FLUID RISE IN C-SHAPED CONDUITS OF SEPARATED BUTTERFLY MOUTHPARTS

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by

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Introduction

Why study the lepidopteran proboscis?

Butterflies and moths (Lepidoptera) represent the second most diverse taxa of animals with approximately 160,000 named species (second only to beetles) (Adler and Fottit 2009) and play an important role in ecological systems, such as pollination (Barth 1991). Lepidoptera employ specialized mouthparts (proboscis, Figure 1) that act as a tool for fluid uptake, thus creating an important ecological interface between the liquid food source and the insect. A functional proboscis is important to lepidopteran fitness (i.e., the ability to survive and reproduce), as it is the means by which they feed from a wide range of food sources, e.g., nectar, sap, blood, rotting fruit (Adler 1982, Krenn 2010), which prompts the importance for studying the functionality of the proboscis.

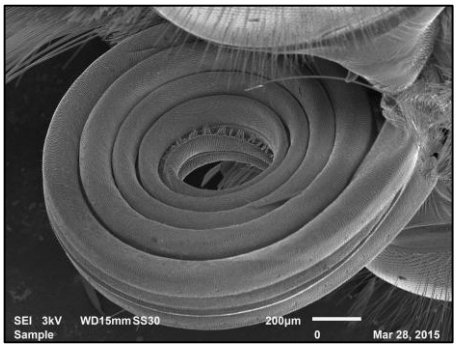

Figure 1. Scanning electron microscope image of a coiled proboscis of *Vanessa cardui*.

The lepidopteran proboscis serves as a model for coevolutionary systems. Coevolution is a reciprocal relationship where the evolution of one species or population affects the evolution of another species or population (Ehrlich and Raven 1964). For example, the founder of the theory of evolution by natural selection, Charles Darwin, famously examined the flower of the Comet Orchid (*Angraecum sesquipedale* Thouars) that is only found in Madagascar and noted its 300mm long nectar spur. Darwin predicted that there must be an insect that can successfully reach the nectar found at the bottom of the corolla (Darwin 1862). The unknown insect would later be discovered in 1903 as a

hawk moth (*Xanthopan morganii praedicta* Rothschild and Jordan, Figure 2), named in honor of Darwin's prediction, which displays a proboscis length to match that of the floral tube (Kritsky 1991). Darwin concluded that the evolution of its proboscis was due to selection pressures exerted by predators on the pollinators (Darwin 1862).

Figure 2. Image of Darwin's sphinx moth. Image acquired from Wikipedia.com.

The proboscis also serves to assist male species in acquisition of nutrients during a behavior known as puddling. Mud puddles, dung, and carrion contain substances such as sodium, which is important for female fertility; however, the act of ovipositing results in females losing a majority of their sodium (Boggs et al. 1991). Males that puddle are able to transfer sodium to the female to help increase the survival rate of the eggs. Thus, the proboscis serves to replenish nutrient deficiencies in both males and, indirectly, females (Boggs et al. 1991).

The proboscis also has come to be a model for microfluidic systems (Monaenkova et al. 2012, Tsai et al. 2012, Lehnert et al. 2013). Interest in lepidopteran proboscis functionality piqued the interest of microfluidic and material science engineers due to the inept ability of butterflies and moths to acquire fluids from micro-sized droplets. With the production of novel microfluidic devices that replicate the feeding ability of Lepidoptera, the proboscis has become a model for biomimicry (Tsai et al. 2011, Lehnert et al. 2013, Tsai et al. 2014, Kornev et al. 2016). The secret to a butterfly's feeding ability provides a

classic example of structure and function relationships, thus proboscis evolution deserves distinctive attention.

The evolution of the proboscis

Insects of Lepidoptera and Trichoptera constitute the superorder Amphiesmenoptera (Kiriakoff 1948). Although they represent sister taxa (last shared a common ancestor approximately 210 mya) (Misof et al. 2014), the evolution of the proboscis is an apomorphic characteristic specific to the Lepidoptera (Krenn et al. 2005, Krenn 2010). The fossil record of the Lepidoptera dates back to the Lower Jurassic (approximately 201-174 mya) (Sohn et al. 2012, 2015). Ancestral Lepidoptera contained short and reduced mouthparts, represented by the extant moth families Eriocraniidae, Heterobathmoidea, Micropterigidae, and Agathiphagidae, which comprise the non-glossatan Lepidoptera (Grimaldi and Engel 2005, Krenn 2010).

Extant members of the Heterobathmoidea, Micropterigidae, and Agathiphagidae retain mandibles for pollen and fern spore consumption, providing evidence (along with the fossil record) that the common ancestor of Lepidoptera and other insect groups, including their sister taxa the Trichoptera, possessed mouthparts classified as chewing mouthparts, which are found in many insect groups today, including grasshoppers (Orthoptera) and beetles (Coleoptera). Chewing mouthparts consist of a suite of different structures that are modified for handling, crushing, and eating solid food particles. The Eriocraniidae represent an ancient extant lepidopteran lineage that completely lacks mandibles (Grimaldi and Engel 2005).

Linked to the reduction of the mandibles, a lepidopteran clade evolved that had a true or derived proboscis, i.e. the Glossata (Krenn and Kristensen 2004). These early proboscises were smaller than those of derived lineages and were likely used to acquire fluids from water droplets and sap (Krenn 2010, Monaenkova et al. 2012). A change to a liquid-only diet reinforced the role of other important feeding structures, such as the sucking pump, which is located in the insect's head (Davis and Hildebrand 2006). The shift towards longer proboscises is coupled with a massive radiation of Lepidoptera that co-occurred with angiosperm diversification during the Early Cretaceous Period (146 – 100 mya) (Misof et al. 2014); the speciation of holometabolous insects (i.e., those with complete metamorphosis) alongside the angiosperms represents a classic example of coevolution (Hu et al. 2008). The benefits of obtaining exclusive access to nectar, a nutritive food source, reduced competition and supported the drive towards the elongated mouthpart (Borrell and Krenn 2006). Lepidoptera continued to diversify throughout the Cretaceous, perhaps giving rise to butterfly lineages during this Period; however, the first fossils of butterflies are from the mid-Eocene epoch approximately 45 million years ago (Sohn et al. 2012).

The structure of the proboscis is often used as a delineating character when determining higher-level lepidopteran phylogenies (Grimaldi and Engel 2005, Kristensen 2007, Lehnert et al. 2016). Proboscises among lepidopteran species have become specialized and host a range of architectures that relate to feeding habits, including mop-like structures for feeding from surfaces (Knopp and Krenn 2003, Molleman et al. 2005, Lehnert et al. 2013) and piercing structures to penetrate tissue to feed on blood (Bänziger

1971, Zaspel et al. 2011). It is these modifications that have allowed Lepidoptera to enter new adaptive feeding zones and likely contributed to their diversification (Monaenkova et. al 2012, Lehnert et al. 2016).

Proboscis Morphology

The evolution of insect mouthparts stem from the anterior legs in ancestral lineages taking on new functions for food handling, a trait favored by natural selection. Insect mouthparts consist of the mandibles, labium, hypopharynx, and the maxillae, which house numerous other structures, such as the lacinicae and galeae. These mouthparts can be modified, enlarged, reduced, or completely absent from some insect lineages, which relates to the types of food from which they feed (Labandeira 1997, Rogers et al. 2002).

