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External Morphology of Stable Fly (Diptera: Muscidae) Larvae

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ABSTRACT Scanning electron microscopy was used to examine the external morphology of first-, second-, and third-instar stable flies (Stomoxys calcitrans (L.)). In the cephalic region, the antennae, labial lobe, and maxillary palpi are morphologically similar among instars. Antennae comprise a prominent anterior dome that is the primary site of olfaction, while the maxillary palpi are innervated with mechanosensilla and scolopidia. The ventral organ and facial mask, also located in the pseudocephalon, are not well-developed in first instars, but become progressively more so in the subsequent instars. When the pseudocephalon is partially retracted, anterior spines cusp around the oral ridges of the facial mask. This indicates the anterior spinose band may be used in conjunction with the facial mask in predigestion. Functional anterior spiracles are absent on first instars, but become evident as a pair of palmate spiracular processes with five to seven lobes in second and third instars. A pair of Keilin’s organs, functioning as hygroreceptors, is located on each thoracic segment. Abdominal segments are marked with ventral creeping welts, the anal pad, anus, papillae, and posterior spiracles. Ventral creeping welts are thought to aid in locomotion, while the anal pad acts as an osmoregulatory structure. Posterior spiracles are modified from round spiracular discs with two straight slits in the first instar to triangular discs with two and three sinuous slits in the second and third instars, respectively.

KEY WORDS Stomoxys calcitrans, integument, sensilla, development, scanning electron microscopy

Stable flies (Stomoxys calcitrans (L.)) are hematophagous flies that require one to two bloodmeals per day as adults for successful mating and egg development (Anderson 1978, Chia et al. 1982). Stable flies are primarily pests of cattle (Anderson and Tempelis 1970), but will attack other animals and humans if given the opportunity (i.e., proximity to host and reduced host defensive behaviors; Hogsette and Farkas 2000, Johnson et al. 2010, Pitzer et al. 2011). Sharp prestomal teeth on the labellum (Stephens and Newstead 1907) inflict pain as adults feed, and in response, cattle exhibit defensive behaviors that ultimately contribute to decreased weight gains and milk production (Mullens et al. 2006, Taylor et al. 2012). Despite continuing efforts to develop effective management options, stable fly reduction remains a challenge.

Relative to adults, little is known about stable fly larval biology. Stable flies develop in moist, decaying vegetation including hay residue, animal bedding, seaweed, and lawn clippings (Simmons and Dove 1941, Haines 1955, Meyer and Petersen 1983, Schmidtmann 1988). Within the substrate, larvae tend to aggregate (Wienhold and Taylor 2012); whether this is due to population interactions, environmental niches, or both is uncertain. However, substrate in which stable flies develop is not physiochemically homogenous and abiotic parameters such as moisture, pH, and temperature may partially explain larval absence or presence (Wienhold and Taylor 2012). Further, the importance of microbial–larval interactions has been established and microbial niches may influence larval movement (Romero et al. 2006, Albuquerque and Zurek 2014). To better understand larval behavior, our objective was to augment previous morphological descriptions of stable fly larvae with an emphasis on cuticular sensilla (Grodowitz et al 1982). In this article, the external morphology of first-, second-, and third-instar stable fly larvae are examined with scanning electron microscopy.

Materials and Methods

Stable flies were obtained from a colony initiated in 2008 and maintained at the U.S. Department of Agriculture, Agricultural Research Service, Agroecosystem Management Research Unit, Lincoln, NE, following the methods described by Berkebile et al. (2009). Four hours after oviposition, eggs were harvested, rinsed with water, and either placed onto moistened filter paper or into larval media consisting of wheat bran, fish meal, wood chips, and water. Eggs on moistened filter paper were incubated at 25°C and, after 30–40 h, first-instar larvae were collected. Eggs inoculated into larval media were allowed to develop for 3 or 6 d, after which
the second- or third-instar larvae, respectively, were collected. Stage was determined by examination of posterior spiracles.

Larvae were heat-killed in boiling water for 1–2 min, then removed, rinsed three times in sterile distilled water, and punctured with a fine needle. Up to 15 processed larvae were placed in 1.5-ml centrifuge tubes containing 2.5% glutaraldehyde for at least 24 h at 5°C. Subsequently, specimens were serially dehydrated by submerging larvae in 30, 50, 70, 80, and 90% ethanol for 15 min per step, followed by two 30-min submersion in 100% ethanol. Specimens were mounted on aluminum studs and sputter coated with gold for examination with field-emission (Hitachi S-4700) and variable pressure (Hitachi S-3000N) scanning electron microscopes (Hitachi Corp., Tokyo, Japan) at the Microscopy Core Research Facility, University of Nebraska, Lincoln.

