Activity of the Extrinsic and Intrinsic Muscle Networks of the Malpighian Tubules and the Ureter in the Female Stable Fly, *Stomoxys calcitrans* (L.)[1]

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ABSTRACT The four Malpighian tubules observed in the female stable fly, *Stomoxys calcitrans* (L.), are divided into an anterior dorsal pair and a posterior ventral pair. Intrinsic muscles were found only in the short proximal section (ureter) of each pair of tubules that emptied into the midgut. Isolated preparations of the ureter demonstrated four types of organized rhythmic activity: compression, peristalsis, reverse peristalsis, and segmentation. Compression caused a momentary (0.4 to 0.6 sec) shortening of the longitudinal muscle fibers in a localized region of the ureter. Peristalsis was the dominant type of activity. Each wave progressed along the duct with a duration that ranged from 0.9 to 1.7 sec. Segmentation consisted of a localized annular constriction of the ureter without progression that lasted for a duration of 0.3 to 0.6 sec. The large distal ends of the dorsal pair of tubules showed spontaneous movement *in situ*. Close examination revealed a network of extrinsic muscle cells attached to the distal tubular ends. The motile properties of the extrinsic muscles were slower and not as complex as those of the ureter. The sequence from the resting state to contraction and return was much longer with durations that ranged from 10.8 to 27.1 sec. Also, the time for peak tension was 5 to 25.9 sec in duration. The extrinsic muscles did not show the organized and rhythmic activity observed in the ureter.

Key Words Visceral muscle, ultrastructure, motility

The muscle networks of Malpighian tubules in insects have attracted the attention of entomologists over the years. Palm (1946) provided one of the most comprehensive surveys (86 species) of their action in his study of Malpighian tubule peristalsis. He concluded that insects can be divided into four groups on the basis of muscular distributions on the Malpighian tubules. The first group consists of those insects that lack muscle fibers on the tubules (Thysanura, Dermaptera, and Thysanoptera). The second group have muscles only at the base of the tubules near the junction with the midgut (Diptera, Lepidoptera, and Trichoptera). In the third group, muscle networks form an almost continuous

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coating over the tubules (Coleoptera and Neuroptera). The fourth group includes those insects that have one or two muscle fibers helically wound about the tubule over its entire length (Orthoptera and Odonata). Davey (1964) relates that of the few studies on tubule movement, only insects in groups 3 and 4 listed above have been considered. The only insects studied since Palm and Davey have been the cockroach, *Periplaneta americana* (L.) (Schaefer 1968, Meyers and Miller 1969, Crowder and Shankland 1972a, 1972b) and the stick insect, *Carausius morosus* (Br.) (Pilcher 1971).

The four Malpighian tubules present in the stable fly arise in pairs from from short ducts (ureters) which open into the midgut much like Palm described for other dipterous insects. In an initial utrastructural survey of this excretory system (Cook and Pryor 1997), we discovered intrinsic muscle cells in the ureters. The ureter appears to serve as a pump to prime the release of urine from the Malpighian tubules. The ultrastructural features of the epithelia cells that line this duct suggest the possibility of some ion transport. More recently, we found a network of extrinsic muscles in close association with the distal regions of the anterior dorsal pair of Malpighian tubules. The following report gives an account of the principal structural features of both muscle networks and the types of spontaneous motility that these groups of muscles display.

Materials and Methods

Stable flies were obtained as pupae from a laboratory-reared colony of the USDA, ARS, Knipling-Bushland U.S. Livestock Insects Laboratory in Kerrville, TX. After adult emergence, the flies were held in screen cages at 27° and 50 to 60% R. H. The flies were fed daily from cotton pads soaked with citrated (5 mg/L) bovine blood and placed on top of the screen cages. At 4 to 7 d after adult emergence, female flies were removed from cages and dissected. The composition of the stable fly saline used for dissection was (in mM): NaCl 105, KNO_3 5, $CaCl_2$ 5, $MgSO_4 \bullet 7H_20$ 3, L-histidine 10, and glucose 28. The pH was adjusted to 6.8 with .1 M NaOH. The presence of L-histidine provided a buffering action. Composition of the saline solution reported here was developed from recent analyses of the cations, anions (Chen 1989) and organic constituents (Chen and Wagner 1992) found in adult stable fly hemolymph.

Preparation of tissue for microscopic examination. Female flies were immobilized by cooling (4°C) and the head, wings, and legs were removed. The thorax and abdomen were placed ventral side up in a small, wax-filled Petri dish and were pinned with a minuten pin through the thorax. The retracted ovipositor was pulled out to its full length with forceps and pinned to the wax preparation dish with a minuten pin through the tip. Then the central portion of the integument on the ventral abdomen was excised. The body cavity was flooded with stable fly saline and the integument of the abdominal side walls was pinned with a minuten pin on each side. The integument of the extended ovipositor was cut along the mid line to fully expose the terminal end of the hindgut. The ventral pair of malpighian tubules were removed carefully from the surface of the ovaries and traced to their junction with the midgut. At this point, the ovaries were removed and the dorsal pair of Malpighian tubules was lifted and severed from attachments to the dorsal wall on either side of the

heart (Fig. 1). The entire Malpighian tubule system with its attachments to the gut could be lifted out of the abdomen by cutting the terminal end of the hindgut and the anterior end of the midgut. Once the system was isolated, it was pinned on a plastic (sylgard) coated slide for examination with Nomarski differential interference contrast microscopy.

