ± 2.4	<i>C. crotalifera</i> (N = 4) <i>L. camara</i> (N = 1)
<i>Damas immaculata</i> NICOLAY, 1973	1	52.7	<i>S. frantzii</i>
<i>Saliana esperi esperi</i> EVANS, 1955	2	35.9 ± 2.2	<i>C. lutea</i> (N = 1) <i>C. crotalifera</i> (N = 1)
<i>Saliana longirostris</i> (SEPP, [1840])	1	42.7	<i>C. lutea</i>
<i>Saliana salius</i> (CRAMER, 1775)	3	47.2 ± 5.7	<i>C. lutea</i>
<i>Saliana severus</i> (MABILLE, 1895)	1	51.8	<i>C. crotalifera</i>
<i>Saliana triangularis</i> (KAYE, 1914)	6	41.5 ± 2.7	<i>C. crotalifera</i> (N = 5) <i>C. lutea</i> (N = 1)
<i>Talides hispa</i> EVANS, 1955	1	45.5	<i>C. lutea</i>
<i>Thracides phidon</i> (CRAMER, 1779)	1	42.0	<i>C. lutea</i>
<i>Tromba xanthura</i> (GODMAN, 1901)	1	48.2	<i>S. frantzii</i>
Anthoptini			
<i>Corticea lysias lysias</i> (PLÖTZ, 1883)	1	14.1	<i>L. camara</i>

Table 1 continued.

Species of HesperIIDae	N	Proboscis length [mm]	Flowers visited
Moncini			
<i>Arita arita</i> (SCHAUS, 1902)	1	27.4	<i>C. crotalifera</i>
<i>Cymaenes alumna</i> (A. BUTLER, 1877)	2	16.5 ± 1.5	<i>L. camara</i>
<i>Lerema ancillaris</i> (A. BUTLER, 1877)	1	20.5	<i>S. frantzii</i>
<i>Morys geisa</i> (MÖSCHLER, 1879)	8	20.1 ± 1.9	<i>S. frantzii</i> (N = 7) <i>L. camara</i> (N = 1)
<i>Morys miccythus</i> (GODMAN, 1900)	2	19.6 ± 0.8	<i>S. frantzii</i> (N = 1) <i>L. camara</i> (N = 1)
<i>Papias phaeomelas</i> (HÜBNER, [1831])	10	17.3 ± 1.4	<i>S. frantzii</i>
<i>Papias phainis</i> GODMAN, 1900	1	16.2	<i>S. frantzii</i>
<i>Papias subcostulata</i> (HERRICH-SCHÄFFER, 1870)	12	25.5 ± 1.4	<i>S. frantzii</i> (N = 11) <i>C. lutea</i> (N = 1)
<i>Vehilius stictomenes illudens</i> (MABILLE, 1891)	2	13.0 ± 0.01	<i>L. camara</i>
Hesperiiini			
<i>Pompeius pompeius</i> (LATREILLE, [1824])	5	15.1 ± 0.3	<i>S. frantzii</i> (N = 4) <i>L. camara</i> (N = 1)
<i>Quinta cannae</i> (HERRICH-SCHÄFFER, 1869)	7	21.7 ± 1.1	<i>S. frantzii</i>

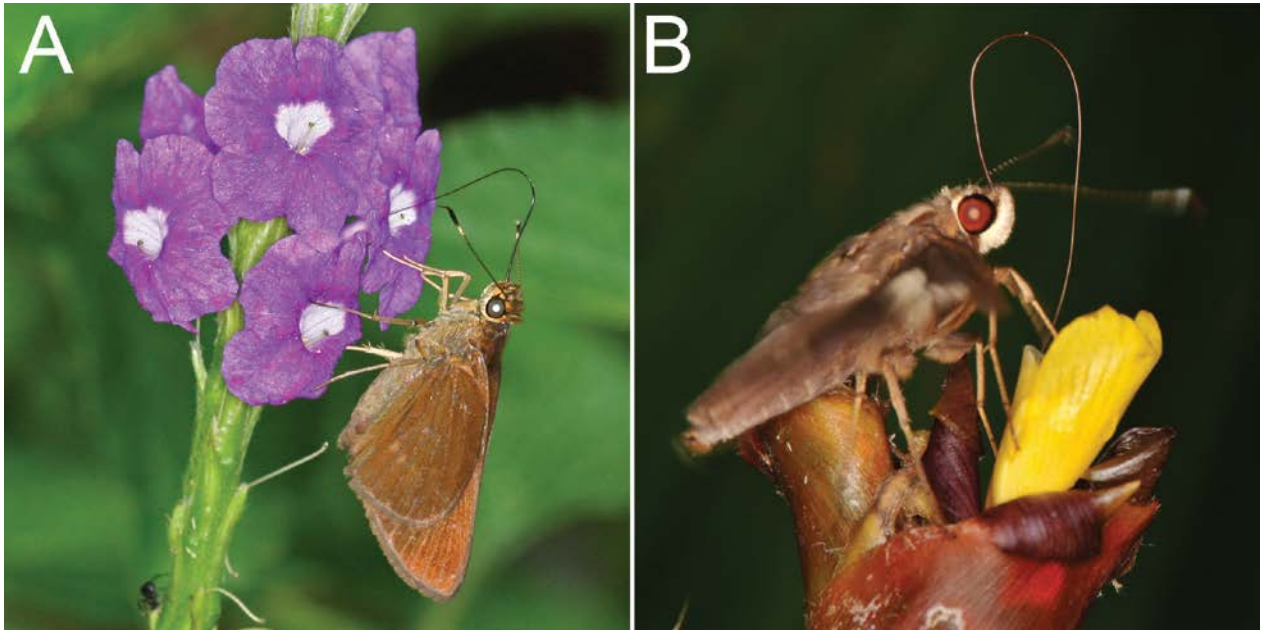


Figure 1. Neotropical skippers with different proboscis length use flowers with matching nectar spur length. **A.** *Papias cf. phainis* (GODMAN, 1900) with a medium-sized proboscis visits flowers of *S. frantzii* (Verbenaceae). Photo courtesy of M. Hepner. **B.** Extremely long-proboscid skipper *Carystoides escalantei* H. FREEMAN, 1969 drinking nectar from a long-spurred flower of *C. lutea* (Marantaceae). Photo courtesy of P. Figueroa.



Figure 2. Flowers of *Lantana camara* (Verbenaceae), *Stachytarpheta frantzii* (Verbenaceae), *Calathea crotalifera* (Marantaceae) and *Calathea lutea* (Marantaceae) are shown from left to right with increasing corolla length. Photo courtesy of P. Figueroa.

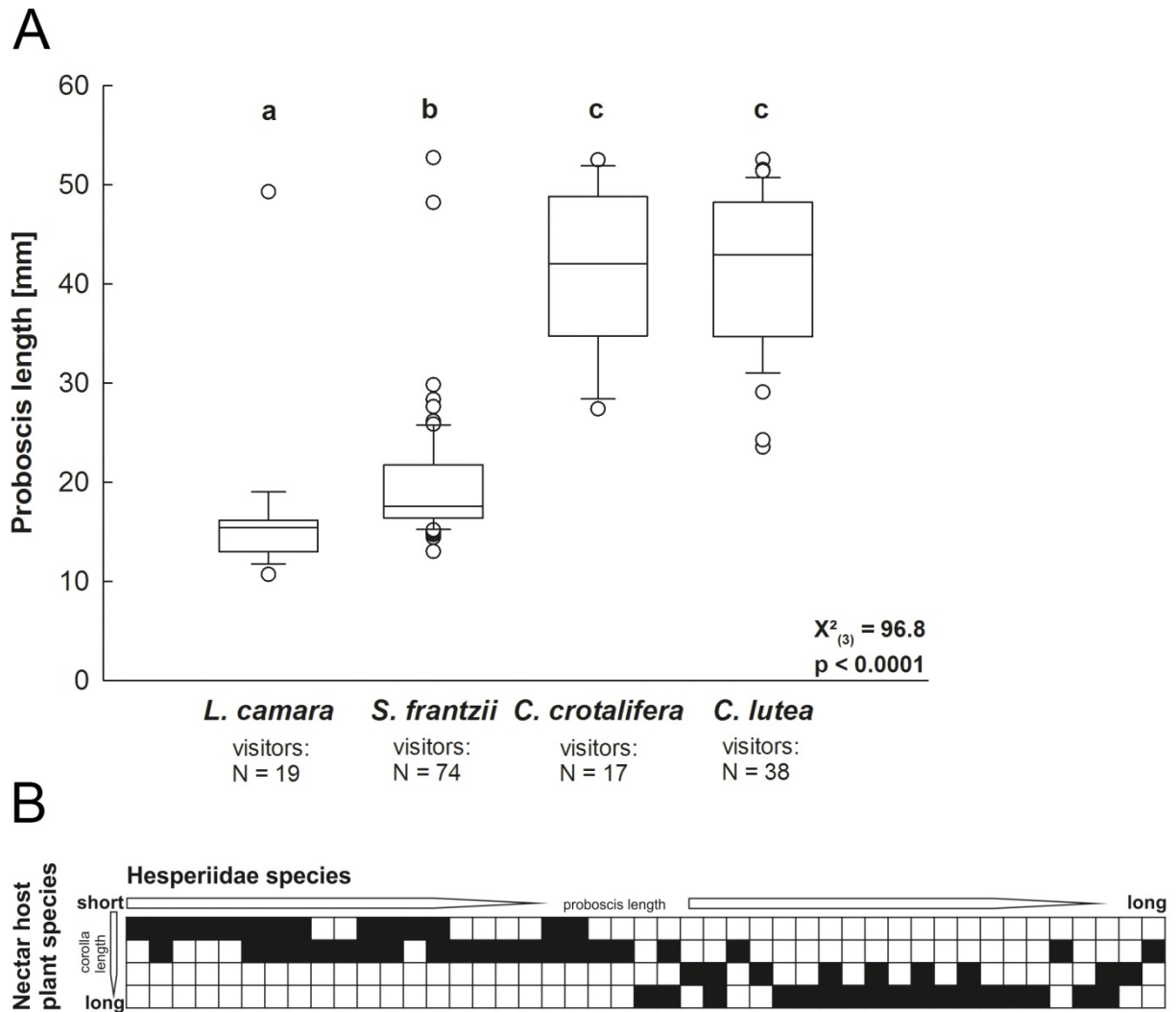


Figure 3. A. Flower use by Hesperidae differed significantly according to proboscis length ($X^2_{(3)} = 96.8$, $p < 0.0001$). Hesperidae having long proboscides visited flowers with deep nectar spurs. Although the corolla length of all nectar host plant species differed significantly, proboscis length of Hesperidae that visited *C. crotalifera* and *C. lutea* did not differ significantly (Post-Hoc Tests: Mann-Whitney U-Tests, different letters above boxplots represent significant differences at $p < 0.05$; Bonferroni-corrected). **B.** Binary interaction matrix (presence or absence of flower visits) of skipper species (columns) and plant species (rows) ranked according to mean proboscis length or mean corolla length, respectively. Increasing proboscis length did not increase the number of visited plant species. Short- and long-proboscid skippers each used a different set of flowering plants.

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CHAPTER V

Functional constraints on the evolution of long butterfly proboscides: Lessons from Neotropical skippers (Lepidoptera: Hesperiiidae).



Carystoides escalantei probing a flower of *Calathea lutea*.

