Annals of the Entomological Society of America, XX(X), 2019, 1–9
doi: 10.1093/aesa/saz041
Research

Research

Self-repair of the Lepidopteran Proboscis

Suellen F. Pometto,[1,4,©] Charles E. Beard,[1] Patrick D. Gerard,[2] Konstantin G. Kornev,[3] and Peter H. Adler[1]

[1]Department of Plant and Environmental Sciences, Clemson University, Clemson, SC 29634, [2]School of Mathematical and Statistical Sciences, Clemson University, Clemson, SC 29634, [3]Department of Materials Science and Engineering, Clemson University, Clemson, SC 29634, and [4]Corresponding author, e-mail: spomett@clemson.edu

Subject Editor: Vonnie Shields

Received 24 May 2019; Editorial decision 8 July 2019

Abstract

Self-repair in the animal world typically involves regeneration of body parts. We present an example featuring the proboscis of butterflies and moths, which after separation of the galeae, undergoes nonregenerative repair. We demonstrated the ability of representative species to completely reunite (repair) the proboscis after total separation of the two galeae, and we showed that the repaired proboscis can take up fluid. Movements of the proboscis during repair were similar to the initial proboscis assembly after emergence from the pupa. We tested the influence of labial palps, wing movements, coiling, and fluid immersion on self-repair. These factors showed no statistically significant influence on the ability to repair the proboscis, with the exception of wing movements in one species. We suggest that the major selection forces driving assembly and repair have been the need to insert the proboscis into restricted openings of floral tubes to obtain nectar and the need for a united, compactly coiled proboscis to reduce air resistance during flight.

Key words: Lepidoptera, proboscis, butterfly, moth, Nymphalidae

Self-assembly occurs at scales from molecular to organismal and beyond, such as in the organization of flocks of birds, schools of fish, and synchronized flashing of fireflies (Camazine et al. 2001). Self-assembly autonomously brings order to disordered components and integrates environmental factors, templates, and behaviors to produce new functions (Whitesides and Grzybowski 2002). Assembly typically involves the initial fitting together of parts, whereas repair involves restoration of parts that have been damaged or become separated. At the tissue level, self-repair compensates for damage from interactions with the environment, and at the level of the organism, it involves remodeling, healing, or growth (Weinkamer et al. 2013). At all levels, self-repair restores a damaged, worn, or deteriorated part to working order.

Among vertebrates, repair is usually a cellular or developmental process, such as wound repair or regeneration of lost limbs. Animal grooming, such as preening of bird feathers, could be considered a form of mechanical repair because it restores or maintains wing function. Self-repair in arthropods can occur by regeneration of lost limbs through molting (Maruzzo and Bortolin 2013). However, the absence of molts in adult pterygote hexapods limits the opportunities for repair of appendages and other body parts to the immature stages (Minelli and Fusco 2013).

The proboscis of butterflies and moths is self-assembled when the insect emerges from the pupa (Krenn 1997) and represents a special case of repair that does not involve molting. We, therefore, asked whether separation of the galeae after assembly could lead to nonregenerative repair. The proboscis consists of two elongate galeae united by overlapping plate-like dorsal legulae and hook-like ventral legulae (Eastham and Eassa 1955, Krenn 2010). Krenn (1997) described the process of assembly as a 'once in a lifetime sequence of events' and proposed that if the galeae became separated after assembly, the loss of flexibility in the sclerotized proboscis would prevent repair and the separated food canal would prevent acquisition of fluids. However, based on the theory of capillarity and wetting, fluid uptake by the open, wettable food channel should occur (Darhuber et al. 2003, Berthier et al. 2016). Uptake of fluid has been confirmed for two butterfly species, *Papilio polyxenes asterius* Stoll and *Pieris rapae* L., when the galeae are separated up to half of their length (Lehnert et al. 2014).

We systematically investigated whether butterflies could repair a proboscis after total separation of the galeae, and if so, whether fluid uptake could be restored. To distinguish initial proboscis assembly from subsequent separation and reunion, we refer to the latter process of galeal gathering and relinking into a single unit as 'repair'. This distinction emphasizes that repair occurs after cuticular sclerotization and tanning, resulting in a change in the material properties of the proboscis.

We then examined the influence of selected structures and behaviors on the process of repair. To identify the primary mechanisms that might play a role in repair, we used the observations of

proboscis assembly reported by Krenn (1997), who demonstrated the involvement of coordinated movements of the wings and labial palps, repeated extension and coiling of the galeae, and saliva production. Additionally, although holding butterflies by their wings for experimental purposes is technically convenient, we wished to know whether this artificial constraint might affect repair. Accordingly, we experimentally tested the following hypotheses: 1) butterflies can repair a totally separated proboscis, 2) the repaired proboscis is functional, 3) labial palps are necessary for repair, 4) wing movements affect repair.

Subsequent studies of assembly have demonstrated that saliva facilitates galeal union by capillary action (Zhang et al. 2018a,b). Proboscis coiling is associated with directional and controlled transport of saliva, which is needed to hold the galeae together by the formed menisci, while the insect links the legulae (Zhang et al. 2018b). When the insect is not able to coil separated galeae, it should still be able to gather and reunite them, with saliva facilitating the capillary adhesion of the two strands (Zhang et al. 2018a). Therefore, we tested the hypothesis that 5) coiling is necessary for repair. Based on preliminary observations that fluid feeding facilitates repair (Pometto 2014), we also simulated feeding from a pool of liquid by submersing the proboscis in water after separation to test the hypothesis that 6) fluid feeding facilitates repair.

We suspect that self-repair among insects is more widespread than currently appreciated. Thus, in the context of our findings, we discuss additional possibilities for repair, as well as the selection pressures that might have driven the evolution of proboscis repair. The mechanical process of self-repair in nature also offers lessons that can be of benefit to engineering. We, therefore, also suggest applications of our research.

Materials and Methods

Insects and Rearing Conditions

We tested five butterfly and two moth species: *Vanessa cardui* (L.) and *Danaus plexippus* (L.) (Nymphalidae), *Papilio cresphontes* Cramer (Papilionidae), *Phoebis sennae* (L.) (Pieridae), *Heliothis virescens* (Fabricius) (Noctuidae), and *Manduca sexta* (L.) (Sphingidae). These include species that have served as models in our previous work and, therefore, provide a foundation for understanding the proboscis (Monaenkova et al. 2012; Lehnert et al. 2016; Zhang et al. 2018a,b). All of these species were laboratory reared except *P. sennae*, which was field collected (Table 1).