For observation under transmission election microscopy, the Malpighian tubules from two insects were fixed in 1% acrolein and 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at room temperature or overnight at 4°C. The buffer was exchanged 3 times after 10-min intervals in the cold and post fixed in 1% OsO_4 buffered with 0.1 M sodium cacodylate for 2 h at 4°C. The tissues were dehydrated in a series of aqueous acetone solutions (30%, 50%, 70%, 95% and 100% acetone; 3 times in 100%) prior to embedding in Spurr's resin as modified by Mollenhauer (1986). Tissues were sectioned with a Reichert Ultracut S microtome (Leica, Deerfield, IL) either at 1 μm and stained with Toluidine Blue or at 70 nm and stained with lead citrate. Appropriate specimens were viewed either under the light microscope or at 75 kV under a Hitachi H 7000 electron microscope (Hitachi, Ltd., Tokyo, Japan).

Video recordings of extrinsic muscle contractions and ureter peristalsis were made with a high resolution 8 mm recorder (EV-C100, Sony, Tokyo) equipped with a high resolution (525 lines) charge-coupled device (CCD) camera (Ultrichip Model JE-7442, Javelin, Torrance, CA) (Chen and Schleider 1996). The camera was mounted to an Olympus IMT-2 inverted microscope (Olympus Corp., Lake Success, NY). Recordings of muscle activity were monitored on a high resolution monitor (BWM 12, Javeline, Torrance, CA) and prints of selected activity from the video recordings were made with a sublimation heat transfer color video printer (CVP-MI, Sony, Tokyo). Motile events were generally timed with a digital stopwatch that could be read to the nearest 0.01 sec. The accuracy of our measurements was in the range of ±0.1 sec. Other events such as peristalsis and segmentation were subjected to image analysis with Image Pro-Plus digital image processing system developed by Media Cybernetics. This system permitted the capture of sequential video images for analysis on a computer. The software provides an array of analytical tools and graphic display options.

Results

Two pairs of Malpighian tubules were observed in female stable flies. Each pair joined a short common duct (ureter) before entering the digestive tract at the junction between the midgut and hindgut (Fig. 1 and 2A). One pair of tubules lay dorsal to the gut in a region parallel to the heart. The distal extremities of these anterior dorsal tubules were enlarged noticeably and contained a large quantity of white spherical granules. The second pair of Malpighian tubules was located between the ventral abdominal wall and the surface of the ovaries (Fig. 1). Although the distal terminations of the posterior ventral set of tubules contained some white spherical granules, their terminations were not enlarged. In addition to these striking terminal differences, the translucent yellow appearances of the posterior set of tubules was darker.

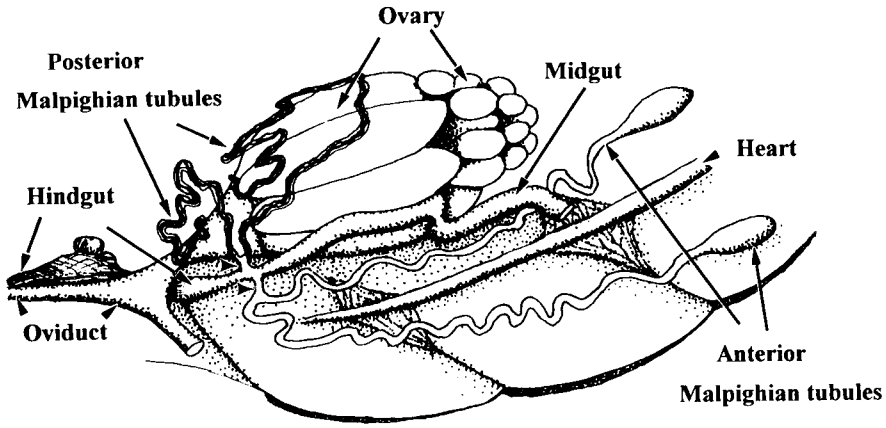


Fig. 1. Ventral lateral aspect of the female stable fly abdomen illustrating the anterior dorsal and posterior ventral pairs of malpighian tubules and their relationship to ovaries and the digestive tract. Unmarked arrowhead = ureter. The posterior set of Malpighian tubules are shown in darker profile to denote the more intense yellow pigmentation present in comparison with the anterior set.

