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Fluid uptake by the lepidopteran proboscis in relation to structure

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FLUID UPTAKE BY THE LEPIDOPTERAN PROBOSCIS
IN RELATION TO STRUCTURE

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Entomology

by
Karena Jia Kwauk
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Dr. Peter Adler, Committee Chair
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Dr. David Tonkyn

ABSTRACT

Three species of butterflies—eastern tiger swallowtail (*Papilio glaucus* L.), monarch (*Danaus plexippus* L.), and painted lady (*Vanessa cardui* L.)—were used to investigate the effect that exposing multiple regions of the proboscis to fluid has on feeding rate. Although the lepidopteran proboscis historically has been considered a sealed tube with a specialized region near the tip for fluid uptake, droplets of 1% sucrose solution were shown to enter multiple locations along the proboscis. Feeding experiments were conducted comparing fluid uptake rates of butterflies with their proboscises either fully or partially submerged in 1% or 15% sucrose solutions. Only one significant relationship was found. Male tiger swallowtails exhibited a faster feeding rate with the 1% sucrose solution than did females, suggesting that within nectar-feeding species with males that also exhibit puddling behavior, males are capable of faster fluid uptake than females. No significant differences were found for feeding rate in relation to the amount of the proboscis exposed to fluid. Instead, interactions among butterfly size (proboscis length, forewing length, and possibly sucking pump size), gender, and the amount of proboscis exposed to fluid compared to feeding rate should be investigated. Further examination of these variables may provide valuable insights into the relation between fluid uptake of the lepidopteran proboscis and fitness.

DEDICATION

I would like to dedicate this thesis first and foremost to my parents who have supported me through all of my educational endeavors, both emotionally and financially; my sister for teaching me the rewards of perseverance; and Michael Bond for being my center and keeping me sane through it all. I would also like to dedicate this to Marianne Dotson for teaching me discipline, motivation, and to embrace my passions with open arms.

ACKNOWLEDGEMENTS

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INTRODUCTION

Adult Lepidoptera feed from a wide spectrum of food sources including floral nectar, ripe and rotting fruit, puddles, dung, sweat, and even blood (Arms et al., 1974; Adler, 1982; Lederhouse et al., 1990; Smedley & Eisner, 1995; Büttiker et al., 1996; Beck et al., 1999; Boggs & Dau, 2004; Krenn et al., 2001; Molleman et al., 2005; Krenn, 2008, 2010). To feed efficiently from these sources, the proboscis must be engineered in a particular structural fashion. Morphological adaptations involving the proboscis can be seen throughout the Lepidoptera, aiding the efficient acquisition of nutrients and water (Molleman et al., 2005; Krenn, 2008).

Proboscis morphology has been associated with feeding behavior, such as nectar feeding, pollen feeding, surface sweeping, and skin piercing (Büttiker et al., 1996; Molleman et al., 2005; Krenn, 2010). An important component of proboscis morphology is the relative length of the tip-region, which is the distal portion of the proboscis with dorsal "drinking" slits that open to the food canal (Krenn, 1998, 2008; Krenn et al., 2001; Molleman et al., 2005). The drinking region of non-nectarivorous nymphalid butterflies is twice as long as it is in the nectarivorous species (Krenn et al., 2001). When feeding from flowers, only a limited portion of the proboscis has access to the nectar. To successfully take up fluid, the entire drinking region putatively needs to be immersed in the fluid (Krenn et al., 2001). Thus, nectar feeders would require a short drinking region so they could still feed on flowers with limited nectar. When feeding from a more open source, such as rotting fruit, a larger area of fluid is available to accommodate a greater portion

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of the proboscis (Krenn et al., 2001). Those species with extended drinking regions would be able to take advantage of these food sources.

Another key component of feeding behavior is the structural modification of the proboscis, particularly around the drinking region. Sensilla styloconica are sensory structures mainly concentrated around the drinking region of the proboscis. These sensilla vary in shape, size, and number, depending on species (Krenn et al., 2001; Krenn, 2010). In nectar-feeding species, the sensilla are relatively small, so the proboscis tip can maintain a slim, tapered shape conducive for flower probing (Krenn et al., 2001). Non-nectar-feeding nymphalids have longer and more sensilla styloconica around the drinking region than do the nectar-feeding species (Krenn et al., 2001). In some species, this area is so densely packed with elongated sensilla that the drinking region has been described as a flat brush (Knopp & Krenn, 2003; Molleman et al., 2005; Krenn, 2010). This brush-like tip of the proboscis is thought to help accumulate fluid while feeding from open surfaces, allowing for more efficient feeding (Krenn et al., 2001; Knopp & Krenn, 2003; Krenn, 2010).

The proboscis is putatively a sealed tube with a designated region near the tip for fluid uptake (Krenn et al., 2001, 2004, 2005; Krenn, 2010). However, some Lepidoptera exhibit water intake along the entire proboscis, rather than only in the drinking region (Monaenkova et al., 2011, Grant et al., In press). If fluid uptake is possible in multiple locations along the proboscis, the individual could potentially increase its feeding rate, minimizing the time spent feeding. Less time devoted to feeding permits more time for other activities critical to fitness such as finding mates, ovipositing, and defending

territories (Rutowski, 1982). In many species, males puddle, in addition to feeding from flowers, to obtain extra nutrients or minerals (Arms et al., 1974; Adler & Pearson, 1982; Lederhouse et al., 1990; Smedley & Eisner, 1995; Sculley & Boggs, 1996; Beck et al., 1999; Boggs & Dau, 2004; Molleman et al., 2005; Krenn, 2010). To maximize fluid uptake from puddles, would males have greater uptake potential along the proboscis than females of the same species? For many species, feeding on substances such as tree sap, rotting fruit, and dung is the primary means of acquiring nutrients and water. Would the proboscis of tree-sap, rotting-fruit and dung feeders be more versatile in fluid uptake compared with that of a nectar feeder? My research is intended to address these questions and further our knowledge of the structure and function of the proboscis in understanding the diverse feeding patterns and behaviors of adult Lepidoptera.