The glossatan Lepidoptera represent approximately 95% of butterflies and moths and have mouthparts that consist of two elongated, C-shaped galeae (Eassa and Eastham 1955, Krenn 2010, Lehnert et al. 2016). The galeae are separate upon the emergence of the adult butterfly or moth from the exuvium (the empty pupae), but then become interconnected, dorsally and ventrally, by cuticular projections known as legulae (Krenn 1997, Lehnert et al. 2014). The assembly of the galeae creates a circular food canal, which acts as a conduit to transport fluids (e.g., nectar, sap, rotting fruit) toward the head during feeding.

Internally, each galea includes a lumen that houses a trachea, nerve, and intrinsic musculature. The lateral intrinsic muscles (*lim*) and median intrinsic muscles (*mim*) overlap and extend along the ventral galeal wall in the lumen. In many species, the

median muscles are absent near the distal regions of the proboscis (Krenn and Muhlberger 2002). Proboscis coiling and uncoiling is a result of an antagonistic relationship between musculature action and the hydrostatic pressure of hemolymph (Krenn 2010, Lehnert et al. 2015).

Externally, the proboscis consists of structurally and positionally-defined regions that relate to function and feeding habits. The uncoiled position of the proboscis, for instance, often displays a knee-bend region that aids in feeding from food sources (Lehnert et al. 2015). Ellipticity of the proboscis also relates to feeding habits. A nectar-feeding butterfly, for instance, has a more round proboscis when compared to the elliptical shape observed in butterflies that feed on sap and rotting fruit, which plays an important role in the fluid dynamics when the proboscis is submerged in liquids (Lehnert et al. 2013, 2016).

The shape and sizes of the dorsal legulae and the chemoreceptors represent noteworthy features of the lepidopteran proboscis. The dorsal legulae are found along the majority of the proboscis length, extending beyond the tip in sap-feeding butterflies (Lehnert et al. 2016). The dorsal legulae are small in width for approximately 10 – 20% of the proboscis length, where they overlap and putatively aid in preventing the proboscis from splitting (Krenn et al. 2001, Lehnert et al. 2014). At the distal region, however, the dorsal legulae are modified for fluid uptake where they are wider with larger interlegulae spaces that support the movement of fluids from food sources into the food canal of the proboscis (Krenn 2010, Monaenkova et al. 2012, Lehnert et al. 2013, 2016).

The proboscis also includes various mechano- and chemoreceptors, known as sensilla, which include the sensillum basiconica, coeloconica, filiformia, campaniformia, and styloconica. The classification of the sensilla depends on the morphology and occupancy of pores (Faucheux 2013). The sensilla styloconica particularly deserve attention due to their role in fluid uptake (Lehnert et al. 2013, Lee and Lee 2014) and their association with the classification of lepidopteran species as flower or non-flower visitors (Petr and Stewart 2005, Krenn et al. 2001, Lehnert et al. 2016). Sensilla styloconica are the largest chemosensilla on the proboscis (Petr and Stewart 2005) and are often restricted to the distal region of the proboscis. The sensilla styloconica have an enlarged stylus with a chemoreceptive peg at the end. When present in high quantities, sensilla styloconica create a mop or brush-like appearance of the proboscis that is a characteristic of sap-feeding butterflies.

Proboscis functionality

Due to its structural architecture, the functionality of the proboscis has been described to operate similarly as a drinking straw, relying only on action of the sucking pump for fluid rise (Kingsolver and Daniel 1995, Krenn 2010). The model stresses the role of establishing a pressure gradient, via sucking pump, as the only source in transporting fluids up the food canal and assumes its shape to be cylindrical and straight (Kingslover and Daniel 1995, Bauder et al. 2013). Therefore, fluid rise can be described with the Hagen-Poiseuille equation:

$$\Delta P = \frac{8 * \mu * L * Q}{\pi * r^4}$$

where P = pressure drop in a cylinder tube, u = viscosity, L = length of tube, Q = flow rate, and r = radius of the tube. The drinking-straw model remained in the literature until recent studies addressed that this model does not fully explain the ability for Lepidoptera to acquire fluids from porous surfaces, such as rotting fruit and sap. Using x-ray phase contrast imaging to view fluid movement in the proboscis, it was revealed that butterflies first employ capillarity to bring fluids from porous surfaces into the food canal of the proboscis, which is then followed by sucking pump action (Monaenkova et al. 2012). The spaces between the dorsal legulae support capillary action, which removes fluids from small pores. The fluids travel into the food canal and begin to circumferentially spread until the film grows into a liquid bulge and collapses into a liquid bridge (Monaenkova et al. 2012, Lehnert et al. 2013) via Plateau instability (Plateau 1873).

The proboscis consists of a dichotomy in wettability (Lehnert et al. 2013). The distal 10-20% of the proboscis length (where the dorsal legulae are modified for fluid uptake) has an overall hydrophilic profile, which is called the drinking regions, as determined using a capillary-rise technique. The proximal region of the proboscis, however, has a hydrophobic profile, which is termed the nondrinking region. The nondrinking region might have adaptive value in providing self-cleaning properties (Lehnert et al. 2013, 2016). The change in the wetting dichotomy is due to the increase in size of the dorsal legulae and the presence of the enlarged sensilla styloconica, which are both hydrophilic, whereas the surface roughness of the galeal surface provides hydrophobic properties.

A subsequent study took the role of capillarity and the importance of wettability (i.e., the hydrophilicity and hydrophobicity of objects and surfaces) a step further by examining the ability for butterflies to feed with a partially split proboscis (Lehnert et al. 2014). In this study, butterflies had proboscises split up to 50% of the total length and the drinking regions were submerged in a 20% sucrose solution with red food coloring. The butterflies were subsequently dissected and the crop (a part of the alimentary canal that stores fluids) was inspected for the presence of the food coloring. It was determined that butterflies can feed with partially split proboscises, again challenging the drinking straw model. However, in this study, proboscises were placed in a horizontal position when feeding, thus preventing gravity from impacting fluid rise.

Purpose of study and hypothesis

The purpose of the present study is to determine if fluid rise can occur in completely split proboscises (i.e., a single, C-shaped galea) against gravity, thus, mimicking capillary rise in a split capillary tube. Fluid rise in C-shaped conduits is an almost completely unstudied aspect of microfluidics (Concus and Finn 1969), thus if capillary rise does occur it not only provides insight into proboscis functionality, but could have interesting implications in the field of microfluidics and the development of microfluidic devices (i.e., biomimicry). If capillary rise does occur, we hypothesize the speed of fluids will relate to the conduit size; we suggest that food canals of smaller radii will exhibit greater capillary rise speeds due to the surface-to-volume ratio. In addition, if capillary rise does occur and the liquid column moves to the head, we hypothesize that butterflies will retain the ability to feed.

