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ALLOMETRY AMONG STRUCTURES OF PROBOSCISES OF *VANESSA CARDUI* L. (NYMPHALIDAE)
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ABSTRACT. The morphometrics of proboscises of *Vanessa cardui* were examined to determine potential allometric growth relationships. Butterfly mass and six proboscis measurements were recorded from *V. cardui* reared on artificial diet treatments, in conjunction with fluid uptake rates. Log10 transformed data revealed negative allometry with proboscis length, butterfly mass, and drinking region length as the independent variables. Fluid uptake rates, however, had a nearly isometric relationship with most proboscis measurements, but not the total number of sensilla styloconica, which indicated inverse allometry with fluid uptake rates. The patterns exposed here show that the studied proboscis structures are important predictors for fluid uptake, but the selection pressures associated with feeding strategies and the substrates from which *V. cardui* feed favor a particular proboscis architecture, deviations from this configuration might require different feeding strategies and food sources.

Additional key words: Allometric growth, isometry, wettability, morphometrics, feeding strategies

The lepidopteran proboscis is composed of an assortment of structures that operate as a functional unit for liquid feeding. Fluid uptake dynamics depends on the orientation and spacing of proboscis structures and wettability dynamics (Monaenkova et al. 2012, Lehnert et al. 2013, Lee and Lee 2014) that work in coordination with the cibarial pump (Lee et al. 2014). This twofold system combined with the porosity along the proboscis legular seam (Kwauk et al. 2014) challenges the paradigm of the proboscis being sealed and straw-like in function (Kingsolver and Daniel 1979, Borrell and Krenn 2006, Bauder et al. 2013).

The structural diversity of the proboscis includes the two concave maxillary galeae that come together at their medial sides by dorsal and ventral legulae, which produces a tubular food canal after the adult emerges from the exuvium (Eastham and Eassa 1955, Krenn 1997, Krenn 2010). The ventral legulae interlink for the majority of the proboscis length (Lehnert unpublished). The lower branches of the dorsal legulae overlap for the proximal 80–95% of the proboscis length (Krenn and Kristensen 2000, Krenn et al. 2001), which represents an overall hydrophobic and structurally demarcated nondrinking region (Lehnert et al. 2013). Larger upper branches of dorsal legulae and enlarged interlegular spaces provide an overall hydrophilic profile that

characterizes the distal region (i.e., drinking region) of the proboscis (Lehnert et al. 2013).

The use of fluorescent stains coupled with studies of surface roughness and contact angle measurements suggests that proboscis structures differ in their wettability and role in the fluid uptake process (Monaenkova et al. 2012, Lehnert et al. 2013, Lee and Lee 2014). The galeal surface, for instance, is hydrophobic and might facilitate self-cleaning and the channeling of liquids to regions where fluid uptake occurs. The hydrophilic sensilla styloconica (chemosensilla) create a brushlike, distal region of the proboscis in some non-flower visiting species (Krenn et al. 2001, Molleman et al. 2005). The sensilla styloconica facilitate the channeling of fluids to the hydrophilic dorsal legulae (Lee and Lee 2014), which is further enhanced due to the sensilla creating an elliptical cross section of the proboscis that increases the meniscus height of liquid surfaces (Lehnert et al. 2013). The modified dorsal legulae combined with intervening spaces in the drinking region provide sponge-like abilities that bring fluids from porous surfaces into the food canal for subsequent delivery to the gut (Monaenkova et al. 2012).

The structural architecture of proboscises relates to feeding habits (Krenn et al. 2001, Molleman et al.