Terminology follows the descriptions by Chu-Wang and Axtell (1971) and Courtney et al. (2000).

**Results and Discussion**

Cyclorrhaphous larvae do not have an externally sclerotized cephalic region and, therefore, are considered acephalic (Courtney et al. 2000). The cephalic region, referred to as the pseudocephalon, is bilobed, each lobe including the dorsolateral antenna and the ventrally located maxillary palpus and ventral organ (Fig. 1). The antenna, also known as the dorsal organ, is composed of the anterior dome, lateral pore receptor, and basal ring (Fig. 2). The most prominent antennal feature is the anterior dome whose entire surface is densely perforated with pore canals <0.01 μm in width, resembling fingerprint ridges. The dome also is characterized by seven proximal pores ~1 μm in width that are evenly spaced along the circumference. Lateral to the dome is a pore receptor, which is flush with the surrounding basal ring. Other than the absolute size, no differences in antennal morphology were observed between instars.

Until the late 1930s, it was widely believed that the antennae functioned as a photoreceptor, despite published evidence to the contrary (Bolwig 1946). In fact, photoreceptors of house fly larvae were later described as a cluster of sense cells located in the optic depression of the cephalopharyngeal skeleton (Bolwig 1946). Behavioral assays demonstrated the olfactory nature of the antennae (Welsh 1937, Bolwig 1946), evidence that was subsequently strengthened by ultrastructure (Chu and Axtell 1970), comparative morphology and behavior (Colwell 1986), and electroantennograms (Oppliger et al. 2000). Therefore, the structure previously referenced as the dorsal organ is now referred to as the antenna. Transmission electron microscopy has revealed that the antennal domes of the house fly (Musca domestica L.) and the fruit fly (Drosophila melanogaster Meigen) larvae, which are similar to the stable fly dome (Szpila and Pape 2008, Akent’eva 2011), are perforated by ~300,000 pore tubules, or 860 tubules per square micrometers (Chu and Axtell 1970, Singh and Singh 1984). Atmospheric air diffuses through the pores that are in close but not direct contact with dendritic branches (Chu and Axtell 1970). Odorant-binding proteins bind and transport compounds from the cuticular surface of the pores to odorant receptors located on the dendritic branches (Vosshall and Stocker 2007), leading to neural transduction (Oppliger et al. 2000). Putative odorant-binding proteins have been sequenced in stable fly larvae (Olañon et al. 2010). An interesting comparison of the antennal morphology and life history of two cattle grubs (Hypoderma lineatum (de Villiers) and Hypoderma bovis (L.)) and a mouse bot (Cuterebra fontinella Clark) also indicates the olfactory nature of the antenna (Colwell 1986). Cattle grubs, which lack antennae, are oviposited directly on the host’s hair shafts. After hatching, larvae follow the hair shaft to the base of the epidermis and penetrate the skin. In contrast, the mouse bot has a comparatively well-developed antenna, and is oviposited on the ground in proximity to potential hosts. Larval mouse bots must locate the host, attach to the hair, and then locate a moist body opening to enter the host.

Although the primary function of antennae appears to be odor detection, antennae are equipped with at least two other types of sensilla. The seven larger pores encircling the dome’s base probably have no sensory function, but rather, are relics of scolopales that are pulled out through these basal spots during molting (Chu and Axtell 1970). The lateral pore receptor likely serves as a contact chemoreceptor (Chu and Axtell 1970).
In close proximity to the antennae are the ventrally located maxillary palpi (Figs. 1 and 3) or terminal organs. Each palpus is composed of two distinct groupings of sensilla. The larger, distal group includes two knob sensilla, three papilla sensilla, and four pit sensilla, while the smaller dorsolateral group includes one modified sensillum, one papilla sensillum, and one spot sensillum. Each group also includes one scolopodium, which is not obvious externally. The lateral group is adjacent to the distal group in the first-instar, but is subsequently separated by a greater distance in the second- and third-instars.