<center>Objectives</center>

My overall goal is to develop a better understanding of the feeding behaviors in glossatan Lepidoptera through insights into the structure and function of the proboscis. My specific objectives were to determine 1) where fluid uptake is possible in the lepidopteran proboscis, 2) if fluid uptake varies along the proboscis between males and females of the same species, and 3) if feeding habits are related to fluid uptake capabilities of the proboscis. I used a comparative approach including species with different proboscis morphologies: smooth proboscis with a short drinking region (9% of total proboscis length), smooth proboscis with a long drinking region (15% of total proboscis length), and a brush-like proboscis with a short drinking region (6% of total proboscis length) (Figure 1).

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<u>Research Hypotheses</u>

1) Fluid uptake is possible in multiple regions of the proboscis.

2) The rate of fluid uptake is faster when more of the proboscis is exposed to the fluid.

3) Fluid uptake is faster for males than for females in species that exhibit puddling behavior.

LITERATURE REVIEW

Adult lepidopterans use the proboscis to feed on a liquid diet. The proboscis is made up of two elongated maxillary galeae linked together dorsally and ventrally by cuticular processes called legulae. The legulae originally were considered to form an air-tight and fluid-tight central food canal opening along the distal tip where the dorsal legulae are spaced farther apart (referred to hereafter as the "drinking region"), essentially functioning as a straw operated by a sucking pump in the head (Krenn, 1998, 2008, 2010; Krenn & Kristensen, 2000, 2004; Krenn et al., 2001; Krenn & Mühlberger, 2002; Molleman et al., 2005). A more recent study demonstrated that the legulae have spaces of varying sizes between them along the length, allowing the proboscis to function like a sponge and a straw (Monaenkova et al., 2011). Mechano- and chemoreceptors are located along the proboscis: sensilla trichodea, sensilla basiconica, and sensilla styloconica (Krenn, 1998). The sensilla styloconica are concentrated mainly around the drinking region and display a variety of number, shapes, and sizes that correlate with feeding behavior (Krenn et al., 2001, Molleman et al., 2005; Krenn, 2010; Zenker et al., 2011).

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The elongated proboscis originally was adapted for imbibing floral nectar, and it has evolved a multitude of ways to feed from other sources (Krenn & Penz, 1998; Knopp & Krenn, 2003; Molleman et al., 2005; Krenn, 2008). With the aid of modified cuticular microstructures along the proboscis, adult Lepidoptera are capable of feeding from sources such as ripe and rotting fruit, tree sap, pollen, mud puddles (puddling), dung, lachrymal secretions, and blood (Arms et al., 1974; Adler, 1982; Lederhouse et al., 1990; Büttiker et al., 1996; Beck et al., 1999; Krenn et al., 2001; Boggs & Dau, 2004; Molleman et al., 2005; Krenn, 2008, 2010). The first food source exploited by the elongated proboscis of butterflies was floral nectar (Krenn et al., 2001; Molleman et al., 2005). This feeding behavior has selected for a slender, tapered proboscis to access the nectar (Krenn, 1990; Büttiker et al., 1996; Krenn et al., 2001; Molleman et al., 2005). Within the Nymphalidae, the sensilla styloconica are fewer in number in nectar-feeders than in non-nectar feeders and the drinking region is shorter (Krenn et al., 2001).

Puddling is a feeding behavior exhibited most commonly by males (Arms et al., 1974; Adler, 1982; Adler & Pearson, 1982; Lederhouse et al., 1990; Smedley & Eisner, 1995; Büttiker et al., 1996; Sculley & Boggs, 1996; Beck et al., 1999; Boggs & Dau, 2004; Molleman et al., 2005; Krenn, 2010). Males frequent mud puddles to supplement their sodium and amino acid levels, which are important for neuromuscular function and, in many species, serve as nuptial gifts (Arms et al., 1974; Adler, 1982; Adler & Pearson, 1982; Smedley & Eisner, 1995; Beck et al., 1999; Scriber, 2002; Boggs & Dau, 2004). For nectar feeders especially, sodium and amino acids are available in limited amounts (Hiebert & Calder, 1983). Puddling males will include these extra nutrients and minerals

in the spermatophores given to the females at mating (Smedley & Eisner, 1996). One study showed this addition of amino acids increased the proportion of fertile eggs (Molleman et al., 2008). A few cases have been documented of females puddling (Scriber, 2002), but these mainly consisted of older females with exhausted spermatophores (Lederhouse et al., 1990; Boggs & Dau, 2004).

In species that feed on rotting fruit, the proboscis is described as "brush-like" because the sensilla styloconica are elongated and more numerous than in nectar feeders (Krenn et al., 2001; Knopp & Krenn, 2003; Krenn, 2010). These densely packed sensilla around the larger drinking region allow the individual to efficiently imbibe fluids from wet surfaces (Knopp & Krenn, 2003). The flexible drinking region is also capable of conforming to irregular shapes (Krenn et al., 2001).