Materials and Methods

Species used for study

Three species of Lepidoptera, *Vanessa cardui* L. (Nymphalidae), *Pieris rapae* L. (Pieridae), *and Manduca sexta* L. (Sphingidae), were used to determine fluid uptake rates. These species were chosen because they are readily available for purchase and because they exhibit different proboscis architectures: *V. cardui* (medium length/semi-brush tip), *P. rapae* (small length/smooth tip) and *M. sexta* (long length/smooth tip). Butterflies were obtained from the Carolina Biological Supply Company© as larvae and held in a humidity chamber (22° C, 18:6 photoperiod) until the pupal stage, with the exception of *M.* sexta, which were ordered as pupae and maintained at room temperature (24°C) until the adult stage. The butterfly chrysalises were hung from a toothpick with hot glue, which was stuck into a Styrofoam sheet inside an insect tent. Adults were placed into glassine envelopes and stored in a 4° C refrigerator. During the feeding trials and functionality tests butterflies were stored in small insect chambers in order to allow them to warm to room temperature (Figure 3).

Figure 3. Proboscis functionality test: Top – butterflies held in individual insect chambers at room temperature (24 degrees C) during feeding trials. Bottom Left = *V. cardui* spp. Feeding 1 mL drop of 20% sucrose solution. *M. sexta* spp. not pictured.

Fluid rise in a single galea (split proboscis)

Adult Lepidoptera were fed water for two minutes to ensure proboscis functionality and then placed into a -80° C freezer. The individuals were removed from the freezer and the head was removed (with the proboscis intact) and secured on a Styrofoam sheet with insect pins. The proboscises were uncoiled and straightened on top of the Styrofoam sheet, using insect pins and strips of glassine paper, and placed into a cabinet to dry for five days. Once dried, the galeae were separated along the legular seam using an insect pin, thus creating two C-shaped conduits for the fluid uptake rate trials.

Each galea (per individual) was placed into a separate treatment: one treatment consisted of proboscises with drinking regions (DR treatment) and the other treatment consisted of proboscises that had the drinking region removed (NDR treatment). Galea in the NDR treatment had the drinking region removed with dissecting scissors under a Leica M205 C stereomicroscope. Hot glue was then administered to the distal end of the galeae in both treatments (for consistency) in order to seal the galeal lumen that was exposed when removing the drinking region in the NDR treatment. The application of the hot glue was needed to prevent capillary rise from occurring in the lumen rather than the exposed food canal.

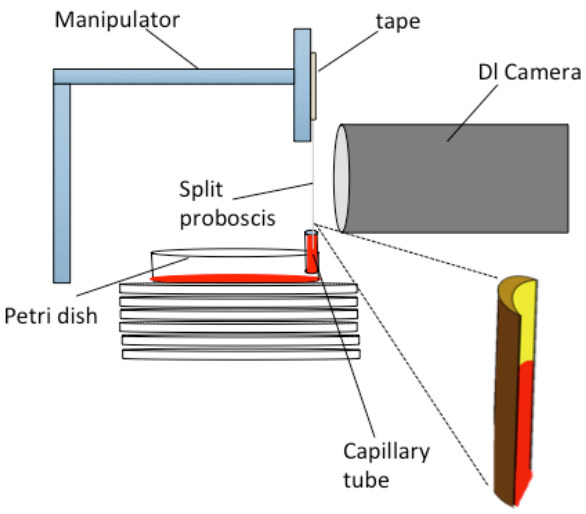

Figure 4. Set up for fluid rise experiments with a single galea for the DR and NDR treatments.

The base of the proboscis was attached to surgical tape, secured to a micromanipulator, and suspended over a small petri dish (Figure 4). The petri dish contained a shortened 1mm capillary tube that was hot glued to the inside perimeter. The distal end (approximately representing the drinking region) of the proboscis was lowered into the shortened capillary tube and positioned so that the food canal was facing a Dino-lite camera (150X magnification, showing a total height of 2.01 mm on the computer screen). Another camera (ImagingSource IC 80HD) was set up adjacent to the petri dishes so that it contained a viewing range of the entire proboscis length.

Once both cameras had the proboscis in focus a measuring tool was placed next to the proboscis and an image was captured using the ImagingSource camera and Capture C software, which would be used later as a reference for determining the final height of the liquid column. Both cameras simultaneously recorded videos of fluid rise (Dino-lite recorded videos at 60 fps, ImagingSource camera at 30 fps). A 1mL pipette was used to administer red food coloring solution (0.5mL deionized water: 1mL red food coloring concentrate) into the petri dish until fluids entered the bottom of the capillary tube, which would travel up the capillary tube, then come into contact with the distal region of the

proboscis that was inside the tube. Videos were recorded until fluids stopped moving for 2 minutes, then each galea was placed into a labeled vial and saved for imaging with a scanning electron microscope (SEM).

Videos from the Dino-lite camera were uploaded into Adobe® Premiere Pro CS6 software and individual frames from each trial for both treatments were captured starting from the initial column height at the top of the capillary tube up to a maximum of 1 sec (a total of 60 frames). Each captured image represented 1/6 sec (i.e., an image was captured every 10 frames). Trials where the top of the liquid column moved out of screen in under 1 second had a fewer number of images captured for analysis. Each saved image was copied to Microsoft® PowerPoint where the proboscis in each image was aligned and the liquid column height was marked with a horizontal line that extended into the other proboscis images, which allowed us to measure the distance the liquid column traveled for every 1/6 sec (Figure 5). Using the lines as markers, the distance traveled by the liquid column was measured using ImageJ® software

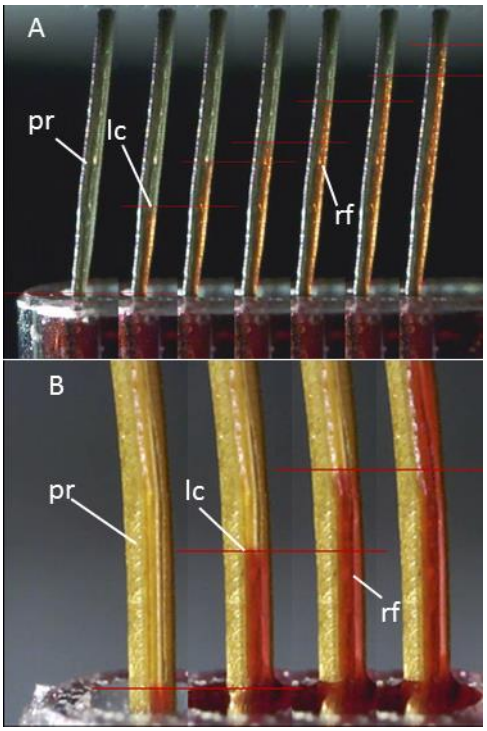

Figure 5. Isolated frames for fluid uptake measurements. The top image (A) shows the rise of the red food coloring (rf) in the proboscis (pr) of *P. rapae*. The bottom image (B) shows the proboscis of *M. sexta*. The horizontal red lines indicate the top of the liquid column (lc). Each red line represents 10 frames (i.e., 1/6 sec). The distances were measured between the red lines to determine the speed of fluid rise. Image B provides an example of when fluid rise to the top of the screen (2.01 mm) occurred in less than 1 sec.

(the total window distance of 2.01 mm was used to set the scale for subsequent measurements).