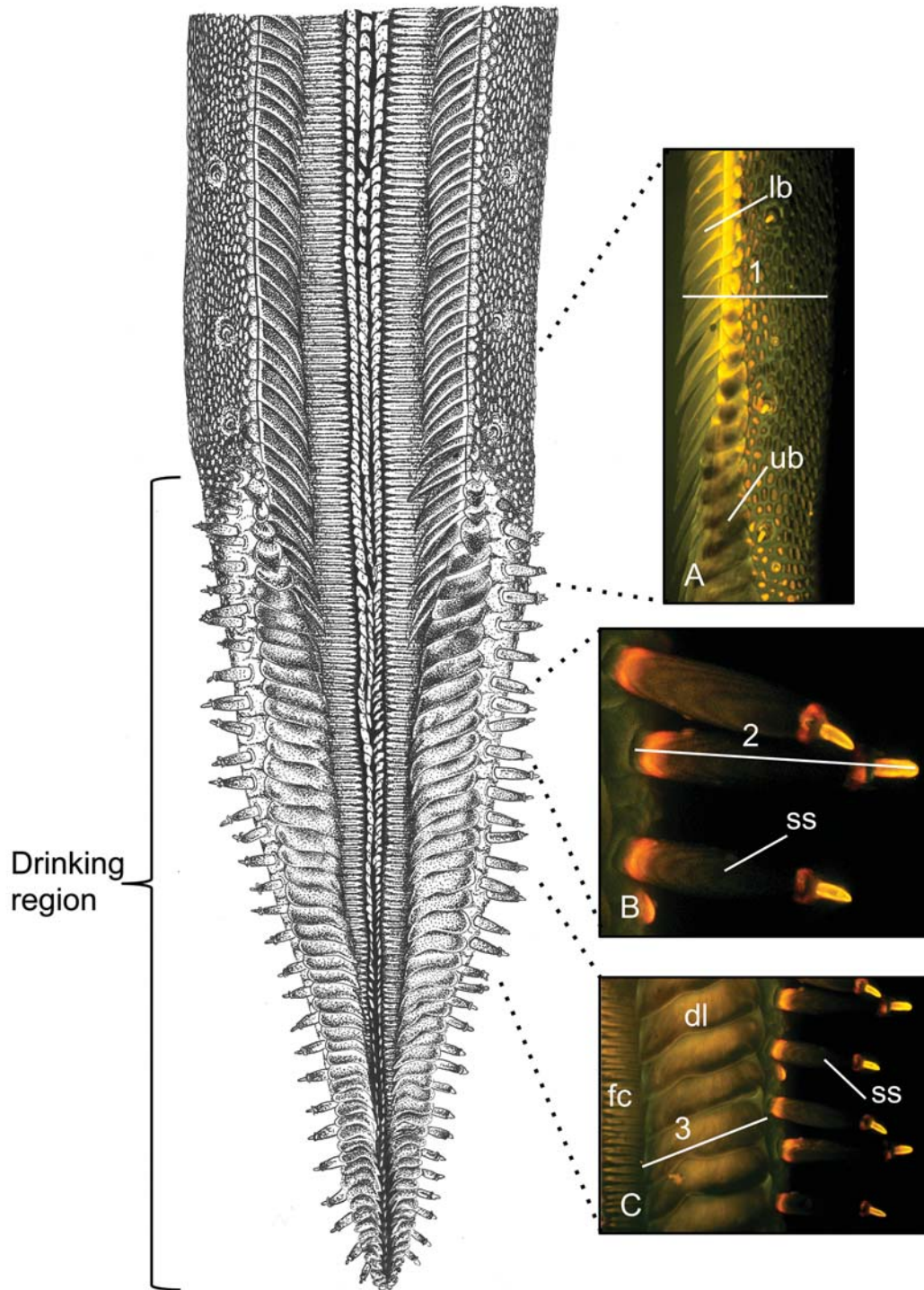


FIG. 1. Schematic showing proboscis measurements. The drinking region was measured from the transition point where the upper branches of dorsal legulae begin to enlarge to the proboscis tip (Lehnert et al. 2013). Insets **A** – **C** are confocal microscopy images showing proboscis measurements of: **1**) galea width, which was measured at the point where the upper branches of dorsal legulae (ub) begin to enlarge (lower branches, lb, shown for reference), **2**) length of sensilla styloconica (ss, measured from the base to the sensillum tip), and **3**) dorsal legulae width (dl, measured from the dorsal legulae base to the tip). The food canal (fc) also is shown in C. The spacing of the dorsal legulae between the opposing galeae, thus showing the ventral legulae in the middle and the food canal is an artifact from the preparation process. Illustration by Miranda Bowman.