Other sources of nutrients that have become available to Lepidoptera through modifications of proboscis microstructure include pollen, lachrymal secretions, and blood. Pollen contains amino acids required by some species for egg production and nuptial gifts (O'Brien et al., 2003; Eberhard et al., 2007). The proboscises of pollen-feeding species are longer than those of non-pollen-feeding species, and have numerous long sensilla trichodea on the proximal region of the proboscis (Krenn & Penz, 1998). The sensilla trichodea hold the pollen bundles in place, which allow the proboscis to agitate the pollen in protease-filled saliva, causing the release of the amino acids through germination pores (Krenn & Penz, 1998; Eberhard et al., 2007; Luo et al., 2011). These pollen-feeding species can access the pollen contents only through the germination pores; they are not capable of breaking down the pollen walls that consist of sporopollenin, a

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complex polymer of fatty acids and phenolic compounds (Luo et al., 2011). Two species of moths (Gelechiidae), however, are capable of breaking down the sporopollenin, using an unknown chemical secreted from the proboscis (Luo et al., 2011).

In species that feed on lachrymal secretions, the proboscis is adapted for accessing the surface of the eye and increasing tear production. The sensilla styloconica and the dorsal legulae around the drinking region are elongated and serrated (Büttiker et al., 1996; Hilgartner et al., 2007; Krenn, 2010). Even when the host's eye is closed, this flexible tip-region can insert between the eye and eyelid, and between the eye and nictitating membrane of birds, irritate the eye surface and conjunctival sac, and imbibe the proteinaceous secretions (Bänziger, 1989a; Büttiker et al., 1996; Hilgartner et al., 2007; Krenn, 2010). The proboscis of blood-feeding Lepidoptera is shorter and more robust than those of other feeding types, and is equipped with short sensilla styloconica modified into tearing hooks (Bänziger, 1989a,b; Krenn & Penz, 1998; Zaspel et al., 2007; Krenn, 2010). These hooks provide resistance as the proboscis pierces into the flesh (Krenn, 2010).

Proboscis structure, thus, plays a crucial role in determining the dietary sources of nutrients and minerals for adult Lepidoptera. A more detailed understanding of the basic structure of the proboscis could provide new insights into the relation between structure and feeding behavior.

MATERIALS AND METHODS

Colony Maintenance

Three species of butterflies were chosen for their different proboscis structure, such as drinking region lengths and sensilla sizes: monarch (*Danaus plexippus* L.), eastern tiger swallowtail (*Papilio glaucus* L.), and painted lady (*Vanessa cardui* L.) (Figure 1).
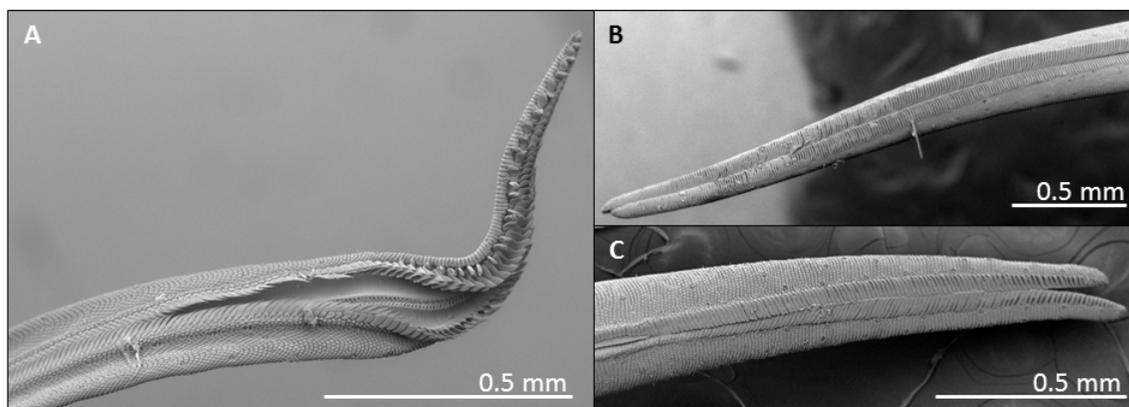
Figure 1. Scanning electron micrographs of proboscis tips, demonstrating different drinking region architecture: (**A**) short drinking region with brush-like tip (*Vanessa cardui*), (**B**) long drinking region with smooth tip (*Papilio glaucus*), and (**C**) short drinking region with smooth tip (*Danaus plexippus*). The straightened proboscis attached to the head was examined for each species. Each specimen was dehydrated through a series of ethanol washes (80-100%, 24 h each), followed by chemical drying using hexamethyldisilazane. Dried heads were mounted on carbon-graphite tape affixed to aluminum stubs, and sputter-coated with gold or platinum for 1-3 minutes at full or partial vacuum and imaged with a Hitachi TM3000 tabletop scanning electron microscope (SEM) (15 or analytical kv).

Female tiger swallowtails were wild-caught and placed in screened tents (BioQuip) with tulip poplar (*Liriodendron tulipifera* L.) for oviposition (Bossart & Scriber, 1995; Lehnert & Scriber, 2012). Tents were 61 cm^3 with two panels of clear vinyl and two panels of 104 x 66 mesh/cm white polyester netting. Tiger swallowtail larvae were placed in Rubbermaid TakeAlongs® with tulip poplar leaves that were

replaced every 1-2 days. Tiger swallowtail pupae also were obtained from Ward's Natural Science (Rochester, New York). Pupae were collected from the containers and hung in screened tents (BioQuip) by hot-gluing the pupal cremaster to toothpicks inserted into a piece of styrofoam oriented vertically. All pupae were kept in this fashion. Monarch pupae were obtained from the Shady Oak Butterfly Farm (Brooker, Florida). Adults were hand-paired (Clarke & Sheppard, 1956) and females were placed in a screened tent (BioQuip) with tropical milkweed (*Asclepias curassavica* L.) for oviposition. Monarch larvae were placed in the same kind of screened tents (BioQuip) as for ovipositing females, with tropical milkweed or common milkweed (*Asclepias syriaca* L.). Tents were kept in a mesh-enclosed butterfly garden in the summer of 2011. Tents with larvae reared after October 2011 were kept in the same rearing chamber as for ovipositing females. Painted lady eggs were obtained from the Carolina Biological Supply Company (Burlington, North Carolina). Eggs were divided into 29.6-mL Solo$^®$ graduated soufflé cups with artificial diet. Adults used for mating were fed a pooled honey-water solution with a sugar concentration of approximately 20% once daily. All butterflies (eggs, larvae, and adults) were reared in a chamber with a 16:8 light:dark cycle at 28°C ± 3°C and relative humidity of 60-70% unless otherwise stated.