Scanning Electron Microscopy

Double-sided carbon graphite tape was placed onto a SEM aluminum stub and a galea was positioned on the tape with the food canal-side up using the Leica stereoscope. On the other galea, a transverse cross section was made proximal to the hot glue, using a scalpel, and positioned on the tape with its cross section end projecting upwards, thus showing the C-shape of the galea. Once the specimen was secured, it was placed into an EMS 150TS sputtercoater and coated with a 7-nm layer of platinum. The sample was then imaged using a JEOL 6010LV SEM at 500x magnification for subsequent food canal measurements. Four measurements were acquired using the SEM's InTouch® software, including the perimeter, the area of the food canal, the diameter, and an estimated area of a circle (representing the area of a capillary tube) (Figure 6).

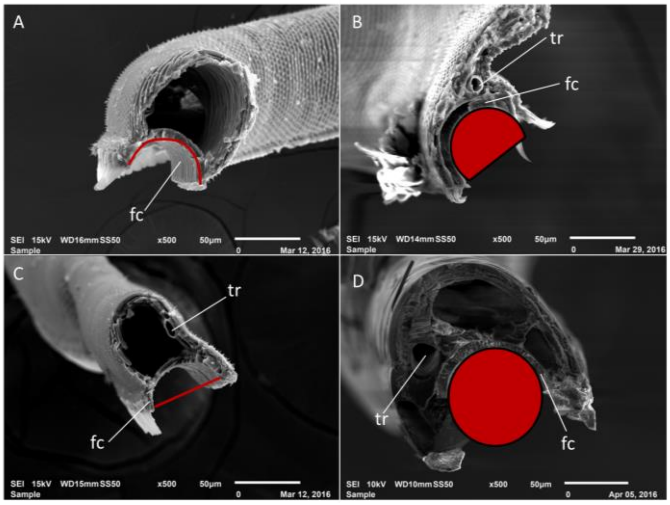

Figure 6. SEM measurements of the food canal; (A) perimeter, (B) volume, (C) diameter, and (D) circle area (representing the area of a typical capillary tube).

Statistical Analyses

A paired t-test was used to test for significant differences in fluid rise speeds between the DR and NDR treatments. The recorded measurements (fluid speed and SEM measurements) were compared among species for significant differences using an ANOVA ($P<0.05$) followed by a Tukey post hoc test. A linear regression analysis was used to test for a potential relationship between the food canal measurements and the speeds of the fluid rise. All statistical analyses were performed using SPSS® software.

Live feeding trial – green food coloring

The feeding trials consisted of two separate experiments. In the first experiment, adults of *V. cardui* were placed into one of four different treatment groups: 1) butterflies with a sealed proboscis and fed green food coloring solution (20% sucrose solution with green food coloring, positive control), 2) sealed proboscis fed only 20% clear sucrose solution (negative control), 3) 100% split proboscis fed green food coloring solution, and 4) 75% split proboscis also fed the green food coloring solution. The galea that was not used as the feeding conduit was fastened to a Styrofoam block with insect pins. All butterflies were fed a 20% sucrose solution 24 hours before the feeding trials to assess proboscis functionality. In addition, all butterflies were weighed immediately before and after each feeding trial.

Butterflies were fastened to a mount by clamping their wings between two microscope slides. The butterflies were then lowered onto a Styrofoam sheet to ensure no interaction between the food source and the butterfly's legs. Once positioned, the proboscis was extended over the edge of the sheet into a petri dish that contained the

feeding solution. An insect pin was used to uncoil the proboscis and lower it into the feeding solution so that the surface of the fluid was in contact with the distal portion of the nondrinking region. The insect pin also was used to keep the proboscis submerged during the feeding trial, which consisted of 10 minutes. After the butterflies were weighed they were dissected to reveal the contents of the crop (food storage region of the alimentary canal) to determine if feeding had occurred. The crop was removed, imaged with the ImagingSource camera, and the average L*, a*, b*, color values were determined using Lenseye software (see Lehnert et al. 2011 for protocol). Proboscis lengths were measured using the Leica stereoscope and a measuring tool (Figure 7). The color values and proboscis lengths were compared among treatments using an ANOVA ($P<0.05$) followed by a Tukey post hoc test with SPSS software.

Figure 7. Image of *V. cardui* acquired for proboscis length measurements.

Live feeding trial – fluorescent microsphere

Adults of *V. cardui* and *P. rapae* were used to determine feeding abilities with split proboscises when fed fluorescent microspheres (ThermoFisher Scientific®). Each butterfly was fed a 20% sucrose solution prior to the feeding trial to ensure the proboscis was functional (determined by weighing the butterflies before and after being fed). Each individual was placed into one of three treatment groups: 1) sealed proboscis (positive control), 2) sealed proboscis (negative control), and 3) split proboscis. The positive control and split treatment groups were fed a 20% sucrose-fluorescent microsphere

solution (1:1 ratio) and the negative control fed only a 20% sucrose solution. All solutions were vortexed for 10 seconds immediately before the feeding trials. Prior to feeding, the forewing length (mm), weight, and sex of each butterfly was determined. The butterflies had their wings closed and placed between two slides that were attached to a stand. A 50-uL droplet of the feeding solution was placed into the center of a concave slide and the proboscis was lowered into the droplet with an insect pin and the butterflies were allowed to feed for 3 minutes. Using a Dino-lite camera, videos of the feeding were recorded at 20x magnification (60 fps). For the positive control and split treatment groups, a UV light was placed underneath the slide for observation of the fluorescence of the microspheres. Immediately following feeding, the butterfly was weighed again and then dissected in a 1X PBS solution using the Leica stereomicroscope. The crop was removed from the dissected butterfly and placed onto a slide and covered with a cover slip. In order to determine intensity values as an indicator of positive feeding of the microspheres, a Z-stack was acquired (3 slices) and processed with the CellSens® software on an Olympus X81 confocal microscope. Intensity values were compared among treatments using an ANOVA ($P<0.05$) followed by a Tukey post hoc test in SPSS software.

Results

Fluid rise speeds in a single galea (split proboscis)

Speed measurements for each treatment (DR and NDR) were compared within species to determine potential differences in fluid rise speed (Table 1). The paired t-test showed no significant differences in fluid rise speeds in one second (60 frames), half a second (the first 30 frames), and the fastest overall speed (Table 2).

Rates of fluid rise in the DR treatment differed significantly among species (ANOVA, $P<0.05$) for all rate measurements, including the whole proboscis length, i.e., the portion of the proboscis shown in the computer screen (F – 17.24; df – 2, 15; $P<0.0001$), speeds for the first 0.5 seconds (F – 13.64; df – 2, 15; $P=0.001$), and the fastest speed (F – 13.79; df – 2, 15; $P=0.001$). Speed measurements for proboscises in the NDR treatment also differed significantly for the whole proboscis (F – 6.74; df – 2, 15; $P=0.010$), first 0.5 seconds (F – 8.31; df – 2, 15; $P=0.005$), and fastest fluid rise (F – 9.32; df – 2, 15; $P=0.003$). For both treatments *P. rapae* exhibited the fastest rates in fluid rise, followed by *M. sexta*, then *V. cardui* (Table 3).

Table 1. Distance measurements (mm) of fluid rise in a single galea per 1/6 second increments. Data are presented per increment, not averaged with previous increments.

