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Journal

ISSN

Authors

Shelomi, Matan
Kimsey, Lynn S

Publication Date

DOI

Peer reviewed

Vital Staining of the Stick Insect Digestive System Identifies Appendices of the Midgut as Novel System of Excretion

Matan Shelomi* and Lynn S. Kimsey

Bohart Museum of Entomology, Department of Entomology and Nematology, University of California, Davis, California

ABSTRACT The stick insects or phasmids (Phamsatodea) have a series of pyriform ampulles with long, thin filaments on the posterior end of their midgut referred to as the "appendices of the midgut." Found only in phasmids, their function had never been determined until now. Their similarity to the Malpighian tubules, which are ubiquitous insect organs of excretion, suggested a similar function. To differentiate between the appendices and the Malpighian tubules and compare functional differences between the two tissue types, vital staining (the injection of histological stains into living organisms) was done in conjunction with light and scanning electron microscopy in multiple phasmid species. The results showed that the appendices originated in the basal phasmids (Timematidae) and grew more numerous in derived species. The appendices stain selectively, notably failing to pick up the indicators of the two known systems of invertebrate excretory function, indigo carmine and ammonium carmine. Appendices sequester stains in the ampule portion before eliminating the compounds into the midgut. We conclude by confirming that the appendices do have an excretory function, but one unlike any other known in invertebrates. Their function is likely cation excretion, playing a role in calcium regulation and/or organic alkaloid sequestration. The appendices must thus be considered distinct organs from the Malpighian tubules. J. Morphol. 275:623–633, 2014. © 2013 Wiley Periodicals, Inc.

KEY WORDS: histochemistry; excretion; digestion; insects; phasmatodea

INTRODUCTION

The stick and leaf insects (Phasmatodea), or phasmids, are an enigmatic order of leaf-chewing insects characterized by their long and thin body plan, which places size and shape constraints on their digestive system. Past anatomical studies of the phasmids (Bordas, 1897; de Sinéty, 1901; Cameron, 1912; Clark, 1976) reveal their gut is straight and narrow with no obvious enlargements or diverticulae. Although the foregut and hindgut are typical, the midgut is modified in ways unlike those of the phasmids' nearest relatives, hypothesized by various authors (Flook and Rowell, 1998; Plazzi et al. 2011) to be either the Orthoptera (grasshoppers and crickets), Embioptera (webspinners), or Notoptera (ice-crawlers and heel-walkers, formerly Grylloblattidae and Mantophasmatodea, respectively). The phasmid midgut is divided into two sections based on morphology: the anterior third to half of the midgut is heavily pleated and folded, whereas the posterior third of the midgut is studded with a variable number of ampules that terminate with long, thin filaments that run toward the posterior of the insect, referred to as the "appendices of the midgut" and not found in any of the proposed Phasmid sister orders (Lacombe, 1971; Nation, 1983; Klass et al. 2002). Although most authors describe the midgut as having two parts (Cameron, 1912; Chopard, 1949; Clark, 1976), undifferentiated regions between the pleated and appendices-containing sections and between the latter and the hindgut are occasionally counted as distinct midgut sections for four sections total (Holtmann and Dorn, 2009). At the junction between the midgut and hindgut, as in most other insects, are over one hundred Malpighian tubules, which are the primary organs of excretion.

The functionality and anatomy of phasmid Malpighian tubules is well known, mostly due to the

*Correspondence to: Matan Shelomi; Department of Entomology and Nematology, 1 Shields Ave, Davis, CA 95616.
E-mail: mshelomi@ucdavis.edu

work of Ramsay (1954, 1955), who developed the most commonly used assays for Malpighian tubule research on a walking stick, *Carausius morosus*. Phasmid Malpighian tubules are differentiated into two types: the superior tubules that function in excretion of nitrogenous wastes plus ion and water homeostasis as in other insects, and the inferior or "calciferous" tubules that store calcium (Savage, 1962), possibly for production of the calcium oxalate layer unique to phasmid eggs (Clark, 1958). These inferior tubules only develop in late-instar phasmids, whereas the primary tubules appear in the embryonic stage (Savage, 1962). Superior and inferior tubules are grouped into clusters at a 1:5 ratio, respectively, and open in conical ampullae exactly at the junction between the midgut and hindgut.