Adults of *D. plexippus* and *P. cresphontes* were reared from pupae obtained from Shady Oak Butterfly Farm, Brooker, FL, or from eggs derived from these cohorts. Adults of *V. cardui* and *M. sexta* were reared from larvae obtained from Carolina Biological Supply Company, Burlington, NC. Adults of *H. virescens* were reared from larvae obtained from Benzon Research, Carlisle, PA. Adults of *P. sennae* were collected at the South Carolina Botanical Gardens, Clemson, SC (34.6760N, 82.8222W) on 23 October 2015 and maintained at 4–8°C until use. Adults of additional families were collected at the Clemson University Cherry Farm in Clemson, SC (34.6532N, 82.8338W). Representatives of species used were deposited in the Clemson University Arthropod Collection (CUAC). Eggs and larvae were maintained in the laboratory until pupation. All pupae were reared with a 12:12 photoperiod (artificial lighting) at 22–31°C and relative humidity of 46–95%. All experiments were run between 0900 and 2100 EDT or EDST. Adult specimens with patent deformities or that did not fully assembly the proboscis after emergence were excluded from experiments.

Specimen Handling and Imaging

Reared specimens were used 20–47 h after emergence from the pupa, except in the fluid immersion experiment (73–81 h after eclosion). No food or water was given before the experiments. Individuals were randomly assigned to treatment or control groups. Each cohort of butterflies and moths was dedicated to a specific experiment to ensure that the rearing conditions within each test were standardized. Experiments were performed at 23–30°C and relative humidity of 26–72%.

We restrained the wings by inserting them vertically over the body into a glassine sleeve and securing them with a clothes pin roughly parallel to the body (Pometto 2015). The legs were restrained by a second sleeve overlapping the first and secured by the same clothes pin. Once secured, each insect was placed laterally on a Parafilm-wrapped glass slide with a translucent ruler inserted in the Parafilm (Pometto 2015). The proboscis was extended and propped in position with insect pins and photographed under low magnification with a Canon EOS Rebel T3i camera (Canon USA, Inc., Melville, NY) mounted on a MEIJI Techno RZ dissecting microscope (MEIJI Techno Co., LTD, San Jose, CA). To separate the galeae, we gently palpated the proboscis with a capillary tube (1.0 mm outer diameter) distal to the bend region to initiate a medial separation of the galeae. We then inserted the capillary tube between the galeae distal to the bend region and moved the tube along the length of the proboscis distally and proximally to separate the galeae totally (Pometto 2014). For the repair experiments, the proboscises of treatment specimens were separated and the proboscises of control specimens were left intact. Krenn (1997) determined that assembly occurs within 210 min after eclosion. We, therefore, worked within a time frame of 1 h. Photographs were taken at 0, 10, 20, 30, 45, and 60 min.

We took measurements from the photographs using ImageJ software (National Institutes of Health, Bethesda, MD) to compute the percent union of the galeae (U), using the formula $U = (L_{united}/L_{total}) \times 100$, where L_{united} is the united length of the proboscis measured from the base of the head, and L_{total} is the total length of the proboscis. When the proboscis presented a medial split consisting of two discontinuous sections united and a separated region in between, we summed the two united regions to compute L_{united} (Fig. 1). General

Table 1. Species and sample sizes (control: treatment) of Lepidoptera used in six experiments involving proboscis repair

Species	Repair	Functionality	Labial palps	Wing restraint	Coiling	Fluid immersion
Danaus plexippus	11:11	9:11	8:8	11:10	9:11	13:13
Vanessa cardui	11:10	9:10	10:10	11:11	9:9	13:13
Papilio cresphontes	11:12	11:12	—	—	—	—
Phoebis sennae[1]	5:6	—	—	—	—	—
Heliothis virescens	11:12	8:11	—	—	—	—
Manduca sexta	7:10	—	—	—	—	—

[1]Field collected; all other species were laboratory reared.

observations of the behaviors used in repair were made during the experiments.

Images of the ventral legulae of at least one individual of each species that had completely reunited the proboscis were taken to confirm linkage. The ventral surface of the proboscis of *D. plexippus* was imaged while the insect fed. For *V. cardui* and *H. virescens*, the extended proboscises of three dispatched specimens of each species were dehydrated in an ethanol series and fixed using hexamethyldisilazane (HMDS). The fixed proboscises of *V. cardui* were viewed with a Zeiss SmartZoom-5 digital microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY), and fixed proboscises of *H. virescens* were viewed on a Hitachi TM3000 Scanning Electron Microscope (Hitachi, Tarrytown, NY). The repaired, coiled proboscis of one individual of *M. sexta* was fixed in Bouin's solution, washed and dehydrated in a tert-butyl alcohol series, infiltrated and embedded in L. R. White acrylic resin, sectioned with an 820 Spencer Microtome (American Optical Corp., Southbridge, MA), stained with methylene blue, and photographed with a Jenoptik ProgRes SpeedXT Core 5 digital camera (Jenoptik, Huntsville, AL) on an Olympus BH-2 compound microscope (Olympus Corporation of the Americas, Miami, FL).

Functionality after Repair

Control and treatment specimens of *D. plexippus*, *V. cardui*, *P. cresphontes*, and *H. virescens* were tested for the ability to take up fluid by feeding them 21–29 h after initial separation of the proboscis (Table 1). Individuals were placed for at least 5 min on a brown paper towel saturated with 12–27% sucrose-water (v:v) with six drops of blue food coloring per 100 ml of water (Southern Home Assorted Food Coloring Set, Jacksonville, FL). The sugar concentration was measured with a Bellingham and Stanley Pocket Refractometer (Epic, Inc., New York, NY). If an individual did not voluntarily extend its proboscis, we extended it with an insect pin to initiate contact with the paper towel. After feeding, the legs and body were rinsed with tap water and blotted dry, and the specimens were placed in separate cages lined with white filter paper (qualitative, medium) to collect gut exudate for 21–29 h at 24–30°C.

Functionality of the proboscis was demonstrated by presence of blue dye in the gut exudate on the filter paper. If filter paper was negative for dye, the specimen was either fed again and observed for colored gut exudate or dissected to determine whether dye was in the gut. At least two individuals of each species were used as color controls by following the same procedure but feeding the specimens with clear sucrose solution.

Repair by Wild-Caught Lepidoptera

To determine the generality of repair among Lepidoptera, we collected representatives of additional families. The previously described separation procedure was adapted for small specimens as follows: 1) instead of using clothes pins, the wings were covered with glassine sleeves and a pin was inserted into the sleeve above and below the wings, parallel to the body; 2) the galeae were separated using a minuten pin mounted on a probe; and 3) measurements were taken at 30 and 60 min to reduce manipulation of the proboscis.