Intrinsic muscles of the ureter were not visible under Nomarski optics at lower magnifications, but their presence was indicated by the spontaneous peristaltic action of this short proximal duct. However, electron microscopic examination of transverse sections of the ureter revealed a large array of small striated muscle fibers (arrow heads) embedded in the basal regions of large epithelial cells Fig. 2B). Although these muscles formed an irregular lattice that covered the entire surface of the ureter, similar networks of such intrinsic muscles were not detected in other regions of the Malpighian tubule system. The majority of intrinsic muscle fibers of the ureter were found in direct contact with the epithelial cells. However, a number of additional fibers were loosely associated with the epithelium through long connective tissue strands composed of basal lamina (Fig. 2C and D). in some instances, cytoplasmic elements were incorporated into these connective tissue attachments near the points of contact.

During Malpighian tubule dissection, the large distal ends of the anterior dorsal pair of tubules often moved spontaneously *in situ*. Once they were completely isolated from the abdomen, closer examination of the distal ends of these tubules under Nomarski optics showed an extensive, but loosely attached network of extrinsic muscle cells near the tubules (Fig. 3A-E). Interference microscopy revealed the stellate and striated character of these muscle cells with their nuclei (Figs. 3A, 3D, and 3E). Some muscle fibers were clearly attached to trachea (Fig. 3C), but the direct attachment of muscle to the tubule was not

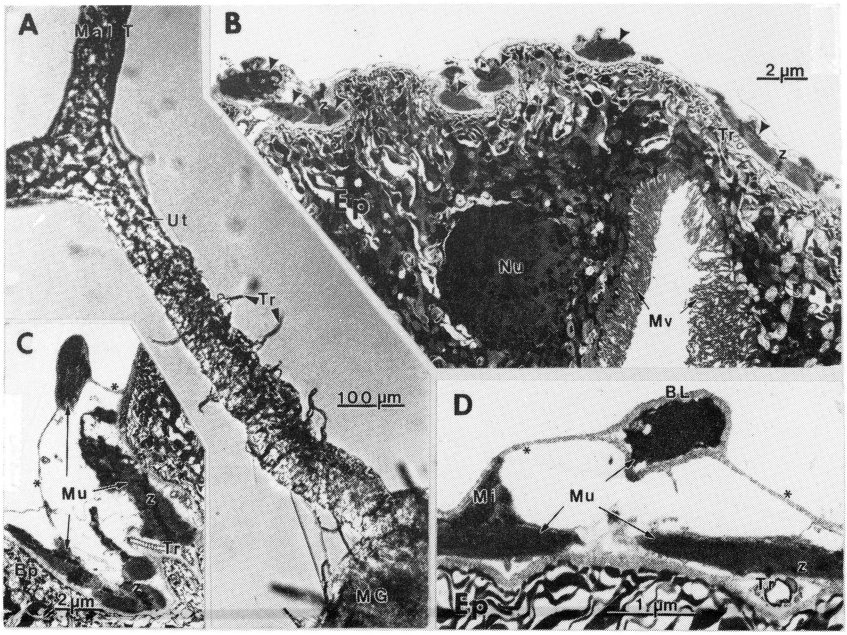


Fig. 2. Principal structural features of the intrinsic muscles of the ureter of the stable fly. (A) Differential interference contrast micrograph of the dorsal ureter (Ut) as it enters the mid-gut (MG). Mal T = Malpighian tubule; Tr = trachea. (B) An electron micrograph which illustrates an array of intrinsic muscle cells (arrow heads) embedded in the basal region of an epithelial cell (Ep) of the ureter. Mv = microvilli; Nu = nucleus; Tr = trachea; Z = z band. (C) Muscle fibers (Mu) in transverse section from another region of the ureter. Two fibers are in close contact with a fold in the epithelial cell (Ep) while a third fiber is only held in loose association with the ureter by several connective tissue strands (*) Z = z band; Tr = trachea. (D) High magnification of another loosely associated muscle cell (Mu) linked to the epithelium (Ep) through connective tissue strands. (*). BL = basal lamina; Mi = mitochondria; Tr = trachea; Z = z band.

