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Authors: Moffett, Stacia B., and Moffett, David F.

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Comparison of immunoreactivity to serotonin, FMRFamide and SCPb in the gut and visceral nervous system of larvae, pupae and adults of the yellow fever mosquito *Aedes aegypti*

Stacia B. Moffett and David F. Moffett

School of Biological Sciences, Washington State University, Pullman, WA 99164-4236 U.S.A
smoffett@mail.wsu.edu

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Abstract

In all life stages, the gut of the mosquito is innervated by a small number (typically 4) of central neurons immunoreactive to serotonin (S-I). The serotonergic system appears to pass through metamorphosis largely intact, despite extensive remodeling of the gut. Axons immunoreactive to antibodies raised against molluscan FMRFamide (RF-I) constitute peptidergic innervation that anatomically parallels the serotonergic system. In the larva, two clusters of 3 neurons project to the anterior regions of the gut, whereas in the pupa and adult, typically two large RF-I neurons located next to the esophagus send several processes posteriorly. In adults, these neurons branch throughout the diverticula and anterior stomach. In pupae, but not in larvae or adults, the gut RF-l system coexpresses reactivity to antibodies raised against a member of another peptide family, molluscan small cardioactive peptide b (SCP-I). SCP-I immunoreactivity is localized independently of RF-l immunoreactivity in the ganglia of all stages and in neurons that project along the gut of the adult. We did not find any colocalization of S-I and the peptide markers. Distinct populations of enteroendocrine cells populate different regions of the gut at different life stages. Changes in staining pattern suggest that these cells are replaced at metamorphosis along with the other gut cells during the extensive remodeling of the tract. Distributed in the gut epithelium are subpopulations that express either RF-I or SCP-I; a small fraction of these cells bind antibodies to both peptides. The stomachs of adult females are larger than those of males, and the numbers of SCP-I and RF-I enteroendocrine cells are proportionately greater in females. In all the life stages, the junctions between different regions of the gut are the focus of regulatory input. The larval cardiac valve possesses a ring of cells, the necklace cells, which appear to receive extensive synaptic inputs from both the serotonergic system and the peptidergic system. Another focus of control is the pyloric valve, which is encircled by axon-like processes. The immunoreactive pattern of this region differs across life stages, expressing SCP-I in larvae, S-I in pupae, and both SCP-I and RF-I in adults.

insect, enteroendocrine cells, metamorphosis, neuropeptides

Abbreviation:

S-I	serotonin-like immunoreactivity
RF-I	FMRFamide-like immunoreactivity
SCP-I	small cardioactive peptide b-like immunoreactivity

Introduction

The neurotransmitter serotonin has been implicated in control of multiple processes related to feeding and nutrition in mosquitoes, including appetite (Novak and Rowley, 1994), gut ion transport (Clark et al., 1999, 2000), gut motility (Onken et al., 2004a) and saliva secretion (Novak et al., 1995). The most commonly identified enteroendocrine factors in insects, including mosquitoes, belong to the FMRFamide, allatostatin and tachykinin families (Agricola and Braunig, 1995; Muren and Nassel, 1996; Nassel, 2002; Veenstra, 1999; Yu et al., 1995; Zitman et al., 1993). In adult female mosquitoes, there are 4 basic types of midgut endocrine cells (c.f. Veenstra, 1999): Type 1 is reactive to antisera to FMRFamide, RFamide, bovine pancreatic polypeptide and perisulfakinin; Type 2 is reactive to antisera to allatostatins, Type 3 is reactive to antisera to urotensin I, and Type 4 is reactive to antisera to locustatachykinins. To the extent that physiological actions of the peptide mediators have been demonstrated, they have been found to involve effects on gut motility or secretion of digestive enzymes. Except for a single paper by Lee et al. (1998) on the lepidopteran *Manduca sexta*, we know of no studies of the effect of regulatory peptides on gut ion transport in any insect.

We originally undertook the present studies to characterize the innervation of the larval gut by the presumed serotonergic inputs and to explore the distribution of other signal molecules that might contribute to sustaining and regulating gut transport function. Previous studies in this laboratory (Clark et al., 1999) revealed that the magnitude of the transepithelial potentials of isolated, perfused

preparations of the larval anterior and posterior stomach decreased substantially within minutes after mounting. This was hypothesized to result from the loss of some tonic neural and/or endocrine inputs that we hoped to be able to identify and add to the isolated preparation. In microelectrode studies of cells of the anterior stomach, the decay of the transepithelial potential was associated with changes in an electrically-distinct subpopulation of epithelial cells called "decaying cells", whereas the membrane potentials of a second subpopulation, the "nondecaying cells", were sustained relatively well in isolation. Serotonin, applied at levels similar to those found in circulating hemolymph, partly restores the gross electrical characteristics of isolated anterior stomach, but the presence of serotonin stimulates the nondecaying cells rather than the decaying cells (Clark et al., 2000). In contrast to these findings from fully isolated, perfused gut, investigators using an *in situ* preparation in which the body wall is opened but the gut and its innervation remain intact, have reported stable transepithelial potentials for up to 6 hours (Boudko et al., 2001) consistent with a persisting, tonic effect of the nervous system on gut ion transport. Together, these results suggest that gut function is controlled and maintained by the cooperative effect of serotonin and one or more additional neurotransmitters or hormones.

For the present studies, antibodies to the peptides FMRFamide and "small cardioactive peptide b" (SCP_B) were chosen because of reports of colocalization of serotonin with these peptides in neurons of several invertebrate taxa (Callaway et al., 1987; Lloyd et al., 1987; Homberg and Hildebrand, 1989; Homberg et al., 1990). Also, FMRFamide has been reported to modulate gut serotonin receptors (Banner and Osborne, 1989), an effect consistent with corelease of FMRFamide from serotonergic terminals. Both of these peptides have been implicated in control of gut motility and feeding in molluscs (Lloyd and Willows, 1988; Willows et al., 1988) and members of the RFamide family are associated with feeding in many other invertebrates as well as vertebrates (Dockray, 2004). Our studies, which were initially directed at larvae, evolved into an examination in all three life stages of the conserved and stage-specific features of the immunoreactivity to serotonin and the two peptides.

The gut of mosquito larvae is a straight tube consisting of (anterior to posterior) pharynx, esophagus, stomach (which is surrounded by six pouch-like gastric caeca at the anterior end), the ileum, and the rectum. The stomach, or midgut, is divisible into anterior and posterior regions. In addition to its role in alimentation, the larval gut plays an important part in fluid and ionic homeostasis, because, unlike most freshwater animals, mosquito larvae are reported to drink their medium (Aly and Dadd, 1989). The proximal gut of *Aedes aegypti* raises the pH of its contents, with values reaching as high as 10 in the anterior stomach. Present evidence suggests that at least the bulk of the alkali secretion occurs in the anterior stomach (Zhuang et al., 1999). This remarkable alkalinization of the proximal gut must be balanced by recovery of alkali in the more distal gut regions, so that the animal remains in acid-base balance.

In pupae, the gut is not used for processing food or fluids because pupae do not eat or drink the medium and are impervious to the medium's content. The pupal gut nevertheless displays regional specializations distinct from those of other life stages. These differences are apparent from the unique pattern of expression of peptide neuromodulators in the pupal gut.

The adult anterior stomach possesses paired dorsal diverticula and a single ventral diverticulum or crop in which nectar meals are stored. The large posterior stomach of *Aedes* females accommodates storage and digestion of blood meals, whereas the stomach of males, which do not consume blood, is much smaller. The transport physiology of the adult gut has not yet been addressed at the cellular or isolated tissue level, but systems must exist to coordinate ingestion with the fluid and solute absorption from the stomach and the activity of the Malpighian tubules. Given the sex-specific demands of processing a blood meal, there are likely to be sexually dimorphic aspects of the regulation.

These studies showed that, in all life stages, the gut of *Ae. aegypti* receives extensive serotonergic input from central neurons that project through axon terminals and varicosities to all gut regions. RF-I is found both within large, branching neurons and in cells with short processes that are distributed in specific regions within the gut. The distribution and abundance of RF-I varies with life stage and the adult sex. SCP-I is located in cells similar to RF-I cells, and the two peptides are localized together in a few regions or identified neurons, but generally are not coexpressed. Regulation of transfer of the gut contents from one region to the next is suggested by the axons concentrated at junctions between different gut regions.