The maxillary palpi are complex; each palpus comprises six types of sensilla: papilla, modified papilla, pit, spot, knob, and scolopodia. The four papilla sensilla are innervated by three or five bipolar neurons (Chu-Wang and Axtell 1971). At least one of the dendritic tips in each papilla sensilla is modified such that, distally, it widens and then tapers before terminating from the pore. The enlarged section of the dendrite houses a tubular body in which microtubules and microfilaments are arranged and is indicative of a mechanoreceptor. The remaining dendrites terminate at the pore and are likely chemoreceptors. The pores of chemoreceptors

Fig. 2. The dominant feature of larval stable fly antennae is a dome (ad) (A—third-instar) that is perforated with numerous pore canals resembling fingerprint ridges. Adjacent to the dome is the lateral pore receptor (lpr) (B).

Fig. 3. The maxillary palpi of first- (A) and third-instar (B) larvae include a distal group of knob (k), papilla (p1), and pit (t) sensilla, as well as two papilla (p2) that are in a lateral grouping. The ventral organ of first- (C) and third-instar (D) stable fly larvae. White arrow indicates fourth pore on the ventral organ on the third-instar that is absent in first-instars.
are continuously open and, to prevent desiccation, are also small. Until the electron microscope was utilized, it was accepted that a specialized cuticle covered the pore (Slifer 1970). The four pit sensilla are innervated by two to five bipolar neurons. One of the pit sensilla includes a mechanoreceptor, otherwise, all other dendrites are considered chemoreceptors. The modified papilla and spot sensilla are each innervated by one bipolar neuron with a tubular body exposed to the outside and are considered contact chemo- and mechanoreceptors. The function of the knob sensilla is unknown. Each of the two knob sensilla is innervated by one bipolar neuron, but has a different internodal structure. One contains granular material and few neurotubules, while the other is filled with dendritic lamellae that become more numerous distally.

Posterior to the maxillary palpus is the ventral organ, which is barely perceptible in the first-instar as one small pore (Fig. 3). The ventral organ becomes easily discernible in the second- and third-instar and, in addition to a papilla sensillum, also has three morphologically distinct pores. The ventral organ is equipped with one gustatory sensillum and three mechanosensilla (Chu-Wang and Axtell 1972). The papilla sensillum, or gustatory sensillum, is innervated by two bipolar neurons open to the environment, neither of which possesses a tubular body. Each of the other three pores is innervated by one bipolar neuron with a tubular body that is also open to the environment. As with the basal pores on the antennal dome, the pores of the mechanosensilla are likely relics of molting rather than actual pores (Chu-Wang and Axtell 1972) and are not seen until the second- and third-instar.

Contrary to Ajidagba et al. (1985), the ventral organ is greatly reduced in the first-instar. In contrast, first-instars of kleptoparasitic sarcophagids (Szpila and Pape 2005) have a large ventral organ. First-instars of these species must search for and acquire a competitor’s food source. If the development of the ventral organ is related to foraging, a reduced ventral organ in first-instar stable flies might be indicative of oviposition in locations in which most of the nutritional requirements are met, while second and third instars, which have a larger ventral organ, may seek alternative food sources.

The pseudocephalon is further characterized by the tripartite labial lobe and surrounding facial mask. The labial lobe (Fig. 4) is situated at the base of the oral opening and has two lateral arms that can fold inward to a closed position and outward in an open position. When closed, the junction where the two arms meet form a longitudinal line down the median and the lobe appears triangular in shape. One set of labial organs are evident on each lobe, ~10 μm posterior to the apex. Each set consists of three papillae, one that is rectangular with tapered points that are perpendicular to the base and two that resemble inverted Vs. At the base of the labial lobe is a trapezoidal structure with four finger-like lateral extensions that are evident in the open and closed positions of the lobe. Labial morphology is similar among larval instars.

Posterior to the ventral organ is the facial mask (Figs. 1 and 5), a series of overlapping oral ridges extending ventrolaterally from the mouth opening. The facial mask is greatly reduced in the first-instar and progressively develops in the second- and third-instars, similar to many other cyclorrhaphan species (Courtney et al. 2000). A unique feature of the stable fly and another muscid, the horn fly (Haematobia irritans (L.)), is that the facial mask only includes oral ridges. The facial mask of other muscid species such as Atherigona orientalis (Schiner) (Grzywacz and Pape 2014), Synthesia myia nudiseta (van der Wulp) (Velasquez et al. 2013), and M. domestica (Szpila and Pape 2008) also includes cirri and suprabucal teeth. The oral ridges likely disseminate salivary fluid onto external surfaces for predigestion (Thomsen 1935) and also may channel partially digested material into the food opening. In this sense, oral ridges are analogous to the adult pseudotracheae (Bolwig 1946). The presence of well-developed oral ridges is thought to be primarily associated with saprophagous diterans. They largely are absent or greatly reduced in parasitic species. For example, facial masks are not present in parasitic oestrids, C. fontinella, H. lineatum, and H. bovis (Colwell 1986), in which first instars acquire nutrients across the cuticle rather than orally (Chamberlain et al. 1969).

