

Original Research Paper

Putative Sensory Structures Associated with the Food Canal of *Hybomitra difficilis* (Diptera: Tabanidae).

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Abstract: The feeding tube of *Hybomitra difficilis* is made up of a short distal vestibule followed by a food canal that leads to the cibarium; the two regions demarcated by the vestibule/food canal junction. Two pairs of sensilla were consistently observed in the vestibular walls, the first pair of basiconic design in the mid-vestibular region, and the latter pair of setiform design at the base of the vestibule. Numbers of setiform sensilla in the food canal varied from 31 to 69 (mean = 48.15; ± 1 SD = 10.02), and were aggregated in the distal and distal median regions of the food canal. This aggregation was significant ($X^2 = 241.49$; $P < 0.0001$), leading to rejection of the null hypothesis that setiform sensilla were evenly distributed throughout the length of the food canal.

Two basiconic sensilla were observed in the food canal of every fly examined. While basiconic sensilla varied in position (i.e., distance) from the vestibule/food canal junction, they were significantly aggregated ($X^2 = 14.42$; $P < 0.0024$) in the two median sections of the distal canal region, thus leading to the rejection of H_0 that basiconic sensilla were evenly distributed in subdivisions (i.e., sections) of the distal canal region.

Keywords: *Hybomitra difficilis*; food canal; labrum; sensilla.

Introduction

Because of their noisy flying behavior and persistent biting activity, horse flies (i.e., “tabanids”) are nuisance pests of humans, livestock and wildlife animals (Snodgrass 1944; Foil and Hogsette 1994; Mullens 2009). Additionally, tabanids have been incriminated as mechanical and biological vectors of pathogenic agents (Krinsky 1976; Mullens 2009). Even so, these flies have received relatively little attention when compared with other hematophagous Diptera (Baldacchino *et al.* 2014).

With their mouthparts adapted for piercing the host’s skin and blood feeding, females are most responsible for nuisance activity and the capacity to transmit pathogenic agents, and yet few published reports on the form and function of North American tabanid mouthparts are available (Lall and Davies 1971). Indeed, Muzari *et al.* (2010) note that tabanid flies remain the least studied of arthropod vectors.

Kettle (1995) also addressed this oversight when he emphasized that mouthparts of medically important insects deserved special attention because they serve as the principle route for transmitting pathogens from one host to another.

The classical approach to studying sensory sensilla of insects has been to classify them by type and attempt to assign function, as mechanoreceptors or chemoreceptors, to those types. Snodgrass (1935), who described seven different types, was the first investigator to devise a classification scheme, and this was ultimately followed by the works of Chapman (1998), and Romoser and Stoffolano (1998) who added SEM observations. Still, precise function of certain sensilla types often remains enigmatic, leading the latter workers to caution that a morphological type does not necessarily imply a particular function. Indeed, Dethier (1963) demonstrated that a sensillum on the labellum of the blow fly, *Phormia regina*, acts as both a mechanoreceptor and chemoreceptor. Conversely,

positioning of sensilla throughout the food canal and proximal regions (e.g., the epipharynx and cibarium) of hemotophagous flies has received little attention. Thus the goal of this study was to map positions of sensilla in the food canal of *Hybomitra difficilis* and test for aggregation patterns rather than attempting to link function with sensilla morphology. We think this different approach to the study of structures that provide sensory information is justified because basic concepts of fluid dynamics demonstrates that the velocity profile of a fluid through a “pipe” changes as distance along the pipe increases. For example, Waite and Fine (2007) note that fluid in the entrance region of a “pipe” has a relatively flat velocity profile which changes into an increasingly parabolic flow profile as distance along the pipe increases. Eventually, flow profile becomes constant and no longer changes with increasing distance from the point of entry. Although information on precise positioning of sensory structures throughout the food canal of hematophagous flies is sketchy, the possible link between sensilla aggregation and fluid dynamics is intriguing. Buerger (1967) was the first to suggest that food canal sensilla were not evenly spaced, noting that spacing between sensilla in *Hybomitra rupestris* increased towards the basal end of the labrum. More recently, Joy and Stephens (2016) corroborated Buerger’s findings that sensilla were more closely spaced in the distal food canal region of the deer fly, *Chrysops callidus*. Additionally, Joy (2017) demonstrated that sensilla were significantly aggregated in the distal food canal of *Tabanus atratus* with significantly fewer sensilla located in the proximal canal region. These findings, while based on but a few examples, compliments what we know of fluid dynamics; i.e., sensory sensilla are aggregated in the entrance region of the food canal to provide the feeding fly with sensory information to monitor flow characteristics where fluid is most subject to changing dynamics. Thus additional studies on positioning of sensory structures in the feeding complex seems warranted to determine if this is a common characteristic for other hematophagous fly species.