2005, Zaspel et al. 2011); however, how proboscis structural configurations differ among individuals of different sizes within species is relatively unstudied and is the purpose of this investigation. Previous studies have indicated an allometric growth relationship between adult body size and structures in some insect species (Stern and Emlen 1999, Shingleton et al. 2008). Allometric growth is the disproportionate relationship between characters (e.g., structural, behavioral, physiological); as one character increases in measurement another character increases at a disproportionate rate (Huxley 1932, Klingenberg 1996). Huxley (1932) proposed the allometric growth equation $y = ax^b$, where y and x are the measured characters and a and b are constants. Log-transformed data results in the equation $\log y = \log a + b \log x$, where b is the slope of the line on a log-log plot between the measured characters and serves as an indicator of positive allometric ($b > 1$), isometric ($b = 1$), negative allometric ($0 < b < 1$), and inverse allometric ($b < 0$) relationships (Huber 1985, Klingenberg 1996). Allometric growth relationships have been studied in a wide variety of organisms, including plants (Weiner 2004), fish (Gisbert 1999), octopuses (Lefkaditou and Bekas 2004), humans (Shea and Bailey 1996), and insects (Nijhout and Wheeler 1996, Simmons and Emlen 2006, Emlen et al. 2007), including Lepidoptera (Agosta and Janzen 2005, Kunte 2007, Bauder et al. 2013).

Here, we investigate allometric growth relationships among the structural configurations of proboscises of Painted lady butterflies (*Vanessa cardui* L.) reared on artificial diet treatments. If allometry is present, we hypothesize that proboscis functionality could be affected by changes in structural conformations, for instance, changes in spacing between structures could impact capillarity and alterations in wettability patterns might affect the channeling of fluids. Fluid uptake rates, therefore, are examined for its relationship to proboscis structures. Fluid uptake rates in this study are based on the assumption that the cibarial pump operates similarly among individuals and scales proportionally in size with butterfly mass.

MATERIALS AND METHODS

Rearing protocol and diet treatments.

Eggs of *V. cardui* were obtained from Carolina Biological Supply Company© and placed into 2 oz plastic containers with lids (3 eggs per cup) and an artificial diet treatment (Educational Science©). Containers were placed into a Percival© environmental chamber maintained at 22° C, 60-65% RH, and a 16:8 photoperiod. In order to induce size differences among

individuals, treatments of artificial diet were prepared by varying the proportion of dH₂O mixed with the dry diet (15, 20, 25, 30, and 35% dH₂O). An additional diet mixture (16.66% dH₂O, recommended concentration by manufacturer) was used as a control. Third instar larvae were placed singly into individual 2 oz containers with their diet treatment (ad libitum) where they remained until adult emergence. Adult butterflies were placed into glassine envelopes approximately 24 hrs after emergence and stored in a 4° C refrigerator for assessment of fluid uptake rates.

Feeding trials.

Butterflies were removed from the refrigerator and kept in the glassine envelopes at room temperature (21° C) for 1 hr prior to each feeding trial. The mass (mg) of each butterfly was determined using a digital scale before each feeding trial. The butterflies were then fed a 20% sucrose solution from a 35 mm petri dish for 60 sec. An insect pin was used to maintain contact between the solution and the distal 25% of the proboscis for the entire feeding period. Butterflies were then weighed again. Fluid uptake rates were calculated by averaging the difference in butterfly weights before and after feeding trials from three feeding periods (one feeding per day) and divided by 1 min. Butterflies were stored in a 4° C refrigerator and allowed to warm for each feeding trial.

Proboscis imaging and structural measurements.

After the feeding trials, butterflies were placed in glassine envelopes and stored for 24 hrs. in a -80° C freezer. The heads were then removed using scissors, placed in glass vials with dH₂O and stored in a 4° C refrigerator. Scissors were used to remove proboscises from the heads, which were then placed on glass slides in dH₂O and imaged at 0.78X magnification with a Leica M205 C stereomicroscope. A measuring tool was placed next to the proboscis and an image of the total proboscis length was acquired with an IC 80 HD camera.