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Functional constraints on the evolution of long butterfly proboscides: Lessons from Neotropical skippers (Lepidoptera: Hesperiiidae)

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Running title: Functional constraints on long proboscides

Key words: suction feeding, morphology, mouthparts, insects, body size, nectar intake rate, flower handling, Costa Rica, wing loading, foraging

Abstract

Extremely long proboscides are rare among butterflies outside of the HesperIIDae, yet representatives of several genera of skipper butterflies possess proboscides longer than 50 mm. Although extremely elongated mouthparts can be regarded as advantageous adaptations to gain access to nectar in deep-tubed flowers, the scarcity of long-proboscid butterflies is a phenomenon that has not been adequately accounted for. So far, the scarceness was explained by functional costs arising from increased flower handling times caused by decelerated nectar intake rates. However, insects can compensate for the negative influence of a long proboscis through changes in the morphological configuration of the feeding apparatus. Here, we measured nectar intake rates in 34 species representing 21 HesperIIDae genera from a Costa Rican lowland rainforest area to explore the impact of proboscis length, cross-sectional area of the food canal and body size on intake rate. Long-proboscid skippers did not suffer from reduced intake rates due to their large body size and enlarged food canals. In addition, video analyses of the flower-visiting behavior revealed that suction times increased with proboscis length, suggesting that long-proboscid skippers drink a larger amount of nectar from deep-tubed flowers. Despite these advantages, we showed that functional costs of exaggerated mouthparts exist in terms of longer manipulation times per flower. Finally, we provided evidence for the hypothesis that the convergent evolution of extremely long proboscides within skippers and hawk moths resulted from high metabolic requirements during flight.

Introduction

Nectar is commonly regarded as the world's most ubiquitous food source and therefore favored by many birds, bats and insects (Nicolson, 2007). These taxa have independently evolved various physiological, morphological and behavioral specializations as adaptations for nectar uptake (Pellmyr, 2002; Muchhala & Thomson, 2009; Johnson & Anderson, 2010; Karolyi *et al.*, 2012; Karolyi *et al.*, 2013). Most conspicuous are elongations of the mouthparts which are often shaped as a proboscis in insects (Krenn *et al.*, 2005). Euglossine bees, certain tabanid and nemestrinid flies and some hawk moths have evolved extremely long mouthparts that exceed twice the body length to gain access to long-tubed flowers (Amsel, 1938; Borrell, 2005; Borrell & Krenn, 2006; Pauw *et al.*, 2009; Karolyi *et al.*, 2012; Karolyi *et al.*, 2014). However, such extremely long mouthparts are rare among butterflies. The proboscis of most European species is medium-sized and measures about two thirds of the body length (Paulus & Krenn, 1996) and averages about 80 % in Neotropical butterflies (Kunte, 2007). However, some remarkably long proboscides have been recorded for Neotropical *Eurybia* butterflies (Riodinidae) and for some Neotropical skipper butterflies (Hesperiidae). In some representatives of these families the proboscis may exceed twice the body length (Kunte, 2007; Bauder *et al.*, 2011; Bauder *et al.*, 2013).

The scarcity of long-proboscid butterflies seems peculiar since they could drink nectar from both short- and long-tubed flowers (Agosta & Janzen, 2005), possibly taking a competitive advantage over short-proboscid butterflies. However, disadvantages of having elongated mouthparts can result in longer flower handling times, as has been recorded for hummingbirds, bumblebees, butterflies and some flies (Hainsworth, 1973; Hainsworth & Wolf, 1976; Inouye, 1980; Harder, 1983; Kunte, 2007; Bauder *et al.*, 2011; Karolyi *et al.*, 2013). Kunte (2007) observed that the flower handling times of butterflies with longer proboscides were significantly longer than the flower handling times of butterflies with normal sized proboscides. Therefore, he regarded the reduced foraging efficiency, i.e., harvesting less nectar per time, experienced by long-proboscid butterflies as a functional constraint for evolving extraordinarily long proboscides (Kunte, 2007).

However, flower handling time depends on the time required to enter and leave a flower and the actual time needed to take up nectar (Karolyi *et al.*, 2013). In butterflies, manipulation time depends on uncoiling the proboscis spiral and finding an entrance into the flower as well as withdrawing and recoiling the proboscis. The suction time is

determined by the nectar intake rate, i. e., nectar volume flow per time unit. Therefore, increased flower handling times of long-proboscid insects could result from problems with flower manipulation, deceleration of nectar intake, or a combination of both.

The energy intake rate during feeding influences foraging efficiency (Wolf *et al.*, 1972; Heinrich, 1975; Whitham, 1977; May, 1988) and reproductive fitness (Hainsworth *et al.*, 1991). Rapid feeding should therefore be favored by natural selection (Emlen, 1966; Schoener, 1971; Pyke *et al.*, 1977). Nectar feeding through a tubular proboscis is subject to physical laws of fluid dynamics, and both the morphological configuration of the feeding apparatus and nectar viscosity modify the rate of nectar intake (Daniel *et al.*, 1989; Kim *et al.*, 2011; Lee *et al.*, 2014). Biophysical models describe factors influencing the speed of fluid feeding and therefore help to understand the constraints regarding the evolution of extremely long proboscides (Kingsolver & Daniel, 1979; Kingsolver & Daniel, 1995; Lee *et al.*, 2014). According to the law of Hagen-Poiseuille, the nectar intake rate of butterflies should increase linearly with increasing pressure difference produced by a suction pump, and increase with the radius of the food canal to the exponent four. By contrast, it is expected to decline linearly with escalating proboscis length (Kingsolver & Daniel, 1979; Kingsolver & Daniel, 1995). Therefore, insects must compensate for the negative influence of a long proboscis through changes in the radius of the food canal or the size of the suction pump, or otherwise bear this cost through a decreased intake rate (Borrell, 2007). However, exact measurements of nectar intake rates combined with quantitative morphological data over a variety of butterfly species are lacking, although there are some studies on a few butterfly species and other animals such as euglossine bees, hummingbirds and honeyeaters (Hainsworth, 1973; Kingsolver & Daniel, 1983; May, 1985; Mitchell & Paton, 1990; Molleman *et al.*, 2005a; Borrell, 2007).

Here, we present an integrative approach combining data obtained from behavioural observations and morphological analyses of Neotropical skipper butterflies which vary widely with regard to proboscis length. Video recordings of skippers foraging in the wild and during standardized feeding experiments help explain whether prolonged flower handling times of long-proboscid butterflies result from decelerated nectar intake rates, prolonged flower manipulation times or both. Alternatively, if both of these behavioural aspects were independent of proboscis length, prolonged flower handling times of long-proboscid butterflies would simply result from taking larger amounts of nectar than short-proboscid butterflies. Furthermore, we aimed to analyse the functional implications

of interspecific morphological variation to improve our knowledge on the evolution of insect pollinator communities. Therefore, we differentiated the impact of varying proboscis length, body size and cross-sectional area of the food canal on nectar intake rate across species. Based on these results, we suggest an evolutionary scenario that explains why Hesperidae, especially, evolved a particularly long proboscis several times independently.

Material & Methods

Species sampling

Sampling of Hesperidae was carried out in the garden and surroundings of the Tropical Station La Gamba (Costa Rica: Puntarenas, Piedras Blancas National Park, 8°45'N, 83°10'W; 81 m a.s.l.) in September-October 2012 and January-February 2013. Morphometric measurements and feeding experiments were performed with 113 specimens representing 34 species of Hesperidae from 21 genera. Skippers were collected with a hand net and stored in 70 % ethanol after the feeding trials. Classification of taxa follows the phylogeny of Hesperidae (Warren *et al.*, 2009).

Measurement of body features

Proboscis length and cross-sectional area of the food canal of each individual was measured to estimate their impact on nectar intake rate. However, measuring the exact size of suction pumps requires time-costly morphological reconstructions (Bauder *et al.*, 2013; Karolyi *et al.*, 2013) and is not manageable for a large sample size. Therefore, body size was measured which scales with size of the suction pumps (Karolyi *et al.*, 2013).

Body length was measured by pinning the body of each ethanol-preserved specimen in a lateral position to a foam mat. After taking a micrograph of the body, the proboscis of each specimen was separated from the head at its base, uncoiled and fixed on a foam mat using insect pins. Micrographs of the body and the proboscis were taken using a Nikon SMZ 1500 stereomicroscope (Nikon, Tokyo, Japan) equipped with an Optocam-I digital camera (Nikon, Tokyo, Japan). Micrographs were imported to ImageJ (U.S. National Institutes of Health, Bethesda USA) and body length as well as proboscis length was measured with the aid of the segmented line tool.

The proboscis was cut off at its base and the galeae were separated from each other. Subsequently, one galea was mounted onto a microscope slide with the food canal facing upwards, embedded in glycerol and covered with a coverslip. The height of the food canal was measured using a Nikon Eclipse E800 light microscope (Nikon, Tokyo, Japan) equipped with a Nikon Fi2-U3 digital camera (Nikon, Tokyo, Japan) and the NIS Elements D software (Nikon, Tokyo, Japan). The width of the food canal was calculated as the distance in μm between two focal planes situated on the lateral wall of the food canal and on the cuticular spines of the dorsal linkage. We measured the height and

width of the food canal in two proboscis regions per galea, located at 10 % (proximal) and 80 % (distal) of the total proboscis length. We estimated the cross sectional area of the food canal of a proboscis in approximation to an ellipse and calculated the mean cross sectional area of the proximal and distal food canal for each proboscis.

Butterfly feeding experiments

Feeding trials were conducted in an outdoor cage (3 x 2 x 2 m) in the Tropical Research Station, La Gamba, Costa Rica, using skippers that had been caught with a hand net just as they were to start taking nectar from flowers, i.e., after the proboscis uncoiled. In this way, we ensured that the captured butterflies were hungry and ready to take food which was obligatory for the subsequent feeding trials. Butterflies were stored in a cage until the end of the sampling session which lasted between two and four hours per plant. Feeding experiments were carried out at average ambient air temperatures ranging from 26-30 °C. A 40 % sugar solution containing sucrose, glucose and fructose, which was prepared in advance and kept refrigerated, was used to imitate the natural nectar of *L. camara* flowers (Alm *et al.*, 1990), which are commonly used as a food source by tropical skipper butterflies. Before each feeding trial, the sugar solution was placed under test conditions for half an hour to warm up to ambient air temperature. Each butterfly was immobilized by pinching its wings closed using a pair of tweezers and placed on a feeding platform beside a glass vial (diameter = 3.64 mm) filled with sugar solution (Figure 1 A). The proboscis was uncoiled manually with a dissection needle and put into the sugar solution. As soon as the proboscis was inserted into the fluid, the butterfly started to feed. The whole feeding session was recorded with a Sony HDR-XR550VE Handycam (Sony Corporation, Tokyo, Japan). Prior to each feeding session, artificial nectar was renewed to avoid an increase in concentration due to evaporation. Each butterfly was tested once and was subsequently fixed in 70 % ethanol.

Assessment of nectar intake rate

We estimated the ingested volume of sugar solution using images taken from the start and the end of a continuous video-recorded feeding trial using the software PMB 5.0.02.11130 (Sony Corporation, Tokyo, Japan). Images were imported to Adobe Photoshop CS4 Extended 11.0.2 (Adobe Systems Incorporated, San Jose, California, U.S.A.), converted to semi-transparency and overlaid. In this way, we measured the difference in fluid level with the ruler tool. We estimated the ingested volume in

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approximation to a cylinder (Figure 1 B). Division of the ingested volume of sugar solution by the elapsed time of the non-stop feeding session gave us the rate of volume intake (nl/sec).

Measurement of flower handling time

Skippers were caught from a flower shortly before they would start taking nectar. They were then set free in an outdoor cage equipped with a freshly cut and watered inflorescence of their preferred nectar host plant, i.e., the plant species that they had visited under natural conditions before being caught (*Stachytarpheta frantzii*: *Autochton longipennis* (N = 4), *Urbanus teleus* (N = 3), *Morys geisa* (N = 2); *Calathea crotalifera*: *Damas clavus* (N = 6), *Saliana triangularis* (N = 5)). Flower visits were recorded with a Sony HDR-XR550VE Handycam (Sony Corporation, Tokyo, Japan). Video recordings were analysed with the software PMB 5.0.02.11130 (Sony Corporation, Tokyo, Japan). Behavioral patterns such as proboscis uncoiling, insertion into the floral tube as well as proboscis extraction and recoiling were assessed as manipulation time. In contrast, the period after successful proboscis insertion when the butterfly remained motionless was evaluated as suction time.