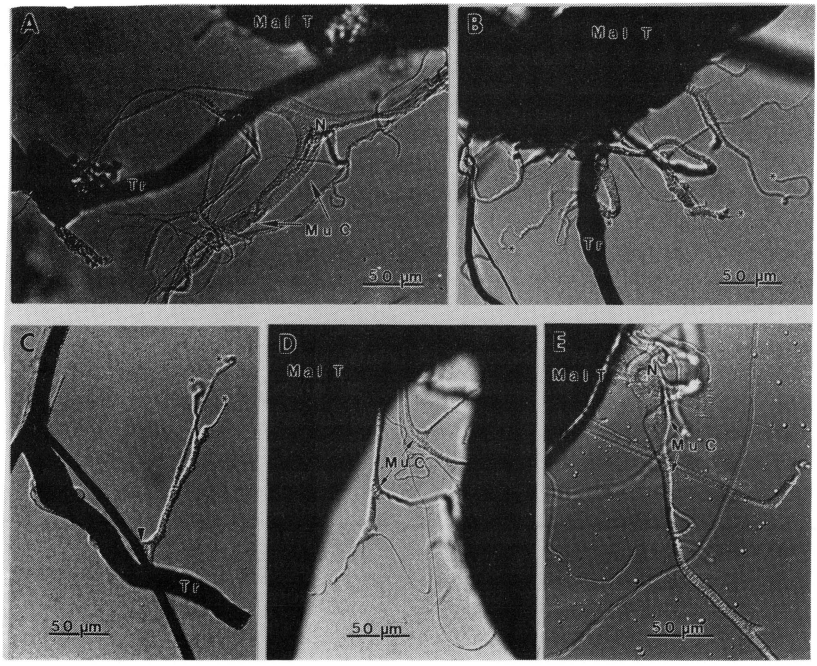


Fig. 3. Differential interference contrast micrographs of extrinsic muscles and their associations with the distal regions of the dorsal Malpighian tubules of the stable fly. (A) Micrograph of a large extrinsic muscle cell (MuC) close to a dorsal malpighian tubule (Mal T). N = nucleus; Tr = trachea. (B) Severed ends (*) of an array of muscle fibers attached to Malpighian tubule (Mal T). Tr = trachea. (C) Attachment of an extrinsic muscle fiber (arrow head) to a trachea (Tr). (D) Two muscle cells (MuC) visible in a large fold of a dorsal Malpighian tubule at its distal end. (E) A large extrinsic muscle cell near the ruptured end of a dorsal tubule (Mal T). A number of crystalline spheres have been released from the tubule.

observed. These extrinsic muscles (8 to 10 μm diam) are obviously much larger than the intrinsic muscles (0.25 to 0.6 μm diam) found in the ureter. The ureters which connect the Malpighian tubule to the midgut in the stable fly occasionally contracted spontaneously after exposure by dissection. However, once the preparations were perfused with saline solution, spontaneous activity appeared either in initially quiescent preparations or increased noticeably in the active ones. Because this activity was also detected in completely isolated Malpighian tubule systems, it demonstrated the inherent myogenic properties of the muscles of the ureter in the absence of any neural stimulation.

Isolated preparations of the ureter with its associated Malpighian tubules demonstrated four types of organized rhythmic activity characteristic of tubular organs in the viscera of insects: compression, peristalsis, reverse peristalsis, and segmentation. In this study, 13 preparations of the ureter (from 7 different flies) were observed and the activity sequences from 10 of these preparations were recorded on video cassette. Compression was occasionally evident in the ureter. In this mode of activity, a localized contraction of longitudinal muscle fibers caused a momentary shortening in one region of the duct. The stretch induced in other regions of the ureter by the primary compression caused secondary compressions. The duration of these sequences was 0.4 to 0.6 sec.

Peristalsis was the dominant type of activity observed in the ureter and was initiated by a localized contraction in the muscle network at some point along the ureter. The contraction progressed as a wave in either a caudal (peristalsis) or an anterior direction (reverse peristalsis). The duration of these events in the ureter ranged from 0.9 to 1.7 sec for peristalsis and 0.8 to 1 sec for reverse peristalsis. In addition to the duration of individual peristaltic events in the ureter, the frequency was determined in 3 preparations by measuring the time for 10 consecutive waves to occur. Nine measurements gave a mean valve of 32 sec with a range of 27.7 - 37.1 sec. Therefore, the frequency is 3.2 sec/peristaltic wave. An example of persistalsis is shown in Fig. $4A_{1-4}$. The progression of the observed peristaltic wave is represented as a hatched zone on the image of the ureter that changes position over time. In the analysis of peristalsis, two approaches were taken as illustrated in Fig. 4B. 1) A specific area was selected for comparison over the 1.2 sec time period of action. The traced area was measured on the captured video image every 150 milliseconds (MS) and the computed changes plotted on the time line of the graph. One complete peristaltic wave was registered with a peak (minimum area) at 0.6 sec. (2) Line profile analysis provided a measure of the change in optical density along a line that crossed the ureter. This change in optical density is expressed in gray scale values from black (0) to white (240). The line across the ureter was traced from the top border to the bottom as shown in the photograph in Fig. 4B. Thus, the line profile graphs were read from left to right to conform to this orientation. In the line profile graph for 0.3 sec all the gray values between 10 and 60 μm were above 95 and all the peaks directed toward the dark end of the scale were shallow. At 0.45 sec, however, a large peak appeared between 30 and 40 μm along the line. At 0.6 sec this central peak has become much sharper as have other peaks along the line. A maximum peak in darkness (70) has also occurred along the border of the ureter at 74 μm. At 0.75 sec the central peak (30-40 μm) has continued to become darker but the other peaks at 54, 44 and 18 μm have