The literature on the appendices of the midgut, by contrast, is sparse and occasionally contradictory. The appendices are the first tubules to appear in the embryonic phasmid, developing before the third cuticulogenesis event with superior tubules developing afterwards (Savage, 1962; Louvet, 1974). To date, no author has determined the function of the tubules: even Ramsay never applied his own methods to the appendices, possibly due to their small size at one third the diameter of Malpighian tubules (Ramsay, 1955). Several authors have referred to the appendices of the midgut as a third type of Malpighian tubule, with Savage (1962) claiming they are "true Malpighian tubules" because their cellular construction is identical to the latters'. Savage also claimed the tubules are untracheated and short, ending "a few millimeters behind the midgut hindgut junction," which contradicts Ramsay's findings. Their shape and similarity to Malpighian tubules has lead many to hypothesize that the appendices are excretory in nature (de Sinéty, 1901), though other possibilities include secretion of digestive enzymes, storage of symbiotic microbes, and sequestration of toxic plant secondary chemicals.

One test for excretory function has been vital staining: a histological technique where stains are injected into a living insect prior to dissection rather than used on preserved tissue in vitro. Vital staining has long been used to demonstrate excretion in insects, resulting in the identification of two excretory systems for most invertebrates (Lison, 1942; Wigglesworth, 1974): one that eliminates indigo carmine (Color Index (CI)[1] # 73015), and one that eliminates ammonium carminates (no CI#). Although the names and responsible organs differ, these two systems appear throughout invertebrates, including molluscs, annelids, and arthropods. In insects, the Malpighian tubules always eliminate indigo carmine (Maddrell et al. 1974), whereas ammonium carmine is sequestered in the pericardial tissue (Palm, 1952). If indigo carmine stains the appendices of the midgut, their status as "true" Malpighian tubules would be confirmed.

In this study, we examined the anatomy of the digestive system of several phasmids to determine the consistency of their morphologies, in particular the number and form of their appendices of the midgut to resolve the confusion over these organs from the literature. We also used vital staining to identify stains that color the appendices differently than the Malpighian tubules and midgut tissue. Such stains would not only facilitate future anatomical work on phasmids (as the appendix tubules are invisible to the naked eye and difficult to discern from among the Malpighian tubules), but would also serve as a functional test of the appendices' excretory ability. In particular, vital staining would demonstrate whether the appendices are part of the indigo carmine (Malpighian tubule-like) or ammonium carmine (pericardial tissue-like) excretory systems.

MATERIALS AND METHODS

Phasmids used were of the following species: ten *Ramulus nematodes*, 15 *R. artemis*, and twenty *Medauroidea extradentata* (Phasmatidae), plus over 100 *Aretaon asperrimus* (Heteropterygidae), and over 50 *Peruphasma schultei* (Pseudophasmatidae). Phasmids were reared in cultures maintained at room temperature in the Bohart Museum of Entomology, University of California, Davis, on an ad libitum diet of privet (*Ligustrum* sp.) for *P. schultei* only and of roses (*Rosa* sp.) for all other species. We also examined ten wild-caught *Timema* sp. (Timematidae) found on *Ceanothus* sp. from Mix Canyon, Vacaville CA. All insects were maintained and used as per the University of California, Davis' Institutional Animal Care and Use Committee guidelines.

For simple light microscopy, phasmids were preserved and dissected in 70% ethanol or in phasmid saline (0.932% NaCl, 0.077% KCl, 0.05% CaCl$_2$). Most photos were taken from Nikon SMZ2B stereomicroscopes. Images were also taken using a Nikon TE2000U fluorescence inverted microscope. For tissue slices, whole insects with longitudinal slits across the body wall were fixed in Bouin's solution for 3 days at room temperature and stored in 70% ethanol. Tissue samples were dissected out, dehydrated at room temperature in a graded ethanol:butanol series (respective percentages in dH$_2$O of 50:20, 50:35, 40:55, 28:69, 23:75, and three exchanges of 0:100) with 12 h per exchange. Samples were then embedded in Paraplast® media, sectioned, mounted onto slides with Meyer's albumin, and stained in Giemsa, Ethyl Green (CI# 42590), or Heidenhaim's haematoxylin (CI# 75290) and eosin (CI# 45400) for light microscopy.

For scanning electron microscopy, dissected tissues were preserved in Karnovsky's fixative at 4°C for at least 24 h, mounted on stubs, sputter coated in gold with a Pelco Auto Sputter Coater SC-7 (Ted Pella, Redding, CA), and viewed using a Philips XL30 TMP scanning electron microscope (F.E.I. Company, Hillsboro OR) with "analysis iTEM Software."