Statistics

All tests were calculated with the statistical package R 3.1.0 (R Development Core Team, 2011). Correlation between variables was assessed using a Pearson correlation with the function *rcorr* of the package *Hmisc* (Harrell Jr., 2012). The influence of proboscis length and food canal area on intake rate was calculated for a set of 21 genera by using a phylogenetic comparative method *comp.gee* as implemented in the package *ape* (Paradis *et al.*, 2004). This method accounts for the phylogenetic relationship between genera since genera cannot be regarded as independent from each other. The phylogenetic matrix implemented in the GEE (generalized estimating equation) was constructed using the phylogenetic tree by Warren *et al.* (2009) and assuming equal branch lengths of one. The usage of standardized branch lengths and of genera as units of analysis was due to the low resolution of the skippers' phylogenetic tree.

Results

Scaling of feeding morphology and intake rate

Intake rates of 40 % sugar solution were measured in a total of 113 HesperIIDae individuals belonging to 34 species and 21 genera (Table 1). Intake rate varied widely between 29.1 ± 3.9 nl per second (*Vehilius stictomenes*, HesperIIDae, N = 2) and 1467 nl per second (*Bungalotis quadratum*, Eudaminae, N = 1).

Proboscis length, food canal area and body size strongly correlated with each other (Figure 2 A – C). Therefore, it was impossible to include all three measured variables simultaneously into a regression model to evaluate their influence on intake rate. However, increasing proboscis length and food canal area are thought to have contrasting effects on nectar intake rate which makes the effect of both variables important. We therefore calculated two separate models with each of these variables. Body size serves as a general description feature for insects and was therefore regarded as the least important variable.

An increase of food canal area resulted in an increasing intake rate of the tested skipper butterflies ($t_{(6.15)} = 15.39$, $P < 0.001$; Figure 3 A). The food canal area is strongly correlated with body size (Figure 2 C). This makes it impossible to separate the effect of the food canal area from other features connected with body size (such as suction pump size). In contrast to biophysical models, we found no negative effect of proboscis length on intake rate. Surprisingly, skippers with long proboscides had the tendency to show high intake rates ($t_{(6.15)} = 2.57$, $P = 0.060$; Figure 3 B).

Flower handling time

Flower handling times were measured in five skipper species equipped with medium-sized to extremely long proboscides. Handling time ranged between 6.5 ± 3.2 seconds on shorter-tubed *Stachytarpheta* flowers and 48.0 ± 14.2 seconds on the long-tubed *Calathea* flowers. Between 1.9 ± 0.8 and 24.5 ± 11.8 seconds of handling time were spent for proboscis uncoiling, the subsequent search for nectar and recoiling, i.e., manipulation time, on *Stachytarpheta* and *Calathea* flowers, respectively (Figure 4 A). Between 2.2 ± 1.3 and 23.4 ± 13.6 seconds were spent for actually taking up nectar, i.e., suction time, on *Stachytarpheta* and *Calathea* flowers, respectively (Figure 4 B). Behavioral observations showed that both manipulation time and suction time increase

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with increasing proboscis length (Figure 4 A - B, Spearman correlation: manipulation time $r = 0.88$; suction time $r = 0.88$).

Discussion

Functional costs of long proboscides?

Our analysis of nectar intake rates showed that long-proboscid skippers had higher intake rates than short-proboscid skippers. Furthermore, our results confirmed theoretical models by certifying that the food canal area is a crucial factor influencing intake rates of nectar feeding insects. By contrast, proboscis length did not negatively influence nectar intake rate as predicted by biophysical models. Here, we show that long-proboscid Neotropical skipper butterflies feature a combination of morphological adaptations which enable an efficient nectar uptake. The evolution of a long proboscis is closely linked to other morphological traits such as a large body size, which probably enables the development of a large suction pump to overcome nectar flow resistance, as well as an enlarged food canal. Further evidence for morphological adaptations that allow for efficient nectar intake comes from long-proboscid *Eurybia* butterflies (Bauder *et al.*, 2013). These butterflies possess larger dilator muscles of the suction pump in relation to the head capsule volume compared to related short-proboscid metalmark species (Bauder *et al.*, 2013). These muscles account for the occurrence of a pressure drop to transport fluid into the gut (Eberhard & Krenn, 2005). In addition, *Eurybia* butterflies were also shown to possess relatively large food canals (Bauder *et al.*, 2013). Behavioral analyses of skippers during flower visitation confirmed the results of Kunte (2007) by showing that long-proboscid skippers require a longer time for a flower visit. This proved true despite their ability to take more nectar in a given time than skippers with shorter proboscides. Further, long-proboscid skipper species spent more time drinking nectar from a flower. These findings indicate that skippers with longer proboscides take higher nectar volumes from the deep-tubed flowers of *Calathea crotalifera* than skippers with shorter proboscides from the flowers of *Stachytarpheta frantzii*. Since the corolla tube of *Calathea crotalifera* is deeper than that of *Stachytarpheta frantzii*, skippers that visit *Calathea* flowers most likely ingest higher amounts of nectar since flowers with deep corollae are known to secrete more nectar than shorter flowers (Harder, 1985; Harder & Cruzan, 1990). Given that assumption, possessing a long proboscis can be regarded as an advantage because it enables skippers to gain access to highly rewarding flowers.

However, the flower manipulation times of butterflies increased with proboscis length. Long manipulation times can lower the energy intake rate by decreasing the proportion of foraging time devoted to actually imbibing nectar (Heinrich, 1983; May, 1985). Therefore, longer manipulation times could constitute functional costs of long

proboscides. Here, we propose two not mutually exclusive explanations for this phenomenon: longer manipulation times of long-proboscid flower visitors may be caused by the difficulty of inserting the long proboscis into a narrow floral tube. This problem may be due to a poor supply of mechano- or chemosensory information, since other long-proboscid butterflies (Riodinidae) are endowed with significantly fewer sensilla on their proboscides than related short-proboscid species (Bauder *et al.*, 2011; Bauder *et al.*, 2013).

Alternatively, longer manipulation times of long-proboscid skippers could also be due to differences in flower morphology: long-proboscid skippers preferred the deep-tubed flowers of *Calathea crotalifera*, while skippers with shorter proboscides visited flowers of *Stachytarpheta frantzii* with shorter floral tubes. It has been shown that bumble bees require more time to learn complex flower designs, such as long floral tubes with concealed nectar, than simple designs (Lavery, 1994). Several studies on the foraging behavior of butterflies showed that individual experience gained by successive attempts to forage on a flower can shorten flower manipulation time (Lewis, 1986; Kandori & Ohsaki, 1996; Goulson *et al.*, 1997). Therefore, learning the floral morphology could serve as an adaptive strategy for increasing the efficiency of nectar collection (Kandori & Ohsaki, 1996). Further, long-proboscid butterflies could compensate for long manipulation times by visiting fewer nectar-rich flowers instead of many flowers with tiny nectar volumes.

Why do skippers need long proboscides?

Among butterflies outside of the HesperIIDae, extremely long proboscides are known only from the genus *Eurybia*. Recently, we studied representatives of several genera of HesperIIDae (Hesperioidea) with equally long or even longer proboscides for the first time (Bauder *et al.*, 2014). Extremely long proboscides evolved several times convergently in HesperIIDae subfamilies (Bauder *et al.*, 2014). This suggests that long proboscides represent important adaptations for some skipper taxa.

Generally, HesperIIDae are taxonomically and morphologically distinct from other butterfly families and typically feature a massive thorax containing powerful flight muscles (Betts & Wootton, 1988; Dudley & Srygley, 1994; Warren, 2006). They are extremely fast and versatile fliers and thus bear a certain resemblance to hawk moths (Betts & Wootton, 1988). Accordingly, skipper butterflies are characterized by extremely high wing loadings (Betts & Wootton, 1988; Wickman, 1992; Corbet, 2000) which are associated with high air speeds during flight (Betts & Wootton, 1988; Chai & Srygley,

1990; Dudley, 1990; Dudley & Srygley, 1994; Hall & Willmott, 2000). In general, wing loading of Lepidoptera has been shown to increase with increasing body mass (Casey, 1976; Wickman, 1992; Corbet, 2000) and energy costs during flight (Casey, 1976). All of these data provide indirect evidence that the flight of skippers is energetically costly (Bartholomew & Casey, 1978; Chai & Srygley, 1990; Dudley & Srygley, 1994) and that they have high metabolic requirements to fuel flight. However, measurements of oxygen consumption as an estimate for metabolic rates of flying HesperIIDae are still lacking. Having a long proboscis opens up access to exclusive food sources and clearly facilitates energy regain by the fast uptake of copious amounts of nectar concealed inside deep flowers.

Other Lepidoptera with energetically expensive hovering flight are the hawk moths (Casey, 1976; Bartholomew & Casey, 1978). The existence of extremely long-proboscid species among large hawk moths, such as the world record holder in proboscis length among insects, *Amphimoea walkeri* (Amsel, 1938), or the famed Darwin's hawk moth, *Xanthopan morgani praedicta* (for a review see Wasserthal, 1997), could be evidence to suggest that convergent evolution of extreme mouthpart lengths may be caused by the similar high metabolic requirements of skippers and hawk moths.

However, gaining access to large amounts of nectar inside deep-tubed flowers with a long proboscis may not suffice to fulfil the high energetic needs of some Lepidoptera. Fruit feeding butterflies, such as Satyrinae and Charaxinae (Nymphalidae), have respectively different modes of feeding that vary significantly in mouthpart morphology, feeding efficiency and food choice. The fruit-piercing technique employed by Charaxinae allows these butterflies to access high quality food, such as juicy, sugar and nitrogen-rich fruits which cannot be utilized by sweeping Satyrinae. In particular, large fruit-piercing species were believed to benefit from their feeding technique, suggesting that the rather short piercing proboscis is a correlate for evolving large body size (Molleman *et al.*, 2005a). Further, the piercing technique was associated with a high foraging efficiency, high flight speed and larger adult size (Molleman *et al.*, 2005a,b).

These examples illustrate that specialized adaptations of the mouthparts often have the same goal: recruiting high energy food sources, which are difficult to access, in order to maintain a costly life style and large body sizes. In addition, morphological, physiological and behavioral variations related to body size can affect the costs of living in a particular environment for insects, and, ultimately, pollination or foraging success (Terblanche & Anderson, 2010). At the same time, size-dependent differences in energy

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constraints can lead to partitioning of resources and provide the preconditions for coexistence of animals.

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Table 1. Body size, proboscis length, nectar intake rate and food canal area were measured in 113 individual butterflies representing 34 species and 21 genera of skippers (Hesperiidae) from Costa Rica. *Note:* Mean values ($\pm$ standard deviation) are given whenever more than 1 individual per species was measured.

Species	N	Body size [mm]	Proboscis length [mm]	Intake rate [nl/sec]	Food canal [μm^2]
Eudaminae					
<i>Astraptes alardus latia</i> EVANS, 1952	1	27.0	23.5	483	5941
<i>Astraptes anaphus anetta</i> EVANS, 1952	1	23.8	19.5	484	4477
<i>Autochton longipennis</i> (PLÖTZ, 1886)	3	18.6 ($\pm$ 0.8)	17.3 ($\pm$ 1.2)	187 ($\pm$ 41)	3343 ($\pm$ 363)
<i>Autochton zarex</i> (HÜBNER, 1818)	2	18.8 ($\pm$ 0.3)	16.3 ($\pm$ 1.5)	174 ($\pm$ 0.5)	3392 ($\pm$ 40)
<i>Bungalotis quadratum quadratum</i> (SEPP, [1845])	1	30.4	39.4	1467	10650
<i>Cogia calchas</i> (HERRICH-SCHÄFFER, 1869)	3	15.5 ($\pm$ 1.1)	12.3 ($\pm$ 0.6)	110 ($\pm$ 27)	2250 ($\pm$ 195)
<i>Spathilepia clonius</i> (CRAMER, 1775)	2	21.9 ($\pm$ 1.3)	16.8 ($\pm$ 0.2)	303 ($\pm$ 48)	4340 ($\pm$ 564)
<i>Urbanus procne</i> (PLÖTZ, 1881)	3	19.9 ($\pm$ 1.1)	15.9 ($\pm$ 0.1)	234 ($\pm$ 129)	3991 ($\pm$ 853)
<i>Urbanus simplicius</i> (STOLL, 1790)	8	19.9 ($\pm$ 0.7)	16.5 ($\pm$ 0.7)	184 ($\pm$ 78)	3570 ($\pm$ 397)
<i>Urbanus tanna</i> EVANS, 1952	7	20.6 ($\pm$ 0.8)	16.7 ($\pm$ 0.3)	252 ($\pm$ 73)	3608 ($\pm$ 487)
<i>Urbanus teleus</i> (HÜBNER, 1821)	4	19.6 ($\pm$ 0.9)	16.3 ($\pm$ 0.6)	168 ($\pm$ 36)	3082 ($\pm$ 386)

Table 1 continued.