The dorsal side of approximately 25% of the distal region of proboscises was imaged at 20X magnification on an Olympus IX81 confocal microscope (GFP channel). Serial images were pieced together using Adobe® Photoshop CS5 into a composite image, which was used to take measurements. We measured: 1) the length of the drinking region (from the proboscis tip to the transition in the width of the upper branches of dorsal legulae), 2) widths of dorsal legulae and 3) sensilla styloconica near the midpoint of the drinking region, and 4) galea width at the transition point of the drinking and nondrinking regions (Fig. 1). The average widths of dorsal legulae and sensilla styloconica were determined by measuring three of each structure from

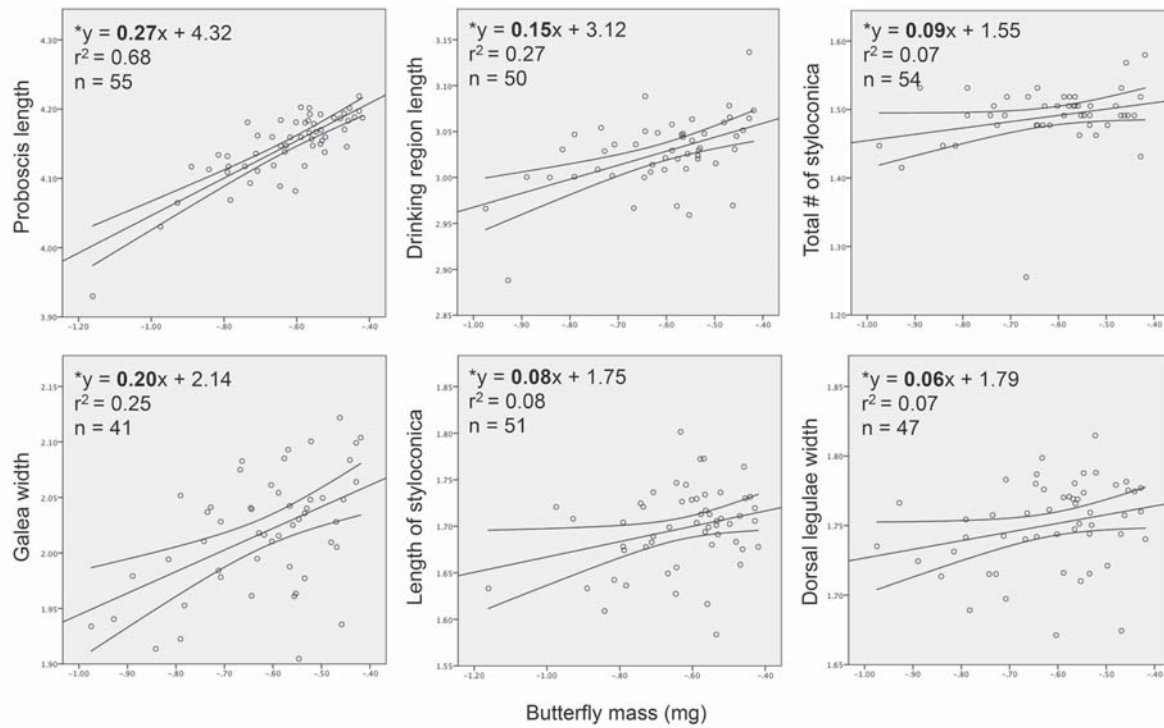


FIG. 2. Log10-Log10 plots indicating relationships between proboscis structures and butterfly mass. The line of best fit is accompanied with lines showing the mean confidence intervals (95%) and the slope for each equation is bolded. The (*) indicates slopes that significantly differed ($p < 0.0001$) from 1 using linear regression analysis.