<div align="center">Droplet Experiments</div>

All experiments were conducted using unfed adults to ensure the proboscis was free of food residues. Upon emergence, each butterfly was placed in a glassine envelope and stored at 4°C for 24-72 hours. Butterflies then were secured to a styrofoam board, using styrofoam strips and insect pins over the spread wings. Droplet experiments were

run in the lab at 25°C ± 1°C. Spread butterflies were held in the lab for 10 minutes to increase body temperature.

The proboscis was straightened and secured with insect pins crisscrossed over it (Figure 2A). A 5-µL syringe was used to dispense a 25-nL droplet of 1% sucrose solution on the proximal end of the proboscis halfway between the head and the knee bend region and halfway between the knee bend and the drinking region. Order of droplet placement was randomized for each individual. A control droplet was placed on a microscope slide next to the proboscis to determine if the droplet was entering the proboscis or evaporating. Each butterfly was given 2 minutes to imbibe the droplet at each location. After 2 minutes, each droplet was scored as imbibed or not to determine if fluid uptake is possible in locations of the proboscis other than the drinking region. Trials were video recorded using a Jenoptik ProgRes® camera mounted on a Meiji Techno Stereo Microscope. Sample size was 10 live adults for each species, unless otherwise stated, with gender chosen at random.

In addition, one trial for each species was conducted using a dead butterfly to determine if the droplet of 1% sucrose solution enters these same locations passively. The head was removed from a live adult and an insect pin was inserted through the head to disable the sucking pump. Trials with dead adults were done immediately after disabling the sucking pump to avoid distortion of the galeae due to desiccation.
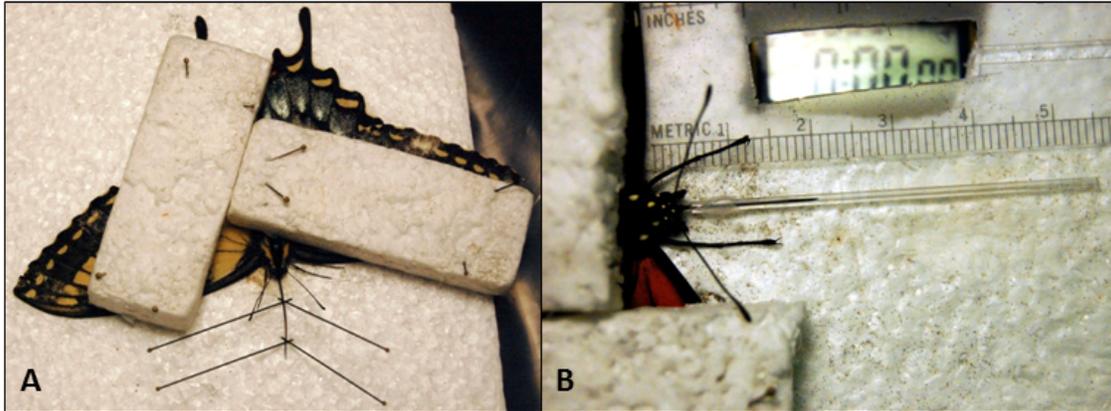
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Figure 2. (**A**) Droplet experiment with the straightened proboscis of *Papilio glaucus* secured by crisscrossed insect pins. (**B**) Feeding experiment with proboscis of *Danaus plexippus* in capillary tube.

<u>Feeding Experiments</u>

All trials were run in a rearing chamber at 28°C ± 3°C and relative humidity of 60-70%. Sucrose solutions were made using pure cane granulated sugar mixed with distilled water. Sucrose concentrations of 1% and 15% were verified using a pocket refractometer (Bellingham & Stanley Ltd.); 1% solutions were used because butterflies would not drink when presented with plain distilled water.

Upon emergence, butterflies were placed in glassine envelopes and spread on a styrofoam board, similar to the procedure in the droplet experiments. Spread butterflies were placed in the rearing chamber for 10 minutes to increase body temperature. For feeding trials, the proboscis was manually uncoiled and directed with an insect pin into a capillary tube adjacent to a metric ruler (Figure 2B). To ensure the fluid emptied from the distal end of the capillary tube, the head-end of the capillary tube was covered by a layer of Parafilm® with a hole made by a size 0 insect pin to insert the proboscis. Initially, trials were conducted with the proboscis completely inserted into the capillary tube or with the

proboscis inserted to the knee bend region. When the proboscis was fully inserted into the capillary tube, the tube appeared to pinch the base of the proboscis, possibly affecting fluid uptake. To avoid this potential problem, full submersion trials were conducted with 2 mm between the head and the end of the capillary tube. To test differences in fluid uptake rates along the proboscis, partial submersion trials were conducted with the proboscis inserted 4 mm into the capillary tube, submerging the drinking region of the proboscis.