Species	ID	Drinking region (y/n)	1/6 sec	2/6 sec	3/6 sec	4/6 sec	5/6 sec	1sec
P. rapae	1	y	0.159	0.102	0.035	0.094	0.093	0.088
	1	n	0.478	0.245	0.107	0.227	0.14	0.17
	2	y	0.701	0.525	0.151	0.293	0.025	~
	2	n	0.301	0.199	0.202	0.161	0.224	0.218
	3	y	0.473	0.444	0.237	0.134	0.191	0.07
	3	n	0.644	0.434	0.555	0.321	~	~
	4	y	0.426	0.242	0.498	0.337	0.197	~
	4	n	0.562	0.533	0.406	0.234	~	~
	5	y	0.141	0.208	0.07	0.141	0.134	0.215
	5	n	0.363	0.537	0.365	0.285	0.287	~
V. cardui	1	y	0.029	0	0	0	0	0
	1	n	0.082	0.067	0	0.023	0	0.036
	2	y	0.023	0.027	0	0.013	0.023	0
	2	n	0	0	0	0	0	0
	3	y	0.047	0.023	0.021	0.01	0.01	0.031
	3	n	0.024	0.035	0.035	0.028	0.042	0.021
	4	y	0.059	0.042	0.028	0.013	0	0
	4	n	0.019	0.008	0	0.013	0	0.013
	5	y	0.028	0	0	0	0	0
	5	n	0	0	0	0	0	0
	6	y	0.02	0.018	0	0.025	0.02	0
	6	n	0	0.015	0.02	0	0.009	0.015
M. sexta	1	y	0.074	0.122	0.056	0.074	0.043	0.028
	1	n	0.756	0.447	0.597	~	~	~
	2	y	0.056	0.031	0.041	0.036	0.031	0.033
	2	n	0.052	0.069	0.026	0.049	0.069	0.037
	3	y	0	0	0	0.017	0	0
	3	n	0.036	0.049	0.052	0.042	0.033	0.036
	4	y	0.071	0.057	0.076	0.043	0.113	0.043
	4	n	0.057	0.075	0.044	0.06	0.065	0.075
	5	y	0.079	0.054	0.064	0.054	0.036	0.044
	5	n	0.152	0.112	0.082	0.075	0.085	0.08

~ represents fluid rise past the 2.01mm window frame in less than 1 second (60 frames).

Table 2. *P*-value results from a paired t-test comparing the speeds of fluid rise in proboscises (mm/sec) in the DR and the NDR treatments.

Species	n	Avg. Speed – 1 sec	Avg. Speed – First 0.5 sec	Fastest Speed
P. rapae	5	0.154832	0.305117	0.513508
V. cardui	6	0.804945	0.754597	0.55104
M. sexta	5	0.29475	0.287393	0.311714

*p-values <0.05 represent significant differences in fluid uptake rates between the drinking region and non-drinking region of the proboscis.

Table 3. Comparison of mean (±S.E.M) fluid uptake rates (mm/sec) among species for each proboscis treatment (DR and NDR).

Species	Drinking region (y/n)	n	Avg. Speed – 1 sec	Avg. Speed – First 0.5 sec	Fastest Speed
P. rapae	y	5	1.47±0.32a	1.84±0.46a	2.56±0.62a
	n	5	2.17±0.34B	2.47±0.38B	3.15±0.36B
V. cardui	y	6	0.10±0.02a	0.13±0.03a	0.22±0.04a
	n	6	0.09±0.04A	0.11±0.05A	0.17±0.08A
M. sexta	y	5	0.31±0.08b	0.33±0.09b	0.48±0.12b
	n	5	1.07±0.67AB	1.09±0.67AB	1.38±0.84AB

Lowercase letters represent significant differences in speeds with a drinking region, whereas uppercase letters represent significant differences in proboscises without a drinking region (Tukey-HSD, p-value <0.05).

Measurements acquired from scanning electron microscopy

There were significant differences in food canal measurements (Table 4) among species (ANOVA, $P<0.05$), including the food canal diameter (F – 53.71; df -2, 15; $P<0.0001$), food canal volume (F – 124.35; df – 2, 15; $P<0.0001$), estimated area of a circle (representing area of a typical capillary tube) (F – 48.11; df – 2, 15; $P<0.0001$), and

food canal perimeter (F – 61.21; df – 2, 15; $P<0.0001$) (Table 5). A linear regression analysis indicated a relationship between food canal measurements and fluid uptake rates for proboscises in both treatments (DR and NDR); however, none of these were significant (Figures 8 and 9). Fastest uptake rates (within 1 second) in proboscises with drinking regions, for instance, showed no significant relationship to food canal diameter (F – 2.53; df – 1, 15; $P=0.134$), area of a circle (F – 2.05; df – 1, 15; $P=0.18$), food canal volume (F – 1.92; df – 1, 15; $P=0.187$), and food canal perimeter (F – 2.03; df – 1, 15; $P=0.176$) measurements among species. Fluid rise in proboscises without drinking regions also displayed no significant differences among speeds and food canal measurements, i.e., food canal diameter (F – 0.52; df – 1, 15; $P=0.482$), circular area (F – 0.30; df – 1, 15; $P=0.596$), food canal volume (F – 0.09; df – 1, 15; $P=0.774$), and food canal perimeter (F – 0.10; df – 1, 15; $P=0.763$).

Table 4. Raw data of food canal measurements (µm) per butterfly species.

Species	n	Food canal diameter	Sealed capillary tube	Food canal volume	Food canal perimeter
P. rapae	1	52.25	1963.51	992.2	82.72
	2	54.78	2169.87	1068.46	83.58
	3	50.95	2147.02	852.61	68.95
	4	45.73	1695.96	563.19	58.52
	5	47.01	2269.58	786.98	72.99
V. cardui	1	56.46	2585.63	1338.04	90.96
	2	53.39	2661.65	932.6	75.9
	3	66.84	3066.78	1367.45	95.54
	4	59.82	2964.79	1258.26	86.9
	5	46.27	1768.42	937.18	76.03
	6	59.94	2560.02	1436.81	97.74
M. sexta	1	59.83	4404.9	4044.94	186.62
	2	80.19	4311.14	3432.22	140.37
	3	95.24	6039.24	5159.77	184.95
	4	82.54	7156.99	4806.34	211.34
	5	95.23	7065.09	4926.6	177.52

Table 5. Mean (±S.E.M.) proboscis measurements (µm) compared among butterfly species.

Species	n	Food canal diameter	Sealed capillary tube	Food canal volume	Food canal perimeter
P. rapae	5	50.14±1.67a	2049.18±101.20a	852.69±87.79a	73.35±4.65a
V. cardui	6	57.12±2.84a	2601.21±186.91a	1211.72±90.61a	87.18±3.86a
M. sexta	5	87.91±3.15b	6080.66±515.89b	4666.33±313.83b	183.10±12.23b

Lowercase letters represent significant differences in food canal size between species, (Tukey-HSD, $P<0.05$).