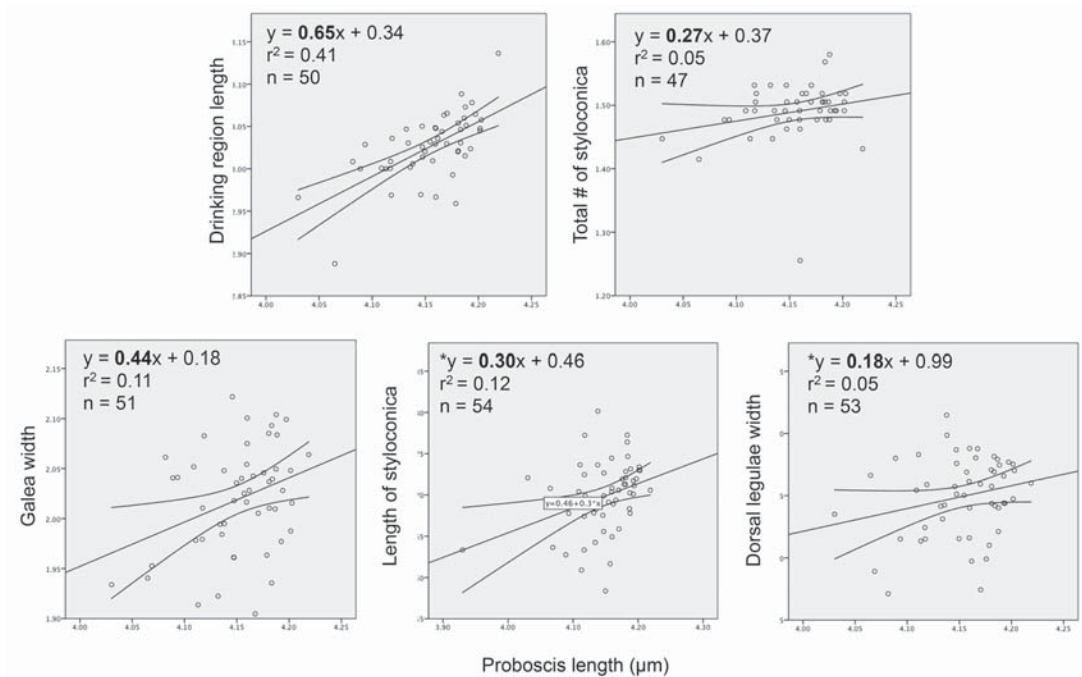


FIG. 3. Log10-Log10 plots indicating relationships between proboscis structures and proboscis length. The line of best fit is accompanied with lines showing the mean confidence intervals (95%) and the slope for each equation is bolded. The (*) indicates slopes that significantly differed ($p < 0.0001$) from 1 using linear regression analysis.

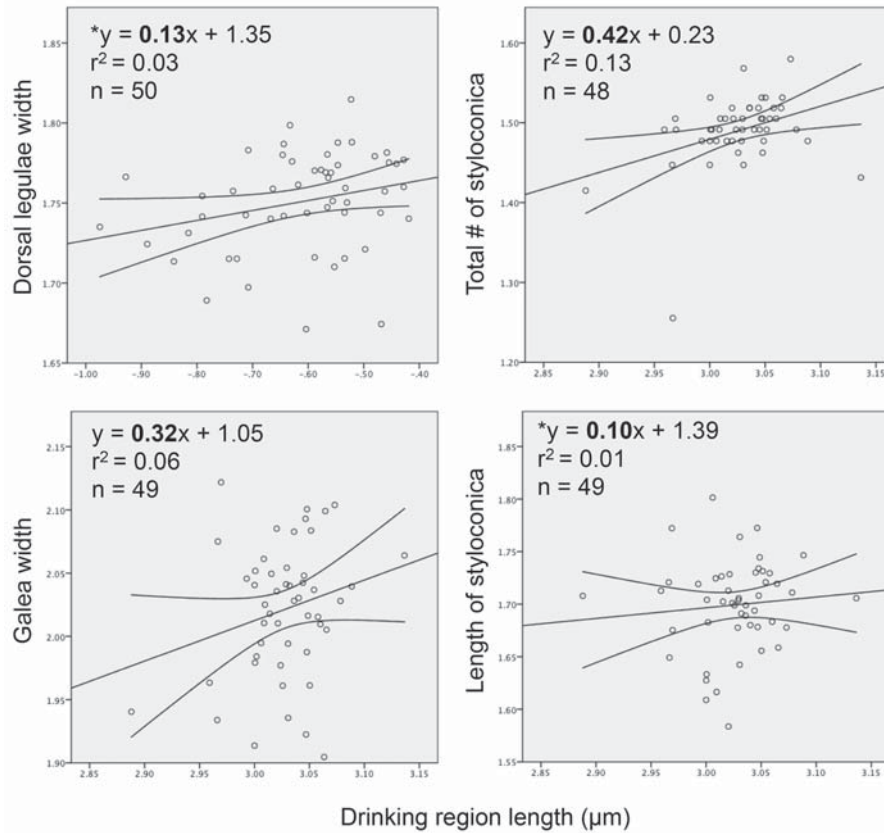


FIG. 4. Log₁₀-Log₁₀ plots indicating relationships between proboscis structures and drinking region length. The line of best fit is accompanied with lines showing the mean confidence intervals (95%) and the slope for each equation is bolded. The (*) indicates slopes that significantly differed ($p < 0.0001$) from 1 using linear regression analysis.

the midpoint of the drinking region length. The total number of sensilla styloconica was counted. All structures were measured using ImageJ software (<http://imagej.nih.gov/ij/>).