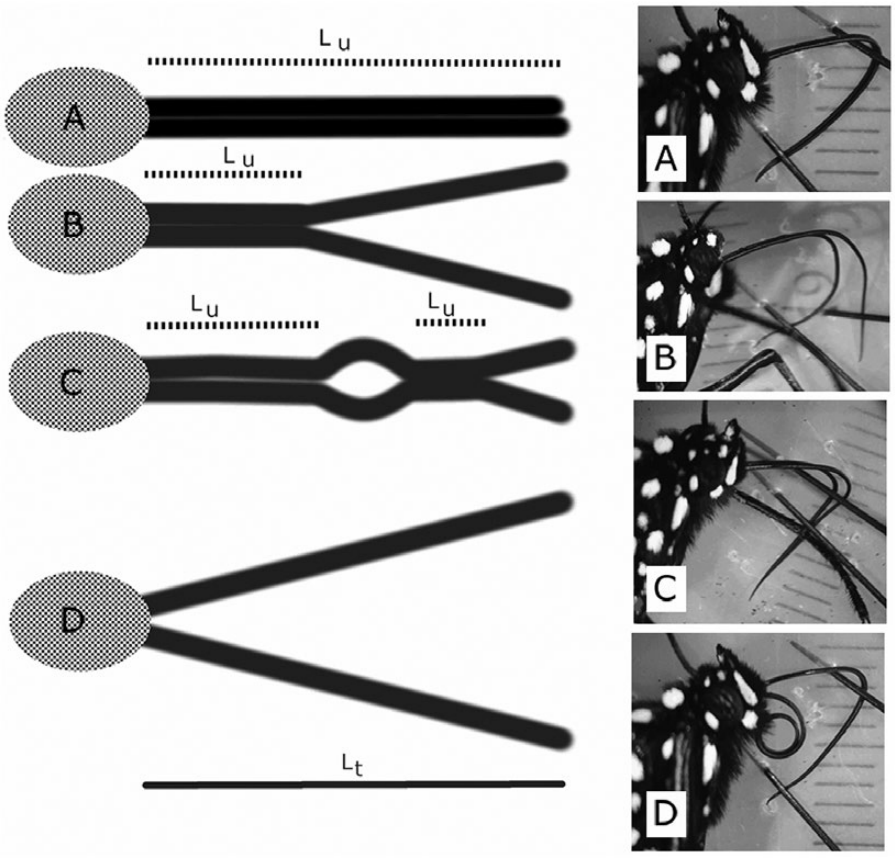

Fig. 1. Types of union of the galeae; schematic drawings in dorsal view on the left, photographs in lateral view from a single specimen of *Danaus plexippus* on the right. (A) Fully united galeae; total length (L_t) equals the united portion of the galeae (L_u), $L_u = L_t$. (B) Partially united galeae, $L_u < L_t$. (C) Partially united galeae with a medial separation; the united portions are summed for L_u. D. Totally separated galeae, $L_u = 0$.

Methodology for Testing the Hypotheses

To study the mechanisms of repair of the proboscises, we used *D. plexippus* and *V. cardui* (Table 1). The methods previously described were followed except 1) the proboscises of both treatment and control specimens were totally separated; 2) the legs were restrained for the labial palp and coiling experiments and unrestrained for the wing restraint and fluid immersion experiments; 3) the proboscises were imaged initially and at an end point based on the time interval in which most repair occurred for that species (10 min unless otherwise stated). To test the role of the labial palps, all three segments of each labial palp were removed with microscissors from treatment specimens; no bleeding occurred. Labial palps of control specimens were not removed. To test whether wing movements assisted repair, the wings of treatment specimens were unrestrained, allowing freedom of movement within a container (14 cm height × 11 cm diameter). The wings of control specimens were restrained throughout the experiment.

To test the influence of coiling on repair, after separating the galeae of all individuals, we positioned each insect vertically on a Styrofoam stage with its body in a groove. For treatment specimens, the separated galeae were extended over a horizontal support consisting of a 7.0-mm contiguous row of insect pins (heads of pins removed) that supported the middle of the galeae but did not contact the proboscis base and allowed the tip to extend beyond the row of pins (Fig. 2). The row of pins was mounted on a soft plastic support that could be attached with two pins to the end of the groove of the Styrofoam stage for treatment specimens or removed for control specimens. For the treatment specimens, this arrangement allowed lateral movement and elevation of the galeae, but not coiling. Elevation of the head was prevented by two insect pins crossing behind the head and inserted into the Styrofoam stage. For control specimens, the support was removed, the head and wings were similarly restrained, and the separated galeae were extended into an open chamber that allowed full movement of the proboscis without contacting the sides of the chamber. At the end point (10 min for *V. cardui* and 5 min for *D. plexippus*), each specimen was removed from the stage and the proboscis was photographed.

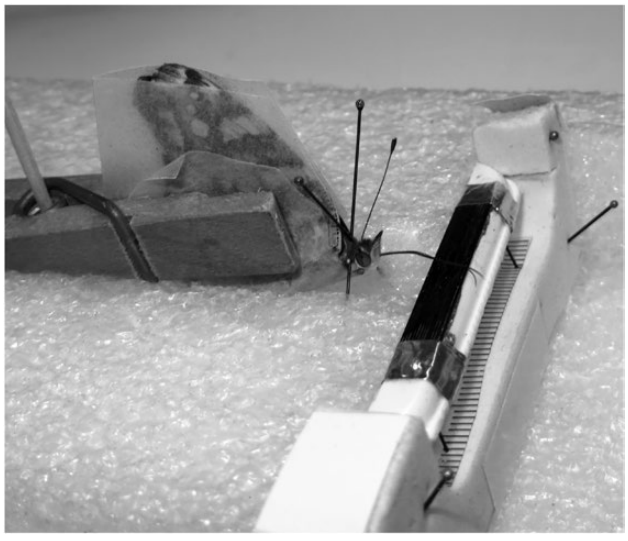

Fig. 2. Experimental setup for testing the effect of coiling on proboscis repair. The butterfly's wings and legs were restrained with two overlapping glassine sleeves held with a clothes pin. The butterfly was positioned on a Styrofoam stage in a groove ending in an open chamber. To prevent coiling, the proboscis was extended over a contiguous row of insect pins mounted on a soft plastic support. Two crossed insect pins prevented elevation of the head. The plastic support with row of pins and the two crossed pins were removed for controls and for the wetting experiments.

To test the effect of fluid immersion, we used unfed specimens 73–81 h after emergence, during which time the specimens remained in the rearing chamber (23–30°C, 58–79% relative humidity). The wings of all specimens were restrained, the legs were free, and the proboscises were separated. For the treatment group, we extended the separated galeae and used flat forceps to gently grasp and immerse at least the distal 50% of both galeae into a watch glass containing approximately 10 ml of distilled water for 1 min. For control specimens, we extended the separated galeae similarly for 1 min without immersion. We positioned individuals of both groups vertically in the groove on the Styrofoam stage described for the previous experiment (with the row of pins and their supporting structure removed), allowing full freedom of movement of the galeae in the open chamber. Pins were inserted through the glassine sleeves and into the Styrofoam to keep the insects in place. At the end point (10 min for *V. cardui* and 5 min for *D. plexippus*), we removed each specimen and photographed the extended proboscis under the dissecting microscope.