Materials and Methods

Mosquitoes

Eggs of *Ae. aegypti* (Vero Beach strain) supplied by Dr. Marc Klowden, University of Idaho, were hatched in a 50:50 mixture of tap water and deionized water to which a small amount of baker's yeast was added to make the water anoxic. Larvae were reared at 30° C in water of the same composition and were fed ground fish food (Tetramin) every second day. Guts of larvae were studied in the 5th instar (a total of 120 were used), pupal guts were collected within 24 hours of pupation (38 were used), and unfed adults were studied within 24-48 hours of emergence (22 males and 15 females were used).

Whole-mount immunocytochemistry

Freshly-dissected gut tissue or wholemounts of the body with gut exposed were pinned with minuten pins on a Silastic-lined dish and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Fixation was carried out overnight at 4° C or for 2-4 hours at room temperature. After 3 rinses in phosphate buffered saline (PBS: .05 M Na_2HPO_4, 0.137M NaCl, pH. 7.4), the fixed tissue was blocked for a minimum of 3 hours in PBS containing 1% donkey serum + 1% Triton X100.

Primary antibody generated against serotonin in rabbit was purchased from Sigma (www.sigmaaldrich.com); serotonin antibody generated in goat and FMRFamide antibody generated in rabbit were from ImmunoStar (www.immunostar.com), and SCPb antibody generated in mouse was purchased from the University of Washington Friday Harbor Laboratories. The antiFMRFamide used in the present studies is broadly reactive to other members of the RFamide family, including FLRFamide (Brown et al., 1986).

Primary antibodies were diluted 1:100 (Serotonin and FMRFamide) or 1:25 (anti SCPb, as recommended by the supplier)

in antiserum diluent (ASD) containing: 1% w/v bovine serum albumin, 1% v/v Triton X100, and 1% v/v normal donkey serum in PBS. Tissues were treated with the primary antibodies (singly or as a mixture) overnight at 4° C. Antibody treatment was followed by 5 quick rinses in PBS followed by two rinses in ASD over 12-24 hours. Secondary antibodies generated in donkey were as follows: fluorescein isothiocyanate anti-rabbit, CY5 anti-goat, CY3 anti-goat and CY3 anti-mouse. Tissues were incubated overnight at 4° C in ASD containing appropriate secondary antibodies and then rinsed for 12-24 hours.

Controls were as follows: Serotonin antibody was preabsorbed overnight with serotonin BSA conjugate (ImmunoStar). The antibodies against the peptides, diluted in antiserum diluent, were preabsorbed overnight at 4° C with the peptide against which they were generated (peptides from Peninsula, www.bachem.com) and also were preabsorbed against the opposite peptide, i.e., anti FMRFamide was preabsorbed with SCPb and vice versa. The resulting solution was used in lieu of antibody in the procedure described above. No vague or confounding patterns of fluorescence accumulation were observed when the secondary antibodies were used without primaries. Preabsorption with the specific conjugate or peptide removed most or all of the binding affinity whereas tests for cross-reactivity left the characteristic binding pattern of RF-I and SCP-I.

Tissues were mounted in a solution consisting of 8.5 ml glycerol / 1.5 ml PBS to which 0.05g N-propyl gallate was added and stirred to promote incorporation. Fluorescence was examined and photographed using a Leitz Laborlux S with Olympus AD exposure control system for conventional fluorescence microscopy or a Bio-Rad MRC 1024 for confocal microscopy. For the latter, the LaserSharp program was employed for 3D z-series reconstruction from single fluorochrome views (to eliminate cross-detection of fluorochromes), which were merged, if appropriate, in processing.

Results

Larva

No S-I-labeled somata were observed within the alimentary tract. The serotonergic innervation of the larval mosquito gut projects from the brain via four axons that pass along the length of the gut from esophagus to ileum (Figure 1). At the cardiac valve, the junction of esophagus and gastric caeca, the axons give off dense arborizations that surround the distal esophagus. The axons extend into the posterior stomach, where they branch to cover the gut with a network of fine processes beaded with varicosities that suggest synaptic release sites.

The cardiac valve is encircled by a ring of peptidergic cells characterized by multiple short processes that give them a stellate shape somewhat reminiscent of vertebrate enteric autonomic neurons (Figure 2A). These "necklace" cells (a term first used by Brown and Cao 2001) express either RF-I or SCP-I and a subpopulation expresses both. The necklace cells appear to be contacted by projections from paired clusters of 3 RF-I neurons located anterior to the necklace (Figure 2B). These RF-I somata appear to be located in the hypocerebral ganglion of the stomatogastric nervous system. Their axons innervate the caeca and some project along the esophagus to the anterior stomach.

Enteroendocrine cells with an elongated spindle shape that exhibit RF-I, SCP-I, or both, are scattered throughout the larval caeca. Similar cells are present in the larval stomach. In the anterior stomach, SCP-I cells predominate (not shown), whereas in the proximal part of the posterior stomach, RF-I cells predominate (Figure 3A and B). The degree of possible colocalization of these two markers was not determined.

At the distal end of the larval stomach, near where the Malpighian tubules join the gut, a ring of axon-like processes that express SCP-I, but neither RF-I nor S-I, is found (Figure 4). The processes that make up this pyloric ring have varicosities that may be transmitter release sites. The cell bodies that give rise to these processes were not detected within the gut.

Results from the studies on larvae are summarized in Figure 5.

Pupa

Pupae examined within 24 hours of pupation exhibit a stage-specific gut morphology in which only remnants or no evidence of the larval stage gastric caeca are seen and the adult stage gastric diverticula are also absent. Often, the smooth and compact gut

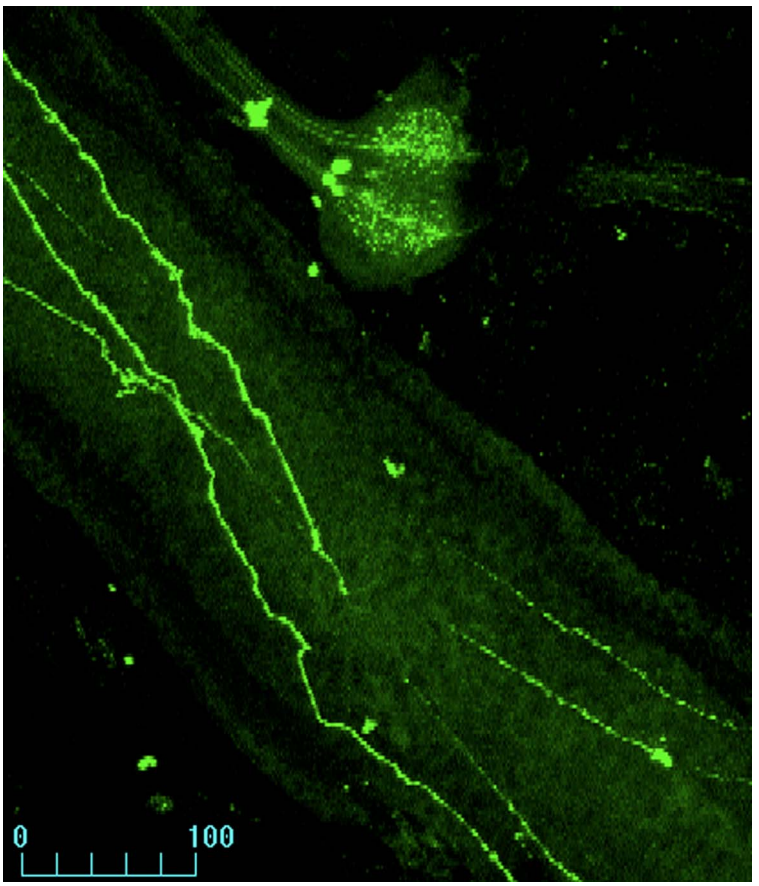

Figure 1. Anterior stomach with 2 pairs of descending S-I axons superimposed in the confocal image; 1st abdominal ganglion visible above (anterior is lower right).