Species	N	Body size [mm]	Proboscis length [mm]	Intake rate [nl/sec]	Food canal [μm ²]
Eudaminae					
<i>Typhedanus undulatus</i> (HEWITSON, 1867)	1	16.2	12.4	89	2725
Pyrginae					
Celaenorrhini					
<i>Celaenorrhinus darius</i> EVANS, 1952	1	21.1	29.8	136	3435
Pyrrhopygini					
<i>Mysoria ambigua</i> (MABILLE & BOULLET, 1908)	4	23.2 (± 1.0)	15.3 (0.6)	387 (± 215)	7270 (± 1462)
Hesperiinae					
Clade 113					
<i>Perichares adela</i> (HEWITSON, 1867)	8	23.2 (± 1.5)	44.5 (± 4.9)	500 (± 249)	5663 (± 1068)
<i>Perichares lotus</i> (A. BUTLER, 1870)	1	22.8	48.3	425	5901
<i>Pyrrhopygopsis socrates orasus</i> (H. DRUCE, 1876)	1	26.1	34.4	544	6792
Calpodini					
<i>Calpodes ethlius</i> (STOLL, 1782)	4	26.1 (± 0.5)	42.2 (± 1.5)	530 (± 61)	5509 (± 725)
<i>Saliana esperi esperi</i> EVANS, 1955	2	18.4 (± 1.7)	35.2 (± 2.2)	174 (± 34)	3082 (± 318)
<i>Saliana longirostris</i> (SEPP, [1840])	1	26.4	42.7	430	6023

Table 1 continued.

Species	N	Body size [mm]	Proboscis length [mm]	Intake rate [nl/sec]	Food canal [µm ²]
Calpodini					
<i>Saliana salius</i> (CRAMER, 1775)	3	23.3 (± 0.6)	47.2 (± 5.7)	199 (± 100)	5197 (± 691)
<i>Saliana severus</i> (MABILLE, 1895)	1	29.6	51.8	747	8510
<i>Saliana triangularis</i> (KAYE, 1914)	6	21.7 (± 1.3)	41.3 (± 2.5)	174.8 (± 71.4)	4234 (± 812)
<i>Talides hispa</i> EVANS, 1955	1	26.0	45.5	349	8171
<i>Thracides phidon</i> (CRAMER, 1779)	1	27.0	42.0	484	7959
Anthoptini					
<i>Corticea lysias lysias</i> (PLÖTZ, 1883)	1	13.7	14.1	149	1797
Moncini					
<i>Cymaenes alumna</i> (A. BUTLER, 1877)	2	13.9 (± 0.6)	16.5 (± 1.5)	65 (± 23)	1490 (± 220)
<i>Morys geisa</i> (MÖSCHLER, 1879)	8	15.0 (± 1.0)	20.1 (± 1.9)	65 (± 19)	1841 (± 489)
<i>Morys miccythus</i> (GODMAN, 1990)	2	14.9 (± 0.2)	19.6 (± 0.8)	118 (± 5)	2310 (± 16)
<i>Papias phaeomelas</i> (HÜBNER, [1831])	10	13.8 (± 0.8)	17.3 (± 1.4)	57 (± 18)	1499 (± 260)
<i>Papias phainis</i> GODMAN, 1900	1	13.7	16.2	80	1311

Table 1 continued.

Species	N	Body size [mm]	Proboscis length [mm]	Intake rate [nl/sec]	Food canal [μm ²]
Moncini					
<i>Papias subcostulata</i> (HERRICH-SCHÄFFER, 1870)	12	18.1 (± 1.0)	25.5 (± 1.4)	96 (± 30)	2199 (± 405)
<i>Vehilius stictomenes illudens</i> (MABILLE, 1891)	2	12.7 (± 0.6)	13.0 (± 0.01)	29 (± 4)	1089 (± 34)
Hesperini					
<i>Pompeius pompeius</i> (LATREILLE, [1824])	5	16.4 (± 0.7)	15.1 (± 0.3)	120 (± 36)	2413 (± 283)

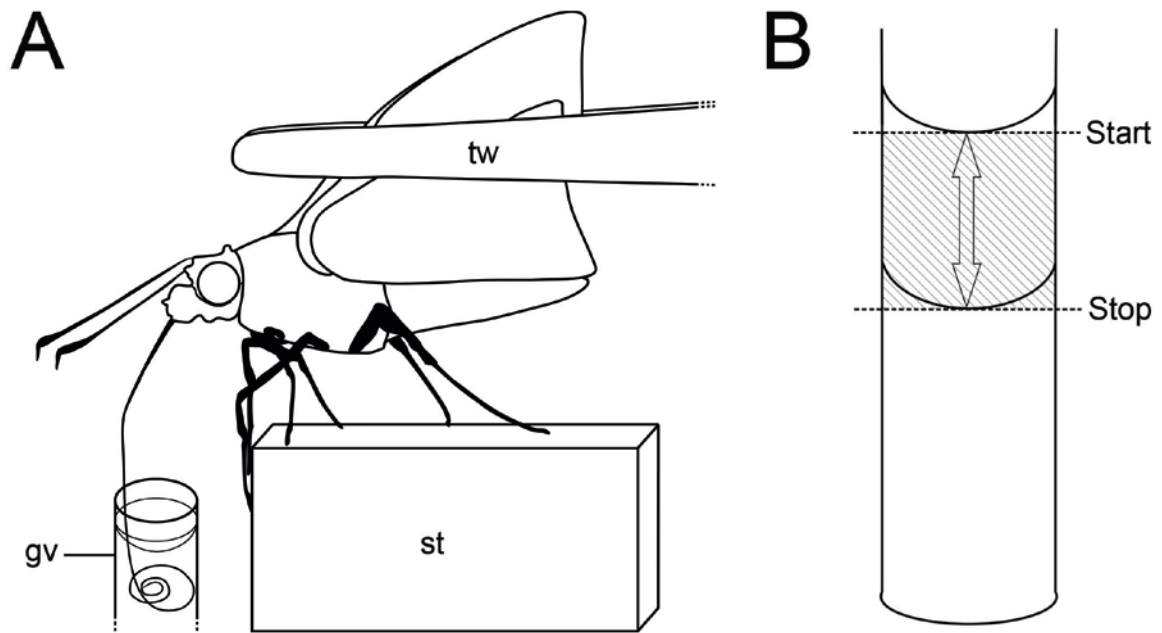


Figure 1. Setup for video-recorded feeding trials. **(A)** Skipper feeding from 40 % sugar solution. Hungry skippers were locked into position on a stage by pinching the wings together with a pair of tweezers. The proboscis was uncoiled manually and inserted into the glass vial filled with sugar solution. **(B)** Measuring the ingested volume of sugar solution on video-footage. The difference of fluid level from the start and the end of a feeding session was estimated in approximation to a cylinder. gv - glass vial, st - stage, tw - tweezers.

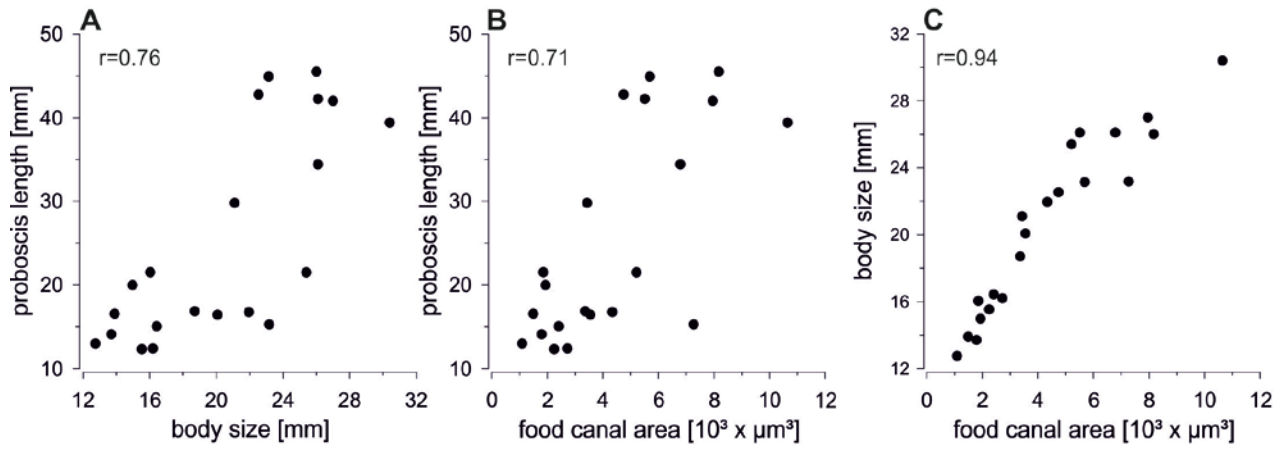


Figure 2. Pearson correlation of all three measured morphological variables suspected to influence intake rate. All variables correlated significantly with each other. **(A)** Body size and proboscis length ($r = 0.76$). **(B)** Food canal cross-section area and proboscis length ($r = 0.71$). **(C)** Food canal cross-section area and body size ($r = 0.94$). Each data point gives the mean value of one genus of Hesperidae ($N = 21$).

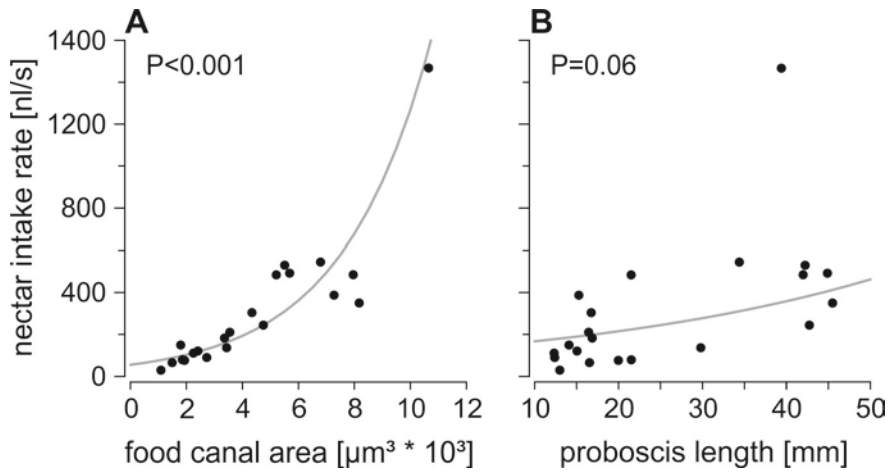


Figure 3. The effect of varying **(A)** food canal cross section area and **(B)** proboscis length on nectar intake rate of skipper butterflies. Each data point gives the mean value of one genus of HesperIIDae (N = 21). P values are from a GEE including a phylogenetic matrix to control for phylogenetic relationship between the measured genera. Bold lines: regression line.

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Morphological and biometrical adaptations of long-proboscid butterflies

Feeding is of direct importance to the individual fitness of a butterfly because the intake of carbohydrates and amino acids is known to have multiple positive effects on longevity, body weight maintenance and fecundity in females (Murphy *et al.* 1983; Hill & Pierce 1989; Fischer *et al.* 2004; Bauernfeind & Fischer 2005). The performance of the feeding apparatus should be subject to a high selective pressure because optimal foraging theory predicts that insects are bound to maximize their rate of food intake (Pyke *et al.* 1977). In contrast, some authors stated that flower visitors with extreme mouthpart lengths pay for the advantage of exploiting highly rewarding deep-tubed flowers, or even in addition to short-tubed flowers (Ranta & Lundberg 1980; Harder 1985; Nilsson *et al.* 1985; Nilsson *et al.* 1987; Nilsson 1988; Haber & Frankie 1989; May 1992; Corbet 2000; Goldblatt & Manning 2000; Agosta & Janzen 2005) with a reduced foraging efficiency (Hainsworth 1973; Hainsworth & Wolf 1976; Inouye 1980; Harder 1983; Kunte 2007; Karolyi *et al.* 2013; **Chapter I**). Thus, the functional morphology of long proboscides is of interest in comparison to short proboscides. Comparative anatomical studies can provide evidence for the performance or, alternatively, the functional failure of long butterfly proboscides, and potentially explain the scarcity of such extreme adaptations, e.g. because of high morphological material investments. Since members of the Neotropical butterfly family Riodinidae are known to comprise species that vary up to ten-fold in proboscis length, these butterflies were chosen to study the evolution of such organs by natural selection and the accompanying constraints on their evolution from a quantitative point of view (**Chapter II**).