The anterior spinose band delineates the first of three thoracic segments and is composed of fan-shaped spines that increase from 5–6 ventrolateral rows in the first-instar to ~16 in second- and third-instar (Figs. 1 and 6). Each spine has ~25 short finger-like extensions and appears to flex in a cupping or half-pipe fashion (Fig. 6). The function of the anterior spinose band is unknown. However, when the pseudocephalon is partially retracted, it appears that the spines cusp to fit over the oral ridges (Fig. 7). This suggests that the anterior spinose band may work in conjunction with the facial mask, possibly in predigestion.

Posterior to the anterior spinose band is a pair of ventrolateral anterior spiracles (Fig. 8). The anterior spiracles were not observed in the first-instar, but in the second- and third-instar spiracles are manifest as a pair of palmate spiracular processes with 5–7 tubular lobes, each lobe with a median slit at the apex (Fig. 9). As is the case with other first-instar cyclorrhaphans, small, nonfunctional pores are present in place of the anterior spiracles (Keilin 1944, Roberts 1981). The number of lobes comprising the second- and third-instar anterior spiracles may vary intra- and interspecifically. Stable fly larvae have 5–7 and are comparable to other muscids. Other diteran larvae may have many more; larvae of the tephridid Anastrepha leptozona Hendel have 16–20 (Lasserre et al. 2009), while sarcophagid larvae may have 14–54 spiracles (Sukontason et al. 2003).

A pair of Keilin’s organs (Figs. 8 and 10), or tuft organs, is located on all thoracic segments in each instar. Each pair comprises three trichoid sensilla with the prothoracic pair positioned relatively closer to each other than the meso- and metathoracic pairs. It is widely cited that Keilin’s organs function as hygroreceptors (Hafez 1950). The morphology and location of Keilin’s organs are common in cyclorrhaphous larvae, although, as a hygroreceptor in general, the morphology and location is unusual. Stable fly larvae possess six
Fig. 4. Labial lobe of third-instar stable fly in closed (A) and open (B) positions. Each arm of the labial lobe has two sets of sensilla of the labial organ (lo) (C). Trapezoidal structure (ts) at the base of the labial lobe in open position (D). Lateral extensions of the trapezoidal structure are evident when the labial lobe is in the closed position (A).

Fig. 5. Spines in the anterior spinose band of a third-instar stable fly (highlighted in white box) appear to flex in a cusping-like fashion.
Keilin’s organs on the ventral side of the thorax. Aporous hygroreceptors are usually few in number compared with other receptors and normally occur in association with either the antennae or mouthparts (Altner and Loftus 1985). Interestingly, larval tsetse flies do not have Keilin’s organs, but are still able to discern relative humidity (Finlayson 1972).

In proximity to Keilin’s organs are campaniform sensilla (Fig. 10), which, although not documented here, probably are present on the abdominal segments as well (Green and Hartenstein 1997). In each instar, thoracic and abdominal segments were perforated with numerous pits or pore canals (Fig. 11). Pore canals pass from the epithelium through the endocuticle and

Fig. 6. The anterior spinose band of a third-instar stable fly is composed of fan-shaped spines (A) with finger-like extensions (B).

Fig. 7. When the pseudocephalon of the third-instar stable fly is partially retracted (A), spines from the anterior spinose band appear to meet and cusp over the oral ridges of the facial mask (highlighted in white box). Magnified view of the highlighted area (B).

Fig. 8. Thoracic segments I, II, and III of first- (A), second- (B), and third-instar (C) stable fly larvae showing Keilin’s organ (ko) and anterior spiracles (asp).
Fig. 9. Anterior spiracles of stable fly larvae that may include 7 (A—second-instar), 6 (B—second-instar; C—third-instar), or 5 (D—third-instar) tubular lobes.