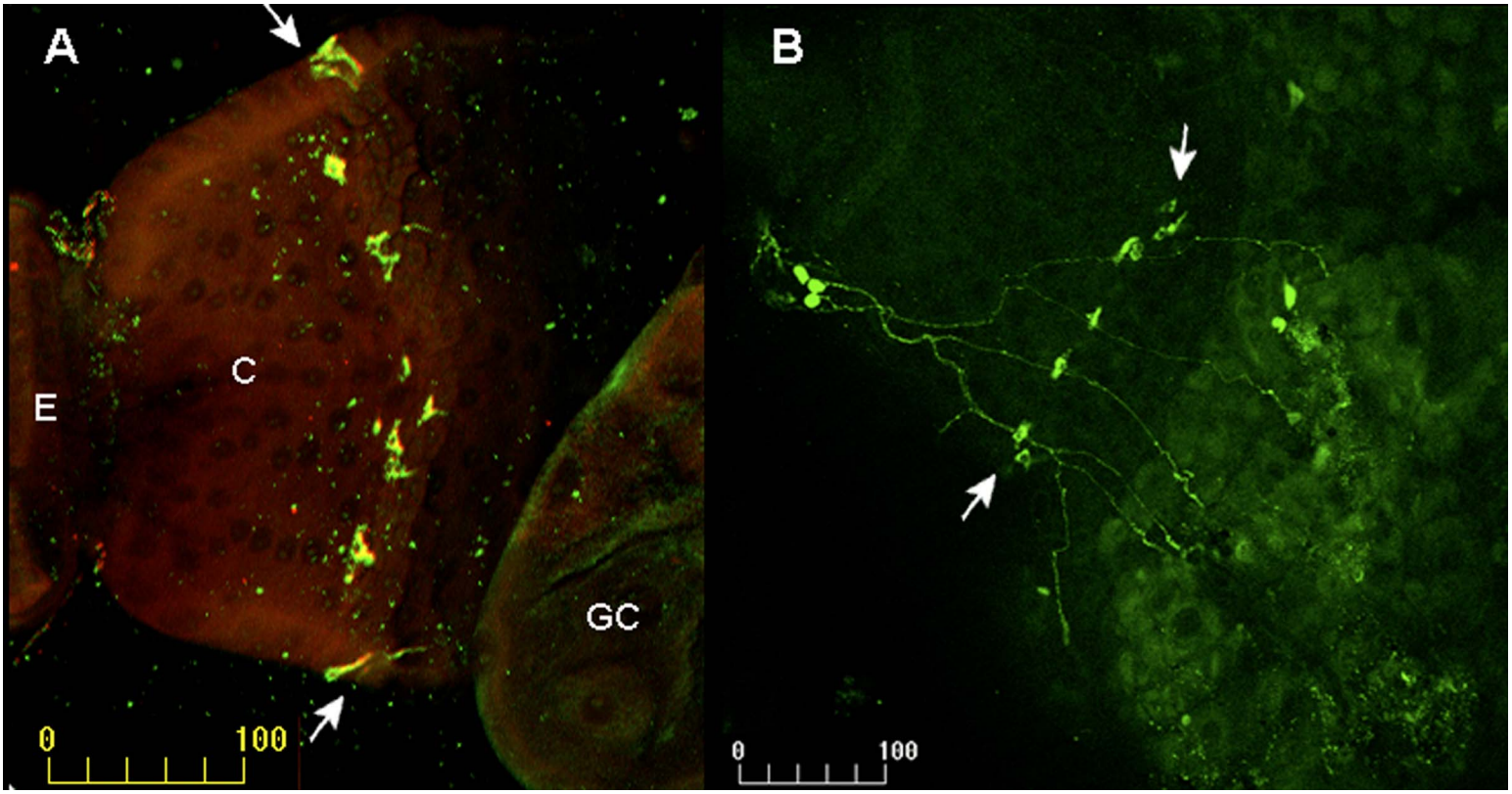

Figure 2. Necklace cells. A Esophagus region (E, left) with cardia (C, middle), showing necklace cells (between arrows); portion of caeca (GC, lower right). The predominantly yellow color of the necklace cells indicates labeling by both RF-I (green) and SCP-I (red). B: RF-I (green) image in plane of axons from one of the two clusters of 3 neurons on the esophagus (upper left) that pass by and possibly make *en passant* connections with necklace cells (between arrows) in route to the caeca (right).

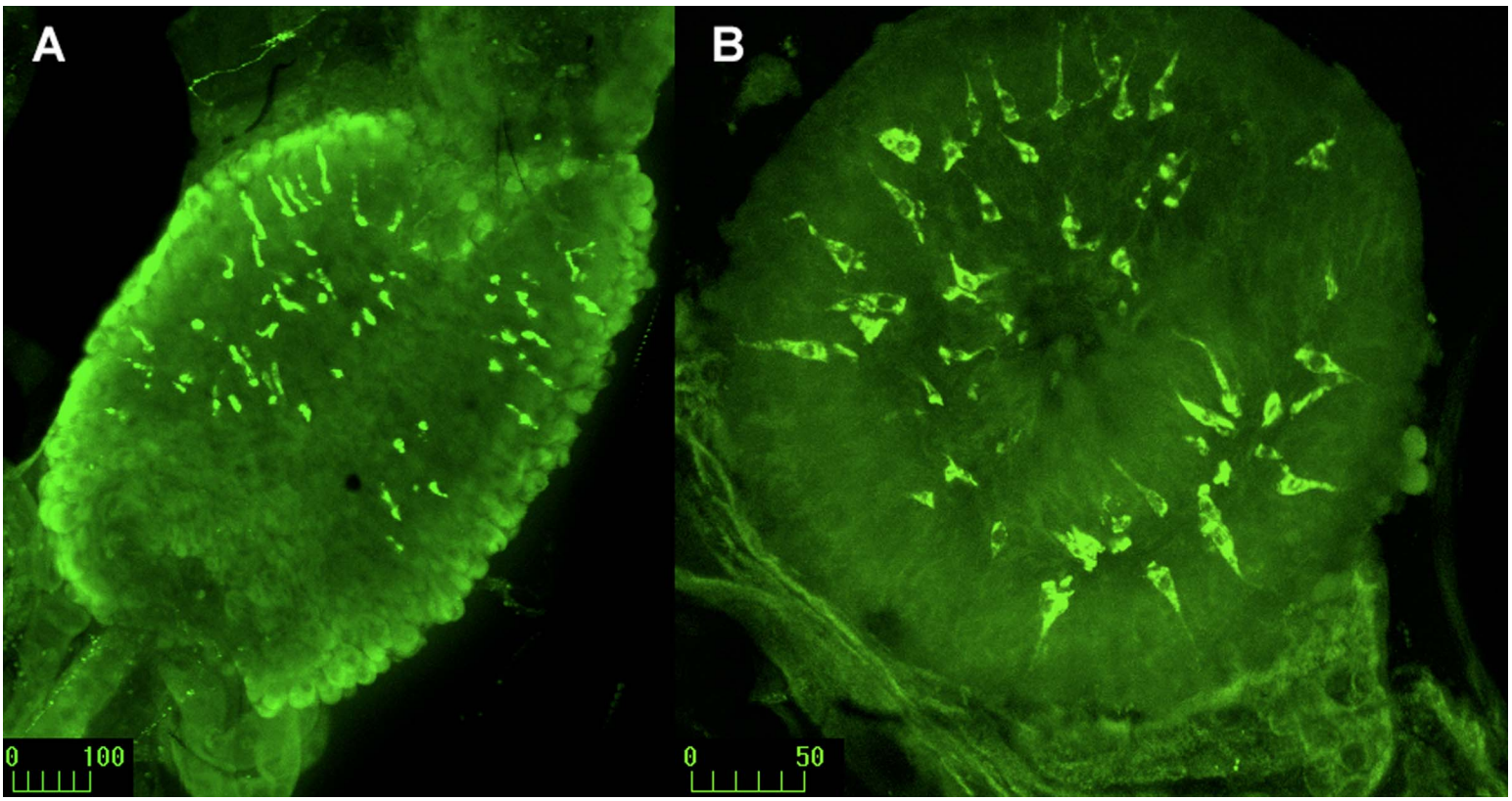

Figure 3. RF-I A. Whole mount of part of anterior stomach, at top, and posterior stomach. B. Posterior stomach enteroendocrine cell showing orientation in tissue: end-on view of posterior stomach segment which has curled outwards; narrow necks of cells always point towards luminal surface.