The extreme size of the proboscis that characterizes species of the genus *Eurybia* comes from the elongation of the proximal and distal regions of the proboscis (**Chapter II**). Comparison of proboscis proportions in related long-proboscid and short-proboscid metalmark butterflies revealed that the tip length is the same in all species, even if the whole proboscis is ten times longer (**Chapter II**).

Extraordinary features of the *Eurybia*-proboscis are a low sensilla density on the outside of the galeae, as well as a short tip region equipped with relatively few and very short sensilla styloconica (**Chapters I and II**). Proboscis sensilla can carry out mechanosensory or chemosensory functions or a combination of both, depending on

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their morphology and structure (Altner & Altner 1986; Krenn 1998; Krenn & Penz 1998; Nagnan-Le Meillour *et al.* 2000; Inoue *et al.* 2009) and are believed to detect the width of the flower's opening as well as to locate nectar and assess the position of the proboscis tip once inside the flower (Krenn & Penz 1998). A reduction in the sensilla equipment may lead to a poor supply of tactile and gustatory information and probably result in longer search times for the corolla opening and nectar concealed inside the floral tube (**Chapters I and V**). Furthermore, as an adaptation to nectar feeding from deep-tubed flowers, short sensilla on the proboscis tip may simply fit better even in narrow floral tubes and simultaneously represent a resolution to prevent the accruelement of material costs coming from proboscis elongation (**Chapter II**).

In addition, the long proboscis of *Eurybia*-butterflies is characterized by a large food canal and thick cuticular walls (**Chapter II**). Similar adaptations regarding the proboscis morphology were proven for long-proboscid skipper butterflies, which were also characterized by having enlarged food canals (**Chapter V**). Especially an increase in the food canal radius is known to dramatically increase the volumetric flow rate of nectar (Kingsolver & Daniel 1979). Therefore, the enlargement of the food canal represents a crucial key-innovation to compensate for lower flow velocities resulting from proboscis elongation, but at the same time comes at the cost of significant cuticular reinforcements of the proboscis walls (**Chapter II**).

Adaptations of the feeding apparatus of *Eurybia*-butterflies were found to include (1) additional and larger stipes muscles (**Chapter II**), which are assumed to improve uncoiling of a very long proboscis, since they function to produce a haemolymph flow from the head into both galeae (Bänziger 1971; Krenn 1990; Wannenmacher & Wasserthal 2003) and (2) reinforced suction pump dilators (**Chapter II**) which raise the flexible roof of the suction pump and thus create a pressure gradient resulting in fluid uptake (Schmitt 1938; Eastham & Eassa 1955; Srivastava & Bogawat 1969; MacFarlane & Eaton 1973; Miles & Booker 1998; Eberhard & Krenn 2005; Davis & Hildebrand 2006). Ultimately, material investments for the long proboscis of *Eurybia*-butterflies can be regarded as relatively low. However, these butterflies must bear significant anatomical costs of traits that are functionally related to feeding performance, such as the pumping organs in the butterfly's head for proboscis uncoiling and nectar uptake. Therefore, such adaptations of the pumping organs and the food canal serve to maintain the feeding performance of long-proboscid butterflies (**Chapter II**).

What limits further proboscis length evolution?

Extremely long proboscides evolved once within Riodinidae and at least three times independently within Hesperidae by allometric scaling with body size (**Chapter III**), indicating that long-proboscid species have higher relative proboscis lengths than short-proboscid species and, in addition, are generally larger (**Chapters II, III and V**). Consequently, record holding species in absolute proboscis length typically have higher relative proboscis lengths: in butterflies, the longest proboscis ever found (**Chapter III**) measured 52.7 mm (*Damas immaculata*, Hesperidae) corresponding to approximately twice the body length; whereas the record holder among insects, a hawk moth (*Amphimoea walkeri*, Sphingidae) is known to have a proboscis measuring 280 mm corresponding to the fourfold body length (Amsel 1938). Several studies on the proboscis length on butterflies showed that relative proboscis length never exceeds 2.5 (Kunte 2007; **Chapters I, II and III**). These results indicate that proboscis length in hawk moths can exceed that of butterflies not only because hawk moths are larger, but also because of a steeper scaling relationship between proboscis length and body size. But what keeps butterflies from evolving equally long mouthparts in relation to body size as hawk moths? A possible explanation for this limit could be found in the flower-visiting behaviour of butterflies and hawk moths: hawk moths typically hover during nectar uptake in front of the flowers, whereas all butterflies need to sit on the flower for feeding. In butterflies, uncoiling a very long proboscis seems to be limited by how far a butterfly can bend back its head and stretch its legs to allow for straightening of the proboscis spiral while sitting on the flower. None of these problems apply to hawk moths, which can modulate the space needed for uncoiling by hovering at an acceptable distance in front of the flower. Although absolute proboscis length determines access to flowers with deep tubes, the scaling relationship between proboscis and body plays a crucial role during the uncoiling process and might constrain butterflies from evolving even longer mouthparts (**Chapter III**). Another limit to proboscis length evolution could be proposed by developmental constraints during metamorphosis (**Chapter III**). In butterflies, proboscis formation takes place in a developmental sheath on the ventral side of the pupa (Lowe *et al.* 2013), where the galeae are straight and arranged parallel to each other. Since this sheath contains the full length of the unfolded proboscis, this organ grows accordingly to accommodate the extreme length of the adult proboscis and may extend a full body length beyond the last abdominal segment (DeVries 1997). Further elongation of this fragile and thin pupal organ might constrain the evolution of

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proboscis length in butterflies. By contrast, the pupae of long-proboscid hawk moths develop a heavily sclerotized hook-shaped external outgrowth that contains a loop of the developing proboscis that allows for the formation of a proboscis of much greater length (Patočka 1993).

Long proboscides for deep tubes?

Food resource partitioning is held to be a driving force for the coexistence of animals (Hespenheide 1973; Schoener 1974; Inouye 1980; Ranta & Lundberg 1980) and is often estimated using morphological traits, such as size differences between animals or differences in mouthparts in relation to the size of food particles. With respect to nectar-drinking flower visitors, such as butterflies, this refers to differences in proboscis length in relation to floral tube length. According to this hypothesis of competition avoidance, on the one hand, extremely long-proboscid butterflies should specialize in visiting flowers that correspond to their proboscis lengths and avoid flower with tubes that are shorter than their proboscis. On the other hand, butterflies are often regarded as generalist flower visitors (Shreeve 1992; Tudor *et al.* 2004; Stefanescu & Traveset 2009), and the number of plant species available to a butterfly or moth could be a function of its proboscis length: the longer the proboscis, the more flowers with varying tube lengths that can be visited (Agosta & Janzen 2005). However, observations on a community of Neotropical HesperIIDae showed that long-proboscid butterflies generally do not visit flowers with short nectar spurs such as *L. camara* or *S. frantzii*, but prefer the deep corollae of two *Calathea* species (**Chapter IV**). Furthermore, long-proboscid skippers did not discriminate between the flowers of *C. crotalifera* and *C. lutea* (**Chapter IV**), probably because these flowers resemble each other in their morphology and colour and grow in the same habitats (Ruppel, unpublished). The deep corollae of *Calathea*-flowers received visits from fewer skipper species (and no visits from other butterflies) than the short corollae of *L. camara* and *S. frantzii*. Therefore, long-proboscid Lepidoptera may be released from the level of competition experienced by short-proboscid Lepidoptera (Agosta *et al.* 2005) and choose deep-tubed flowers which cannot be used by short-proboscid butterflies. The same was confirmed earlier for British butterfly assemblages (Porter *et al.* 1992; Corbet 2000).

The role of long-proboscid butterflies in a plant-pollinator network

One precondition for successful pollination is that the floral tube is slightly longer than/or at least matches the proboscis length of the pollinator (Darwin 1862; Johnson & Anderson 2010) since it ensures safe placement of pollen on the basal proboscis or the head of the insect, where it cannot be removed during the phase of proboscis withdrawal from the flower or when cleaning the mouthparts with the forelegs (Darwin 1862; Nilsson 1988; Wasserthal 1997). Many studies have demonstrated a close match between plant and pollinator morphology that serve as supposed examples for coevolution (Grant & Grant 1965; Grant & Grant 1983; Harder 1985; Nilsson *et al.* 1985; Nilsson 1988; Nilsson 1998; Johnson & Steiner 1997; Alexandersson & Johnson 2002). Although the proboscis lengths of skipper butterflies differed significantly among the visited flower species, no close match exists between extremely long proboscides and deep tubes: Extremely long-proboscid skipper butterflies that used *C. crotalifera* and *C. lutea* as a nectar source, had a mean proboscis length of 43.4 mm, exceeding the corolla depth of *C. crotalifera* by 18.4 mm and *C. lutea* by 12 mm (**Chapter IV**). Similarly, *E. lycisca* butterflies from Costa Rica that fed on the nectar of *C. crotalifera* and *C. lutea* plants were characterized by proboscides measuring 36.5 mm on average (**Chapter I**), exceeding the corolla depth of *C. crotalifera* and *C. lutea* by 11.5 mm or 5.1 mm, respectively (**Chapter IV**). Since the butterflies' proboscides are longer than the floral tube, they are not forced to plunge their head into the corolla entrance and successful pollination is highly unlikely. Apart from that, all of the observations conducted in the course of this project, demonstrate that neither skipper nor metalmark butterflies were able to release the trigger mechanism of *Calathea*-flowers (**Chapter I**) in contrast to their legitimate pollinators, euglossine bees (Janzen 1971; Borrell 2005). Euglossine bees feature extraordinarily long proboscides ranging between 13.8 and 38.9 mm length (Borrell 2005). However, these bees are generalistic flower visitors since the number of visited plant species increases with proboscis length (Borrell 2005). These results raise the questions, why have these deep-tubed flowers specialized on generalistic bees and how do nectar-thieving butterflies fit into this network? Schemske (1981) and Borrell (2005) hypothesized that many steady-state understorey plants, such as *Calathea* or *Costus*, are unable to support the energetic needs of an entire pollinator population because of their low density, small size of some species and few flowers blooming simultaneously (Janzen 1971). They argued that increasing nectar production would be energetically costly, but by morphologically excluding short-proboscid insects

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as potential nectar consumers, these plants can still provide plenty of nectar to individual euglossine bees which learn to include these plants in the same nectar traplines daily (Janzen 1971; Borrell 2005). Given that the long nectar spurs of *Calathea*-plants evolved not via directional selection exerted by a specialized pollinator (Darwin 1862), but by competition among sympatric flowers for inclusion on a pollinator's foraging route (Garrison & Gass 1999). Nectar thieving long-proboscid butterflies would ransack *Calathea*-nectar stores, leaving less nectar for pollinating Euglossini. If long-proboscid butterflies were abundant visitors of *Calathea* and had a serious impact on the nectar availability for the legitimate pollinators of *Calathea*-plants, pollinator sharing (Macior 1971) would be adaptive for two sympatric *Calathea* species, such as *C. lutea* and *C. crotalifera*. Plant species with restricted distributions and low densities increase the effective density of food rewards to pollinators and pollination probabilities by converging in floral characters (floral mimicry: Macior 1971). Since the flowers of *C. lutea* and *C. crotalifera* are both coloured yellow and share similar morphologies, further studies focussing on their local distribution, flowering phenology, nectar secretion patterns and interspecific hybridization (Schemske 1981) could elucidate whether these similarities arise from pollinator sharing or phylogenetic relationship. The pollinator sharing - hypothesis is especially tantalizing keeping in mind that these plants not only suffer from nectar robbery by long-proboscid skipper and metalmark butterflies, but also from flower predation exerted by the larvae of *Eurybia*-butterflies which feed on the flowers of *Calathea* but not on the foliage (DeVries 1997).