Statistical Analyses

For each species, we used repeated-measures analysis of variance (ANOVA) to evaluate when significant repair occurred. We omitted values that remained constant at a given time (for all treatment individuals when the proboscises were initially 100% separated, for *M. sexta* at 10 min when no repair had occurred, and for unseparated controls). The confidence intervals (95% CIs) for mean repair at 60 min were computed to determine whether the mean for the controls (100%) was included in each interval. In experiments of the mechanisms of repair, the variances did not differ significantly, so a two-sample *t*-test assuming equal variances was used to compare means of treatments and controls for each species at the end point. An alpha of 0.05 was used for all *t*-tests. Analyses were performed with SAS software (SAS Institute, Inc. 2008).

Results

Repair of the Proboscis

All six species were capable of total repair of the proboscis after complete separation of the galeae. At 60 min after separation, total repair was accomplished by 91% of *D. plexippus* (n = 11), 75% of *P. cresphontes* (n = 12), 33% of *P. sennae* (n = 6), 20% of *V. cardui* (n = 10), 8% of *H. virescens* (n = 12), and 0% of *M. sexta* (n = 10) (Fig. 3). When viewed the next day (21–29 h after separation), the number of specimens with totally reunited proboscises had increased (except for *P. sennae*, which remained the same): 100% of *D. plexippus*, 92% of *P. cresphontes*, 80% of *V. cardui*, 45% of *H. virescens*, and 30% of *M. sexta*. The proboscises of all control specimens remained entirely intact during the experiment and at 21–29 h after separation. The 95% CIs for the treatment specimens at 60 min included 100% for *D. plexippus* and *P. cresphontes*, suggesting that self-repair reached a condition similar to the intact controls.

Repeated-measures ANOVA within each species demonstrated that the greatest rate of repair occurred in the initial 10 min for *D. plexippus*, *P. cresphontes*, *V. cardui*, and *H. virescens* (Table 2). For example, for *D. plexippus*, 9 out of 11 individuals repaired the proboscis totally during the first 10 min after total galeal separation, with a rate of reunion of 9.23% per minute. *Phoebis sennae* showed the fastest rate during the first 20 min. *Manduca sexta* showed no repair until 20 min and accomplished only partial repair by 4 of 10 specimens by 60 min (8–20% reunion of galeae), with the fastest rate

of repair occurring during the 30- to 45-min interval (Table 2). Of the four partially repaired specimens of *M. sexta*, three were completely repaired by the next day (23–24 h after separation).

Linkage of the ventral legulae of the repaired proboscises was confirmed by observations of live specimens of the butterfly species and *H. virescens* while feeding, and by viewing the fixed, repaired proboscises of *D. plexippus*, *V. cardui*, *H. virescens*, and *M. sexta* microscopically.

Fluid Uptake after Repair

The repair process resulted in a functional proboscis. Of the four reared species that were fed 21–29 h after the initial separation, 35 (79.6%) of 44 treatment specimens of *D. plexippus*, *V. cardui*, *P. cresphontes*, and *H. virescens* were functional. Of these 35 repaired individuals positive for fluid uptake, the proboscises of 29 (66%) were fully reunited, and six (13.6%) were partially repaired (27–93% union of galeae). The proboscises of all controls (n = 37), which were not separated initially, were still intact at 21–29 h past-initiation of the repair experiments, and 36 (97.3%) of the controls were functional (one *H. virescens* control did not take up fluid).

Behaviors of Repair

Proboscis repair generally proceeded by repeated extension and coiling of the proboscis. Movements of the extended galeae included vibrations, crossing, and flicking. A single galea or the loose coil could be held or repeatedly pressed against the thorax. We noted that the proboscis can fully coil even when the galeae are not fully united; therefore, we extended the proboscis to determine whether the galeae were united. Saliva was observed as menisci at the point of separation or as droplets on the proboscis anywhere along the united portion, typically dorsally in the region proximal to the bend and dorsally or ventrally distal to the bend. The behavioral sequence of repair typically ended with the proboscis fully coiled.

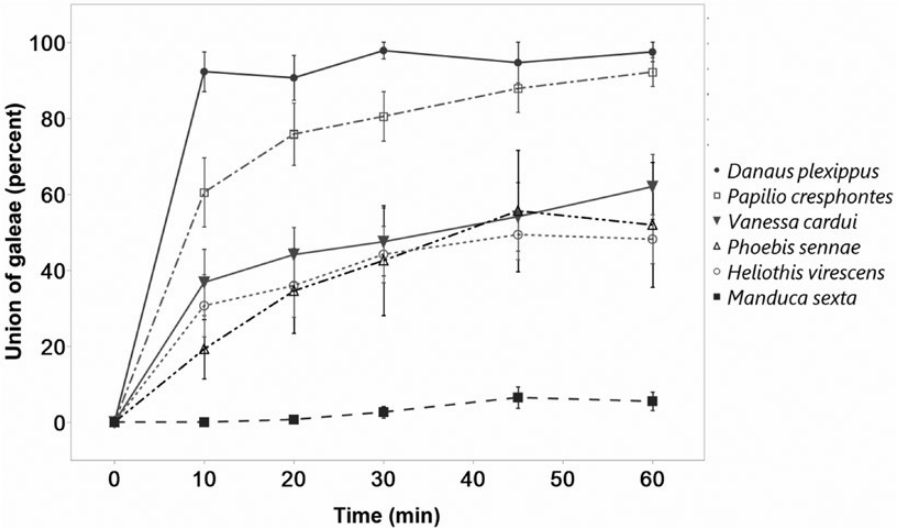

Fig. 3. Percent union of galeae (mean ± SE), measured as $U = (L_{united}/L_{total}) \times 100$, where L_{united} is the united length of the proboscis and L_{total} is the total length of the proboscis, for six species of Lepidoptera after total galeal separation (controls: treatments): *Danaus plexippus* (11:11), *Vanessa cardui* (11:10), *Papilio cresphontes* (11:12), *Phoebis sennae* (5:6), *Heliothis virescens* (11:12), and *Manduca sexta* (7:10). Proboscises of controls were not separated (total n = 56) and persisted at 100% (not shown).