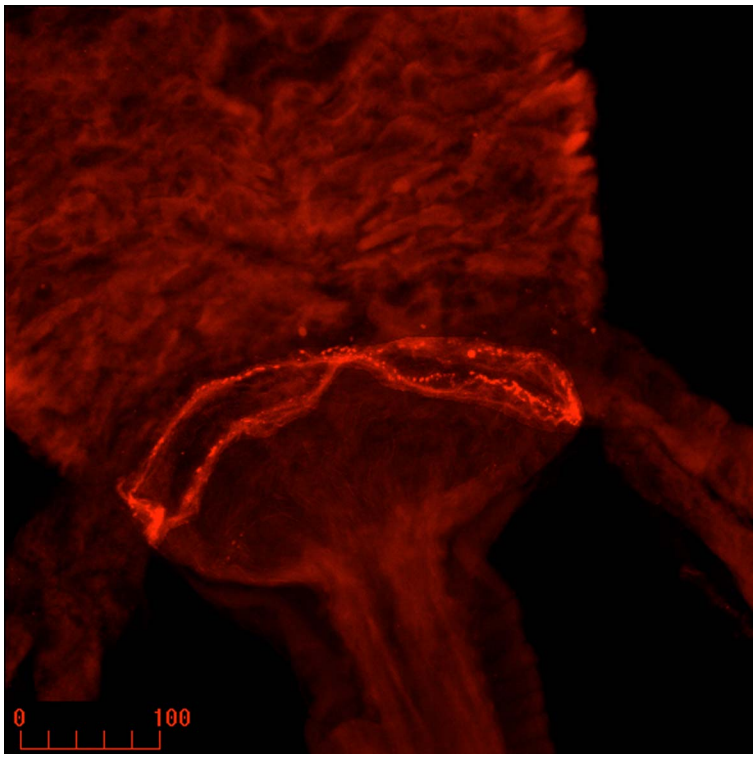

Figure 4. Pyloric ring of larva labeled with SCP-I; posterior stomach is visible above the ring and ileum below it.

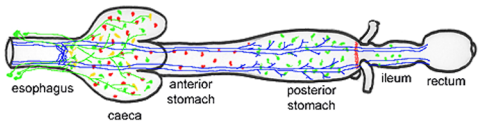

Figure 5. Summary of results from larvae. Blue: S-I, Red: SCP-I, Green: RF-I; Yellow: colocalized RF-I and SCP-I.

encloses pigmented material derived from tissue remnants of the larval gut; during the pupal stage this material gradually disappears (Christophers 1960). So, despite the fact that the pupae do not feed or drink, the gut plays a digestive role and its functional morphology includes a distinctive endocrine and neural presence.

As in larvae, pupae display an S-I labeled network of axons that form prominent arborizations at the head of the anterior stomach; thus this feature appears to persist, either through retraction and regrowth (for which we saw no evidence) or by maintaining its presence even though the stomach itself has undergone extensive remodeling. As in larvae, the axons extend from this nexus and course along the anterior and posterior stomach, branching profusely. A distinctive feature of pupae is that the S-I axons also enter the pyloric ring, where they appear to form synaptic projections (Figure 6). In contrast to the situation in both larvae and adults, neither RF-I nor SCP-I are apparent in the pyloric region and the expanded S-I localization was the only evidence of neural control that we detected in this region.

A prominent feature of the pupal stage is a pair of large neuron somata bilaterally positioned at the posterior margin of the esophagus. These neurons colocalize SCP-I and RF-I (Figure 7A, B). The two neurons arborize anteriorly as well as across the midline (Figure 8). The main axons descend into the stomach and branch extensively in the posterior stomach (Figure 9), projecting into much of the same area served by the S-I axons.

In the pupal gut, a scattering of SCP-I enteroendocrine cells are seen mainly in the anterior half of the posterior stomach. These cells are indicated on the summary drawing for pupae, Figure 10.

Adult

The gut of adults is characterized by the presence of two small dorsal diverticula and a larger ventral diverticulum or crop. In adults of both sexes, as in larvae and pupae, 2 pairs of S-I axons form a prominent nexus at the head of the anterior stomach and richly innervate the slender anterior stomach, the diverticula, and the posterior stomach (Figure 11). These same S-I axons project along the ileum and rectum, where a dense pattern of beaded varicosities is apparent at the ileo-rectal junction and around the anus (Figure 12).

As in pupae, the anterior stomach of adults is innervated

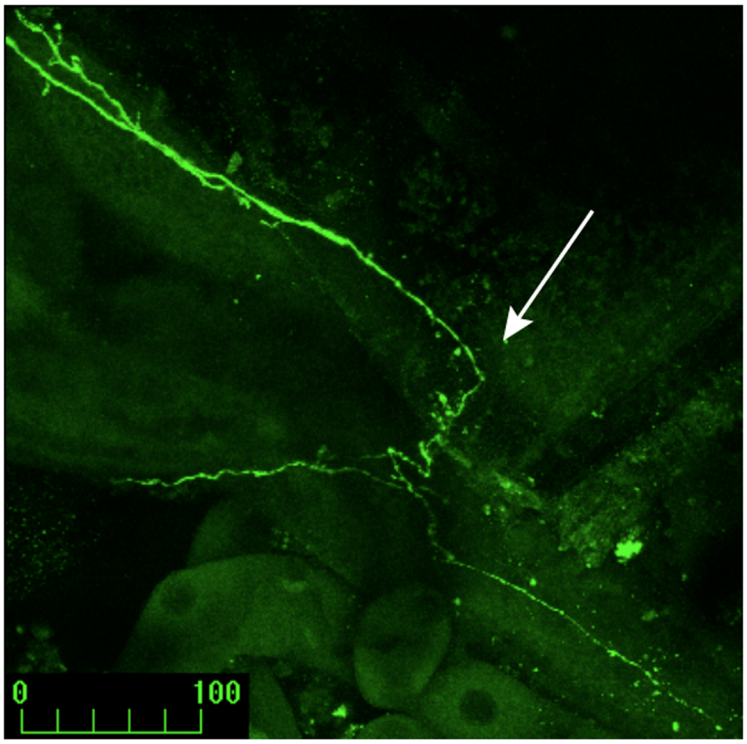

Figure 6. Descending (upper left toward lower right) S-I axons in pupae pass along stomach, connect with the pyloric ring (arrow) and continue along the ileum. Large Malpighian tubule cells are visible below gut.

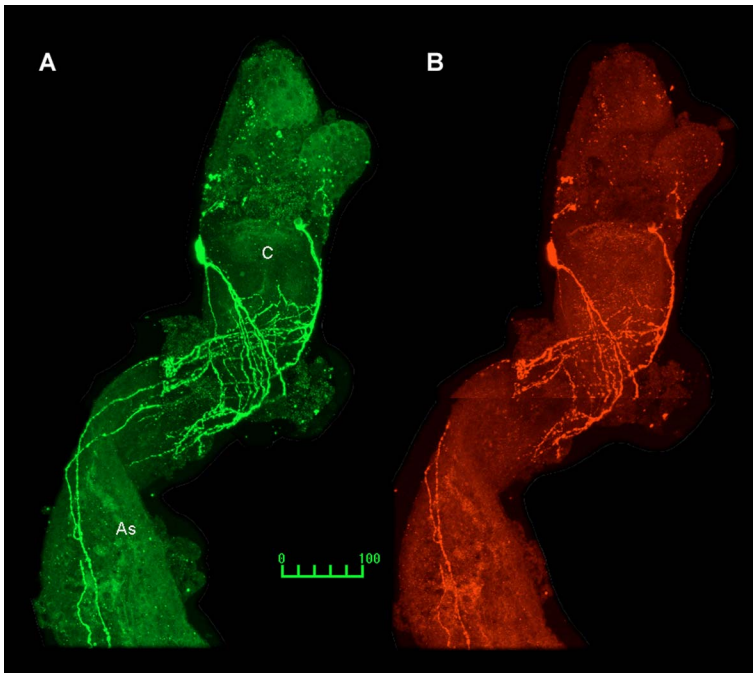

Figure 7. Same views of pupal esophagus and stomach with RF-I (A) and SCP-I (B) showing colocalization of the markers in the two neurons innervating the proximal gut. C, cardial region posterior to disintegrating caeca; As, anterior stomach.

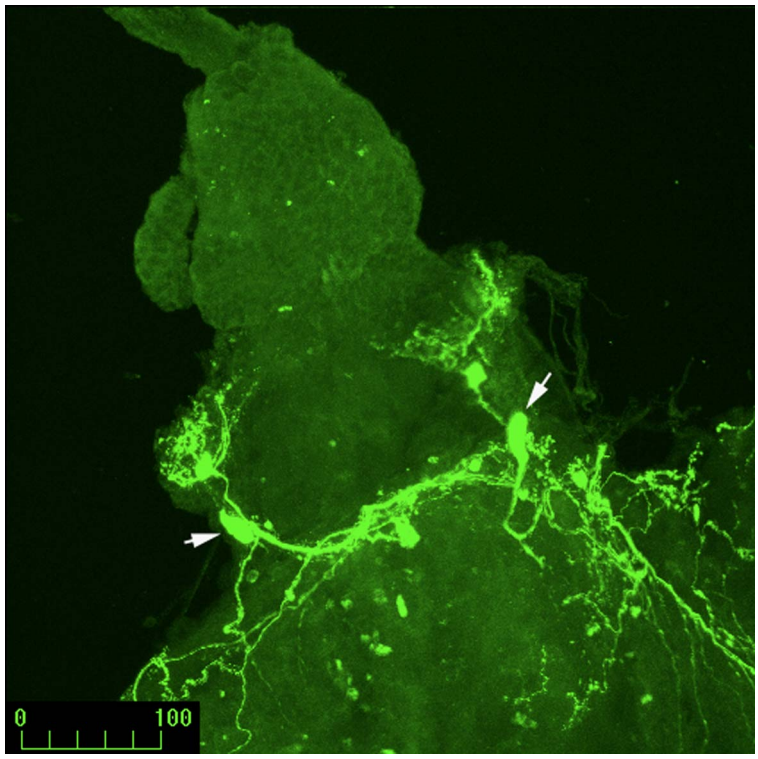

Figure 8. Details of the anterior projections and trans-midline branching pattern of the pupal RF-I/SCP-I neurons. Arrows indicate neuron somata. In this case, the neurons are visualized by their RF-I.