Materials and Methods

Specimen Preparation

A sample of 20 *Hybomitra difficilis* (females) was selected from museum pinned specimens housed in the Marshall University entomology collection. Flies had been collected by C. Coffman and P. van Buskirk from a “Tabanid” trap set in Hampshire Co., WV in 1976. Species determination was made by L. L. Pechuman and J. Hacker. Each fly was removed from

its pin, measured from the tip of the head to the tip of the abdomen to the nearest 0.1 mm with vernier calipers, and placed in a 5% solution of KOH for six to 18 h to soften mouthparts. After softening, the head of each fly was removed and placed in a separate stentor dish containing 70% ethanol, to ensure that data on mouthparts could be matched with the individual body length of each fly, if necessary.

The mouthparts (hypopharynx, labium, labrum, mandibles, and maxillary laciniae) were dissected from the head using microforceps with the aid of a Zeiss stereomicroscope. Mouthparts were then dehydrated in an ethanol series (85%, 95%, 99%), cleared in methyl salicylate in two stages (1 to 1 solution of 99% ethanol and methyl salicylate, followed by 100% methyl salicylate), then mounted on glass slides in Permount[®]. Only the labrum with attending food canal and cibarium region (Fig. 1) of each fly was used for this study. The labrum/cibarium complex was mounted on a separate slide, ventral side up, to allow viewing of sensory sensilla. All other mouthparts from each fly were mounted together on a second slide for additional study if desired.

Two regions of the feeding tube, the vestibule and food canal, were readily identifiable in the labrum of *Hybomitra difficilis*. The food canal (Fig. 1), being the principle focus of this study because of the numerous sensilla lining its walls, was subdivided into four equidistant regions: 1) distal (D), beginning at the vestibule/food canal junction; 2) distal median (DM); 3) proximal median (PM); and proximal (P), where the food canal merged into the area of the cibarial pump (Fig. 1). Every sensilla along both right and left walls in the food canal of each fly labrum, beginning at the vestibule/food canal junction, was measured to the nearest 1.0 μm using a Zeiss compound microscope with a calibrated ocular micrometer. Thus sensilla in both walls of the food canal were identified by position, and numbered, from the distal to proximal end of the canal. Additionally, three aggregations of sensilla (one median, two lateral groups) in the epipharyngeal region leading to the cibarium were identified (Fig. 2A – C). Numbers of basiconic and setiform sensilla were determined for each of these three aggregations. This protocol allowed for precisely assigning a position for all food canal sensilla, and for determining type of sensilla in the epipharyngeal area, in $n = 20$ flies of the sample population.

The distal region of the food canal (D in Fig. 1) was further subdivided into four sections because most basiconic sensilla were positioned in this region of every fly examined.