Vital stains were prepared at 1% solutions of dye in phasmid saline (unless stated otherwise). These were injected into living phasmids in the soft cuticle posterior to the hind coxa, or between the thorax and the first abdominal sternite, until the phasmids appeared fully engorged. Anesthetics could not be

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[1]When available, stains used in this study will be identified by their Color Index (CI) number as declared by the Biological Stain Commission (Horobin and Kiernan, 2002).

used as they change the results of vital staining and impede normal excretory function (Palm, 1952). The phasmids could survive such injections for days depending on the toxicity of the stain. After a period of 30 min (unless stated otherwise), the insect's head and posterior-most abdominal segments were removed and the entire gut pulled through one of the openings using fine-tipped forceps and viewed under phasmid saline. The list of dyes used and their results are in Table 1, sorted by CI number.

RESULTS

All species of walking stick examined except *Timema* have the same general digestive system morphology (Fig. 1). The foregut consists of a transparent crop filled with torn pieces of ingested leaves. It terminates in a small, muscular proventriculus with approximately 45 dorsolaterally arranged ridges covered in small, chitinous denticles. Paired salivary glands are present around the crop. Small, rudimentary gastric caecae fully or partially obscure the proventriculus, which extends into the midgut (Fig. 1, dotted lines). The anterior midgut is covered in a muscular thickening of a variable number folds perpendicular to the body axis and with a longitudinodorsal groove. These pleats, which are more numerous in longer species, become less apparent moving posteriorly and then disappear. At a point usually corresponding to the third abdominal segment, 30–60 small conical ampules that terminate in long filiform tubules appear on the midgut's outer surface. These "appendices of the midgut" demarcate the posterior midgut. At the junction between the midgut and hindgut are the Malpighian tubules.

The appendices of the midgut (Fig. 2) are irregularly spaced along the posterior midgut, arising on or between the longitudinal muscles of the midgut (Fig. 3). The ampules are approximately 300–500 μm long and open into the midgut via small pores (Fig. 4), and are filled with a yellow, noncellular substance (Fig. 2). The tubules are one-third the diameter of Malpighian tubules and nearly invisible without staining, occasionally containing small grains or crystals. They originate at the ampules pointing backward, where they run among and underneath the Malpighian tubules all the way to the posterior end of the insect. They appear to end blindly and do not attach to the hindgut. The appendices do not terminate shortly after the midgut-hindgut junction and were also found to be highly tracheated (Fig. 5). Ampules are wrapped in tracheoles arising from a single tracheal trunk, continuing into a tracheole that runs up the proximal part of the tubule (Figs. 3, 5). Different tracheal filaments serve progressive lengths of the distal portions of the appendix tubules. Ampules are coiled like a corkscrew and are motile, periodically compressing and extending like a spring (Supporting Information, Video S1).

Timema are exceptions to the above, in that they have fewer Malpighian tubules (approximately 30) and only have one or two pairs of appendices symmetrically arranged on their midgut (Fig. 6), which are larger in size and with larger pores into the midgut than those of other phasmid species. Also, in *P. schultei* only, some ampules contained a dark, amorphous, and lipophilic substance of an unknown composition (Fig. 2).

The appendices of the midgut stained differently from the Malpighian tubules (Table 1). Most of the stains that affected appendices also stained all other tissues equally ("nonelective" stains), such as orange G (CI# 16230), brilliant green (CI# 42040), and eosin B (CI# 45400). Several stains proved useful in highlighting the appendices. Acridine orange (CI# 46005) stains the whole gut, but the appendix ampules are more noticeably orange. Janus green B (CI# 11050) colors the appendix tubules and outer wall of the ampules blue, but also stains the Malpighian tubule wall and the midgut longitudinal muscles. Methylene blue (CI# 52015) stains similarly but more strongly. The latter two stains are also useful in staining nervous tissue (Yack, 1993; Horobin and Kiernan, 2002), but no evidence that the appendices are innervated was found. Crystal violet (CI# 52555) stains the appendices and Malpighian tubules completely purple, but not the midgut tissue. Coomassie brilliant blue R (CI# 42660) stains the walls of the appendices, contents of the Malpighian tubules, and longitudinal muscles of the midgut purple-blue. Brilliant cresyl blue (CI# 51010) stains the Malpighian tubule contents dark blue and the walls of the ampules with blue specks: it is unclear if the tubules of the appendices were not stained or had just transferred any absorbed stain proximally to the ampule. Notably, the appendices failed to absorb the stains that "define" Malpighian tubule-like activity, such as indigo carmine and phenol red (no CI#); nor could they pick up the stains absorbed by pericardial tissue like ammonium carmine and trypan blue (CI# 23850).