by RF-I axons that parallel the S-I axons, and the location and appearance of the 2 large neuronal cell bodies is similar in pupae and adults (Figure 13A). This system innervates the anterior stomach and diverticula most extensively (Figure 13B), with only minor ramification in the posterior stomach. The similarity of these neurons to those that appear in pupae is striking, but we did not determine that these cells colocalize SCP-I, although SCP-I axons detected in tissue examined with only that marker may have originated from these cells (see Fig. 16). We also found that abdominal segmental ganglia may be a source of some SCP-I axonal projections to the gut. Each ganglion contains a single prominent SCP-I neuron soma located on the posterior midline of the ganglion. This neuron ramifies extensively within the neuropil of the same ganglion, but also projects across and may innervate the gut (Figure 14A, B).

Enteroendocrine cells showing RF-I are found in the posterior stomach of both sexes (Figure 15). The total number of such cells is approximately 100 in males and perhaps 500 in females. The numerical difference reflects both the fact that the empty posterior stomach of females is more than twice the size of the male stomach (Figure 15A, B) and the fact that the density and size of cells identified by RF-I is greater in females. SCP-I labeled enteroendocrine cells are present mainly in the anterior two-thirds of the posterior stomach (Figure 16), appearing at about half the density of the RF-I labeled cells. Few enteroendocrine cells of either type are found in the anterior stomach of either sex, although the diverticula contain SCP-I cells.

In adults of both sexes, the pyloric ring is encircled by both RF-I (Figure 17A, B) and SCP-I (Figure 16, arrow). We did

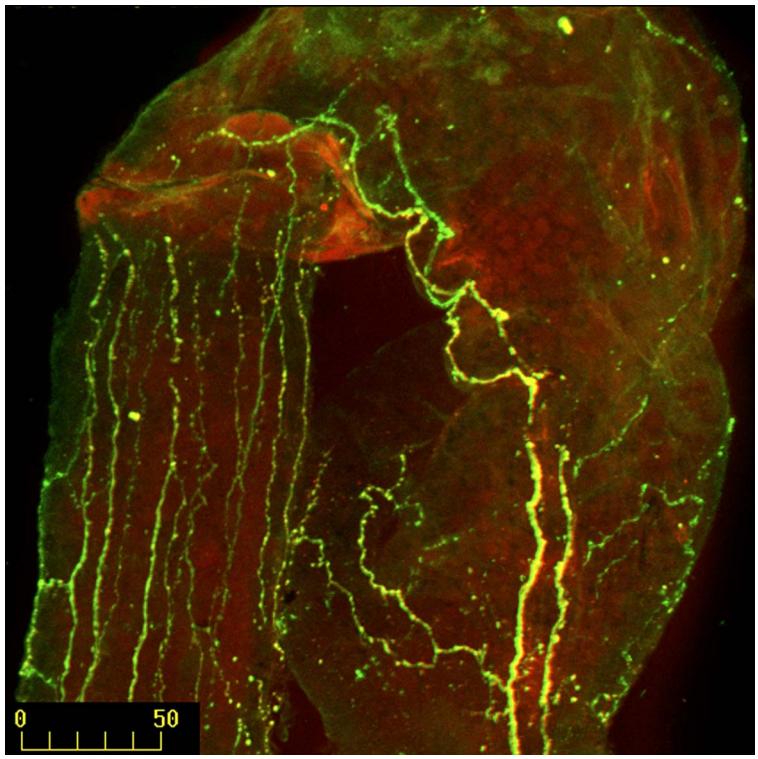

Figure 9. Pupal stomach bent into loop, with anterior on right and posterior stomach on left. Major axons branch into many parallel projections at junction between anterior and posterior stomach. Yellow fluorescence of axons indicates colocalization of RF-I and SCP-I.

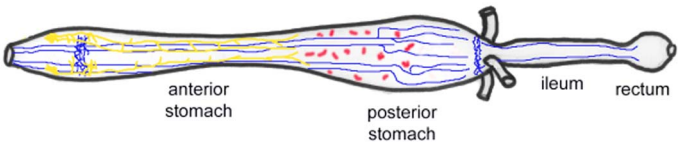

Figure 10. Summary of results on pupae. Blue: S-I; Yellow: colocalization of RF-I and SCP-I; red: SCP-I.

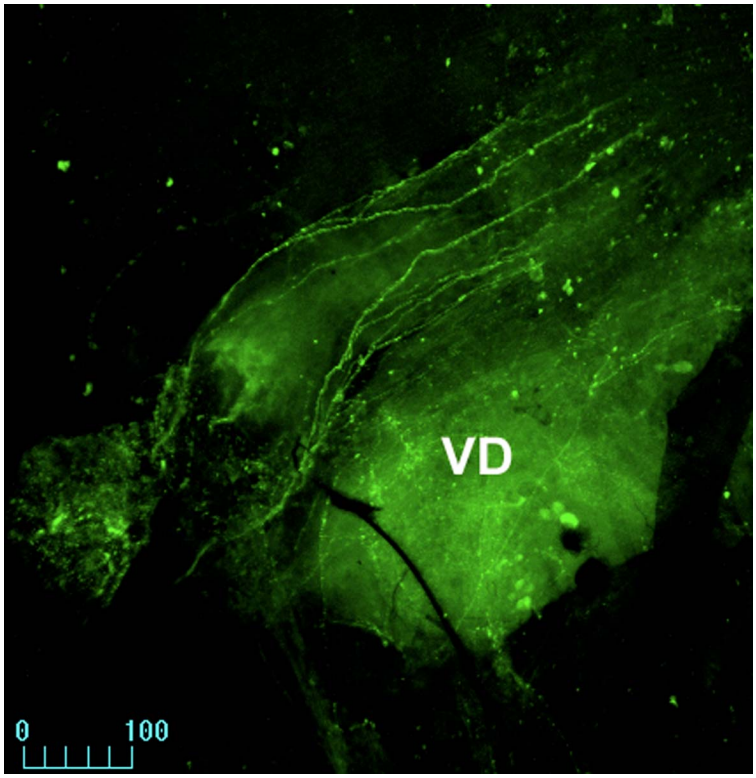

Figure 11. Axons (with S-I fluorescence) on adult anterior stomach above ventral diverticulum (VD).

not determine whether the labeling with the two peptide antibodies colocalizes at this site. This innervation pattern for the pyloric ring differs from the region's ring of SCP-I in larvae and S-I in pupae. As in larvae and pupae, the neurons giving rise to the axons in the pyloric ring were not visible in the gut itself, but several RF-I positive axons that course along the surface of the ileum join the ring (arrows, Figure 17B). These axons could originate from cell bodies located in one or more of the ganglia posterior to the pylorus, since each of these ganglia has several large RF-I cell bodies.

Results from adults are summarized in Figure 18.

Discussion

This study provides insight into how the gut regulatory systems change to meet the differing needs of the larva, pupa and adult mosquito. There is evidence from *Drosophila melanogaster* that FMRFamide-like immunoreactive neurons both persist and retain their immunoreactivity through metamorphosis (White et al. 1986), although the survival of peripheral neuroendocrine cells is less certain. In mosquitoes, the differentiated epithelia of the larval gut is undermined by a layer of regenerative cells during the larval-pupal molt, leading to their detachment from the basement membrane of the gut, and the larval epithelial cells subsequently deteriorate in the pupal gut lumen (Clements 1992). This material possibly provides nutrients that fuel the transition to the adult state, and to the extent that the gut functions at all, it would require neural and endocrine coordination, even though the pupal stage is technically a non-feeding stage. Presumably the larval enteroendocrine cells share the fate of the surrounding epithelial cells, so the enteroendocrine cells that we observed in pupae most likely represent a new population that arises from regenerative cells, and that pattern is repeated as the pupal gut is refashioned into the adult tract.