Functional costs of long proboscides: flower handling time

Several studies measured various aspects of flower handling time of nectar feeding insects and related handling time to morphological traits of the visited flowers and/or of the insects (Inouye 1980; Harder 1983; Harder 1985; Herrera 1989; Kunte 2007; Karolyi *et al.* 2013; **Chapter I**). The results are manifold and strongly dependent on the taxa and methods used, and even differ among similar studies on a single taxon. Contradictory results concerning handling times of nectar feeding insects may also arise from intraspecific variations of corolla length, or nectar concentration and nectar volume of individual flowers. Furthermore, conflicting impacts on handling time, e. g. handling time correlating negatively with proboscis length when measured on deep-tubed flowers (Inouye 1980) and at the same time correlating positively with corolla length of the used flowers (Harder 1983) may overlap and mask the cause for the results, since long-

proboscid flower visitors are generally known to visit deeper flowers. Despite behavioural observations of long-proboscid *Eurybia lycisca* butterflies remaining on a single flower for about one minute (**Chapter I**) confirmed the findings of Kunte (2007), the reasons for these results, e. g. slow nectar intake rates, taking up high amounts of nectar or increased flower manipulation times, remained unclear. Feeding experiments with Neotropical HesperIIDae using standardized sugar solution (**Chapter V**) revealed that long-proboscid skippers have higher nectar intake rates than short-tongued skippers and confirm theoretical models which state that the food canal cross-sectional area is a crucial factor influencing intake rates of nectar feeding insects: Biophysical models predict that an increase in the food canal radius leads to an increase of volumetric flow rate, because the latter depends on the fourth power of the radius. Long-proboscid skipper species feature a combination of morphological adaptations which enable an efficient nectar uptake. The evolution of a long butterfly proboscis is closely linked to a large body size, which probably enables the development of a large suction pump to overcome nectar flow resistance, as well as enlarged food canals (**Chapter V**). Such adaptations were also confirmed for long-proboscid *Eurybia*-butterflies (**Chapter II**). Nonetheless, behavioural observations of skippers foraging in the wild confirmed the results of Kunte (2007) by showing that long-proboscid skippers require a longer time for a flower visit (**Chapter V**). Despite their ability to take more nectar in a given time than skippers with shorter proboscides, they spend more time drinking from a flower, indicating that they take higher nectar volumes from their preferred nectar plants, the deep-tubed *Calathea*-flowers, than short-proboscid skippers from *Stachytarpheta*-flowers which have shorter tubes. Since deep-tubed flowers are known to produce more nectar than short-tubed flowers, a long proboscis is advantageous because it enables skippers to gain access to highly rewarding flowers. However, the flower manipulation times of skipper butterflies increased with proboscis length and therefore constitute functional costs of long proboscides. These could be due to difficulties with inserting the long proboscis into a narrow floral tube. Such difficulties could come from a poor supply of mechano- or chemosensory information, since similarly long-proboscid *Eurybia*-butterflies were found to have significantly less sensilla on their proboscides than related short-proboscid species (**Chapters I and II**) and had slower phases of searching and insertion than butterflies with average proboscis lengths (Krenn 1990; Krenn 2008; **Chapter I**). Furthermore, longer manipulation times may also arise from slower proboscis uncoiling as was proven earlier for long-proboscid *Eurybia*-

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butterflies (**Chapter I**). Alternatively, the longer flower manipulation times of long-proboscid skipper butterflies could also be due to differences in the flower morphology: long-proboscid skippers preferred deep-tubed flowers, whereas short-proboscid skippers visited flowers with shorter tubes. It has been shown that bumble bees require more time to learn complex flower designs, such as long floral tubes with concealed nectar, than simple designs (Lavery 1994). Several studies on butterflies showed that individual experience gained by successive attempts to forage on a flower can shorten flower manipulation time (Lewis 1986; Kandori & Ohsaki 1996; Goulson *et al.* 1997). Therefore on the one hand, learning of floral morphologies could serve as an adaptive strategy for increasing the efficiency of nectar collection (Kandori & Ohsaki 1996). On the other hand, long-proboscid butterflies could compensate for long manipulation times by visiting fewer nectar-rich flowers instead of many flowers with tiny nectar volumes.

Why do large skippers need a long proboscis?

Among butterflies outside of the Hesperiidae, extremely long proboscides are known only from the genus *Eurybia*. However, extremely long proboscides evolved several times convergently in subfamilies of the Hesperiidae (**Chapter III**). This suggests that long proboscides represent important adaptations for some skipper taxa.

Generally, Hesperiidae are taxonomically and morphologically distinct from other families of butterflies and typically feature a massive thorax containing powerful flight muscles (Betts & Wootton 1988; Dudley & Srygley 1994; Warren 2006). They are extremely fast and versatile fliers and thus bear a certain resemblance to hawk moths (Betts & Wootton 1988). Accordingly, skipper butterflies are characterized by extremely high wing loadings (Betts & Wootton 1988; Wickman 1992; Corbet 2000) which are associated with high air speeds during flight (Betts & Wootton 1988; Chai & Srygley 1990; Dudley 1990; Dudley & Srygley 1994; Hall & Willmott 2000). In general, wing loading of Lepidoptera has been shown to increase with increasing body mass (Casey 1976; Wickman 1992; Corbet 2000) and energy costs during flight (Casey 1976). All of these data provide indirect evidence that the flight of skippers is energetically costly (Bartholomew & Casey 1978; Chai & Srygley 1990; Dudley & Srygley 1994), and that they have high metabolic requirements to fuel flight. However, measurements of oxygen consumption as an estimate for metabolic rates of flying Hesperiidae are still lacking. Having a long proboscis opens up access to exclusive food sources and clearly

facilitates energy regain by the fast uptake of copious amounts of nectar concealed inside deep-tubed flowers.

Other Lepidoptera with energetically expensive hovering flight are the hawk moths (Casey 1976; Bartholomew & Casey 1978). The existence of extremely long-proboscid species among large hawk moths, such as the world record holder in proboscis length among insects, *Amphimoea walkeri* (Amsel 1938), or the famed Darwin's hawk moth, *Xanthopan morgani praedicta* (for a review see Wasserthal, 1997), could suggest that convergent evolution of extreme mouthpart lengths is caused by the similar high metabolic requirements of skippers and hawk moths.

However, gaining access to large amounts of nectar inside deep-tubed flowers with a long proboscis may not suffice to fulfil the high energetic needs of some Lepidoptera. Fruit-feeding butterflies, such as Satyrinae and Charaxinae (Nymphalidae), have respectively different modes of feeding that vary significantly in mouthpart morphology, feeding efficiency and food choice. The fruit-piercing technique employed by Charaxinae allows these butterflies to access high quality food, such as juicy, sugar and nitrogen-rich fruits which cannot be utilized by sweeping Satyrinae. In particular, large fruit-piercing species were believed to benefit from their feeding technique, suggesting that the rather short piercing proboscis is a correlate for evolving large body size (Molleman *et al.*, 2005a). Further, the piercing technique was associated with a high foraging efficiency, high flight speed and larger adult size (Molleman *et al.*, 2005a,b).

These examples illustrate that specialized adaptations of the mouthparts often have the same goal: recruiting high energy food sources, which are difficult to access, in order to maintain a costly life style and large body sizes.

Contributions to the scientific field

Studies on nutritional ecology – the acquisition of resources and their allocation to different tasks – are essential for the understanding of life histories. Because differences in mouthpart morphology are correlated with diet, the study of feeding techniques on the one hand and of feeding efficiency and food choice on the other is highly relevant (Molleman *et al.* 2005a). This study represents the first detailed analyses of the morphology of disproportionately long and slender proboscides in context with nectar feeding in metalmark and skipper butterflies. Further, this survey provides insight into the foraging efficiency and functional adaptations, including their benefits and costs, required for long-proboscid nectar feeding butterflies. Consequently, an integrative

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approach combining behavioural, physiological and morphological data gained from a wide range of species presents a significant advance to our understanding on the evolution of flower-visiting insects. In particular, morphological, physiological and behavioural variation in respect to body size, i.e., scaling, has received little attention so far with regard to foraging butterflies. This study aimed to explain how the variation of traits in respect to body size affects costs of foraging and its efficiency for insects. In contrast to other long-proboscid insects, such as South African nemestrinid flies and hawk moths, the role of butterflies as important pollinators remains doubtful. This study provides the first evidence that long-proboscid butterflies are, in fact, nectar thieves, rather than pollinators and therefore challenges the traditional theory of coevolution, which implies reciprocal adaptations and fitness dependence of both pollinators and plants. In this context, the study at hand contributes to a better understanding of the role of butterflies in plant-pollinator networks, and the consequences on other flower visitors that live in the same environment and share resources.

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__6. APPENDIX

6.1. Summary

Extremely long mouthparts of insect flower visitors evolved by natural selection to gain access to highly rewarding deep-tubed flowers. Such morphological specializations have been regarded as the outcome of a mutualistic coevolutionary arms race, assuming that these insects act as efficient pollinators for their nectar host plants. Butterflies have evolved a tubular proboscis to drink nectar from various flowers, but their role as efficient pollinators remains ambiguous. Furthermore, previous studies largely focussed on the pollinator-mediated selection on flowers, whereas specializations of the mouthparts have been neglected so far, except for proboscis length. Extremely long butterfly proboscides that greatly exceed the length of the body and measure up to 52.7 mm are rare and occur only in some species of Neotropical metalmarks (Riodinidae) and skippers (Hesperiidae). So far, the scarceness of long proboscid species among butterflies was explained by functional costs arising from increased flower handling times caused by decelerated nectar intake rates, but can also be the result of costly anatomical investments that serve to maintain the functionality of long proboscides. This study presents an integrative approach combining morphological, biometrical and behavioral data obtained from various butterfly species from a Costa Rican lowland rainforest area differing substantially in proboscis length to shed light on the form, function and (limits to the) evolution of extremely long butterfly proboscides and the ecological role of these insects in plant-pollinator networks. Long butterfly proboscides result from an allometric scaling relationship with body size which differs between phylogenetic lineages on the subfamily level among Hesperiidae. Disproportionally long proboscides evolved once within Riodinidae and at least three times convergently within Hesperiidae. Long-proboscid butterflies experience relatively low extra expense on the proboscis musculature and sensilla equipment, but significant anatomical costs in terms of reinforced haemolymph muscles for proboscis uncoiling and enlarged suction pump musculature for creating a pressure gradient to take up nectar, as well as thick cuticular proboscis walls. Furthermore, feeding experiments with standardized sugar solution reveal that long-proboscid butterflies do not suffer from reduced intake rates due to a combination of morphological adaptations, such as large body size and an enlarged food canal. Video analyses of foraging long-proboscid

butterflies in the wild show that suction times increase with proboscis length, suggesting that long-proboscid butterflies drink larger amounts of nectar from deep-tubed flowers. Despite these advantages, functional costs of exaggerated mouthparts exist in terms of longer manipulation times per flower. Further, analyses of the flower-visiting behavior show that long-proboscid skipper and metalmark butterflies prefer the deep-tubed flowers of *Calathea* plants. However, these butterflies do not contribute to the pollination of their nectar host plant. By contrast, the adult butterflies steal nectar from flowers. In addition, the larvae of long-proboscid metalmark butterflies feed on the flowers of their nectar host plants. These findings indicate an antagonistic relationship between long-proboscid butterflies and their host plants and challenge the traditional view on the coevolution of long-proboscid flower visitors and deep-tubed flowers. Rather, the extraordinarily long proboscides of some Neotropical butterflies represent adaptations for capitalizing on pre-existing mutualistic interactions of the nectar host plants with their long-proboscid nectar feeding pollinators, such as euglossine bees. The study at hand provides evidence that constraints on the evolution of increasingly long butterfly proboscides may come from the underlying scaling relationships, i.e., relative proboscis length, combined with the butterfly's flight style and flower-visiting behaviour and/or developmental processes during the pupal phase. Finally, there is evidence for the hypothesis that the convergent evolution of extremely long proboscides within skippers and hawk moths results from a combination of fuelling a metabolically costly flight and maintaining the high energetic requirements of large body sizes.