Data Analysis

We established two null hypotheses: (Ho1) that setiform sensilla were equally distributed throughout the food canal; and (Ho2) that basiconic sensilla were equally distributed throughout sections of the distal canal region. To test Ho1 the food canal was divided into four equidistant regions (Fig. 1) with a Chi-square goodness-of-fit test (<http://www.vassarstats.net/csfit.html>) employed to assess the distribution of setiform sensilla within each canal region (Table 1). To test Ho2 the distal food canal region was subdivided into four equidistant sections (Fig. 1) and, again, the Chi-square goodness-of-fit test was used for determination of aggregation (Table 2). Levels of significance for Ho1 and Ho2 are given in the appropriate tables.

Results

The feeding tube traversing the labrum of *Hybomitra difficilis* was subdivided into a vestibule and food canal; the two regions demarcated by the vestibule/food canal junction (Fig. 1). Two pairs of sensilla were observed in the vestibular walls, the first pair of basiconic design in the mid-vestibular region, and the latter pair of setiform design at the base of the vestibule. The two pairs of vestibular sensilla were relatively constant in type, number and position from one fly to another.

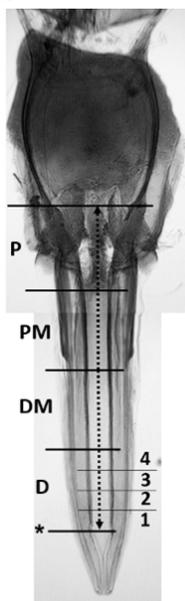


Figure 1. *Hybomitra difficilis* labrum with food canal (dashed arrows), ventral view. Food canal equidistant regions: D, distal; DM, distal median; PM, proximal median; P, proximal. Equidistant sections of distal region (D) depicted by roman numerals. Asterisk indicates vestibule/food canal junction.

A total of 963 setiform sensilla were observed in n = 20 individuals of the sample population. While

these food canal sensilla varied in number (range 31 – 69; mean = 48.2; ± 1SD = 10.02) and position from one fly specimen to another, they were aggregated in the distal and distal median regions of the food canal, with relatively few sensilla observed in the proximal canal region. This aggregation was significant ($X^2 = 241.49$, Table 1), leading to rejection of Ho1 that setiform sensilla were evenly distribution throughout the length of the food canal.

Table 1. Setiform sensilla distribution in four equidistant regions of the *Hybomitra difficilis* food canal (see Fig. 1). Obs. f and Exp. f are observed and expected frequencies; Exp. p, expected proportion; % dev., percentage deviation; Std. res., standardized residuals. Total observed frequencies = 963.

Region	Obs. f	Exp. f	Exp. p	% dev.	Std. res.
D	314	240.75	0.25	+30.43	+4.72
DM	380	240.75	0.25	+57.84	+8.97
PM	208	240.75	0.25	-13.60	-2.11
P	61	240.75	0.25	-74.66	-11.58

$X^2 = 241.49$; $df = 3$; $P < 0.0001$

A total of 40 basiconic sensilla, two in each fly, were observed in the sample population. With but two exceptions, these basiconic sensilla were located in the distal-most food canal region (i.e., D in Fig. 1). While basiconic sensilla varied in position (i.e., distance) from the vestibule/food canal junction, they were significantly aggregated ($X^2 = 14.42$, Table 2) in the two median sections of the distal canal region, thus leading to the rejection of Ho2 that basiconic sensilla were evenly distributed in subdivisions (i.e., sections) of the distal canal region.

Table 2. Basiconic sensilla distribution in four equidistant sections of the *Hybomitra difficilis* distal food canal region (see Fig. 1). Obs. f and Exp. f are observed and expected frequencies; Exp. p, expected proportion; % dev., percent deviation; Std. res., standardized residuals. Total observed frequencies = 38. Note; two basiconic sensilla fell outside the distal region of the food canal.