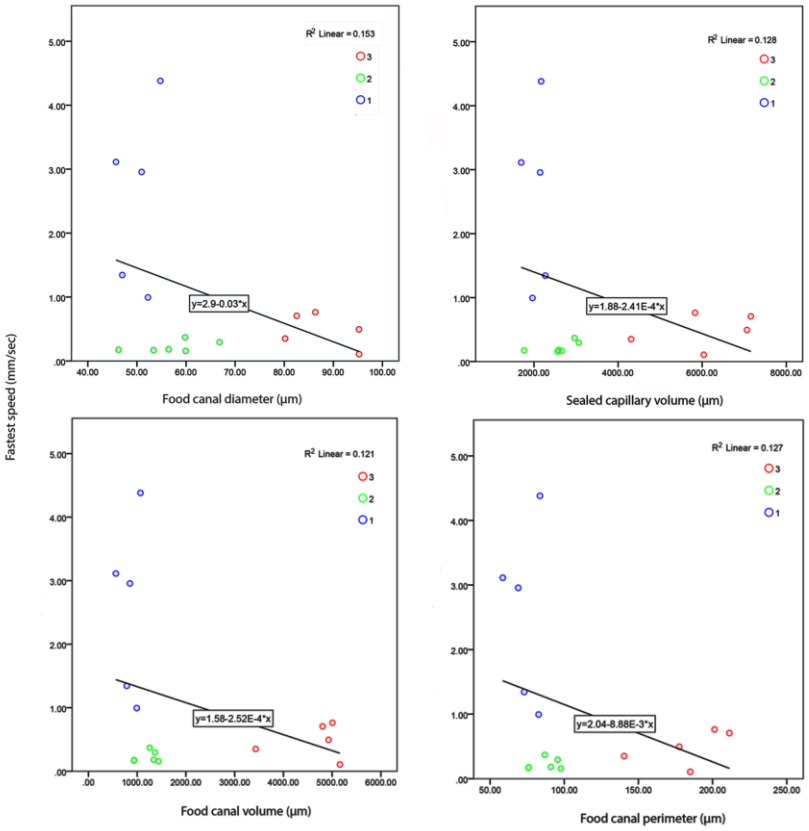

Figure 8. Regression analysis comparing fluid rise rates with food canal measurements in the DR treatment. The red circles represent *M. sexta*, green represents *V. cardui*, and the blue circles represent individuals of *P. rapae*.

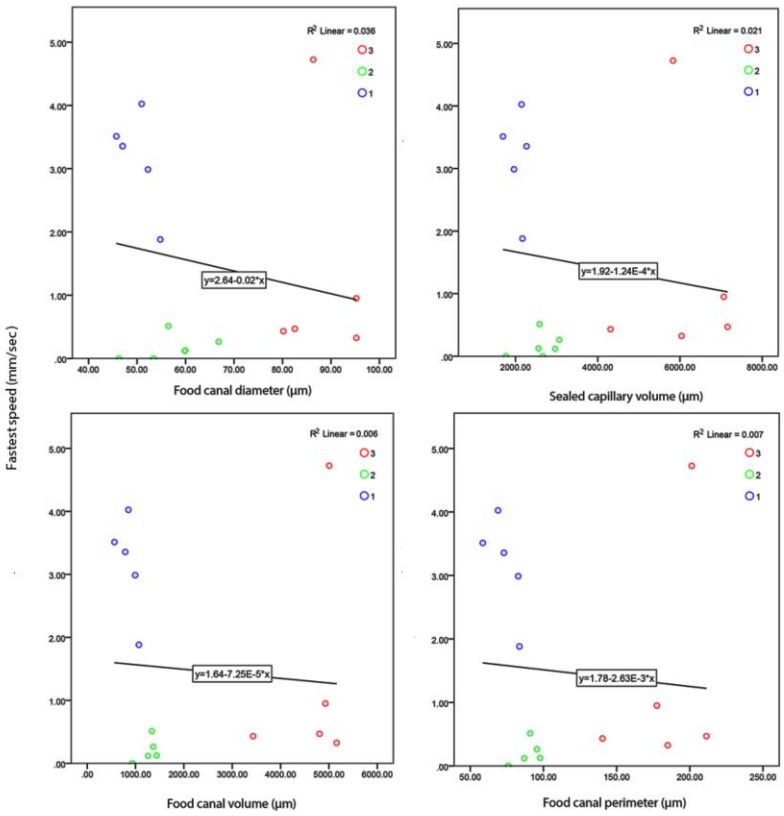

Figure 9. Regression analysis comparing fluid rise rates with food canal measurements in the NDR treatment. The red circles represent *M. sexta*, green represents *V. cardui*, and the blue circles represent individuals of *P. rapae*.

Live feeding trial – green food coloring

Both treatments of separated proboscises showed evidence of feeding, but with reduced abilities. Treatment 1 (positive control), as expected, displayed a bright green color, while treatments 3 (100% split) and 4 (75% split) exhibited a light green color accompanied by tiny air bubbles (Figure 10). Treatment 2 (negative control) exhibited an engorged crop filled with clear fluids. A color analysis followed by an ANOVA indicated significant differences (P<0.05) in L*, a*, and b* color values among treatments (Table 6). The bright green color presented in the crop of treatment 1 was significantly different from treatment 2, but was not significantly different from either split treatment groups, despite the low weight gain.

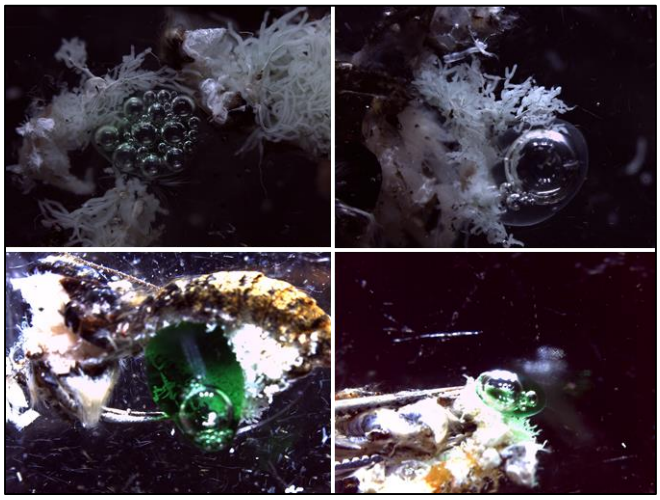

Figure 10. Crop Dissections in PBSx for *V. cardui*. Left to Right: (Top) Treatment 3 and Treatment 2, (Bottom) Treatment 1, and Treatment 4.

Table 6. Fluid uptake abilities of *V. cardui* according to different proboscis treatments when feeding on green food coloring solution. Measurements (mean±s.e.m.) indicate averages among each treatment group.

Treatment	n	Weight change (g)	L*	a*	b*	Proboscis length (mm)
1	3	0.05±0.00 ab	62.48±10.14 a 100.84±0.15	-34.07±5.15 a	24.64±3.20	13.82±0.85
2	3	0.06±0.03 b	b	-15.67±2.82 b	16.61±3.79	13.84±0.69
3	5	0.00±0.00 a	76.96±3.29 a	-29.44±1.55 a	23.10±2.24	13.66±0.30
4	4	0.01±0.01 a	58.90±5.82 a	-36.61±3.18 a	25.46±2.27	13.01±0.31

Lowercase letters represent significant differences using Tukey-HSD (P < 0.05).