Table 2. Repeated-measures ANOVA for rate of repair (change in percent galeal union per minute) in each of six species of Lepidoptera (controls: treatments): *Danaus plexippus* (11:11), *Vanessa cardui* (11:10), *Papilio cresphontes* (11:12), *Phoebis sennae* (5:6), *Heliothis virescens* (11:12), and *Manduca sexta* (7:10)

Time interval	Rate of proboscis repair (%/min)					
	Danaus plexippus	*Papilio cresphontes*	*Vanessa cardui*	*Phoebis sennae*	*Heliothis virescens*	*Manduca sexta*
0–10 min	9.23[a]	6.05[a]	3.68[a]	1.92[a]	3.07[a]	0.00[b]
10–20 min	–0.16[b]	1.53[b]	0.73[b]	1.53[a]	0.53[b]	0.071b[ba]
20–30 min	0.72[b]	0.47[b]	0.34[b]	0.80[ab]	0.82[b]	0.20[ba]
30–45 min	–0.21[b]	0.49[b]	0.44[b]	0.87[ab]	0.35[b]	0.26[a]
45–60 min	0.19[b]	0.29[b]	0.53[b]	–0.25[b]	–0.079[b]	–0.067[c]
df	5, 40	5, 44	5, 36	5, 20	5, 44	5, 36
F value	156.00	24.45	11.86	3.72	10.48	2.93
P value	<0.0001	<0.0001	<0.0001	0.0153	<0.0001	0.0257
95% CI at 60 min	91.78–103.10	83.88–100.50	42.72–81.28	9.54–94.21	33.96–62.39	0.06–10.94

Proboscises of controls were not separated (total n = 56) and persisted at 100% union. Change in percent galeal union (U) per minute was computed for each time interval as $(U_{final} - U_{initial})/(t_{final} - t_{initial})$ where t is the time moment of observation. Values followed by the same letter are not significantly different (least-squares means, $P < 0.05$). df, degrees of freedom; CI, confidence interval.

Repair by Wild-Caught Lepidoptera

At least one specimen of the following taxa completely reunited the totally separated galeae within 24 h of separation (treatments: controls): Nymphalidae: *Eutoieta claudia* (Cramer) (1:0); Lycaenidae: *Cupido comyntas* (Godart) (1:0); Hesperiidae: *Urbanus proteus* (L.) (2:2); Geometridae: *Epimecis hortaria* (Fabricius) (4:1), *Prochoerodes lineola* (Drury) (2:1), *Nemoria lixaria* (Guenée) (5:3); Erebidae: *Halysidota tessellaris* (Smith) (5:4), *Hypoprepia fucosa* Hübner (6:5), *Catocala* spp. (2:1), *Hyphantria cunea* (Drury) (4:4); and Notodontidae: *Heterocampa obliqua* Packard (1:0). For the following species, at least one specimen partially repaired the proboscis within 60 min: Pyralidae: *Hypsopygia olinalis* (Guenée) (4:2) and Attevidae: *Atteva aurea* (Fitch) (6:3). No repair was observed for *Nadata gibbosa* (Smith) (Notodontidae) (1:0). One reared tortricid showed no repair by 60 min but was partially repaired 4 d later and able to acquire fluid. Controls maintained complete union of the galeae with the exception of two individuals of *H. cunea*, two of *A. aurea*, and two of *H. olinalis*.

Mechanisms of Repair

Danaus plexippus and *V. cardui* showed no significant difference in repair, relative to controls, when the labial palps were removed, coiling was prevented, or the proboscis was wetted, and *D. plexippus* showed no significant difference when the wings were free to move (df = 14–24, $P > 0.05$). However, when *V. cardui* was free to move its wings, repair occurred significantly faster than for controls with wings restrained ($P = 0.0300$, $t = -2.53$, df = 10), and we confirmed the result with an independent repetition of the experiment using a larger sample size ($P = 0.0002$, $t = -4.55$, df = 20).

Discussion

Repair of the Proboscis

All tested butterfly species and the moth, *H. virescens*, are able to fully reunite the proboscis within 60 min. The first 10 min predicts the outcome for *D. plexippus* and *P. cresphontes* and shows the most progress in all species except *M. sexta*. The diel activity rhythm of each species might influence repair. Of the two nocturnal species, no specimens of *M. sexta* showed total galeal reunion until viewed the next day (23–24 h after separation) at which time three individuals were completely reunited. One of 12 specimens of *H. virescens* was fully reunited by 60 min, whereas four additional specimens were totally reunited by 21–25 h.

Structural characteristics of the proboscis might influence repair. *Danaus plexippus* and *P. cresphontes* are nectarivores with relatively smooth proboscises. Species that feed from surface films, such as overripe fruit and tree sap, are characterized by sensilla styloconica along the drinking region, which give the proboscis a brushy appearance (Krenn 1998, Lehnert et al. 2016). *Vanessa cardui* and *H. virescens* have brushy proboscises and repair more slowly than do *D. plexippus* and *P. cresphontes*. *Phoebis sennae*, which lacks a sensilla brush and uses its long proboscis to obtain nectar and drink from damp soil, follows a pattern of repair similar to that of the two species with brushy proboscises.

Our survey of locally collected species represents seven lepidopteran families in the Apoditrysia (sensu Mitter et al. 2017), especially of the Papilionoidea and Macroheterocera, and one family in the apodytrisian sister group, the Yponomeutoidea. The survey confirms the trends in the families used in our repair experiments and shows that the ability to repair is widely shared among the Ditrysia. Although the time required for repair and the degree of success vary among species, we do not know if these attributes are species specific or phylogenetically informative. The family Attevidae is nested in Yponomeutoidea (Mitter et al. 2017), and *Atteva aurea* can partially repair the proboscis. Two of the three controls for this species did not maintain full union of the galeae, and the proboscises of two specimens were partially separated when collected. Similarly, none of the four controls of *Hypsopygia olinalis* (Pyralidae) maintained total union of the galeae, suggesting that some species naturally do not maintain tight linkage of the galeae but readily separate and reunite them.

Fluid Uptake

We showed that specimens with partially (as little as 27% reunited) and fully reunited proboscises can acquire fluid. We have found specimens in our laboratory colonies and in nature with separated proboscises. A laboratory-reared individual of *D. plexippus* that did not assemble the proboscis completely due to deformed galeal tips was able to acquire fluid by pressing the proboscis at the point of separation to the substrate; it lived 6 wk (Pometto 2014). One tortricid moth was collected with 50% separation of the proboscis in nature. Saliva along the hydrophilic inner surface of the food canal helps bring together and holds the galeae in close association during linkage of the ventral legulae (Zhang et al. 2018a). The implication is that the hydrophilic cuticle of the food canal can draw up fluid by capillarity even when the galeae are partially separated (Lehnert et al. 2013, 2014; Zhang et al. 2018a). Thus, we infer that the survival of specimens with partially assembled proboscises in nature is possible.