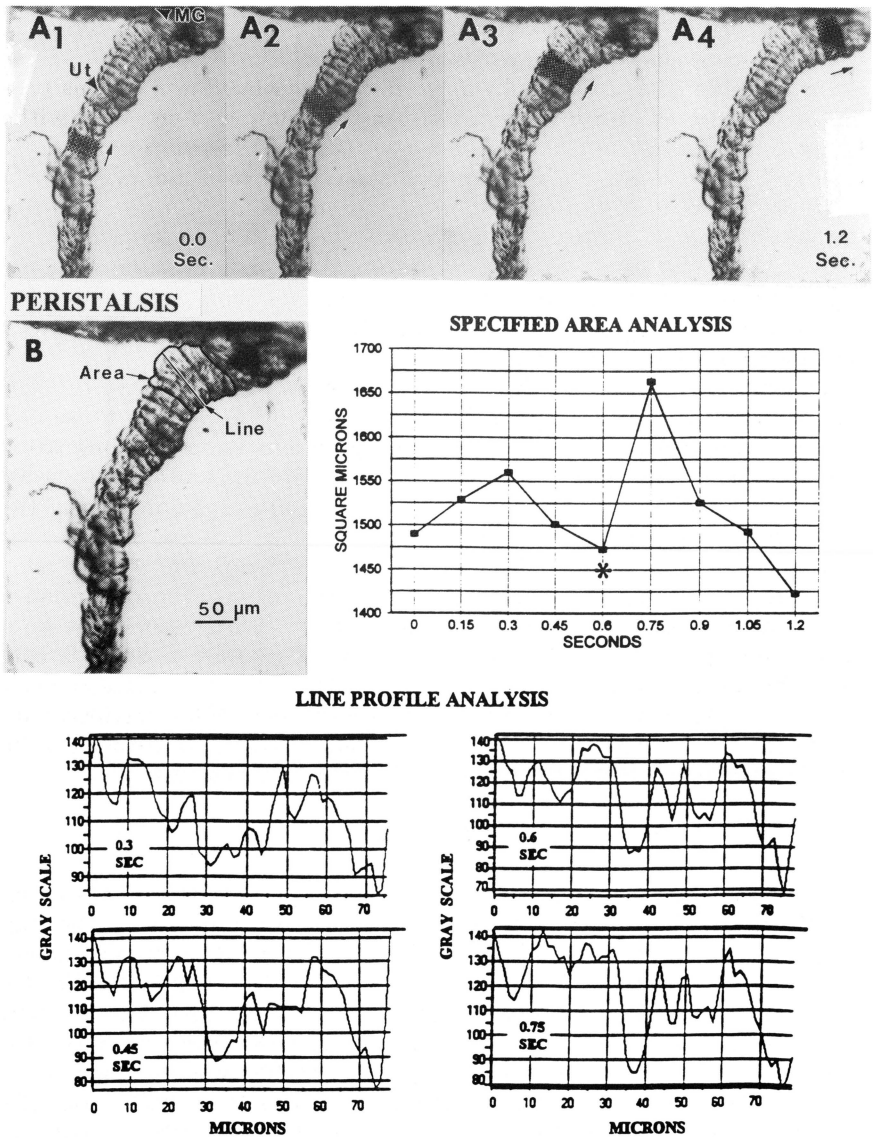


Fig. 4. Analysis of peristalsis in the proximal ureter (Ut). (A_{1-4}) A sequence of video images which illustrate the progression of a peristaltic wave (hatched area) down the ureter toward the midgut (MG) over a 1.2 second interval. B) Area and line profile locations for the analysis of peristaltic action. the specified area analysis graph shows the progression of a peristaltic wave through a given area of the ureter over time. * marks the peak of the wave. The line profile analysis shows the changes in lightness and darkness of a line across the ureter during the course of the peristaltic wave. Further details are explained in the text.

become lighter. The line profile graphs correspond to the progression of the peristaltic wave shown in the area analysis graph.

Segmentation was occasionally detected in the isolated ureter. This type of motility consisted of a localized annular constriction of the duct without progression. Such events had a duration of 0.3 to 0.6 sec. An example of this activity is shown in Fig. 5. Two separate regions of segmentation were observed in the distal ureter. The location of each was indicated by lines L_1 and L_2 in Fig. 5A. The graph in Fig. 5B illustrates the change in the diameter of the ureter at each of the two loci over the course of 1.2 sec. A total of four complete segmentation cycles were detected at the two locations. This analysis was achieved by the capture of a video image for diameter measurement on the computer every 150 milliseconds.

The motile properties of the extrinsic muscles associated with the dorsal Malpighian tubules were much slower and less complex than those of the ureter. In observations from 3 muscle preparations (from two insects), the sequence from the resting state to contraction and return ranged in duration from 10.8 to 27.1 sec. Moreover, the time for peak tension during these contractions was remarkably long (5 to 25.9 sec). An example of one of these spontaneous contractions is illustrated in Fig. 6. A series of 3 video images captures the principle phases of the initial contraction sequence of such a muscle fiber. The full time-course is depicted graphically and shows a peak tension of 5 sec duration. The relaxation phase took 16 sec to return to within 7% of the resting state. The series of 3 video images also illustrates an evident change in Malpighian tubular contour as a consequence of the extrinsic muscle fiber contraction. Motile properties were only evident in intact extrinsic muscles. Those that had been severed or torn showed no activity.