Three stains in particular are well suited for differentiation of the appendices. New methylene blue N (CI# 52030) colors the appendices a dark purple (Fig. 7A), but colors the Malpighian tubules from purple to green-blue (metachromasy). Ethyl green (CI# 42590; synonymous with methyl green, CI# 42585), when used directly from the packaging, is more striking, coloring the appendices purple and the Malpighian tubules varying shades of green (Fig. 8). This phenomenon is due to ethyl green being derived from crystal violet and occasionally reverting back, such that formulations of ethyl green invariably contain crystal violet as an impurity (Kasten and Sandritter, 1962). Protocols involving ethyl green typically call for the crystal violet to be removed using chloroform: when phasmids are stained with purified ethyl green, the appendices are unstained. Purple crystals were

TABLE 1. Results of the vital staining performed on the Phasmatodea

Stain	Midgut wall	Ampule contents	Appendix tubes	Malpighian tubule tissue	Malpighian tubule lumen	CI #	Chemical class	Ionisation	Relative molar mass	Solubility in water (%)
Picric acid	•	•	•	•	+	10305	Nitro	Anionic	229	1.3
Janus green B	−	•	+	+	•	11050	Monoazo	Cationic	511	5.2
Methyl orange	+	+	+	+	+	13025	Monoazo	Anionic	327	0.5
Alizarin yellow R	•	•	•	•	+	14030	Monoazo	Anionic	287	N.A.
Orange G	+	+	+	+	+	16230	Monoazo	Anionic	452	10.9
Chromotrop 2R	+	−	•	•	+	16570	Monoazo	Anionic	468	19.3
Congo red	•	•	•	•	−	22120	Diazo	Anionic	697	5
Trypan blue	+	•	•	•	++	23850	Diazo	Anionic	961	1
Sudan black B in Ethanol	•	•	+	+	+	26150	Diazo	Uncharged	457	N.A.
Biebrich scarlet	•	•	•	•	+	26905	Diazo	Anionic	556	4
Chlorazol black E	−	•	−	−	•	30235	Polyazo	Anionic	782	6
Variamine blue B	−	•	•	•	−	37255	Diazonium Salt	Cationic	262	N.A.
Malachite green	−	•	•	•	−	42000	Triarylmethane	Cationic	365	4
Brilliant green	+	+	+	+	+	42040	Triarylmethane	Cationic	483	N.A.
Fast green Fcf	−	−	−	−	+	42053	Triarylmethane	Anionic	809	6
Light green SF [Yellow]	−	•	•	−	+	42095	Triarylmethane	Anionic	793	10–20
Xylene cyanol FF	•	•	•	•	+	42135	Triarylmethane	Anionic	534	N.A.
Crystal/gentian violet	−	+	+	−	+	42555	Triarylmethane	Cationic	408	0.2–1.7
Coomassie brilliant blue R	•	−	−	−	+	42660	Triarylmethane	Anionic	854	N.A.
Acid fuchsin[e]	•	•	•	−	+	42685	Triarylmethane	Anionic	586	10–12.5
Methyl blue	•	•	•	•	+	42780	Triarylmethane	Anionic	800	N.A.
Eosin Y	−	•	+	−	+	45380	Xanthene	Anionic	692	0.08
Eosin B	+	+	+	+	+	45400	Xanthene	Anionic	624	N.A.
Rose Bengal	−	−	−	+	+	45440	Xanthene	Anionic	974	N.A.
Acridine orange	+	++	+	+	+	46005	Acridine	Cationic	320	5
Acridine yellow	+	+	+	+	−	46025	Acridine	Cationic	273	N.A.
Neutral red	+	+	+	+	+	50040	Azine	Cationic	289	4
Azocarmine G	−	•	•	+	+	50085	Azine	Anionic	580	1
Azocarmine B	+	•	•	+	+	50090	Azine	Anionic	682	2
Phenosafranine	+	+	•	+	+	50200	Azine	Cationic	323	6.5
Safranin[e] O	+	+	+	+	+	50240	Azine	Cationic	351	4.5
Nigrosin[e]	−	•	•	•	•	50420	Azine	Anionic	N.A.	5–10
Brilliant cresyl blue	•	+	•	•	++	51010	Oxazine	Cationic	386	3
Celestine blue	•	•	•	•	++	51050	Oxazine	Cationic	364	2
Nile blue [Sulfate]	•	•	•	•	•	51180	Oxazine	Cationic	733	2
Thionin	•	•	•	•	+	52000	Thiazine	Cationic	287	3
Azure A/I	•	•	•	•	+	52005	Thiazine	Cationic	292	N.A.
Azure B	−	−	•	•	+	52010	Thiazine	Cationic	306	5
Methylene blue	+	+	+	+	+	52015	Thiazine	Cationic	374	9.5
New methylene blue N	+	+	++	+	+	52030	Thiazine	Cationic	348	4
Toluidine blue [O]	+	+	+	+	+	52040	Thiazine	Cationic	306	3.3
Toluidine blue [O] (0.01%)	•	++	++	+	+	52040	Thiazine	Cationic	306	3.3
Alizarin[e]	•	•	•	•	•	58000	Anthraquinone	Anionic	240	N.A.
Alizarin red S	•	•	•	•	+	58005	Anthraquinone	Anionic	360	7.7
Indigo carmine	•	•	•	•	+	73015	Indigoid	Anionic	466	1.5
Aclian blue 8G	•	•	•	•	•	74240	Phthalocyanine	Cationic	1299	0.8–9.5