Live feeding trial – fluorescent microsphere

Butterflies of both species, *V. cardui* and *P. rapae*, displayed patterns where the positive controls had significantly higher intensity values than the split-proboscis treatment and the negative controls (ANOVA, $P<0.05$). The split-proboscis treatment and the negative controls did not significantly differ from each in their intensity values (Figure 11). In addition, butterflies with sealed proboscises tended to gain more weight during the feeding trials (Table 7).

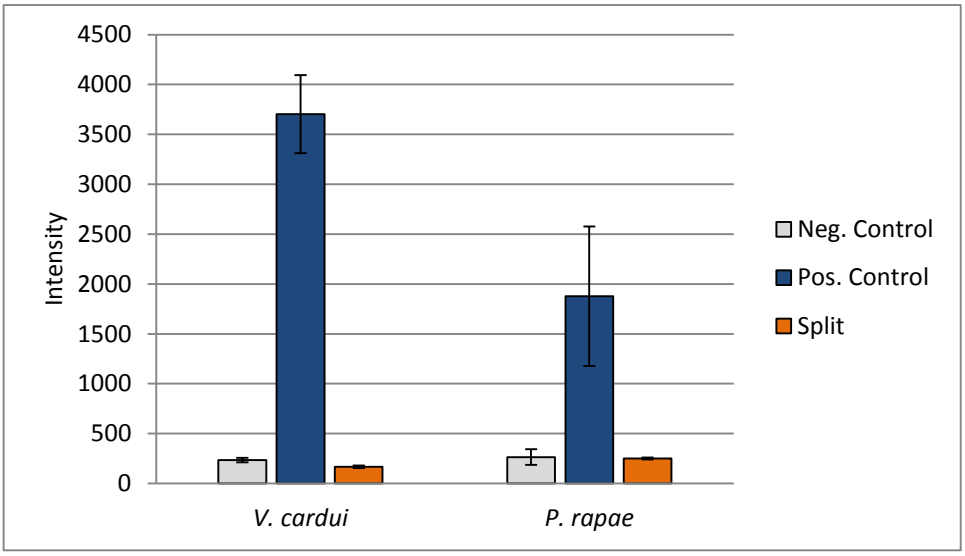

Figure 11. Mean (±S.E.M.) intensity values of the dissected crop determined with confocal microscopy. Butterflies with sealed proboscises were fed either a 20% sucrose solution mixed with fluorescent microspheres (positive control) or no microspheres (negative control. The split treatment represents intensity values of butterflies fed the microsphere solution with only a single galea.

Table 7. Butterflies were placed into treatment groups (+/sealed, -/sealed, or split/galae) and fed a 50% microsphere and sucrose solution (+ and split) for 3 minutes (RH = 16%). Weights were measured (g) before and after feeding to determine if feeding was successful.

Species	Treatment	FW length (mm)	Weight change	Sex	Temperature (C°)
P. rapae	+	24.9	0.0065	♂	24
	+	23.2	(-) 0.0009	♂	24
	+	22.1	0.0011	♂	24
	+	24.9	0.0031	♀	24
	-	25.6	0.0050	♂	24
	-	23.5	0.0011	♀	24
	-	23.9	0.0055	♀	24
	-	25.2	0.0064	♂	24
	split	23.1	0.0014	♀	24
	split	23.8	0.0010	♀	24
	split	25.3	0.0011	♂	24
	split	23.5	0.0011	♀	24
V. cardui	+	31.5	0.0459	♀	25
	+	28.9	0.0438	♀	25
	+	27.8	0.0303	♀	25
	+	30.8	0.0372	♀	25
	-	27.1	0.0271	♀	25
	-	30.7	0.0155	♂	25
	-	32.1	0.0263	♀	25
	-	30.1	0.0065	♂	25
	split	29.8	0.0007	♀	25
	split	27.4	(-) 0.0021	♀	25
	split	30.5	(-) 0.0021	♂	25
	split	29.9	0.0293	♀	25

Discussion

Capillarity responsible for fluid rise in C-shaped conduits

The fluid rise in a single galea is attributed to capillary action. The drinking straw model, as it applies to lepidopteran proboscises, indicates that capillarity plays only a minor role in fluid uptake (Kingsolver and Daniel 1995). Other studies that have indicated the importance of capillarity for feeding provide evidence that capillary action is an essential part of feeding from porous surfaces (Monaenkova et al. 2012, Lehnert et al. 2012). These studies have not fully investigated the methods of fluid uptake when Lepidoptera are presented with liquid pools. The evidence acquired here indicates that capillarity might play a much more important role in fluid acquisition from pools than previously thought.

Most studies of capillarity consist of a relationship between conduit sizes and the final heights reach by the liquid column (Morrow 1970). We used speed measurements rather than fluid height measurements because the fluids in the galea would sometimes travel the entire proboscis length, thus preventing a total height measurement. Proboscises taper distally, thus different treatments were tested because the smaller food canal size at the distal tip could influence the rate at which fluids come into contact with the food canal walls, thus influencing our speed measurements. The rates of fluid rise showed no significant differences within species between proboscises that contain or do not contain the drinking region. It is worth noting, however, that the application of hot glue to the proboscis tip in the DR treatment covered most of the drinking region. Future

studies might examine fluid rise in proboscises that retain the drinking region without the application of hot glue.

There were significant differences in rates of fluid rise among species, which likely relates to the different food canal sizes, however, *V. cardui* (medium-sized food canal; semi-brushy tip) had the slowest fluid rise. Considering that capillarity is likely the responsible phenomenon for fluid rise, we expected that *M. sexta* (largest food canal diameter, smooth tip) would have the slowest uptake rates. The slow rates in proboscises of *V. cardui* that contained a drinking region may be due to the accompanying sensilla styloconica that function as a mop (Lee and Lee 2014) and can retain fluids (Kramer et al. 2015). However, this behavior of sensilla hindering fluid uptake does not adequately explain the similarly slow rates in proboscises without the drinking region. Our preliminary experiments with *V. cardui* indicated faster fluid rise speeds than those reported here; therefore, future experiments are needed to determine if proboscis contamination occurred here, resulting in the slower speeds. In conjunction with capillarity, proboscis ellipticity might influence fluid rise (Lehnert et al. 2013, Tsai et al. 2014, Lehnert et al. 2016), despite the seemingly circular food canal cross section observed among species as shown here (Figure 6).

In addition, there was an enormous range in the heights and speeds at which liquid columns would travel, which caused difficulties in establishing patterns for interpretation. Fluids sometimes would start traveling quickly only to abruptly stop, and then start traveling again (Table 1). It is unclear at this point why this occurs; it could be

due to contamination, such as dust or other particles, impacting flow species, but also could be due to structural artifacts acquired during the proboscis straightening process.

There was considerable variation in the total height fluids traveled. Although the data is not presented here, fluids would sometimes travel the entire proboscis length, even the entire length of the *M. sexta* proboscis, which is more than 65 mm long. Conversely, liquid columns would sometimes have difficulty traveling half the length of the short proboscis of *P. rapae*, which also has the smallest food canal diameter and should have the highest rise in the liquid column.