Statistical analyses.

Measurements were compared for significant differences among the diet treatments using analysis of variance (ANOVA, $p < 0.05$) and a Tukey post hoc test (SPSS software). Data were log₁₀ transformed and allometry was determined using the equation: $\log_{10} y = \log_{10} a + b \log_{10} x$, which is derived from the allometry equation $y = ax^b$ where b is the slope of the log₁₀-log₁₀ plots of x and y , and a is the y -intercept. Body mass, proboscis length, and the drinking region length were used as the independent variables in a linear regression analysis. A separate linear regression analysis was performed with fluid uptake rates as the dependent variable. Departures from isometry (i.e., slope $b = 1$) were tested for significance ($p < 0.05$) with a Student's t test. A multiple regression analysis was conducted to

determine the impact of structures on fluid uptake rates. All data were analyzed using SAS (unless noted otherwise) and figures for log₁₀-log₁₀ plots were produced in SPSS.

RESULTS

A total of 56 *V. cardui* were reared; however, there were individuals that did not qualify for some recorded measurements for analysis. Butterflies with split proboscises, for instance, were not used to assess fluid uptake rates. The variables: butterfly mass, proboscis length, drinking region length, dorsal legulae width, and total number of sensilla styloconica indicated significant differences among butterflies of different diet treatments (Table 1). Butterflies reared on the 25 and 30% diet treatments were larger in mass and proboscis structure measurements than individuals reared on other diet treatments.

Linear regression analysis indicated that all slopes (b) of the log₁₀-log₁₀ plots significantly deviated from 1

TABLE 1. Measurements of variables used to determine allometric growth relationships. The treatments are named according to the percentage of dH₂O mixed with the dry diet.

Variable	Treatment	n	Minimum	Maximum	Mean \pm s.e.m.
Butterfly mass (mg)	15	6	0.11	0.25	0.16 \pm 0.02d
	16.66	9	0.16	0.34	0.22 \pm 0.02bcd
	20	7	0.14	0.28	0.20 \pm 0.02cd
	25	11	0.2	0.38	0.30 \pm 0.02ab
	30	9	0.26	0.37	0.31 \pm 0.01a
	35	13	0.16	0.36	0.27 \pm 0.01abc
Proboscis length (μ m)	15	6	10722.2	13090.63	11934.48 \pm 326.13c
	16.66	10	8511.97	14431.91	12943.76 \pm 534.08bc
	20	7	12969.37	15582.44	14177.07 \pm 377.91ab
	25	10	13734.07	16548.48	15223.09 \pm 261.09a
	30	10	13121.76	15748.42	14894.71 \pm 230.63a
	35	13	13096.65	15905.36	14639.66 \pm 236.96a
Drinking region length (μ m)	15	5	772.72	1068.32	957.29 \pm 51.59b
	16.66	8	932.34	1118.58	1037.45 \pm 22.80ab
	20	6	926.13	1132.58	1028.96 \pm 28.40ab
	25	10	1035.96	1368.93	1123.64 \pm 30.78a
	30	10	930.93	1197.2	1095.47 \pm 26.65a
	35	12	910.04	1225.79	1080.29 \pm 22.66ab
Dorsal legulae width (μ m)	15	6	46.9	58.39	52.24 \pm 1.66b
	16.66	9	55.15	62.89	58.65 \pm 0.80a
	20	7	51.7	58.74	55.24 \pm 1.10ab
	25	11	49.81	65.27	57.63 \pm 1.28a
	30	9	47.25	61.34	56.77 \pm 1.66ab
	35	12	51.3	61.22	55.88 \pm 0.93ab
# of sensilla styloconica	15	4	26	34	29.5 \pm 1.71ab
	16.66	8	30	34	31.38 \pm 0.53ab
	20	6	18	32	28.17 \pm 2.17b
	25	10	27	38	31.70 \pm 0.93ab
	30	9	30	37	32.67 \pm 0.67a
	35	11	29	34	31.09 \pm 0.39ab
Sensilla styloconica width (μ m)	15	5	42.99	52.58	47.50 \pm 1.95
	16.66	10	42.42	63.31	50.77 \pm 1.98
	20	7	40.64	53.03	46.89 \pm 2.08
	25	11	47.62	54.49	51.24 \pm 0.73
	30	9	45.58	59.23	53.17 \pm 1.58
	35	13	38.34	55.8	49.43 \pm 1.24
Galea width (μ m)	15	6	85.88	115.15	97.19 \pm 5.08
	16.66	9	83.65	132.37	106.82 \pm 4.79
	20	6	81.97	118.85	102.80 \pm 5.02
	25	11	102.41	127.03	113.28 \pm 2.57
	30	8	80.29	125.64	105.66 \pm 5.71
	35	12	91.42	121.25	102.32 \pm 2.87
Fluid uptake rate (mg/min)	15	1	0.012	-	-
	16.66	7	0.008	0.016	0.012 \pm 0.001
	20	6	0.007	0.017	0.011 \pm 0.001
	25	9	0.008	0.015	0.011 \pm 0.002
	30	7	0.006	0.015	0.011 \pm 0.003
	35	11	0.003	0.013	0.009 \pm 0.001