Fig. 10. Keilin’s organ (ko) and campaniform sensilla (p) on thoracic segment of stable fly larva (A—first-instar). Magnified view of Keilin’s organ (B—second-instar) and campaniform sensilla (C—first-instar; D—second-instar).
up to the epicuticle (Locke 1961). In some insects, wax is transported in the pore canals through the epicuticle, but in others, the canals may be used to secrete a superficial layer that camouflages the larval integument from a host’s immune system, thereby preventing an immune response (Innocenti et al. 1997).

Abdominal segments bear ventral creeping welts (Fig. 12). Each welt generally consists of rounded spines in five to seven rows that taper laterally into three rows. The second lateral row includes five to seven oblong spines that run longitudinally. It was previously thought that larval movement occurred through use of the mouth hooks. If correct, movement would begin anteriorly and proceed posteriorly. Roberts (1971) demonstrated that movement actually begins posteriorly. As waves of contraction proceed anteriorly, parts of the body are elevated, and to prevent the body from slipping backwards, creeping welts anchor the rest of the body, from which the waves have already passed, to the substrate.

The last posterior segment, or anal division, includes the anal pad, anus, three papillae, and a pair of posterior spiracles (Figs. 12 and 13). The ventrally located anal pad is triangular with rounded corners and is bordered by modified spines, which, on the anterior edge, form what is termed the preanal welt. The anal pad is an osmoregulatory structure with a distinct, thin cuticle (Stoffolano 1970). The anus is a longitudinal slit separating two bulbous portions of the anal pad. Posterior to the anus is a postanal papilla with a mace-like morphology (Fig. 14). A subanal papilla is lateral to each side of the anus and anterior to the postanal papilla.
On the dorsal side of the anal division are two posterior spiracles (Fig. 15). In the first-instar, the posterior spiracles are visible as two straight slits in the middle of a round spiracular plate. In the second- and third-instar, two and three sinuous slits, respectively, form the spiracles and the spiracular plate becomes triangular. The remnant of the previous instar’s spiracular plate is visible as the ecdysial scar in the second- and third-instar stable fly, ventral view. The pre-anal welt (pw), anal pad (ap), anus (an), and subanal papilla (sa) are indicated.

Fig. 13. Posterior region of second-instar stable fly, ventral view. The pre-anal welt (pw), anal pad (ap), anus (an), and subanal papilla (sa) are indicated.

Fig. 14. Ventral view of the posterior region of third-instar stable fly. The anus (an), anal pad (ap), and mace-like post-anal papilla (pa) are indicated.
Fig. 15. Posterior region of stable fly larvae indicating the posterior spiracular discs (psd) (A—first-instar). Spiracular discs of first-instars (B) are round with small slits (s) and broad posterior spiracular hairs (psh). In the third-instar, the slits become “S” shaped (C) and the posterior spiracular discs are triangular (D).

Fig. 16. Stable fly larvae were boiled and rinsed three times in sterile distilled water. After processing, bacteria were observed in dense matrices in the thoracic segmental folds (A, region highlighted by white rectangle magnified in B) and ridges near the anus (C, region highlighted by white rectangle magnified in D) on third-instars.
third-instar. In each instar, four sets of peristigmatic hairs stem from the edge of the spiracular plate.

During molting, the entire spiracular plate is pulled out and shed, forming the ecdisial scar in the subsequent instar. Each slit, or peritreme, is associated with a perispiracular glandular cell, which produces an oily secretion that coats the spiracular hairs, creating a protective covering from water. Each peritreme is connected to the tracheal system by a trunk largely characterized by taenidia. As the trunk approaches the opening of the peritreme, the trunk no longer has taenidia and becomes a slightly enlarged chamber with dense chitinous felt, forming a filter called the felt chamber (Keilin 1944).

It is also worth noting that although the objective of this study was to investigate external morphology of stable fly larvae, we observed what potentially appear to be remnants of bacterial biofilms on the integument (Fig. 16). For preparation, larvae were boiled and rinsed three times in sterile distilled water. Still, a dense matrix of bacteria was observed in the thoracic segmental folds as well as between the ridges proximal to the anal organ. The role of fifth fly adults as mechanical vectors of pathogens has been investigated (Cohen et al. 1991, Doud et al. 2013, Wasala et al. 2013). In this capacity, potential pathogens are collected on the fly’s external body structures and are subsequently deposited on uncontaminated sources through contact with the fly. Observations in the current study merit investigations of the role of larvae as mechanical vectors.

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