6.2. Zusammenfassung

Die extrem langen Mundwerkzeuge von blütenbesuchenden Insekten sind durch natürliche Selektion entstanden, damit diese Zugang zu den reichhaltigen Nektarvorkommen in langröhrigen Blüten erhalten. Diese morphologischen Spezialanpassungen werden als das Ergebnis eines mutualistischen coevolutionären Wettlaufs betrachtet, vorausgesetzt dass diese langrüsseligen Blütenbesucher als effiziente Bestäuber ihrer bevorzugten Nektarpflanzen fungieren. Schmetterlinge haben einen röhrenförmigen Rüssel, mit dem sie Nektar aus den verschiedensten Blüten trinken können, aber bis heute ist umstritten, ob sie diese auch bestäuben. Frühere Studien haben hauptsächlich die von Bestäubern ausgehende Selektion auf Blüten untersucht. Spezialisierungen der Mundwerkzeuge der Blütenbesucher, abgesehen von der Rüssellänge selbst, wurden jedoch bisher kaum untersucht. Außerordentlich lange Schmetterlingsrüssel überragen die Körperlänge um das Doppelte und werden bis zu 52.7 mm lang. Diese sind allerdings sehr selten und kommen ausschließlich in zwei Familien Neotropischer Schmetterlingsarten vor. Bisher wurde die Seltenheit langrüsseliger Schmetterlingsarten einerseits durch funktionelle Kosten des langen Rüssels erklärt, wie längere Blütenbesuchszeiten, die durch langsamere Nektaraufnahmeraten verursacht werden. Andererseits könnte das seltene Vorkommen solcher Arten auch in hohen anatomischen Investitionen begründet sein, welche die einwandfreie Funktion des langen Rüssels gewährleisten. Diese Arbeit stellt einen integrativen Ansatz dar, welcher morphologische, biometrische und verhaltensbiologische Daten verschiedener Schmetterlingsarten kombiniert. Die untersuchten Arten aus einem Tieflandregenwald in Costa Rica unterscheiden sich erheblich in der Rüssellänge. Dadurch können neue Erkenntnisse bezüglich der Form, Funktion und (den Grenzen der) Evolution von extrem langen Schmetterlingsrüssel gewonnen werden. Die Ergebnisse ermöglichen, Aussagen bezüglich der Rolle dieser Insekten in Pflanzen-Bestäuber Netzwerken zu treffen. Lange Schmetterlingsrüssel sind durch eine allometrische Verlängerung mit der Körpergröße entstanden. Das Verhältnis zwischen Körperlänge und Rüssellänge charakterisiert die phylogenetischen Abstammungslinien und unterscheidet sich signifikant zwischen verschiedenen Unterfamilien der HesperIIDae. Überproportional lange Rüssel sind ein einziges Mal innerhalb der Riodinidae, aber zumindest drei Mal unabhängig voneinander innerhalb der HesperIIDae entstanden. Langrüsselige Schmetterlinge müssen relativ wenig in die Rüsselmuskulatur und die Sensillenausstattung investieren. Jedoch entstehen

signifikante anatomische Kosten durch die verstärkte Muskulatur der Haemolymphpumpe, die den Rüssel ausrollt, und durch die vergrößerte Saugpumpenmuskulatur, die einen Unterdruck zum Aufsaugen von Nektar erzeugt. Außerdem müssen die Wände des Rüssels durch Cuticula verstärkt werden. Fütterungsexperimente mit standardisierter Zuckerlösung zeigten, dass langrüsselige Schmetterlinge keine verlangsamten Nektaraufnahmeraten aufweisen, da sie eine Kombination von morphologischen Anpassungen, wie eine große Körpergröße und vergrößerte Nahrungskanalquerschnittsflächen, besitzen. Anhand von Videoanalysen frei lebender langrüsseliger Dickkopffalter bei der Nahrungssuche konnte bewiesen werden, dass die Zeitspanne in der Nektar aufgenommen wird, mit der Rüssellänge ansteigt. Langrüsselige Schmetterlinge können dadurch größere Nektarmengen aus langröhrigen Blüten aufnehmen. Trotz dieses Vorteils, gibt es dennoch funktionelle Nachteile eines langen Rüssels: langrüsselige Tagfalter benötigen mehr Zeit, um ihren Rüssel auszurollen und in Blüten einzufädeln. Beobachtungen des Verhaltens der Schmetterlinge während des Blütenbesuchs zeigten zwar, dass langrüsselige Arten die langröhrigen Blüten von Pflanzen der Gattung *Calathea* bevorzugten und diese am liebsten besuchten, jedoch nicht zur ihrer Bestäubung beitragen. Folglich müssen sie als Nektardiebe betrachtet werden. Zusätzlich fressen die Raupen der langrüsseligen *Eurybia*-Schmetterlinge das Blütengewebe der *Calathea*-Pflanzen. Diese Beobachtungen deuten auf eine antagonistische Beziehung zwischen den langrüsseligen Tagfaltern und dieser Nektarpflanzen hin und stellen damit die traditionelle Ansicht über die Entstehung von langen Mundwerkzeugen bei Insekten und tiefen Kronröhren durch mutualistische Coevolution in Frage. Stattdessen sind die extrem langen Rüssel mancher Neotropischer Tagfalter Anpassungen, um schon bestehende, mutualistische Interaktionen zwischen Nektarpflanzen und deren langrüsseligen Bestäubern, auszubeuten. Diese Arbeit liefert Hinweise darauf, dass es Grenzen für die Evolution von langen Rüsseln gibt, da das Verhältnis von Körperlänge zu Rüssellänge nicht beliebig gesteigert werden kann. Die Gründe dafür liegen einerseits im Verhalten der Schmetterlinge während des Blütenbesuchs und andererseits in der Entwicklung der Puppe während der Metamorphose. Lange Rüssel von großen Vertretern der HesperIIDae und Sphingidae sind wahrscheinlich unabhängig voneinander entstanden, um Zugang zu reichhaltigen Nektarquellen zu erhalten, welche ihren energieaufwändigen Flug zu ermöglichen.

6.3. Review of Chapter V

„Functional constraints on the evolution of long butterfly proboscides: Lessons from Neotropical skippers (Lepidoptera: Hesperiidae)“

Submitted to the Journal of Evolutionary Biology on 29th of October, 2014

Manuscript Number JEB ms # JEB-2014-00687

Decision letter of the editor:

Dear Dr. Bauder,

Thank you for submitting your manuscript "Functional constraints on the evolution of long butterfly proboscides: Lessons from Neotropical skippers (Lepidoptera: Hesperiidae)" (JEB ms JEB-2014-00687) to the Journal of Evolutionary Biology. Your work has now been considered by two reviewers, whose comments are enclosed. As you will see, while both found merit in your manuscript and would ultimately like to see it published in the journal, both have also raised a number of points that preclude it from being acceptable for publication in its present form. I also liked your paper, and I am therefore willing to consider it further for acceptance provided that you revise it appropriately along the lines recommended by the reviewers.

When you submit the revision, please include with it a letter in which you describe how you have responded to each of the referees' comments. Please number the comments and refer to line numbers in the original and revised paper for easy reference. Often a marked-up revision is helpful. Ensure you upload this letter with your other documents so it forms part of the overall PDF document (note there is a file designation called "Author response to Reviewer").

Please pay careful attention to the formatting of tables, figures and references, as well as the style used for reporting the results of statistical tests (see Instructions for Authors, JEB inside back cover, or [http://onlinelibrary.wiley.com/journal/10.1111/\(ISSN\)1420-9101/submiss.htm](http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1420-9101/submiss.htm)) Please submit the revised paper on the

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JEB website <http://mc.manuscriptcentral.com/jeb>, in an editable format, preferably Word, RTF or WP, within eight weeks, since otherwise it will be considered as a newly submitted manuscript. You can access the revision submission by clicking on the link Manuscripts with Decisions in your Author Center.

Please make sure any in-line statistics conform to the Instructions for authors ("In-line statistical results should be presented as Test-statistics: degrees of freedom as subscript(s) to test-statistics (e.g. $F_{1,12} = \dots$ or $t_8 = \dots$), followed by P-value., e.g. ($F_{1,12} = 4.931$, $P = 0.0464$). Statistical results in tables should be comprehensive, allowing future meta-analyses. Depending on the details of the analyses, results reported may include parameter estimates, test-statistics, degrees of freedom, significance levels and err/residual model information (e.g. error MS's and df's in ANOVA or regression models). Since exact P-values can be useful for meta-analyses, we recommend that these are quoted even when non-significant, e.g. $t_{23}=0.25$, $P=0.34$, or $F_{2,32}=1.12$, $P=0.55$. However, non significant tests (i.e. $P > 0.05$) should always be interpreted as such.

Sincerely,
Thomas Flatt

Response of reviewer 1:

Comments to the Author

Title: Functional constraints on the evolution of long butterfly proboscides: Lessons from Neotropical skippers (Lepidoptera: Hesperidae)

Authors: Bauder, Morawetz, Warren & Krenn

This is a well-written manuscript focusing on the evolution of long proboscides in skipper butterflies. The authors used a combination of morphological and behavioral data to demonstrate that while long proboscides allow access to deep-tubed flowers, there is a tradeoff in flower handling time. The study is well designed and the data is well presented. A few comments/suggestions are given below.

- Nowhere in the manuscript the authors explain whether they examined males and females, or individuals of one sex only. Do they expect that both sexes would have the same feeding behavior/nectar consumption?
- Lines 95, 99-100: (Kingsolver & Daniel, 1979; Kingsolver & Daniel, 1995) = (Kingsolver & Daniel, 1979, 1995).
- Lines 122-123, quote: “Based on these results, we suggest an evolutionary scenario that explains why Hesperiidæ, especially, evolved a particularly long proboscis several times independently.” The word ‘especially’ does not make much sense here (or did you mean ‘in particular’?)
- Lines 227-229: This sentence is a bit muddled because it blends two factors that are independent from one another: (1) branch lengths and (2) genera as units of analysis. It is clear how the lack of resolution would affect (2). However, how would lack of resolution require the use of BL=1 is not so clear.
- Line 238, quote: “Proboscis length, food canal area and body size strongly correlated with each other (Figure 2 A – C).” This means that there is an allometry effect in this dataset. Fig. 2A makes perfect sense with body size as the dependent variable. However, it seems more logical to use body size as a dependent variable in Fig. 2C (and make it 2B), and use proboscis length as a dependent variable for the next comparison. The correlations would not change at all – this is just a presentation suggestion. Nonetheless, how would the allometry effects affect your interpretation of the data?
- Lines 247-253: Interesting result. Are the two outliers in Fig. 3A and B the same species? What is special about such species?
- Lines 277-287, quote: “The evolution of a long proboscis is closely linked to other morphological traits such as a large body size, which probably enables the development of a large suction pump to overcome nectar flow resistance, as well as an enlarged food canal.” Although the adaptationist view is plausible (long proboscis allows for sampling deep flowers with more nectar), the authors clearly show that proboscis length is

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correlated with body size. So, the long proboscis may simply be a corollary of selective pressures for increased body size. *Eurybia* is large compared to close relatives, but its proboscis is longer than what would be expected for its body size -- so, here there are reasons to infer selection on proboscis length. That does not seem to be the case for skippers. The authors should use caution when writing about this.

- Lines 350-357: The record holders matter less than the general trend: sphinx moths have long proboscides and energetically demanding flight, and so do skippers (a chicken and egg paradigm, perhaps?). This paragraph is not quite convincing and should be re-written, particularly because *Amphimoea* and *Xanthopan* only are being used as evidence for convergence at a macroevolutionary level.