Variable means with lowercase letters indicate significant differences ($p < 0.05$) and a Tukey post hoc test.

Table 2. Results from linear regression analysis to determine allometry between proboscis characters (lengths and widths measured in μm) and fluid uptake rate (mg/min) (dependent variable). The slope values are shown in bold.

Independent variable	n	Equation	r ²	p-value	Allometry
Drinking region length	36	y = 1.13 x - 5.42	0.06	0.858	0
Dorsal legulae width	37	y = 2.85 x - 6.99	0.17	0.0928	0
Total # sensilla styloconica*	34	y = -0.45 x - 1.32	0.02	0.0194	- -
Sensilla styloconica width	38	y = 1.74 x - 4.93	0.24	0.159	0
Galea width	35	y = 0.87 x - 3.75	0.09	0.7919	0
Butterfly mass*	39	y = 0.04 x - 1.96	0	<0.0001	-
Proboscis length	38	y = 1.10 x - 6.57	0.05	0.8951	0

- - indicates inverse allometry, - indicates negative allometry, 0 = isometry

* indicates variables with slope values that significantly deviate from 1 (isometric relationship) (p-value < 0.05)

when butterfly weight, proboscis length, and drinking region length are used as independent variables ($p < 0.05$), and all showed negative allometric relationships ($0 < b < 1$) (Figs. 2 – 4). When butterfly weight was used as the independent variable the r^2 values ranged from 0.07 (total numbers of sensilla styloconica and dorsal legulae width) to 0.68 (proboscis length) (Fig. 2). Using the proboscis length as an independent variable also produced a range of r^2 values (0.41 for drinking region length to 0.05 for total number of sensilla styloconica and dorsal legulae width) (Fig. 3); all r^2 values were small when using drinking region length as the independent variable (Fig. 4).

When fluid uptake rate was used as the dependent variable, only the total number of sensilla styloconica and butterfly mass produced slopes that significantly differed from 1 (Table 2). The slope between the total number of sensilla styloconica and fluid uptake rate indicated inverse allometry ($b < 0$, $n = 34$, $p = 0.0194$) and the slope with butterfly mass showed negative allometry ($n = 39$, $p < 0.0001$). The r^2 values were low for comparisons that used fluid uptake rate as the dependent variable, and was 0 when butterfly mass was used as a potential predictor.

A multiple regression analysis was conducted to predict the overall fluid uptake rate with proboscis measurements and butterfly mass as the variables (Table

3). The combination of these measures was significantly related to fluid uptake rate ($F_{7,24} = 8.29$, $p < 0.0001$). The coefficient of determination indicated that the studied variables account for 70.74% of the variability in fluid uptake rate. Fluid uptake rate increased for each unit increase in proboscis length, dorsal legulae width, length of sensilla styloconica, and galea width when holding each variable constant (Table 3). Fluid uptake rate decreased, however, with increasing drinking region length, number of sensilla styloconica, and butterfly mass. Only dorsal legulae width, the total number of sensilla styloconica, and butterfly mass had a slope that significantly differed from 0.