Trials were conducted using 20-μL capillary tubes shortened to 5 cm. Using a 1-mL syringe, I injected 8 μL of sucrose solution into each capillary tube, immediately removing the syringe after injection. Each drinking butterfly was timed with a stopwatch until the capillary tube was empty. Trials were video recorded using a Jenoptik ProgRes® camera. Butterflies and capillary tubes were used only once. Forewing length and proboscis length were measured for each butterfly to determine if body size correlated with fluid uptake rate. Sample size was 10 adults per sex per treatment for each species, unless otherwise stated.

<u>Field Observations</u>

Butterflies were observed feeding on lantana (*Lantana camara* L.) and butterfly bush (*Buddleia davidii* Franch.) around the Cherry Farm Insectary, Clemson, SC, from 30 May 2012 to 13 June 2012. The species of butterfly was recorded as well as the percent of the proboscis inserted into the flower. Flowers from the plants were collected to measure the length of the corolla.

The rate of fluid uptake (µL/second) was calculated by using the time each butterfly took to drink 3.2 µL of sucrose solution. Mean rates were compared for males and females within a species for partial versus full submersion. Statistical analyses were done using JMP$^©$ v.10, Minitab 15 Statistical Software$^©$, and SAS$^©$ v.9.2. Two-way ANOVAs were performed to determine if gender and extent of proboscis submersion had a significant effect on fluid uptake rate for the two sucrose concentrations within each species. Proboscis and forewing length measurements were used to determine if size was a covariate for the impact of gender and extent of proboscis submersion on fluid uptake rate.

A series of Pearson's correlations were run to determine the relations between proboscis length and forewing length within each species, proboscis length or forewing length and fluid uptake rate, and proboscis movements and the time taken to imbibe droplets. T-tests were used to compare the means for times for droplet entry between genders and between locations (proximal versus distal to the knee bend), the amounts of time taken to start feeding between males and females, forewing or proboscis lengths between males and females, and the time for droplet entry between butterflies with and without proboscis movements.

RESULTS

Droplet Experiments

All droplets placed along the proboscis entered the proboscis, including passively, for each species (painted lady n=6 live, 1 dead; monarch n=10 live, 1 dead; tiger

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swallowtail n=5 live, 1 dead). No statistical differences in amount of time taken for the droplet to enter the proboscis could be determined between live and dead butterflies due to the small sample size of dead individuals. For painted ladies and monarchs, droplet entry was faster for live individuals than dead. However, the solution entered the proboscis of tiger swallowtails faster when they were dead than alive (Table 1). No gender differences in the average time to imbibe the droplets were found for live painted ladies or monarchs (painted lady: df = 7, t = 1.08, P = 0.32; monarch: df = 14, t = 2.14, P = 0.05). No location differences (proximal versus distal to the knee bend) in the average time to imbibe the droplets were found for any of the live species (painted lady: df = 6, t = -0.77, P = 0.47; monarch: df = 15, t = 1.16, P = 0.27; tiger swallowtail: df = 7, t = 1.74, P = 0.13). The size of the control droplet remained unchanged during the time taken for the droplets to enter the proboscis.

For 33% of the trials, two species displayed distinctive proboscis movements while the droplet was being imbibed. Both male and female monarchs displayed anti-parallel movements of the galeae and alternate pulsing of individual galea. Anti-parallel movements were characterized by the galeae sliding laterally alongside each other in opposite directions. Pulsing involved alternating contractions of each galea and no anti-parallel movements. Within the monarchs, the number of proboscis movements was not significantly correlated (r = 0.28; P = 0.21) with time taken to imbibe the droplet. Only anti-parallel movements occurred for painted ladies. No proboscis movements were observed during trials with tiger swallowtails. No significant differences were found between the presence or absence of proboscis movements and the time taken to imbibe

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the droplet (monarch: df = 18, t = -0.61, P = 0.55; painted lady: df = 5, t = -0.63, P = 0.56).

Table 1. Time (s) to imbibe a 25-nl droplet of 1% sucrose solution for three butterfly species; males and females were combined. Sample sizes given in parentheses. Droplets were placed between the head and the knee bend region (proximal) and between the knee bend region and the drinking region (distal).

		Location	Time to imbibe
Painted lady	Alive (6)	Proximal	13.3 ± 1.94
		Distal	16.3 ± 2.07
	Dead (1)	Proximal	20.0
		Distal	24.6
Tiger swallowtail	Alive (5)	Proximal	26.0 ± 2.26
		Distal	19.6 ± 2.12
	Dead (1)	Proximal	13.6
		Distal	18.5
Monarch	Alive (10)	Proximal	17.4 ± 1.94
		Distal	13.1 ± 1.82
	Dead (1)	Proximal	22.6
		Distal	17.4

<u>Feeding Experiments</u>

Overall, males had a faster fluid uptake rate than did females for both sucrose solutions, although the difference was significant in only one case (Table 2). Painted ladies showed no significant difference between genders (1% sucrose: $F_{1,36}$ = 0.12, P = 0.73; 15% sucrose: $F_{1,37}$ = 0.04, P = 0.84) or the extent (length) of the proboscis submerged (1% sucrose: $F_{1,36}$ = 4.11, P = 0.05; 15% sucrose: $F_{1,37}$ = 0.8, P = 0.38) (Figure 3). The interaction between gender and length submerged was not significant (1% sucrose: $F_{1,36}$ = 1.26, P = 0.27; 15% sucrose: $F_{1,37}$ = 1.76, P = 0.19).