The accessory structures associated with the gut in the three life stages are specialized for each stage and are discarded and new structures created as the gut is transformed from a structure suited to processing a continuous input of detritus to the adult stage feeding on intermittent fluid meals. The cardia and caeca of the larva are lost during the early pupal transition, and the diverticula arise in the transition to adult (Christophers 1960). We found that the necklace cells encircling the cardia are gone by the pupal stage, along with the pattern of neurons that project across them toward the caeca. The set of neurons that appears in the pupal stage may both play a role in the pupal gut, considering the extensive ramifications of the axons in the pupae, and also persist through the pupal-adult transition to serve a significant role in adult gut function.

Towards the other end of the gut, the Malpighian tubules are retained from larva through pupa to adult, and axonal

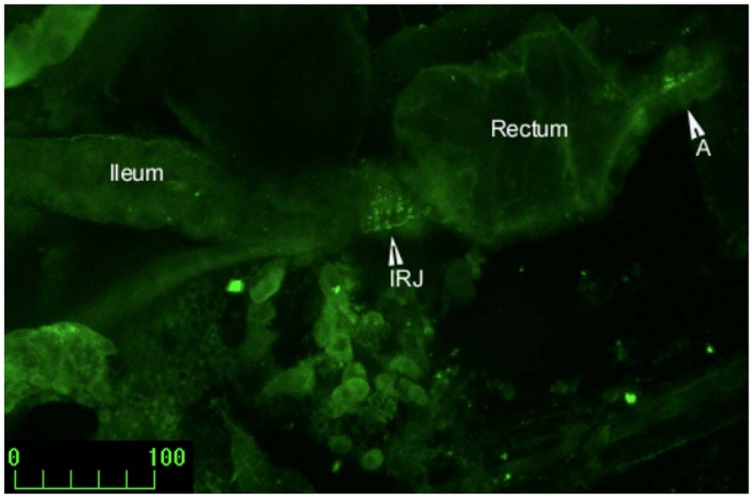

Figure 12. Adult Ileum (left) and rectum (right) with apparent S-I terminals at ileo-rectal junction (IRJ) and anus (A, arrowhead).

concentration at the pyloric valve is a feature of all three stages. Although this region would appear to be relatively conserved in the three stages, its functional transitions are marked by the disappearance of the SCP-I characteristic of the larval ring, the concentration of S-I in the pupal ring, and a significant representation of peptidergic axons (SCP-I and RF-I) in the adult ring. Other peptides have been identified with the regulation of this region, since Brown and Cao (2001) found that in *Anopheles gambiae*, but not in *Ae. aegypti*, at least some pyloric ring axons of both larvae and adults were immunopositive for ovarian ecdysteroidogenic hormone I. The same marker was found in the necklace cells of *An gambiae* larvae. In adult females, ecdysteroidogenic hormone I is released into the hemolymph after a blood meal and serves to stimulate ecdysteroid synthesis by the ovaries, but its functions in larvae are unknown.

In all three life stages of *Ae. aegypti*, all parts of the mosquito gut are innervated by S-I projections from centrally located neurons, suggesting that serotonin has as pervasive a role in control of the mosquito gut as acetylcholine does in the vertebrate gut. The constancy of serotonergic innervation was striking, considering the many changes that the peptide markers revealed for both neurons and enteroendocrine cells. Although we did not confirm that the same central serotonergic neurons persist through each stage, it is the most likely explanation. Clements (1992) describes a framework of tissues that that could support the persisting axons while the gut is remolded during metamorphosis. Although there are significant differences in the details of the serotonergic projections in each life stage, we discovered no sexual dimorphism in the serotonergic innervation of the stomach or diverticula to add to the differences in the salivary gland serotonergic innervation of females and males described by Novak et al. (1995).

Since the serotonergic innervation of the entire gut derives from a small number of central neurons, it is likely that this system facilitates generalized, state-specific control rather than regulating temporally distinct activities or sequential functions. Serotonin has many potential effects on gut function. The levels normally found in circulating hemolymph have been shown to be of sufficient magnitude to affect the transepithelial potentials of anterior and posterior stomach, and the levels change in response to salinity acclimatization (Clark and Bradley 1997; Clark et al. 1999). Isolated gut preparations lose their initial transepithelial potential rapidly after mounting, but part of this can be restored by bath application of serotonin. Our view of the extensive direct serotonergic innervation of the gut is that these axons are the source of the primary serotonergic regulation of gut function, both directly within the tissue and as a blood-borne messenger, although neurons in the

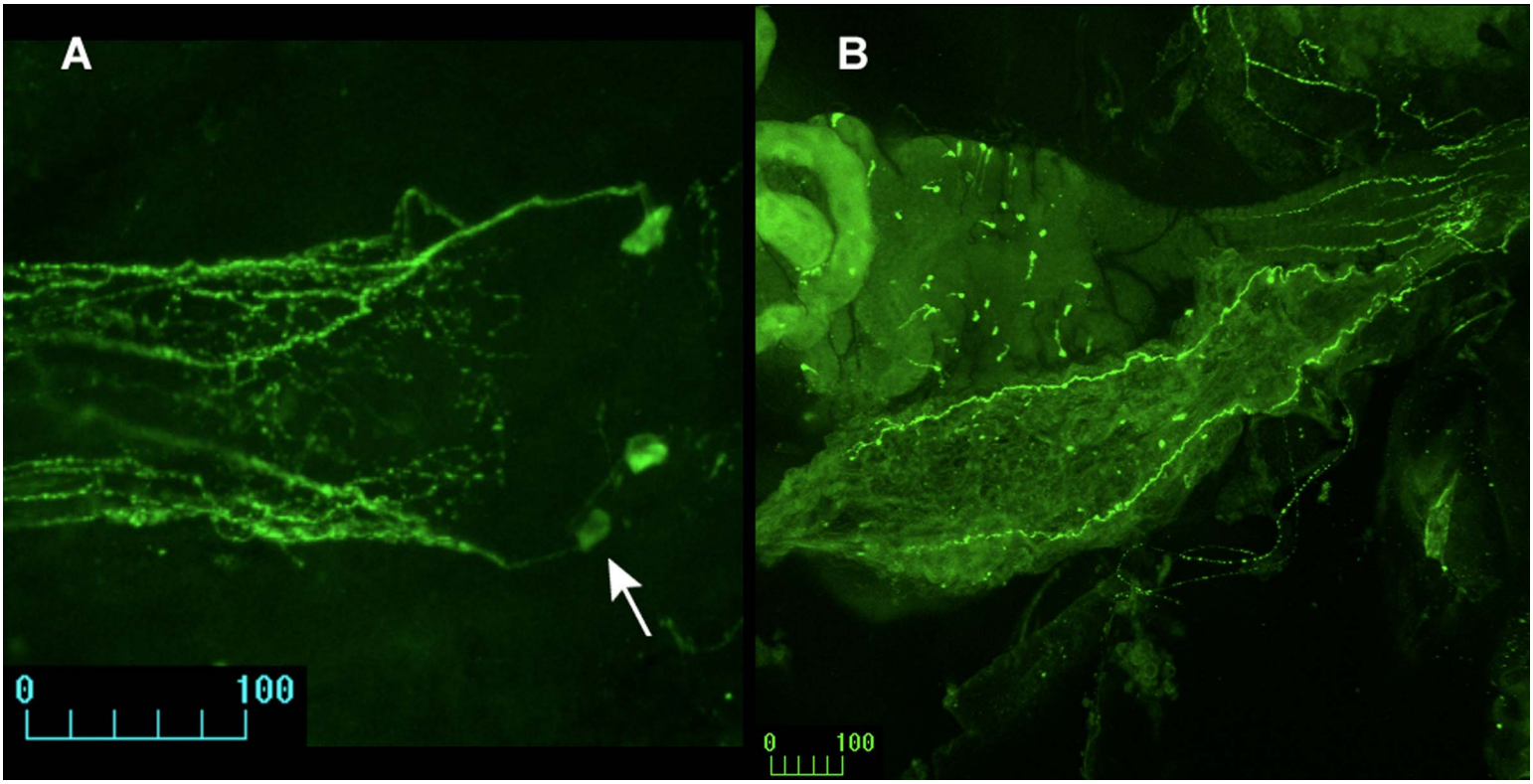

Figure 13. RF-I A. Neurons located on the esophagus of an adult male; typically two somata are present but in this case there is an additional, more faintly marked soma (arrow). Anterior is to the right. B. RF-I axons branch posterior to the esophagus to innervate the anterior stomach and outline the ventral diverticulum of an adult female. Anterior is to the right. Scattered FR-I enteroendocrine cells are seen in the posterior stomach (upper middle); Malpighian tubules join the gut at the left and part of dorsal diverticulum is visible to upper right of stomach.