Comparison of Repair and Assembly

Krenn (1997) reported that assembly takes place within 30–210 min for five nymphalid species, with the longer time required when the galeae become entangled. Our observations indicate that total repair can occur more rapidly, i.e., within 10 min (at 23–30°C). The primary behaviors for assembly include repeated extension of the galeae, crossing or entanglement of the galeae, coiling with or without the separated galeae forming lateral spirals, lateral movements of the coil, repetitive tightening and loosening of the coil, antiparallel motions of the galeae, vibrations of the labial palps, coordinated movements of the labial palps and wings, and discharge of saliva (Krenn 1997). These behaviors are common to repair, with the exception of entanglement of the galeae and coordinated movements of palps and wings. Lateral coils were seen only in repair by *M. sexta*. Thus, we suggest that, like assembly, repair progresses through the following steps: 1) the galeae are aligned by repeated extensions, 2) saliva draws and holds the galeae together by capillary forces (Zhang et al. 2018a), and 3) the ventral legulae are linked during extension and coiling of the galeae or while it is coiled.

Mechanisms of Repair

We falsified the hypotheses that the labial palps, coiling, and fluid immersion are independently necessary for repair. Although repair can be accomplished without the palps, we do not rule out their putative role as mechanical guides for alignment of the galeae (Krenn 1997). The coil is hypothesized to act as a mold for linking the distal portions of the galeae (Krenn 1997). Our results, however, indicate that coiling is not essential to repair; other mechanisms might compensate when coiling is prevented. Saliva plays a critical role in proboscis repair. Coiling of the proboscis or the galeae controls the flow and delivery of saliva to a particular location, and capillary forces guide saliva toward the legular bands and facilitate repair (Zhang

et al. 2018b). Wing movements assist repair in at least some species; for example, *V. cardui* repairs the proboscis faster with unrestrained wings. The physical mechanism responsible for this effect is not known. However, species (e.g., *D. plexippus*) in which proboscis repair is not significantly affected by wing restraint might serve as ideal models not only for future studies of repair, but also for other experiments involving a variety of proboscis functions.

The capillary action of saliva during assembly and repair is similar to the wet hair effect in bringing fibers together (Bico et al. 2004). Menisci formed at the edges of separated galeae on the dorsal and ventral sides create suction pressure that forces the galeae to adhere to each other (Zhang et al. 2018a). However, we found that immersing the separated galeae does not improve the ability to repair the proboscis. We conclude that supplemental fluid beyond the production of saliva is not necessary for repair, although we recognize that it might help maintain hydration and pliability of the proboscis. Nonetheless, by drinking water from a pool, a butterfly or moth creates a pressure differential that would force partially separated galeae together. However, this action would require energy input and, hence, would not be as efficient as passive capillary adhesion with saliva menisci (Zhang et al. 2018a).

The mobility of the galeae might account for the ability to repair with or without the other mechanisms we tested. The musculature of the head and galeae enable a broad range of motions, such as retraction and elevation of the base and coiling of the proboscis (Eastham and Eassa 1955, Krenn and Mühlberger 2002, Krenn 2010). We observed both vertical and lateral movements of the coil, as reported by Krenn (1997), and the individual galeae. Fine movements of the proboscis include antiparallel movements of the galeae and flexing of the distal drinking region in all directions (Krenn 1997, Lehnert et al. 2014, Tsai et al. 2014). The fine and rapid movements of the proboscis that facilitate efficient foraging from flowers and other food sources are evident in both assembly and repair (Krenn 1997, 2010).

Selection Pressures: Paradox of Repair

The primary function of the lepidopteran proboscis is the acquisition of fluid for hydration and energy (Eastham and Eassa 1955, Snodgrass 1961, Kingsolver and Daniel 1995, Krenn 2010). In this context, repair could be viewed as ensuring the uptake of fluid. However, as follows from the science of capillarity and wetting, the insect does not need to have a closed food channel to acquire fluid; having a hydrophilic, indented channel of any shape would be sufficient to draw fluid in by capillary forces (Casavant et al. 2013; Berthier et al. 2015, 2016; Zhang et al. 2018a,b). The earliest fluid-feeding Lepidoptera had weakly linked galeae (Krenn and Kristensen 2000). And among derived lineages of Lepidoptera, particularly within the Notodontidae, unlinked or weakly linked proboscises have secondarily evolved with remarkable ability for fluid uptake (Miller 1991, Smedley and Eisner 1995, Kornev et al. 2017). Thus, repair and assembly, perhaps paradoxically, are not required for fluid uptake.

A single origin of the lepidopteran proboscis has been hypothesized (Kristensen et al. 2007, Krenn 2010). Early glossatan families are present in the fossil record of the early Cretaceous when the proboscis might first have been used to acquire fluid via capillarity from gymnosperm pollen droplets, water, and sap (Scoble 1995, Labandeira 2003, Kristensen et al. 2007, Monaenkova et al. 2012). As an adaptation to nectar feeding from angiosperm flowers, the proboscis became longer, linkage of the galeae became tighter, and development of intrinsic musculature enhanced fine movements (Krenn and Kristensen 2000, Krenn 2010). We suggest that feeding from longer and more restricted corollas was a principal force selecting for assembly and repair. The restricted opening of a floral tube requires the proboscis to enter as a single functional unit (Kwauk et al. 2014); splayed galeae would be difficult to thread into the opening of a floral tube. Accordingly, selection should favor efficiency of repair and the ability to maintain galeal linkage in dedicated nectar feeders. Thus, the linkage mechanism ensures effective entry of flowers while allowing micro (e.g., antiparallel) movements of the galeae that enhance fluid uptake (Tsai et al. 2014).

Members of the Sphingidae uncoil and insert their long proboscises into tubular flowers while hovering, relying on tightly linked galeae. Of the species in our study, the sphingid *M. sexta* had proboscises most resistant to separation. The proboscis of *M. sexta* is smooth and long (69.4 ± 2.4 mm, $n = 9$), two to seven times the length of the proboscises of the other species tested. For *M. sexta* and some other sphingids, the proximal two thirds of the proboscis develop in a pupal tube separate from the body of the pupa (Eaton 1988), and the galea in this portion are linked before eclosion. The distal third of the proboscis develops inside the main body of the pupa, and the associated galeal portion is assembled after eclosion. The unique development and assembly of the proboscis of *M. sexta* possibly make its proboscis less likely to separate and less likely to require repair.

If feeding from floral tubes was a selection force driving assembly and repair, why do some species of Lepidoptera that do not feed from flowers also assemble the proboscis? Adult lepdiopteran feeding habits are extraordinarily diverse (Norris 1936, Adler 1982, Hall and Willmott 2000, Kristensen 2003). For example, entire taxonomic groups, such as the charaxine nymphalids, feed from animal excreta and rotten fruit rather than from flowers (DeVries 1987). Thus, multiple pressures must have selected for assembly and repair. As an additional example, we suggest that selection for a compact proboscis with united galeae neatly coiled and tucked against the body would significantly reduce the drag associated with flailing galeae during flight.