Butterflies with split proboscises retain feeding abilities

Given that fluid rise was possible in split proboscises (C-shaped conduits), I hypothesized that butterflies would retain the ability to feed. The feeding experiments with the 20% green sucrose solution revealed there were no significant differences in the L*, a*, and b* color values among the treatments with sealed proboscises (treatment 1) and split proboscises (75%, 100%); they were both green. Treatment 1, however, indicated a crop full of green fluids, whereas butterflies in the split proboscis treatments had crops that were green, but full of bubbles. The presence of bubbles in the crop suggests that the butterflies might have been swallowing air through the sucking pump rather than only liquids. It is possible that the process of splitting proboscises also disables the oral valve that leads to the sucking pump (Eberhard and Krenn 2005). A disabled oral valve would prevent the closing of the sucking pump when the compressor muscles constrict the sucking pump, thus possibly pushing some fluids back down the food canal. In addition, an open oral valve exposed to the air (possible in our split

proboscis treatments) would allow air to enter the sucking pump. The ability of fluids to enter the sucking pump is likely due to capillarity and wettability dynamics, as the sucking pump would not be able to act fully on a liquid column in a C-shaped conduit. Incoming fluids likely moved as a film along the hydrophilic sucking pump and were swallowed by the pump into into the alimentary canal and move into the crop along with air bubbles.

The feeding trial performed with fluorescent microspheres revealed results that differed from those with the green food coloring; there were no significant differences between treatments with a split proboscis (100%) and the negative controls. We suspect the contradictory results between the two studies are due to differences between the lengths of the feeding times. The green food coloring feeding experiment allotted butterflies to feed for 10 minutes, whereas only 3 minutes were provided in the microsphere feeding experiments. Liquid columns might have traveled along the food canal (via capillarity) and into the sucking pump in both feeding trials, but were not able to reach the crop within the shorter time frame provided in the microsphere feeding trials. Although butterflies with split proboscises retain feeding abilities (Lehnert et al. 2014), the reduced fluid uptake rates likely result in those individuals having a lower fitness.

A new model for proboscis evolution

Capillarity might have played an essential role in the evolution of the lepidopteran proboscis. An ancestral proboscis, perhaps missing interlocking legulae, might have lacked the ability to create a semi-sealed conduit observed in derived species. These ancestral proboscises would be short in length (similar to extant Eriocraniidae), but have

the hydrophilic and hydrophobic dynamics observed on derived species, i.e., hydrophilic food canal and a hydrophobic galeal surface (Monaenkova et al. 2012, Lehnert et al. 2013, 2014) (Figure 12). The sucking pump in these ancestral groups might have been used more as a swallowing pump for incoming fluids rather than a pump to create a pressure differential for fluid rise. As the angiosperms were experiencing their radiation approximately 120 mya, the flower architectures were diversifying and flower corollas were lengthening, thus there were selective advantages to those insects that had longer proboscises that were tube-like. An increased proboscis length, however, also requires the ability to transport liquids over greater distances. As proboscis lengths increased, capillarity might have maintained an important role for the initial rise of fluids, which would have been amplified in a sealed tube rather than a C-shaped conduit (Raphaël 1989), but the sucking pump became necessary to bring fluids in remainder of the length and to increase the flow rate of those liquids. It is important to note, however, the capillary rise in the food canal might be restricted to when Lepidoptera feed from pools, not liquid films on porous surfaces.

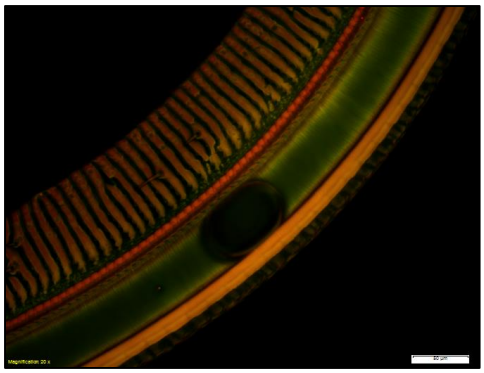

Figure 12. Confocal microscopy image of air bubble in the food canal of a lepidopteran proboscis. The contact angle indicates the hydrophilicity of the food canal.

Future studies and applications to microfluidics

We have research plans to continue exploring the phenomenon of capillarity in C-shaped conduits and lepidopteran proboscises. We have begun using laser ablation tomography (LAT) to create 3D images of the proboscis (Figure 13), which can be used to create proboscis models with a 3D printer. We intend to use a clear polymer to make the 3D models, thus we will be able to visualize the flow of fluids in closed proboscises and split proboscises in order to better understand the fluid dynamics.

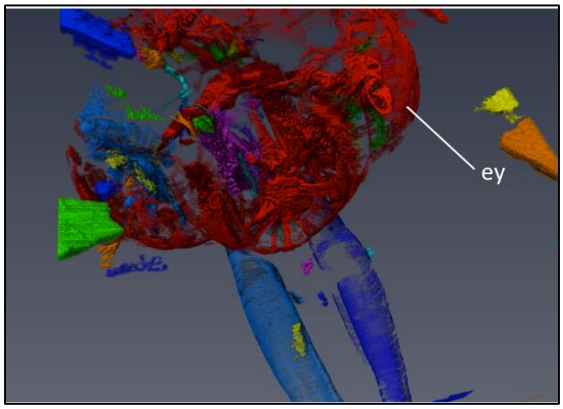

Figure 13. Laser ablation tomography of a vampire moth, *Calyptra thalictri*, head and proboscis. The (ey) indicates the eye. The image can be used to observe the internal anatomy of the head and corresponding structures.

The ability of fluids to travel against gravity in C-shaped conduits has applications to fields of microfluidics, material sciences, biomimicry, and the production of microfluidic devices. There are few studies that investigate capillary action in c-shaped conduits (Raphaël 1989). Due to its ability to acquire minute amounts of fluids, the lepidopteran proboscis is currently being used as a model for the production of novel microfluidic devices (Tsai et al. 2011, 2014). These devices are intended for applications in the medical and genomic fields and could have the abilities to acquire fluids, such as cytoplasm, from a single cell. Our findings here provide an alternative method for the design of the tip. A tip, for instance, could be C-shaped, thus reducing the affective area of the tip that is needed to pierce tissues or cell membranes, but would still retain capillarity action abilities. In addition, a tip with a star-shaped cross section would provide numerous C-shaped grooves that also could employ capillarity (Figure 14). The potential benefits to a tip of this type is 1) increased capillary potential and 2) the tip would not have to have a cylindrical cross-section (for capillarity in a tube-like conduit), thus allowing the production of a sharper tip for piercing membranes.

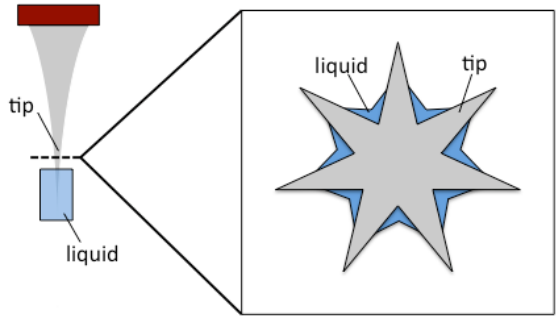

Figure 14. Micro-sized tip of a microfluidic device that could exploit capillarity in C-shaped conduits.

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