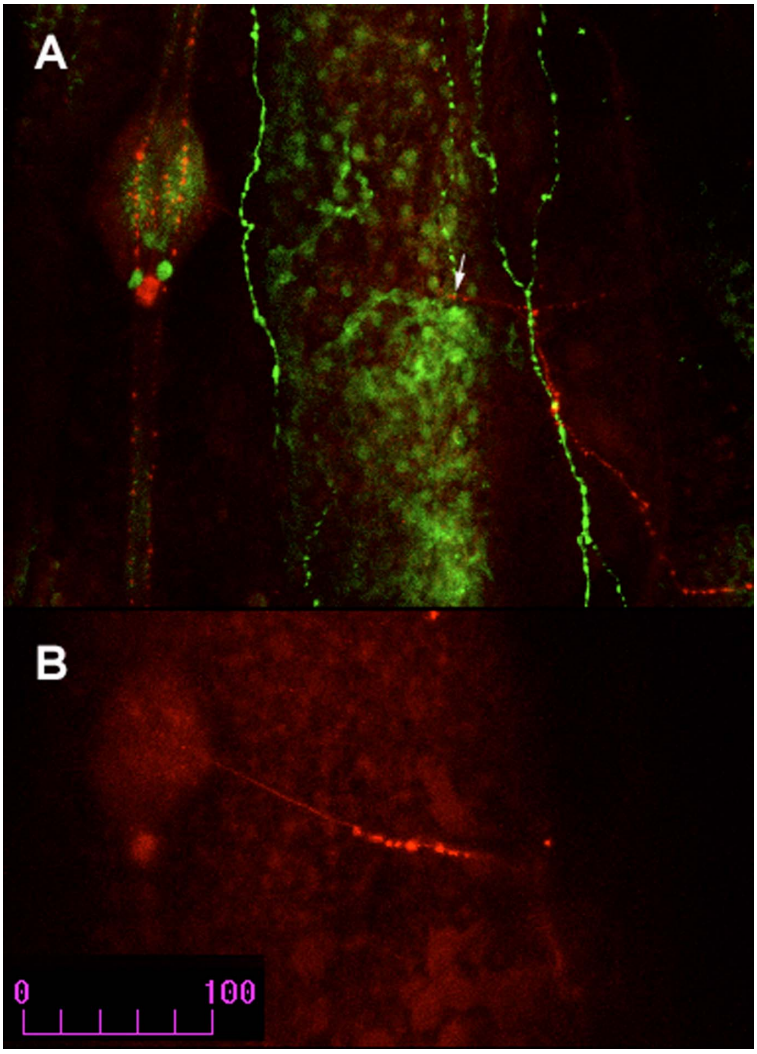

Figure 14. S-I (green) and SCP-I (red) neurons in abdominal ganglion at left of gut. The S-I neurons in the ganglia did not appear to project to the gut, which is only innervated by the bilateral pair of S-I axons from the CNS; the SCP-I axon running across the gut (arrow) could be traced to the ganglion as shown in B, the same view showing only the SCP-I and with the focus on the gut surface.

segmental ganglia are also able to contribute to the level of serotonin in the blood.

Like serotonin, RFamides and SCPb are implicated in control of gut contraction and control of feeding (Lange and Orchard 1998; Dockray 2004). In contrast to the pervasive presence of serotonin throughout the gut, the diversity of the peptide innervation of the different regions of the gut seen in this and other studies (Stracker et al. 2002; Veenstra et al. 1995; Stanek et al. 2002) suggests that differential control probably resides in these systems. For the larval stomach, the present results are consistent with a local role for RFamides, possibly to oppose or modulate an excitatory effect of serotonin (Peef et al. 1993; Lange and Orchard 1998). The paired clusters of three neurons that project to the necklace cells in larvae and the RF-I/SCP-I neurons that appear in the pupae and probably survive in the adults may act directly on muscle, secretory or transport target cells or may interact with neurons or enteroendocrine cells to influence regional gut function indirectly.

Although application of the peptides FMRFamide and SCPb did not have a consistent effect on anterior stomach motility (unpublished) and Clark (2002 personal communication) found that the commercially available forms of FMRFamide and SCPb are

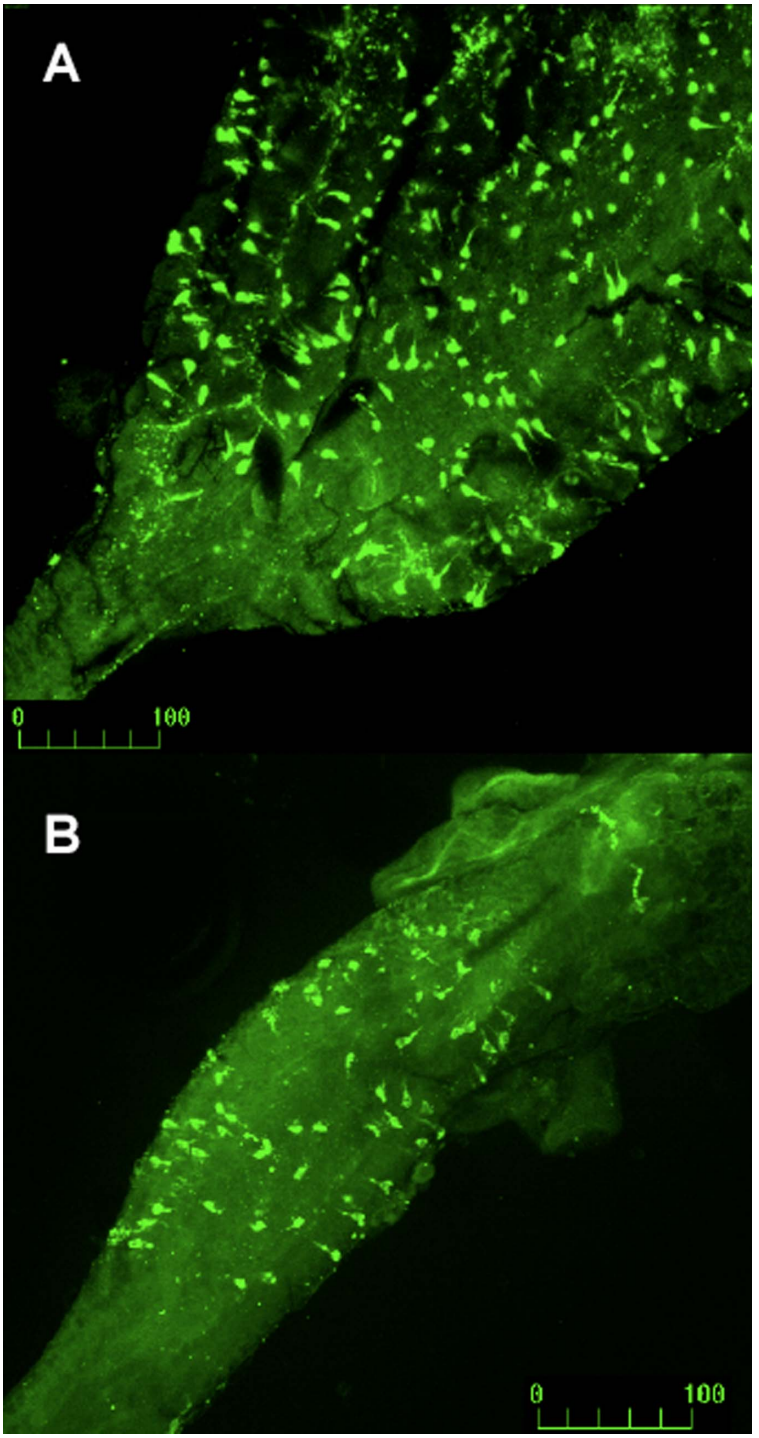

Figure 15. A: RF-I enteroendocrine cells in adult female posterior stomach; anterior stomach is at lower left. B: corresponding view of RF-I cells in stomach of male; anterior end is at lower left. RF-I in pyloric ring is also visible encircling the gut at upper right.