Discussion

Two differences were recognized in the structural properties of the anterior dorsal pair and the posterior ventral pair of Malpighian tubules in the female stable fly. The first was the striking distinction between the terminal diameters of the two sets of tubules. Diameters of the dorsal set were at least twice those of the ventral set. This difference reflected the number and size of spherical granules present in these respective distal regions (Cook and Pryor 1997, Kongoro et al. 1991). The second structural distinction was the discovery of a network of extrinsic muscle fibers associated with the terminal regions of the anterior dorsal set of Malpighian tubules. Such a network was not found associated with the posterior ventral pair. Instead, the extensive tracheation in the distal regions of the posterior ventral pair tied these tubules to the surface of the ovaries (Fig. 1) which contain a large superficial network of muscles (Cook and Peterson 1989). Thus, both pairs of Malpighian tubules have functional motility at their extremities. Wigglesworth (1972) suggested that the twisting movements brought about by the intrinsic muscles of the tubules increase contact between the tubes and the body fluid. This same suggestion might apply to the actions of the extrinsic muscles associated with the tubules and the anchorage of tubules to other active organs.

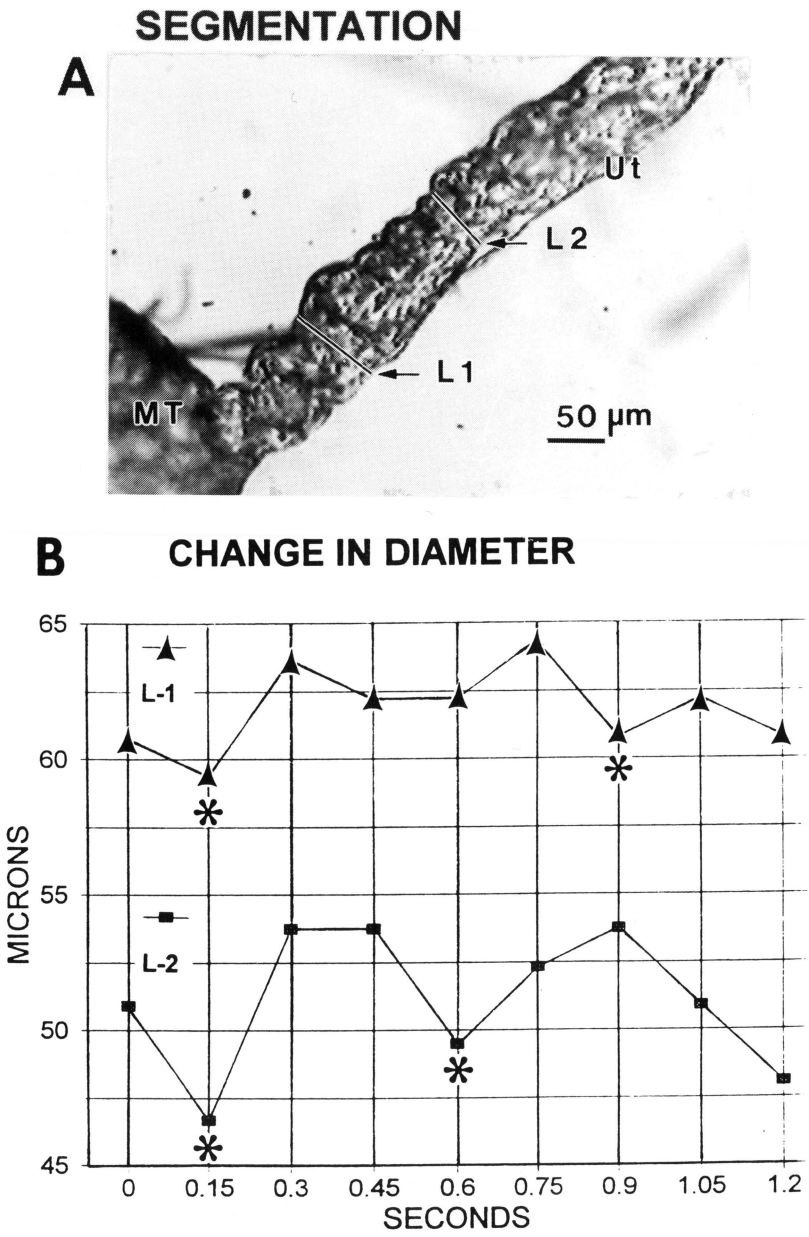
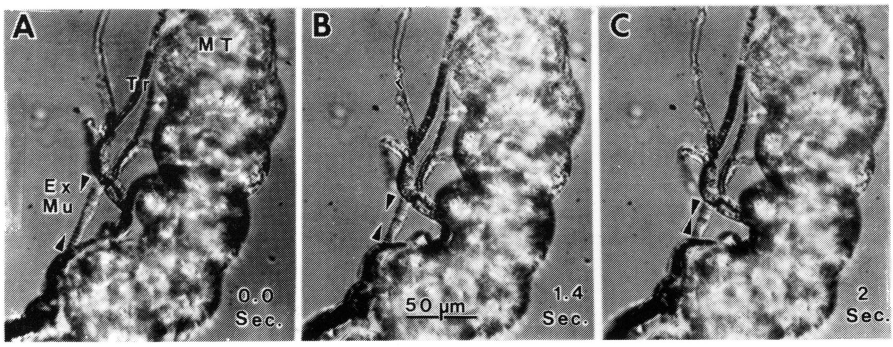