Nature-Inspired Applications

The lepidopteran proboscis offers a novel opportunity to further explore self-assembly and repair. Our findings demonstrate that galeal union is not restricted to the initial assembly after emergence from the pupa, but rather the galeae can be conveniently separated on-demand and the repair process evaluated under varied experimental conditions. The ledpidopteran proboscis, as a set of assembled fibers, also suggests that self-repair might occur in other arthropod systems with constituent parts, including other fiber-based proboscises, such as those of true bugs and mosquitoes. The ovipositors of some insects, such as those of parasitoid wasps (Quicke 2015), also might experience some separation in nature, whether of the valves forming the egg tube proper or the sheath that protects them. Similarly, wing-coupling mechanisms (Stocks 2010) offer possibilities for separation and repair. We are not aware that separation and repair have been explored for other insect proboscises, ovipositors, or wing-coupling devices. We suggest, however, that the mechanism of proboscis repair without remodeling, healing, or growth of new tissue deserves greater attention in organismal biology.

Structural and functional properties of the lepidopteran proboscis, such as musculature, cuticular arrangement, and saliva, have inspired applications in microfluidic devices (Tsai et al. 2011, 2014). Thus, the ability of the proboscis to self-repair would be a prime resource for nature-inspired applications in microfluidics. More generally, lepidopteran proboscis repair opens new possibilities for applications

that involve self-sufficient structural elements. In Lepidoptera, these structural elements are the galeae—natural fibers. Proboscis assembly and repair involve physical mechanisms acting on the fibers, such as adhesion via elastocapillary effects (Zhang et al. 2018a). Physical mechanisms of repair differ significantly from chemical means of repair that have long been used in many aspects of engineering (e.g., Dry 2001). Thus, physical repair, much like in the lepidopteran proboscis, might find use in aerodynamic, biomedical, mechanical, and materials engineering in which functional fibers serve multiple purposes, such as in robotics (Jafferis et al. 2019), drug delivery, and sensing and monitoring of an organism's physiological state (Hongu et al. 2005, Yildirim et al. 2014, Yunusa et al. 2017).

Conclusions

The ability to repair the proboscis is widespread in the ditrysian Lepidoptera and is probably found in all species that assemble the proboscis after emergence from the pupa. Repair of a proboscis that becomes separated, e.g., by mechanical stresses such as probing substrates or predator attacks, is aided by saliva but can be accomplished without labial palps, coiling, or immersion in fluid, and in some species, without wing movements. Yet, these factors are typically observed during repair. We suggest that proboscis repair is accomplished by multiple mechanisms acting synergistically and as back-up systems to ensure the structural integrity of the proboscis. Interactions of factors that might optimize repair could be a focus of future study. Repair is adaptive for nectar feeders that insert their proboscises into floral corollas and for all Lepidoptera in maintaining a compact proboscis during flight to reduce air resistance (drag). In addition to the adaptive value of repair, the mechanisms of repair provide inspiration for the artificial design of microfluidic devices.

Acknowledgments

This is Technical Contribution No. 6751 of the Clemson University Experiment Station and is based, in part, on work supported by National Institute of Food and Agriculture/U.S. Department of Agriculture under project number SC-1700527. Funding was provided, in part, by National Science Foundation grants PoLS-1305338 and IOS-1354956. We thank Alison Arling for fixing, sectioning, and photographing proboscises of *Manduca sexta*; Brian Scholtens (College of Charleston) for assistance with identification of wild-caught moths; and Richard Blob (Clemson University) for comments on an earlier draft of the manuscript.

References Cited

Adler, P. H. 1982. Soil and puddle visiting habits of moths. J. Lepidopter. Soc. 36: 161–173.

Berthier, J., D. Gosselin, and E. Berthier. 2015. A generalization of the Lucas–Washburn–Rideal law to composite microchannels of arbitrary cross section. Microfluid. Nanofluid. 19: 497–507.

Berthier, J., K. A. Brakke, and E. Berthier. 2016. Open microfluidics. Wiley-Scrivener, Hoboken, NJ and Beverly, MA. 309 pp.

Bico, J., B. Roman, L. Moulin, and A. Boudaoud. 2004. Adhesion: elastocapillary coalescence in wet hair. Nature 432: 690.

Camazine, S., J.-L. Deneubourg, N. R. Franks, J. Sneyd, G. Theraulaz, and E. Bonabeau. 2001. Self-organization in biological systems. Princeton University Press, Princeton, NJ. 538 pp.

Casavant, B. P., E. Berthier, A. B. Theberge, J. Berthier, S. I. Montanez-Sauri, L. L. Bischel, K. Brakke, C. J. Hedman, W. Bushman, N. P. Keller, et al. 2013. Suspended microfluidics. Proc. Natl. Acad. Sci. USA 110: 10111–10116.

Darhuber, A. A., J. P. Valentino, J. M. Davis, S. M. Troian, and S. Wagner. 2003. Microfluidic actuation by modulation of surface stresses. Appl. Phys. Lett. 82: 657–659.

DeVries, P. J. 1987. The butterflies of Costa Rica and their natural history. Papilionidae, Pieridae and Nymphalidae. I. Princeton University Press, Princeton, NJ. 327 pp.

Dry, C. 2001. Design of self-growing, self-sensing, and self-repairing materials for engineering applications. Proc. SPIE (Smart Mater.) 4234: 23–29.

Eastham, L. E. S., and Y. E. E. Eassa. 1955. The feeding mechanism of the butterfly *Pieris brassicae* L. Philos. Trans. R. Soc. Lond. B 239: 1–43.

Eaton, J. L. 1988. Lepidopteran anatomy. John Wiley and Sons, New York. 257 pp.

Hall, J. P. W., and K. R. Willmott. 2000. Patterns of feeding behaviour in adult male riodinid butterflies and their relationship to morphology and ecology. Biol. J. Linn. Soc. 64: 1–23.

Hongu, T., G. O. Phillips, and M. Takigami. 2005. New millenium fibers. Woodhead Publishing Ltd, Cambridge, United Kingdom. 312 pp.

Jafferis, N. T., E. F. Helbling, M. Karpelson, and R. J. Wood. 2019. Untethered flight of an insect-sized flapping-wing microscale aerial vehicle. Nature 570: 491–495.

Kingsolver, J. G., and T. L. Daniel. 1995. Mechanics of food handling by fluid-feeding insects, pp. 32–74. In R. F. Chapman and G. de Boer (eds.), Regulatory mechanisms in insect feeding. Springer, New York.

Kornev, K. G., A. A. Salamatin, P. H. Adler, and C. E. Beard. 2017. Structural and physical determinants of the proboscis-sucking pump complex in the evolution of fluid-feeding insects. Sci. Rep. 7: 6582.

Krenn, H. W. 1997. Proboscis assembly in butterflies (Lepidoptera) – a once in a lifetime sequence of events. Euro. J. Entomol. 94: 495–501.

Krenn, H. W. 1998. Proboscis sensilla in *Vanessa cardui* (Nymphalidae: Lepidoptera) – functional morphology and significance in flower probing. Zoomorphology 118: 23–30.