For tiger swallowtails, gender was significant for fluid uptake rate, but only with the 1% sucrose ($F_{1,28}$ = 9.37, P = 0.005; 15% sucrose: $F_{1,19}$ = 0.45, P = 0.511) (Figure 4). There was no significant difference between length of proboscis submerged (1% sucrose: $F_{1,28}$ = 0.59, P = 0.45; 15% sucrose: $F_{1,19}$ = 0.14, P = 0.71), and the interaction between

gender and length submerged was not significant (1% sucrose: $F_{1,28} = 1.07$, P = 0.31; 15% sucrose: $F_{1,19} = 0.02$, P = 0.89).

Feeding trials with the monarchs were similar to those for the painted ladies. Gender was not significant for fluid uptake rate (1% sucrose: $F_{1,25} = 1.64$, P = 0.21; 15% sucrose: $F_{1,28} = 0.41$, P = 0.53), nor was length of proboscis submerged (1% sucrose: $F_{1,25} = 0.24$, P = 0.63; 15% sucrose: $F_{1,28} = 3.38$, P = 0.08) (Figure 5). The interaction between gender and length submerged was not significant (1% sucrose: $F_{1,25} = 0.50$, P = 0.49; 15% sucrose: $F_{1,28} = 0.46$, P = 0.50).

Trials of monarchs with fully submerged proboscises were analyzed to determine if there was a relationship between gender and the amount of time taken to start feeding for the two sucrose solutions. Males took similar amounts of time to start feeding for both sucrose concentrations (1%: 1.62 ± 0.87 seconds; 15%: 2.27 ± 0.96 seconds) (P = 0.41), whereas females took slightly longer to start feeding on the 1% sucrose solution than on the 15% solution (1%: 4.51 ± 2.11 seconds; 15%: 1.07 ± 0.28 seconds) (P = 0.06).

Forewing length was compared between sexes within each species. No significant difference was found in forewing lengths between sexes except painted ladies in which females on average were larger than males (painted lady: df = 78, t = 2.36, P = 0.02; monarch: df = 52, t = 0.08, P = 0.93; tiger swallowtail: df = 39, t = 1.38, P = 0.18). Average proboscis length also was compared between sexes within each species, and no significant differences were found (painted lady: df = 70, t = 0.60, P = 0.55; monarch: df = 53, t = -0.12, P = 0.90; tiger swallowtail: df = 47, t = -0.08, P = 0.93). Forewing lengths and proboscis lengths were evaluated to determine if proboscis length is a proxy for

butterfly size. Males and females were combined for monarchs and tiger swallowtails, but analyzed separately for painted ladies. A correlation was present for monarchs and painted ladies (monarch: $r = 0.74$, P < 0.0001; painted lady: males $r = 0.93$, P < 0.0001, females $r = 0.86$, P < 0.0001), but not for tiger swallowtails ($r = 0.01$, P = 0.94). No correlation was found between size (proboscis length) and fluid uptake rate for monarchs (1% sucrose solution: $r = 0.30$, P = 0.11; 15% sucrose solution: $r = 0.14$, P = 0.48). Painted lady size was correlated with fluid uptake rate, but only with the 1% sucrose ($r = 0.62$, P < 0.0001; 15% sucrose: $r = 0.10$, P = 0.48).

Tiger swallowtail uptake rates were compared between trials with the proboscis inserted fully into the capillary tube versus inserted with 2 mm between the head and the capillary tube, using 15% sucrose solution to determine if possible pinching by the capillary tube would affect uptake rates. Comparisons within males and females resulted in faster rates with the 2 mm space, but not significantly faster (males: df = 8, $t = 1.72$, P = 0.12; females: df = 4, $t = 1.24$, P = 0.28).

Table 2. Average fluid uptake rate (µL/second) for three butterfly species. Fluid uptake rates were calculated according to the length of proboscis inserted into a capillary tube (full, partial) for each aqueous sucrose solution (1%, 15%). Sample size given in parentheses.

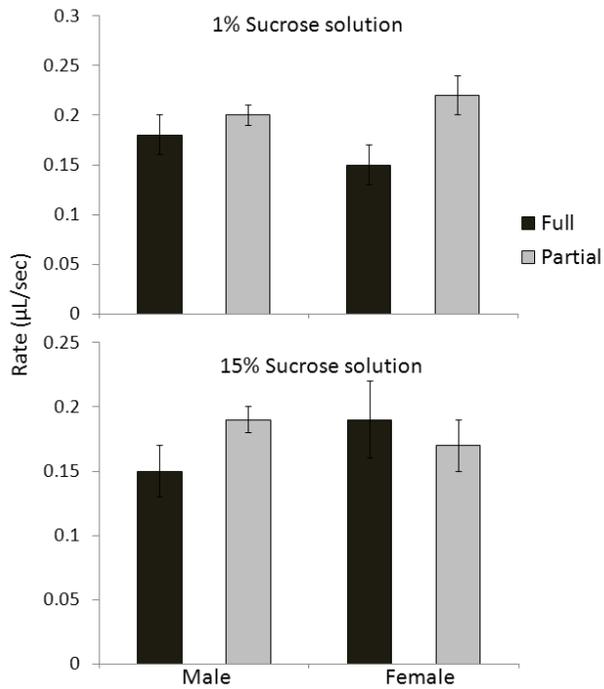|		1%		15%	
		Full	Partial	Full	Partial
Vanessa cardui	Male	0.18 ± 0.02 (10)	0.20 ± 0.01 (10)	0.15 ± 0.02 (10)	0.19 ± 0.01 (10)
	Female	0.15 ± 0.02 (10)	0.22 ± 0.02 (10)	0.19 ± 0.03 (10)	0.17 ± 0.02 (10)
Papilio glaucus	Male	0.93 ± 0.05 (8)	0.91 ± 0.06 (8)	0.71 ± 0.20 (8)	0.71 ± 0.10 (8)
	Female	0.66 ± 0.10 (8)	0.79 ± 0.09 (8)	0.64 ± 0.18 (4)	0.54 ± 0.13 (3)
Danaus plexippus	Male	0.31 ± 0.04 (9)	0.26 ± 0.02 (8)	0.36 ± 0.05 (8)	0.46 ± 0.02 (8)
	Female	0.23 ± 0.05 (5)	0.24 ± 0.04 (7)	0.36 ± 0.04 (8)	0.41 ± 0.04 (8)