- Lines 358-359: My guess is that the authors meant to say that there is more than one solution to the problem of gaining enough energy from food to sustain flight energy demands. This is a bit of a 'dangerous' reasoning in the absence of information about how much energy actually comes directly from adult feeding vs. other metabolic functions (storage). Moreover, fruit juices have a different composition than nectar (more Nitrogen, correct?) which has uses beyond the immediate 'energy demand'. Again, my suggestion would be to re-think and perhaps re-write this portion of the text (from line 350 over to the end).

- The tradeoff between flower handling and proboscis length (which allows the use of deep flowers) is no surprise. And field biologists also know that certain activities, feeding included, can make an individual more vulnerable to successful predator attacks (the samples were collected at flowers). However, if skipper fly fast, could that help offset the tradeoff...?

Response of reviewer 2:

Comments to the Author

This is a carefully planned study and a well-organized manuscript. I was pleased to see that the authors collected various kinds of very useful data under semi-natural conditions and also more controlled experiments under captive conditions. Their approach has contributed useful insights into the evolution of long probosces in

butterflies, and also into the biomechanics of feeding through long probosces. Statistical analyses are adequate, and the data are summarized and presented in suitable tables and figures. Overall, I think this is a good paper that contributes to its field substantially.

I do have a major concern, though, regarding the last part of the manuscript. The abstract states, "Finally, we provided evidence for the hypothesis that the convergent evolution of extremely long proboscides within skippers and hawk moths resulted from high metabolic requirements during flight." More extended discussion of this is found on lines 333 to 377. These authors actually do not provide any evidence for skipper butterflies and hawk moths having greater metabolic rates, and these higher rates being responsible for longer probosces. They only point out from other studies that skippers have swift flight and presumably require greater energy to sustain these flights, and therefore they may benefit from feeding from nectar-rich deep flowers. The authors invoke a similar explanation for hawk moths. However, contrary to their claim, they neither synthesize available evidence into a useful comparison between the two groups, nor do they provide satisfactory data or argument in support this hypothesis. The phylogenetic distribution of long and normal probosces in several groups of skipper and other butterflies also does not seem to support their argument. For example, many skippers with robust thoraxes, extremely fast flight and therefore high energy requirements have normal-sized probosces and not long probosces (examples include skipper butterflies from subfamily Coeliadinae). On the contrary, *Eurybia*, studied by Bauder et al and mentioned in this manuscript, have relatively small bodies and presumably low energy demands, and they still have very long probosces. So the explanation given in Discussion for the evolution of long probosces in Hesperidae and hawk moths is not general and therefore satisfactory. There appear to be many exceptions. May be the explanation lies somewhere else, e.g., in the distribution of corolla tube depths among available flowers and probosces lengths in competitors in evergreen forests where these butterflies occur. It may be advisable for the authors to completely drop the entire paragraph in the discussion, which is the only weak part in this manuscript. They can perhaps tackle this in a separate paper where they specifically test the hypothesis concerning energy demands, nectar/food intake and proboscis morphology/length.

I have two more specific suggestions:

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- (1) In Table 1, move the column “Food Canal” before “Intake Rate” to keep all morphological variables together.
- (2) In Fig. 1c, it is more logical to have body size on the x axis and food canal area on the y axis.

6.4. Acknowledgements

First of all, I want to express my most sincere gratitude to my supervisor Harald Krenn for turning my interest from ecology to the fascinating realms of functional morphology, for all his support throughout the years and for making this project possible in the first place. As to that, I am greatly indebted to the Austrian Science Fund for funding the project during the first three years.

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I am very grateful to all of my co-authors: Many thanks to Andy Warren from the Florida Museum of Natural History for all of his time spent with looking at the rear parts of my skipper butterflies, and to Brian Metscher and Stephan Handschuh for introducing me into the world of Micro-CT as well as to Linde Morawetz for tricking the statistical computer package R into calculating suitable tests and models and Nora Lieskonig for her profound knowledge of proboscis bristles and merry dialogues while waiting for the butterflies in the shade of our favourite plants.

My transitory field assistant Martin Hepner deserves my deepest gratitude for the time spent with me during one of my field trips to Costa Rica. Without your commitment to my work, your elaborate skills in catching butterflies and, even more important, your inexhaustible patience waiting for them, none of this would have been possible or not that much fun.

Furthermore, I want to thank Kerstin Stejskal for her support regarding the production of graphical illustrations and for reformatting my thesis a thousand times. In addition, without the technical assistance of her and Nora Lieskonig in the laboratory, this thesis would be far from finished yet and the world record holder of proboscis length among butterflies still unknown.

Many thanks go out to John Plant, who corrected the English language of every single manuscript and grew never tired of smoothing and polishing the wording.

Konrad Fiedler deserves my gratitude for his valuable discussions, suggestions and for sharing some of his knowledge on the mysteries of statistics with me.

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I am very grateful to our head of the department Andreas Wanninger, who supported and accepted my divided occupation as technical assistant and PhD student ever since and gave me time for my thesis whenever it was short.

To the health of my colleagues and friends that went along with me and cheered up my time at the department! In no particular order: Joseph Gokcezade, Florian Karolyi, Ilja Bründl-Tschulenck, Norbert Milasowszky, Irene Zweimüller, Ursula Adelpoller, Andreas Wilhelmi, Daniela Bartel, Nikola Szucsich, Sarah Pfabigan, Martin Streinzer, Sonja Matuš, Gerald Timelthaler, Walter Lechner, Thomas Schwaha.

Cheers to my favourite non-biologist Johannes Pardatscher, who was always interested in my work, never tired of trying to understand what I was going on about and in doing so helped me, to organize and sort through the chaos in my mind and finally put it all in a nutshell. However, sadly, I think I failed to convince him that not all of my figures look alike and just comprise a disorganized cloud of dots.

To my parents, Hans and Linde: Thank you for all aspects of your support throughout the years and for making it possible to follow my interests!

Saving the best for last, to my beloved 'Hesprid-Mann, der die besten Hesprids fangen kann' Philipp, who accompanied und supported me all along. Thanks for sharing my interest in biology, for sweetening my time as a student during boring lectures, exploring rainforests together, the most creative techniques for catching butterflies, endless and fruitful conversations about various aspects of life in general, and last, but not least, for your patience, good nature and your unrivalled talent to radiate positive vibes even in the darkest hours.

6.5. Curriculum Vitae

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PERSONAL DATA

Born in Vienna, Austria on 6th of August, 1985
Austrian citizenship

RESEARCH INTERESTS

Morphology, ecology and evolution of flower-visiting insects, with special focus on
Lepidoptera

EDUCATION

2010	present	PhD study in Zoology at the University of Vienna, Austria (supervisor: Harald Krenn): Extremely long mouthparts in butterflies (Lepidoptera): form, function and evolution.
2009	2010	Study course of Genetics and Developmental Biology at the University of Vienna, Austria
2003	2008	Study course of Biology / Ecology at the University of Vienna, Austria Diploma thesis: Proboscis musculature in pollen feeding and non-pollen feeding Heliconiini (Lepidoptera: Nymphalidae)
1995	2003	High school (Klosterneuburg, Austria)

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PROFESSIONAL EXPERIENCES/FIELD TRIPS/RESEARCH VISITS

2014	present	Technical assistant, Department of Integrative Zoology, University of Vienna
2008	2013	Technical assistant, Department of Evolutionary Biology, University of Vienna
2008	2009	Collaborator in the project “Pollen feeding in butterflies” funded by the Austrian Science Fund (FWF) at the Department of Evolutionary Biology, University of Vienna
2007	2008	Collaborator in the project “Microbial communities” funded by the Austrian Science Fund (FWF) at the Department of Microbiology and Ecosystem Science, University of Vienna
2009	2013	Field trips to La Gamba, Costa Rica for the collection and measurements of Lepidoptera, behavioural observations of butterflies during flower visitation, feeding experiments with butterflies

TEACHING EXPERIENCE

2011	2013	Advisor in a Practical Lab Course on Animal Morphology at the Department of Evolutionary Biology, University of Vienna
2005	2007	Tutor in the course “Diversität der Höheren Pflanzen” at the Department of Biogeography, University of Vienna
2006	2007	Study assistant of a physically challenged student at the Department of Microbiology and Ecosystem Science, University of Vienna

LANGUAGE SKILLS

German (native), English (fluently), Spanish (basic)

AWARDS

2009 Sponsorship-award of the Dr. Schaumayer-Foundation,
received for diploma thesis

PUBLICATIONS

Peer reviewed journals

Bauder J. A. S., Warren A. D., Krenn H. W. (2014) Evolution of extreme proboscis lengths in Neotropical HesperIIDae (Lepidoptera). *The Journal of Research on the Lepidoptera* **47**: 65 - 71.

Bauder J. A. S., Handschuh S., Metscher B. D., Krenn H. W. (2013) Functional morphology of the feeding apparatus and evolution of proboscis length in metalmark butterflies (Lepidoptera: Riodinidae). *Biological Journal of the Linnean Society* **110**: 291 - 304.

Bauder J. A. S., Lieskonig N. R., Krenn H. W. (2011) The extremely long-tongued Neotropical butterfly *Eurybia lycisca* (Riodinidae): Proboscis morphology and flower handling. *Arthropod Structure & Development* **40**: 122 - 127.

Bauder J., Waitzbauer W., Lampropoulos M., Nemec A., Kainz M. & Scheiblhofer H. (2008) Auswirkungen der Beweidung im Nationalpark Neusiedler See-Seewinkel auf ausgewählte tagaktive Schmetterlingsgruppen (Lepidoptera). *Abhandlungen der Zoologisch-Botanischen Gesellschaft in Österreich* **37**: 261 - 291.

Books and book chapters

Bauder J. A.-S. & Krenn H. W. (2013) La Gamba – Ein Restaurant für Schmetterlinge. In: Roland A., Werner H., Pamperl S., Wanek W., Weber A. & Weissenhofer A. (eds) 20 Jahre Tropenstation La Gamba. *Verein zur Förderung der Tropenstation La Gamba, University of Vienna*: 80-81.

Conference contributions

Krenn H. W., Bauder J. A.-S. & Warren A. D. (2014) The world record holder in proboscis length of butterflies: the Neotropical skipper *Damas immaculata*. 7th

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International Conference on the Biology of Butterflies. Department of Biology, University of Turku. (poster)

Krenn H. W., Bauder J. A.-S., Karolyi F. & Gruber M. (2014) Extremely long mouthparts in flower-visiting insects: form, function and evolution. *Entomologia Austriaca* 21: 241. ÖEG Kolloquium, Universalmuseum Joanneum, Graz. (poster)

Krenn H. W., Bauder J. A.-S., Karolyi F. & Gruber M. (2013) Extremely long mouthparts in flower-visiting insects: form, function and evolution. 106th Annual Meeting of the German Zoological Society, Ludwig-Maxilian University, Munich. (abstract)

Bauder J. (2012) The feeding apparatus in metalmarks with different proboscis lengths (Lepidoptera: Riodinidae). *Entomologia Austriaca* 19: 49. ÖEG Kolloquium, Department of Tropical Biology and Animal Biodiversity, University of Vienna. (talk)

Bauder J. (2011) Der extrem langrüsselige Tagfalter *Eurybia lycisca* (Riodinidae): Rüsselmorphologie und Blütenbesuch. *Entomologia Austriaca*: 18:147. ÖEG Kolloquium, Institute of Zoology, Karl-Franzens University, Graz. (talk)

Bauder J. A.-S. & Krenn H. W. (2011) Die außergewöhnlichen Mundwerkzeuge der Tagfalter La Gambas. Annual Meeting of the Tropical Research Station La Gamba, Department of Tropical Biology and Animal Biodiversity, University of Vienna. (talk)

Bauder J. A.-S., Lieskonig N. & Krenn H. W. (2010) Extremely long proboscis in the Neotropical butterfly *Eurybia lycisca* (Riodinidae): morphology and flower-visiting behavior. Program & Abstracts: 27. 6th International Conference on the Biology of Butterflies, Department of Biological Sciences, University of Alberta. (poster)

Bauder J. & Krenn H. W. (2009) Muskelanordnung im Rüssel von pollenfressenden und nektarsaugenden Heliconiini (Lepidoptera: Nymphalidae). *Entomologia Austriaca* 16: 159-160. ÖEG Kolloquium, Institute of Zoology, Karl-Franzens University, Graz. (talk)