Section	Obs. f	Exp. f	Exp. p	% dev.	Std. res.
1	8	9.5	0.25	-15.79	-0.49
2	12	9.5	0.25	+26.32	+0.81
3	17	9.5	0.25	+78.95	+2.43
4	1	9.5	0.25	-89.47	-2.76

$X^2 = 14.42$; $df = 3$; $P < 0.0024$

In addition to food canal sensilla there were three groups of sensilla – two lateral and one medial –

positioned in the epipharyngeal region (Fig. 2A - C). All sensilla in the lateral groups were of setiform design, with a mean (\pm 1 SD) of 18.6 (\pm 2.94) for the right wall and 18.2 (\pm 3.12) for the left wall. The medial group consisted of 26.9 (\pm 6.66) setiform sensilla and, in every case, 6.0 basiconic sensilla.

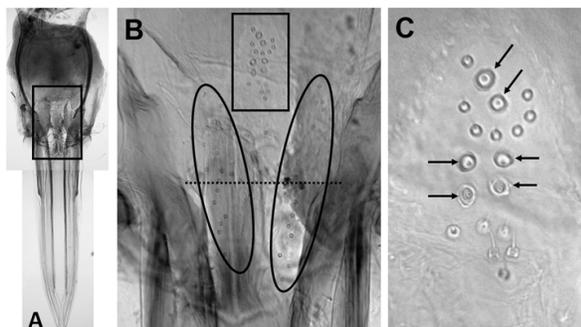


Figure 2. *Hybomitra difficilis* labrum, entire (A). Box in A highlights epipharyngeal region (enlarged in B); dotted line in B represents proximal extent of food canal. Box and ovals in B depict “median patch” and “lateral patches” of sensilla, respectively (described by Buerger 1967). Box in B enlarged in C to show six basiconic sensilla (arrows).

Discussion

Sensilla of the Vestibule

Sensory structures associated with the labrum of biting flies have been known since Stephens and Newstead (1906) described a pair of “hair-bearing papillae” with long fine hairs (shown in their Fig. 28) that were “...very constant in position...”, near the tip of the labrum in *Glossina palpalis*. This description comports with a pair of setiform mechanoreceptors similarly positioned in the vestibule of *Hybomitra difficilis* near the vestibule/food canal junction. In contrast to *G. palpalis*, however, there was a second pair of basiconic sensilla positioned in the mid region of the *H. difficilis* vestibule anterior to the mechanoreceptors. The occurrence of both basiconic and setiform sensilla in the vestibule of *H. difficilis* is reminiscent of Faucheux (1975) who described two pairs of lateral sensilla in the opening of the food canal of the tabanid, *Ochrops fulvus*. Faucheux (1975) also noted that five to six sensilla were present in *Tabanus bovinus* (although only four are shown in his Fig. 27), two of which were short, globular (basiconic?), and positioned distally, and four others that were fine and elongated (setiform?). Thus in tabanids, unlike glossinids, the first sensilla of the vestibule/food canal complex to come in contact with imbibed blood are of basiconic design.

Sensilla of the Food Canal

After establishing the presence of a pair of “papillae” at the labrum tip in *G. palpalis*, Stephens and Newstead (1906) then noted that such “...papillae are not again encountered until the labrum approaches the bulb”, before becoming numerous at the labrum base near the bulb of the proboscis (their Figs. 27 & 29). This arrangement was corroborated by Rice et al. (1973) who observed 25 to 30 thin, elongated (i.e., setiform), sensilla along each side of the labrum clustered proximally (i.e., near the head) in *Glossina austeni*. Clearly, positioning of these setiform sensilla in *Glossina* spp. is in contrast with *H. difficilis* where, in the latter species, these sensilla are significantly aggregated in the distal (Table 1), rather than proximal, regions of the food canal.