Figure 3. Feeding treatments (full versus partial submersion of proboscis in a capillary tube) per sucrose concentration for *Vanessa cardui*. No significant differences (ANOVA, $p > 0.05$) were found between treatments per concentration. Trials were run at 28°C ± 3°C.
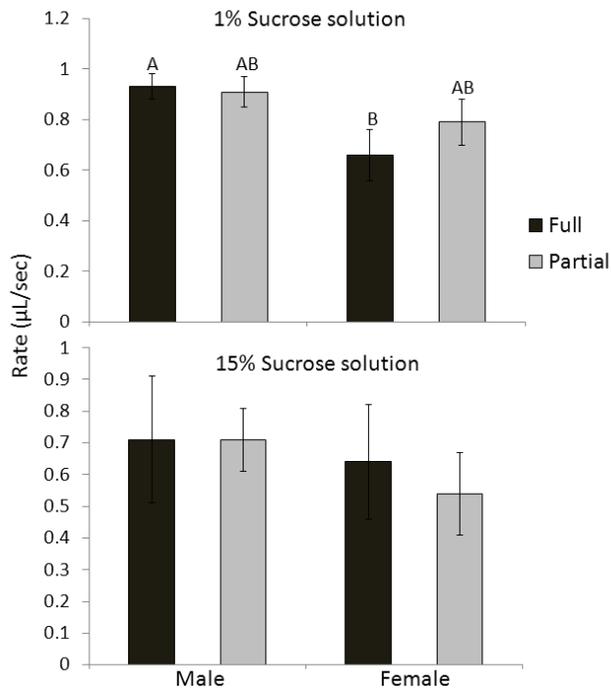
Figure 4. Feeding treatments (full versus partial submersion of proboscis in a capillary tube) per sucrose solution for *Papilio glaucus*. Significant differences (ANOVA, $p < 0.05$) were found among treatments using 1% sucrose solution. Trials were run at 28°C ± 3°C.
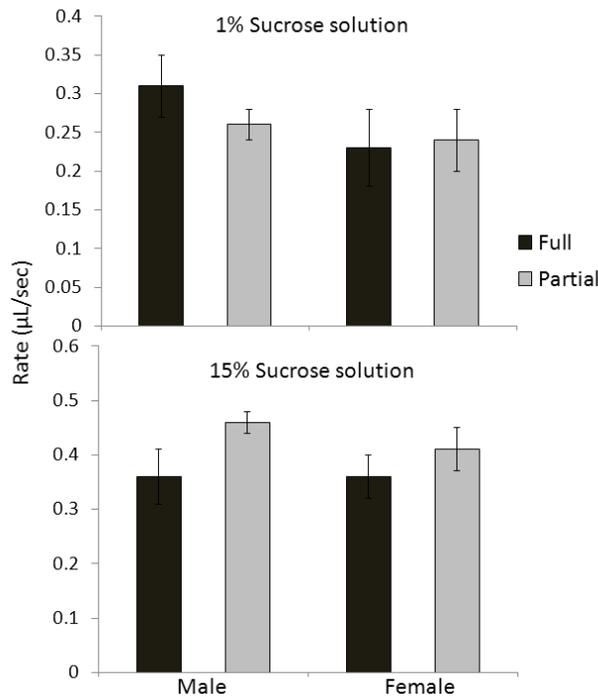
Figure 5. Feeding treatments (full versus partial submersion of proboscis in a capillary tube) per sucrose solution for *Danaus plexippus*. No significant differences (ANOVA, p > 0.05) were found between treatments per concentration. Trials were run at 28°C ± 3°C.

Field Observations

The average corolla lengths for butterfly bush and lantana were 1 cm (n=6 for each). Three species were observed feeding on butterfly bush: tiger swallowtail, silver-spotted skipper (*Epargyreus clarus* Cramer), and great spangled fritillary (*Speyeria cybele* Fabricius); and four species were observed feeding on lantana: painted lady, common buckeye (*Junonia coenia* Hübner), variegated fritillary (*Euptoieta claudia* Cramer), and Delaware skipper (*Anatrytone logan* W.H. Edwards) (Table 3). All butterflies inserted more than half of the proboscis into the corolla. Butterflies feeding on lantana inserted about 25%, on average, more of the proboscis into the corolla than did butterflies feeding on butterfly bush.

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Table 3. Field observations of percent proboscis length inserted in flowers by 7 butterfly species in Clemson, SC, May-June 2012.