DISCUSSION

A functional proboscis plays an essential role in butterfly fitness, including sodium intake associated with puddling behavior (Arms et al. 1974) and the acquisition of amino acids and carbohydrates (Bauernfeind and Fischer 2005). Previous lepidopteran studies of allometric growth of proboscises have primarily focused on relationships between proboscis length and body size with respect to the cibarial pump, feeding strategies, and fitness trade-offs (Kunte 2007, Bauder et al. 2013). Intraspecific allometric studies provide insight into the importance of structures for specific functions; they also offer predictive parameters regarding how structures

TABLE 3. Multiple linear regression analysis of proboscis structures (lengths and widths measured in μm) with fluid uptake rate as the dependent variable (32 observations used, $r^2 = 0.7074$, $df = 1$).

Variable	Parameter estimate	St. error	t value	pr > t
Intercept	-0.45225	0.14827	-3.05	0.0055
Proboscis length	0.06311	0.03613	1.75	0.0934
Drinking region length	(-)0.0237	0.02075	-1.14	0.2647
Dorsal legulae width*	0.12992	0.0264	4.92	<0.0001
Total # of sensilla styloconica*	(-)0.03000	0.01347	-2.23	0.0355
Sensilla styloconica width	0.02929	0.01957	1.5	0.1475
Galea width	0.01221	0.01535	0.8	0.434
Butterfly mass*	(-)0.02432	0.00805	-3.02	0.0059

* indicates parameter estimates that differ significantly from 0

might be selected for or against under particular environmental conditions. Here, we addressed how structures of proboscises vary in *V. cardui* of different body masses and if these variations relate to fluid uptake rates.

All measured proboscis structures exhibited a similar trend of negative allometry ($0 < b < 1$) when related to butterfly mass, proboscis length, and drinking region length. We suspect that the lack of an isometric relationship is due to the intense selection pressures that act on the proboscis architecture. Butterfly feeding strategies are complex and have been optimized for efficient food searching and handling (Boggs 1988, Krenn 1998), in addition to fluid uptake. Extensive alterations in proboscis architectures might prevent feeding from the flowers from which *V. cardui* use as nectar sources, i.e., adding one μm to proboscis width per one μm added to proboscis length could result in a proboscis too wide to reach nectar sources in narrow floral corollas. When put into context of fitness trade-offs (Berenbaum et al. 1986, Futuyama 1997, Agrawal 2000), the data indicate that similar amounts of resources are allocated to proboscis structures to maintain feeding habits and an optimal fluid uptake rate within species, regardless of butterfly mass. The results of the linear regression, however, conflict with those of the multiple linear regression, suggesting there is some evidence of multicollinearity among the independent variables. The fluid uptake rates measured in this study are similar to those reported in previous studies of *V. cardui* feeding abilities (Hainsworth et al. 1991; Kwauk et al. 2014).

Sensilla styloconica, in addition to their chemosensory abilities (Altner and Altner 1986, Petr and Stewart 2004), are often attributed to function similarly as a mop, adapted for acquiring liquids from films on porous surfaces (Molleman et al. 2005, Lehnert et al. 2013, Lee and Lee 2014); however, this study indicated that increasing the number of sensilla styloconica decreased fluid uptake rates. We suggest that proboscis movements (Krenn 1998, Knopp and Krenn 2003, Tsai et al. 2014) that resemble mopping on surfaces might assist with the channeling of fluids from the sensilla styloconica to the dorsal legulae where fluid uptake can occur. The pool feeding system used here to test fluid uptake rates eliminated proboscis-to-surface contact; therefore, liquids might become trapped in the spaces between the hydrophilic sensilla rather than move to the dorsal legulae. The generalized feeding habits of *V. cardui* might favor maintaining sensilla styloconica for surface feeding, but these are fewer in number compared to other butterfly species that primarily visit exposed liquids on porous surfaces (Petr and Stewart 2004, Krenn et al. 2001).

The presence of a nearly isometric slope between most studied proboscis structures and fluid uptake rates indicates that these variables are important for fluid uptake, which could have important attributes to biomimetic studies. The lepidopteran proboscis has become a model for the production of microfluidic devices (Tsai et al. 2011). We envision the next generation of microfluidic devices capitalizing on the structures and their role in fluid uptake described here. Additional studies of allometry in butterflies with other feeding habits also could contribute to a pool of knowledge of proboscis structure and function relationships; therefore, setting the stage for microfluidic probes that vary in their structures for particular functions.

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