In general, the type, number, and position of sensilla lining the food canal of tabanids remains poorly described. Lall and Davies (1971), working with *Chrysops vittatus*, *Hybomitra lasiophthalma*, and *Tabanus lineola* (their samples being a “...total of 5 – 6 flies of each species...”), noted only that; “...small, thin-walled trichoid hairs are found in pairs along the food groove.” Faucheux (1975) also noted that the alimentary canal of *T. bovinus*, comparable to the food canal of *H. difficilis*, contained other, regularly spaced, sensilla along its length. Neither Lall and Davies (1971), nor Faucheux (1975) provided information on the number, category, or position, of sensilla in the food canal of the tabanid species they studied, nor did they mention the presence of basiconic sensilla in the food canal proper of any flies in their samples. So, contrary to the pairing described by Lall and Davies (1971), and the regular spacing reported by Faucheux (1975), sensilla in *H. difficilis* exhibited little evidence of occurring in pairs, and they were significantly aggregated in the distal region of the food canal rather than being “regularly spaced.” Moreover, basiconic sensilla in the food canal of *H. difficilis* exhibited little evidence of pairing, and were, with but two exceptions, aggregated in the distal-most distal region of the food canal.

Additional information on numbers of sensilla associated with the food canal of tabanids is available. Scudder (1953) observed “roughly 30” sensilla (his “rheometric trichodes”) on each side of the food channel of *Tabanus quinquevittatus*. Buerger (1967) reported a mean of 67 sensilla (ranging from 45 to 102) in $n = 36$ *Hybomitra rupestris* females, but offered little insight on the spacing/aggregation of these structures other than commenting that the; “...space between the individual sensilla increases towards the basal end of the labrum.” The “spacing” of sensilla noted by Buerger (1967) suggests that aggregation of food canal sensilla in *H. rupestris* is similar to that found in *H. difficilis* (Table 1). Increased spacing of sensilla in the food canal, from distal to proximal, has also been reported in

the deer fly, *Chrysops callidus* (Joy and Stephens 2016). Mean numbers of food canal sensilla provided by Scudder (1953) and Buerger (1967) appear somewhat greater than the mean \pm 1SD (50.15 ± 10.02) observed for *H. difficilis*, but without measures of variability in the *T. quinquevittatus* or *H. rupestris* data, statistical comparisons between numbers of labral sensilla in those species with the *H. difficilis* sample population cannot be made.

Sensilla Type, Position and Function

Rice et al. (1973) observed 25 to 30 thin, elongated sensilla (their LC1 sensilla) clustered proximally in lateral walls of the labrum, close to the head of *Glossina austeni*. Furthermore, Rice et al. (1973) noted that, "...on structural grounds alone they can be classified as mechanoreceptors", adding that; "The setae of the LC1 sensilla project far across the food canal and they are certain to be stimulated by the passage of food during ingestion." Mechanoreceptor (i.e., setiform sensilla) structure in the food canal of *H. difficilis* in this study appears homologous to those elongated sensilla described by Rice et al. (1973) (the "LC1 Receptors" in their Fig. 1), although mechanoreceptor setae of *H. difficilis* with lengths of $\approx 15 \mu\text{m}$ are shorter than those of *G. austeni* which often exceed $30 \mu\text{m}$. Moreover, food canal sensilla of *H. difficilis* are significantly aggregated in the two distal regions of the canal (Table 1), whereas *G. austeni* receptors are clustered proximally. Clearly, there are differences in length, and positioning of setiform sensilla in *H. difficilis* vis-a-vis *G. austeni*, but however positioned in the food canal, such mechanoreceptors appear designed to monitor movement of fluids in tabanids and tsetse flies. This comports with Scudder (1953) who posited that such irregularly placed "rheometric trichodes" were positioned in the food canal to record "...the impact and subsequent deflection caused by the flow of liquid past them on its upward course into the cibarium." Rice et al. (1973) took a more cautionary tone in noting that it is "a matter of speculation" whether or not neurons emanating from these mechanoreceptor setae, "can monitor vibrations caused by erythrocytes striking the delicate ends of the setae." Such speculation, however, seems plausible, given the numbers and positioning of food canal setiform sensilla in tabanids.