Lepidopteran species (n)	Plant species	Approximate proboscis length (cm)	% proboscis inserted
Eastern tiger swallowtail (*Papilio glaucus*) (1)	*Buddleia davidii*	2.0	65
Silver-spotted skipper (*Epargyreus clarus*) (1)	*Buddleia davidii*	1.6	75
Great spangled fritillary (*Speyeria cybele*) (1)	*Buddleia davidii*	1.8	65
Painted Lady (*Vanessa cardui*) (6)	*Lantana camara*	1.5	81.6 ± 3.57
Common buckeye (*Junonia coenia*) (7)	*Lantana camara*	1.2	92.8 ± 3.59
Variegated fritillary (*Euptoieta claudia*) (4)	*Lantana camara*	1.3	86.6 ± 7.26
Delaware skipper (*Anatrytone logan*) (3)	*Lantana camara*	1.5	100

DISCUSSION

In the field, all observed butterflies inserted more than 65% of their proboscis into the flower's corolla, suggesting that a length of the proboscis was inserted into the corolla greater than the length of the corolla itself. The proboscis tip, therefore, must curl back on itself at the base of the corolla. The diameter of the lantana corolla is larger than that of the butterfly bush, allowing more space for the proboscis to curl, in turn allowing butterflies to insert their entire proboscis into the corolla. If so, more of the proboscis potentially would be exposed to the nectar, providing an opportunity for fluid uptake in locations other than just the drinking region. Further studies should be conducted, perhaps using a transparent, artificial flower, to see the behavior of the proboscis while inserted in the corolla.

The droplet experiments indicate the proboscis is not an air-tight or fluid-tight tube as previously described (Borrell & Krenn, 2006; Krenn, 2010). Instead, the butterfly has control over the permeability along the proboscis. The painted lady provides an example because the proboscis cuticle is more transparent than in the other two species. After

placing the droplet on the proboscis, liquid bridges can be seen moving through the proboscis directly under the droplet. These liquid bridges are most likely saliva because no fluid was introduced to the drinking region. In this situation, the butterfly might be actively creating a fluid-tight tube through muscles in the galeae. When the droplets are imbibed, the butterfly presumably can change the pore sizes between the dorsal legulae by relaxing the galea muscles or through proboscis activities (anti-parallel movements and pulsing of individual galea). Also, the sucking pump could assist droplet movements, explaining the faster droplet movement into the proboscis with live painted ladies and monarchs, compared with dead individuals. For the dead individuals of these species, the passive movement of the droplet could occur because an active seal is no longer present. Movement of droplets into the proboscis is slower than in live individuals perhaps because the pore sizes between the dorsal legulae cannot change through proboscis activities and the sucking pump is no longer active. No proboscis activity was seen with the live tiger swallowtails, suggesting that the droplet movement was entirely accomplished through the active use of the sucking pump and only a slight release of the seal. This would result in slower droplet movement than for a dead individual with a disengaged seal. This experiment should be repeated while observing the proboscis at a finer scale to determine how anti-parallel movements, pulsing, and the sucking pump affect droplet movement into the proboscis.

The overall faster fluid uptake rate of males could reflect the need for males to defend territories and find mates without spending additional time feeding. The only feeding treatments with significant differences in uptake rates were tiger swallowtails

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with fully submerged proboscises in the 1% sucrose solution. Males had a significantly higher fluid uptake rate than did females. This might be explained by the puddling behavior common in males of this species (Arms et al., 1974; Lederhouse et al., 1990; Otis et al., 2006). Males possibly require fewer stimuli to induce feeding along the length of the proboscis than do females. This needs to be verified by comparing the feeding rates, using solutions with contents mimicking puddles and nectar. Future tests could clarify if there is a gender difference in stimulus response when a droplet is introduced along the proboscis. The gender difference with the fully submerged proboscis disappears with the 15% sucrose solution potentially because the solution is too viscous to be imbibed in locations other than the drinking region.

When analyzing the amount of time taken to begin feeding, male monarchs differed by less than 1 second between the 1% and 15% solutions, whereas females took about 3 seconds longer with the 1% solution than with the 15% solution. The faster time for the females with the 15% solution could be a response to greater sucrose stimulus. The amount of stimulus should correlate with the quality of the food source. Therefore, the greater the stimulus, the better the quality of the food source and the faster the individual would begin feeding upon detection. Searching for high-energy foods would be important for females as they develop eggs. Additional studies comparing the time taken to start feeding on a 15% sucrose solution with that using the suggested optimal sucrose concentrations of 20-40% (Kingsolver & Daniel, 1979; May, 1985) could provide insights into the relation between amount of stimuli and feeding behavior. Based on these

observations, I predict monarch females would begin feeding faster on a 20-40% sucrose solution than on a 15% solution.

To determine if a larger butterfly, presumably with a proportionally larger sucking pump, would have a faster fluid uptake rate than a smaller butterfly, the relationship between butterfly size (forewing length and proboscis length) and rate was examined. The only treatments with a significant correlation between size and rate were for the painted ladies fed 1% sucrose solution. Proboscis length was correlated with feeding rate, indicating that larger painted ladies, regardless of gender, are capable of feeding faster than smaller butterflies. Forewing lengths of females were significantly larger than those of males, suggesting that females might have larger sucking pumps than males, even though proboscis length did not differ between genders. Therefore, females should have a faster feeding rate than males, but this is not necessarily represented in the data. The relationship between butterfly size, based on proboscis and forewing lengths, and fluid uptake rate remains unclear. Future feeding experiments should include measurements of the sucking pump (e.g., overall size, volume change per contraction, amount of pressure induced) to determine its relationship with proboscis length, forewing length, and fluid uptake rate.

SUMMARY

This study demonstrated that the butterfly proboscis is capable of fluid uptake in locations other than the drinking region. The permeability of the proboscis potentially can be controlled by the butterfly. Fluid uptake was faster for males than females in species that exhibit puddling behavior when fed a 1% sucrose solution. The rate of fluid uptake

was not significantly related to the amount of proboscis exposed to fluid. The relationships among butterfly size (proboscis length, forewing length, and sucking pump size), gender, and amount of proboscis exposed to the fluid need to be more thoroughly examined.

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