Fig. 5. Analysis of segmentation of the distal ureter (Ut). A) Video image of the ureter that shows the location of two zones of segmentation (lines L_1 and L_2). MT = Malpighian tubule. B) Time-course graph of change in the diameter of the ureter during cycles of segmentation. * marks complete cycles.



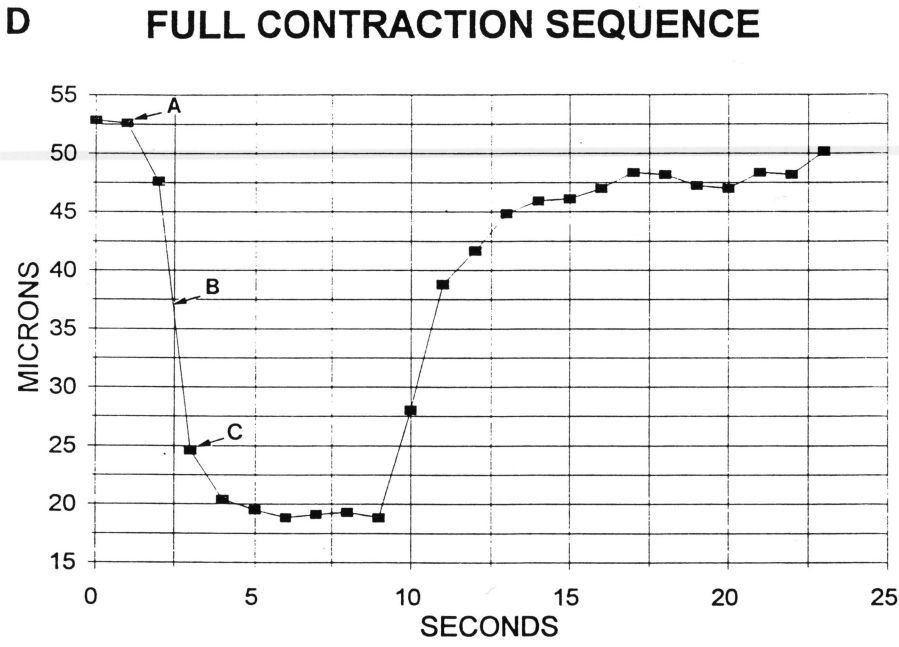


Fig. 6. Analysis of the contractile properties of an extrinsic muscle fiber (Ex Mu) associated with the anterior dorsal Malpighian tubule (MT). Tr = trachea. A) Muscle fiber in the resting state (opposed arrow heads). B) Fiber partially contracted (47%). C) fiber almost fully contracted (79%). D) Image analysis of the full contraction sequence obtained by measuring the distance from the base of one arrow head to the other on the image for each sec. Over the 25 sec. time course. The arrows with the designations A, B & C identify the locus of the above photographs in the time-course graph for contraction.

A careful analysis of video recordings of the ureter showed that four basic types of motility occurred: compression, peristalsis, reverse peristalsis, and segmentation. These forms of movement are common to many tubular visceral organs in the stable fly (Cook and Peterson 1989, Cook et al. 1991, Cook 1992) and other insects (Cook and Reinecke 1973, Cook et al. 1984). The individual and joint actions of the movements give rise to a variety of functional events. These events include: (1) lateral transport of materials in the lumen of the tube; (2) mixing of contents in the lumen of the tube by alternating contractions at different loci along the tube; and (3) changes in intratubular pressure, which may affect transport mechanisms across secretory cell boundaries. both the lateral transport of spherical granules and the oscillation of granules in the lumen of the ureter have been observed. Although not tested, one might suspect that changes in intralumenal pressures facilitate secretory transport mechanisms.

In the absence of innervation to the ureter, the presence of organized muscle activity might seem unexpected. However, the stands of basal lamina with their sarcoplasmic extensions between cells of the muscle network (Fig. 2C and D) provided a structural pathway for the transmission of excitation which led to the coordination of the observed rhythmic contractions. Although the intrinsic muscles of the ureter are independent of the nervous system, they may be stimulated by substances released from the neuroendocrine system as suggested by Cameron (1953) and Pilcher (1971). This prospect has not been tested in the stable fly.