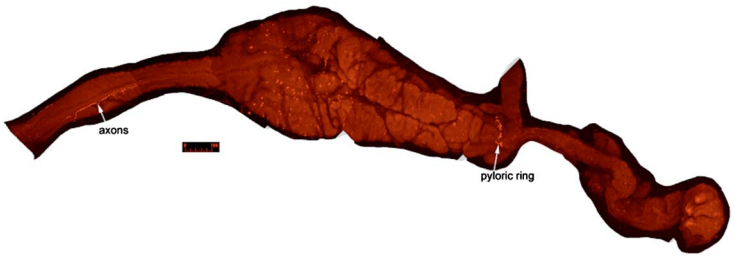

Figure 16. Montage of gut of female adult. SCP-I labeling appears in axons and pyloric ring (arrow) as well as enteroendocrine cells of posterior stomach. A concentration is also visible in the rectum (right).

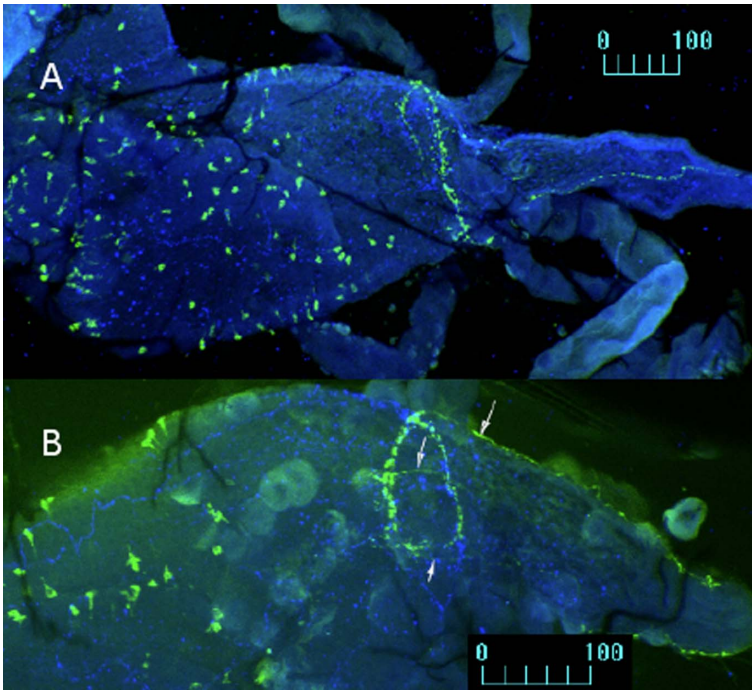

Figure 17. RF-I (green) and S-I (blue) in posterior stomach and ileum of female and male adults: A: female; ileum is to right; two RF-I axons (arrows) are visible coursing along ileum towards pyloric ring. B: male; ileum is to right and posterior stomach to left; network of S-I axons are visible in posterior stomach; three RF-I axonal connections to the ring are indicated by arrows.

without effect on the transepithelial potentials of anterior and posterior midgut, other peptides are effective. Our group has found that application of some of the *Ae. aegypti* peptides to anterior stomach preparations antagonize the effects of serotonin, reducing the transepithelial potential and inhibiting the pattern of spontaneous contractions (Onken et al. 2004b). For instance, all the allatostatins, neuropeptide F and proctolin reduced the transepithelial voltage and the head peptides I and III and short neuropeptide F and neuropeptide F reduced the peristaltic movements. Of these, the head peptides and 2 neuropeptide F's fall into the RFamide peptide family, and were probably visualized by the antibody that we used. Although we could not find a sequence homology for SCPb in the *An.gambiae* genome, the genome includes an extensive list of peptides, many of which may be present in the gut (Riehle et al., 2002), and, as Riehle points out, the smaller the peptide, the less likely that it can be identified by such an approach.

We used the term enteroendocrine cells for those cells that lie within or on the gut epithelium and lack obvious axons. However, the shapes of these cells are diverse and could reflect a heterogeneous population, including some purely endocrine cells and sensory or nerve-net cells with small-diameter axons. RFamides and SCPb-like peptides secreted by enteroendocrine cells might operate at the paracrine level, regulating digestive functions of the immediately adjacent epithelium and muscule bands, and also might pass into the hemolymph as hormonal signals feeding back to the CNS, other gut regions, or the fat body, carrying information about dietary volume and composition. Particularly intriguing are the cells that have thin processes projecting to the gut lumen, as shown in Figure 3B. These might represent either release sites for a modulator acting through the peritrophic space, or the projection might serve a sensory function that regulated peptide release from the other end of the cell.

Classical estimates of the total number of such endocrine cells in the stomach of adult females are of the order of 500 (Clements 1992); if this estimate is accurate, cells identified by our markers for RF-I and SCP-I clearly account for the great majority of the population. How the cells marked by these two antibodies compare with the similar number estimated from the four categories of peptide markers described by Veenstra (1999) remains to be determined, although those in the RFamide family may all have been labeled by our broadly reactive antibody. Stracker et al. (2002) report that hundreds of neurosecretory cells in the midgut of *Ae. aegypti*

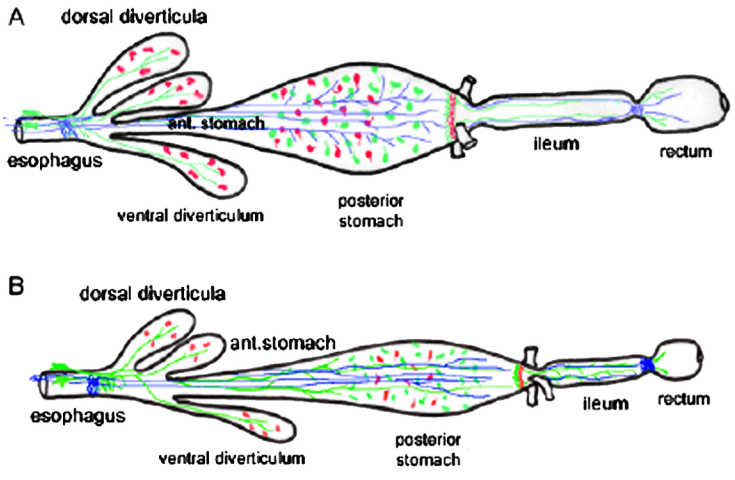

Figure 18. Summary of results from females (A) and males (B). View is from side. blue: S-I; green: RF-I; red: SCP-I.

adult females were labeled by antibodies reactive with the Aea-HP-I, a hormone known to inhibit host-seeking behavior. Counts are difficult to reconcile, because the cell populations identified by individual peptide antibodies often represent overlapping subsets of the total number of cells present in a tissue, due to expression of more than one of the peptides.

The SCPb family is not so widespread as the RFamide family, but has been found in insects and crustaceans as well as mollusks (Langworthy et al. 1997; Homberg et al. 1990). The sequence of SCPb, Met-Asn-Tyr-Leu-Ala-Phe-Pro-Arg-Met-NH2 (Morris et al. 1982), suggests that this peptide is not a member of the RFamide family, but it could conceivably be recognized by some portion of the population of polyclonal antibodies against FMRFamide or the monoclonal antibodies generated against SCPb might recognize some RFamide-like molecules. Our localization pattern argues for distinct localization phenomena for the two antibodies we used, although we do not know the range of peptides that each antibody may bind. In some studies in crustaceans and insects (Arbiser and Beltz 1991; Callaway et al. 1987; Homberg and Hildebrand 1989; Homberg *et al.* 1990, 1991), SCP-I and RF-I were found to colocalize after preabsorption of antibodies, and this was interpreted as evidence that they were both expressed in those cells. Although there is low crossreactivity between FMRFamide and SCPb antibodies in ELISAs, such crossreactivity is not expected when immunocytochemical approaches are used (Miao et al. 1998).

We found colocalization consistently in a small subpopulation of the peptidergic cells, including the larval necklace cells surrounding the cardial region of the esophagus, the paired central neurons of the pupae that innervate the gut, and in some enteroendocrine-like cells of the gastric caeca of larvae. When such colocalization is clear in some cells and absent in adjacent cells, the colocalization is unlikely to be the result of cross-reacting antibodies, although the possibility exists that those cells express a distinct peptide to which both antibodies bind. Additional evidence for specificity of the detection system was seen in the thoracic and abdominal ganglia, in which the neurons we classed as RF-I and SCP-I are distinctly labeled.

Although neither of the specific peptides used to generate the antibodies we employed in this study is likely to be present in *Ae. aegypti*, these markers for broad peptide families, together with the description of serotonergic and peptidergic neurons, have given an overview of the changing distribution of identifiable control elements in the gut of *Ae. aegypti* in its life transitions between different habitats. More comprehensive mapping of natural peptide distributions, already available for some peptides in larvae and adults, as noted above, can be combined with physiological testing of roles played by the neural and hormonal modulation of the gut at each stage. The importance of mosquitoes as vectors of disease makes understanding of vulnerable systems, including the neural, endocrine and digestive systems, an important goal for public health.

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