Distal positioning of basiconic sensilla (i.e., chemoreceptors?) in the vestibule and food canal of *H. difficilis* is also quite different from the proximal chemoreceptor position in *Glossina austeni* described by Rice et al. (1973). Those investigators noted that chemoreceptors of tsetse flies are the most proximal of mouthpart sensilla, adding that; "They are the only chemoreceptors actually within the food canal of the

tsetse and thus have a unique role to play; that of deciding whether the fluids sucked up should be swallowed or not." ATP (Galun and Margalit 1969) and ADP (Friend and Stoffolano 1983) have been reported as significant phagostimulants for probing *G. austeni*, and *Tabanus nigrovittatus*, respectively. Since the first sensilla of *H. difficilis* to come in contact with imbibed fluids are the vestibular basiconic sensilla (chemoreceptors?), these sensilla appear ideally positioned to signal the presence of blood to the probing fly. This is reminiscent of a similar positioning of vestibular chemoreceptors shown by Faucheux (1975) (in his Fig. 27) for *T. bovinus*.

Our observation of putative chemoreceptors aggregated in the distal-most region of the *H. difficilis* food canal (Table 2) is not unique, since Scudder (1953) mentioned, somewhat ambiguously, the presence of "occasional basicones" among "Group A Trichodes" (his Group A Trichodes being sensilla lining the food canal walls). Other investigators (Buerger 1967; Lall and Davies 1971; Faucheux 1975), however, failed to mention the presence of such chemoreceptors outside the area of the vestibule.

Neither Lall and Davies (1971) or Faucheux (1975) provided information on number and relative position of "trichoid hairs" in the food canal of their tabanid study specimens. Buerger (1967), however, noted that in *H. rupestris*, "...space between the individual sensilla increases towards the basal end of the labrum." The latter finding comports with the aggregation of sensilla in distal regions of the *H. difficilis* food canal and lack of aggregation (i.e., increased spacing) for sensilla in the proximal canal region (Table 1).

Epipharyngeal Sensilla

In addition to the food canal sensilla, some investigators have observed additional sets of sensilla at the base of the canal (i.e., the epipharynx or prepharynx). Buerger (1967) wrote of "three patches" of sensilla in the epipharyngeal region of *H. rupestris*; two lateral (each with "about 17 setiform sensilla"), and one median ("...consisting in most individuals of 6 basiconic sensilla and 16 setiform sensilla..."). Similarly, Scudder (1953), working with *Tabanus quinquevittatus*, describes two short rows (= Buerger's "lateral patches") made up of a dozen or fewer simple tactile hairs in the inner antero-lateral wall of the cibarial region (i.e., "prepharynx"), and a third grouping (his "palatal papillae", comparable to Buerger's "medial patch") consisting of "a dozen or more specialized basicones with occasional trichodes intermixed." Our findings of epipharyngeal sensilla in *H. difficilis* agree closely with Buerger (1967); most notably the consistency of six basiconic sensilla in the

medial grouping. Conversely, numbers of basicone and setiform sensilla reported for *T. quinquevittatus* appear decidedly different from those seen in *Hybomitra* species.

From an ecological perspective, studies on tabanid mouthparts may yield important information on potential hosts and the epidemiology of pathogen transmission (Gouteux *et al.* 1989). For example it has been suggested that the longer, more slender feeding apparatus possessed by deer flies (e.g., *Chrysops*), is well suited for feeding on a wider range of hosts, including “animals with thick fur”, whereas larger tabanids (e.g., *Tabanus*, *Hybomitra*), may be restricted by their short, broad proboscis, to feeding on animals with sparse or short hair (Lall and Davies 1971). Of course, dispersal of adult tabanids is probably influenced by the availability of suitable hosts (Gouteux *et al.* 1989; Mullens 2009; Muzari *et al.* 2010). Tabanid feeding strategies, presumably, could be equally affected by the arrangement of sensory structures in the food canal designed to detect the presence of blood, or monitor blood flow, and thus a productive avenue for continued research would be the examination of these sensory structures in a wider range of tabanid species.

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