The action of extrinsic muscle fibers attached to the tracheal networks that invest the distal ends of the anterior dorsal pair of Malpighian tubules produced a gentle oscillatory motion with a much slower rate of contraction. The sequence from the resting state to contraction and return was also much slower with a range of duration from 10.8 to 27.1 sec. In fact, the peak tension for these contractions was quite long (from 5 to 25.9 sec in duration), similar to muscles with "catch-like" properties (Hoyle 1983). Unfortunately, it was not possible to establish the presence of paramyosin in these muscles by ultrastructural analysis.

In summary, more attention must be given to the functional and structural aspects of both extrinsic and intrinsic muscles of the malpighian tubular system before a full understanding of the process of excretion in insects can be achieved. The details of muscle structure and activity presented in this study on the stable fly make it much easier to consider the effects of diuretic peptides and other agents on peristalsis of the ureter and the movements of the extrinsic muscles in close association with the Malpighian tubules.

References Cited

Cameron, M. L. 1953. Secretion of an O-diphenol in the corpus cardiacum of insects. Nature, Lond. 172: 349-350.

Chen, A. C. 1989. Changes in the hemolymph of the stable fly, *Stomoxys calcitrans*, after a blood meal. Arch. Insect Biochem. Physiol. 11: 147-158.

Chen, A. C. and P. G. Schleider. 1996. An analysis of excretion in the stable fly, *Stomoxys calcitrans* (L.). Southwest. Entomol. 21: 43-48.

Chen, A. C. and R. M. Wagner. 1992. Hemolymph constituents of the stable fly, *Stomoxys calcitrans.* Comp.Biochem. Physiol. 192A (1): 133-137.

Cook, B. J. 1992. The oviduct musculature of the stable fly *Stomoxys calcitrans*: Properties of its spontaneous motility and neural regulation. Arch. Insect Biochem. Physiol. 19: 119-132.

Cook, B. J., G. M. Holman and S. M. Meola. 1984. The oviduct musculature of the cockroach *Leucophaea maderae* and its response to various neurotransmitters and hormones. Arch. Insect Biochem. Physiol. 1(2): 167-178.

Cook, B. J. and T. Peterson. 1989. Ovarian muscularis of the stable fly, *Stomoxys calcitrans:* its structural, motile, and pharmacological properties. Arch. Insect. Biochem. Physiol. 12: 15-30.

Cook, B. J. and N.W. Pryor. 1997. Structural properties of the intrinsic muscles of the Malpighian tubules of the female stable fly *Stomoxys calcitrans.* J. Entomol. Sci. 32 (2): 138-147.

Cook, B. J. and J. P. Reinecke. 1973. Visceral muscles and myogenic activity in the hindgut of the cockroach, *Leucophaea maderae.* J. Comp. Physiol. 84: 95-118.

Cook, B. J., R. M. Wagner and T. L. Peterson. 1991. The hindgut muscularis of the stable fly, *Stomoxys calcitrans*; some of its structural, motile and pharmacological properties. J. Insect Physiol. 37: 635-645.

Crowder, L. A. and D. L. Shankland. 1972a. Structure of the Malpighian tubule muscle of the American cockroach, *Periplaneta americana.* Ann. Ent. Soc. 65: 614-619.

1972b. Pharmacology of the malpighian tubule muscle of the American cockroach *Periplaneta americana.* J. Insect Physiol. 18: 929-936.

Davey, K. G. 1964. The control of visceral muscles in insects, pg 219-245. *In* Beament J. W. L. and J. E. Treherne (eds.), Advances in insect physiology. Academic Press, New York.

Hoyle, G. 1983. Muscles and Their Neural Control. John Wiley & Sons, New York.

Kongoro, J., T.R. Odhiambo and J. R. Mainoya. 1991. An ultrastructural study of Malpighian tubules of the stable fly, *Stomoxys calcitrans* (L.) (Diptera: Muscidae): cell types and structural zonation. Insect Sci. Appl. 12: 517-525.

Meyers, J.and T. Miller. 1969. Starvation-induced activity in cockroach Malpighian tubules. Ann. Ent. Soc. 62: 725-729.

Mollenhauer, H. H. 1986. Surfactants as resin modifiers and their effect on sectioning. J. Microsc. Tech. 3: 217-222.

Palm, N-B. 1946. Studies on the peristalsis of the Malpighian tubes in insects. Acta. Univ. Lund. 42: 1-39.

Pilcher, D. E. M. 1971. Stimulation of movements of Malpighian tubules of *Carausius* by pharmacologically active substances and tissue extracts. J. Insect Physiol. 17: 463-470.

Schaefer, C. W. 1968. Inhibition by acetylcholine of Malpighian tubule muscle of the cockroach *Periplaneta americana.* Ann. Entomol. Soc. 61(1): 226-227.

Wigglesworth, V. B. 1972. The principles of insect physiology. Chapman and Hall, New York.