Krenn, H. W. 2010. Feeding mechanisms of adult Lepidoptera: structure, function, and evolution of the mouthparts. Annu. Rev. Entomol. 55: 307–327.

Krenn, H. W., and N. P. Kristensen. 2000. Early evolution of the proboscis of Lepidoptera: external morphology of the galea in basal glossatan moths, with remarks on the origin of the pilifers. Zool. Anz. 239: 179–196.

Krenn, H. W., and N. Mühlberger. 2002. Groundplan anatomy of the proboscis of butterflies (Papilionoidea, Lepidoptera). Zool. Anz. 241: 369–380.

Kristensen, N. P. 2003. Skeleton and muscles: adults, pp. 39–122. *In* N. P. Kristensen (ed.), Part 36 Lepidoptera, Moths and Butterflies, Vol. 2: Morphology, physiology, and development, In Maximillian Fischer (ed.), Handbook of Zoology, Vol. 4, Arthropoda: Insecta. Walter de Gruyter, Inc., Hawthorne, NY.

Kristensen, N. P., M. J. Scoble, and O. Karsholt. 2007. Lepidoptera phylogeny and systematics: the state of inventorying moth and butterfly diversity. Zootaxa 1668: 699–747.

Kwauk, K. J., D. K. Hasegawa, M. S. Lehnert, C. E. Beard, P. D. Gerard, K. G. Kornev, and P. H. Adler. 2014. Drinking with an unsealed tube: fluid uptake along the butterfly proboscis. Ann. Entomol. Soc. Am. 107: 886–892.

Labandeira, C. 2003. Insect mouthparts: ascertaining the paleobiology of insect feeding strategies. Annu. Rev. Ecol. Syst. 28: 153–193.

Lehnert, M. S., C. E. Beard, P. D. Gerard, K. G. Kornev, and P. H. Adler. 2016. Structure of the lepidopteran proboscis in relation to feeding guild. J. Morphol. 277: 167–182.

Lehnert, M. S., D. Monaenkova, T. Andrukh, C. E. Beard, P. H. Adler, and K. G. Kornev. 2013. Hydrophobic-hydrophilic dichotomy of the butterfly proboscis. J. R. Soc. Interface 10: 20130336.

Lehnert, M. S., C. P. Mulvane, and A. Brothers. 2014. Mouthpart separation does not impede butterfly feeding. Arthropod Struct. Dev. 43: 97–102.

Maruzzo, D., and F. Bortolin. 2013. Arthropod regeneration, pp. 149–169. *In* A. Minelli, G. Boxcall, and G. Fusco (eds.), Arthropod biology and evolution. Springer-Verlag, Heidelberg, Germany.

Miller, J. S. 1991. Cladistics and classification of the Notodontidae (Lepidoptera: Noctuoidea) based on larval and adult morphology. Bull. Am. Mus. Nat. Hist. 204: 1–230.

Minelli, A., and G. Fusco. 2013. Arthropod post-embryonic development, pp. 91–122. *In* A. Minelli, G. Boxcall, and G. Fusco (eds.), Arthropod biology and evolution, Springer-Verlag, Heidelberg, Germany.

Mitter, C., D. R. Davis, and M. P. Cummings. 2017. Phylogeny and evolution of Lepidoptera. Annu. Rev. Entomol. 62: 265–283.

Monaenkova, D., M. S. Lehnert, T. Andrukh, C. E. Beard, B. Rubin, A. Tokarev, W. K. Lee, P. H. Adler, and K. G. Kornev. 2012. Butterfly proboscis: combining a drinking straw with a nanosponge facilitated diversification of feeding habits. J. R. Soc. Interface 9: 720–726.

Norris, M. J. 1936. The feeding-habits of the adult Lepidoptera Heteroneura. Trans. R. Entomol. Soc. Lond. 85: 61–90.

Pometto, S. F. 2014. Repair of the proboscis of brush-footed butterflies (Lepidoptera: Nymphalidae). M.S. thesis, Clemson University, Clemson, SC. 119 pp.

Pometto, S. F. 2015. Saliva collection and quantification from adult butterflies (Lepidoptera). Entomol. News 124: 305–309.

Quicke, D. L. J. 2015. The braconid and ichneumonid parasitoid wasps: biology, systematics, evolution and ecology. Wiley-Blackwell, West Sussex, United Kingdom. 704 pp.

SAS Institute, Inc. 2008. Base SAS® 9.4 procedures guide: statistical procedures, 2nd ed. SAS Institute, Cary, NC.

Scoble, M. J. 1995. The Lepidoptera: form, function and diversity. Oxford University Press, Oxford, United Kingdom. 404 pp.

Smedley, S. R., and T. Eisner. 1995. Sodium uptake by puddling in a moth. Science 270: 1816–1818.

Snodgrass, R. E. 1961. The caterpillar and the butterfly. Smithsonian Misc. Coll. 143: 1–51.

Stocks, I. C. 2010. Comparative and functional morphology of wing coupling structures in Trichoptera: Annulipalpia. J. Morphol. 271: 152–168.

Tsai, C. C., P. Mikes, T. Andrukh, E. White, D. Monaenkova, O. Burtovyy, R. Burtovyy, B. Rubin, D. Lukas, I. Luzinov, et al. 2011. Nanoporous artificial proboscis for probing minute amount of liquids. Nanoscale 3: 4685–4695.

Tsai, C. C., D. Monaenkova, C. E. Beard, P. H. Adler, and K. G. Kornev. 2014. Paradox of the drinking-straw model of the butterfly proboscis. J. Exp. Biol. 217: 2130–2138.

Weinkamer, R., J. W. C. Dunlop, Y. Bréchet, and P. Fratzl. 2013. All but diamonds – biological materials are not forever. Acta Mater. 61: 880–889.

Whitesides, G. M., and B. Grzybowski. 2002. Self-assembly at all scales. Science 295: 2418–2421.

Yildirim, A., M. Yunusa, F. E. Ozturk, M. Kanik, and M. Bayindir. 2014. Surface textured polymer fibers for microfluidics. Adv. Funct. Mater. 24: 4569–4576.

Yunusa, M., F. E. Ozturk, A. Yildirim, U. Tuvshindorj, M. Kanik, and M. Bayindir. 2017. Bio-inspired hierarchically structured polymer fibers for anisotropic non-wetting surfaces. RSC Adv. 7: 15553–15560.

Zhang, C., P. H. Adler, D. Monaenkova, T. Andrukh, S. Pometto, C. E. Beard, and K. G. Kornev. 2018a. Self-assembly of the butterfly proboscis: the role of capillary forces. J. R. Soc. Interface 15: 20180229.

Zhang, C., C. E. Beard, P. H. Adler, and K. G. Kornev. 2018b. Effect of curvature on wetting and dewetting of proboscises of butterflies and moths. R. Soc. Open Sci. 5: 171241.