Table 1. (continued).

Stain	Midgut wall	Ampule contents	Appendix tubes	Malpighian tubule tissue	Malpighian tubule lumen	CI #	Chemical class	Ionisation	Relative molar mass	Solubility in water (%)
Haematein	•	•	•	•	+	75290	Natural	Anionic	300	1.5
Haematoxylin	•	•	•	•	–	75290	Natural	Anionic	302	3
Carmine/Carminic acid	+	•	•	•	+	75470	Natural	Anionic	492	0.4
Carbon (India/China ink)	•	•	•	•	•	77266	Inorganic	Uncharged	12	0
Basic fuchsin[e]	–	•	•	+	+	42500, 42501, 42520	Triarylmethane	Cationic	N.A.	0.3–2.4
[M]ethyl green–Pure	+	•	•	+	+	42590=42585	Triarylmethane	Cationic	517	7
[M]ethyl green–Unpure	+	++	++	+	++	42590=42585, 42555	Triarylmethane	Cationic	517	7
Aniline blue	+	–	•	•	++	42780, 42755	Triarylmethane	Anionic	800	7
Azure II	+	•	•	•	+	52005, 52010, 52015	Thiazine	Cationic	N.A.	N.A.
Borax carmine	•	•	•	•	•	N.A.	Natural	Anionic	N.A.	N.A.
Carmine, Alum lake	•	•	•	•	•	N.A.	Natural	Anionic	492	3
Brom[o]phenol blue	•	•	•	–	+	N.A.	Triarylmethane	Anionic	692	3
Phenol red	–	•	•	+	+	N.A.	Triarylmethane	Anionic	376	0.3
Bromocresol green	+	•	•	+	•	N.A.	Triarylmethane	Anionic	698	N.A.
Iodine, Gram's	+	•	•	•	•	N.A.	Inorganic	Uncharged	254	0.03
Ammonium carmine	+	•	•	•	+	N.A.	Natural	Uncharged	509	N.A.
Nile red in ethanol	+	+	•	•	•	N.A.	Oxazine	Uncharged	318	N.A.
Ethanol (70%)	•	+	+	+	+	N.A.	N.A.	Uncharged	N.A.	N.A.

Stains are sorted by CI number. Stains were mixed at 1% in saline and injected into the insect 30 min prior to dissection unless stated otherwise. Relative molar mass and solubility in water, when provided, came from Conn's Biological Stains (Horobin and Kiernan 2002). Bracketed text reflects variation in spelling internationally and over time. Tissues marked with a • were unstained, – were weakly stained, + were strongly stained, and ++ stained strongly but differently from other tissues, indicating a metachromasy or effect of multistain formulations (polychromatism). Note that [m]ethyl green is contaminated with crystal violet unless purified via chloroform (Kasten and Sandritter, 1962). Note that "gentian violet" produced in the USA, as our stains were, is identical to crystal violet, but "gentian violet" produced in Europe may refer to other stains (Conn, 1922).
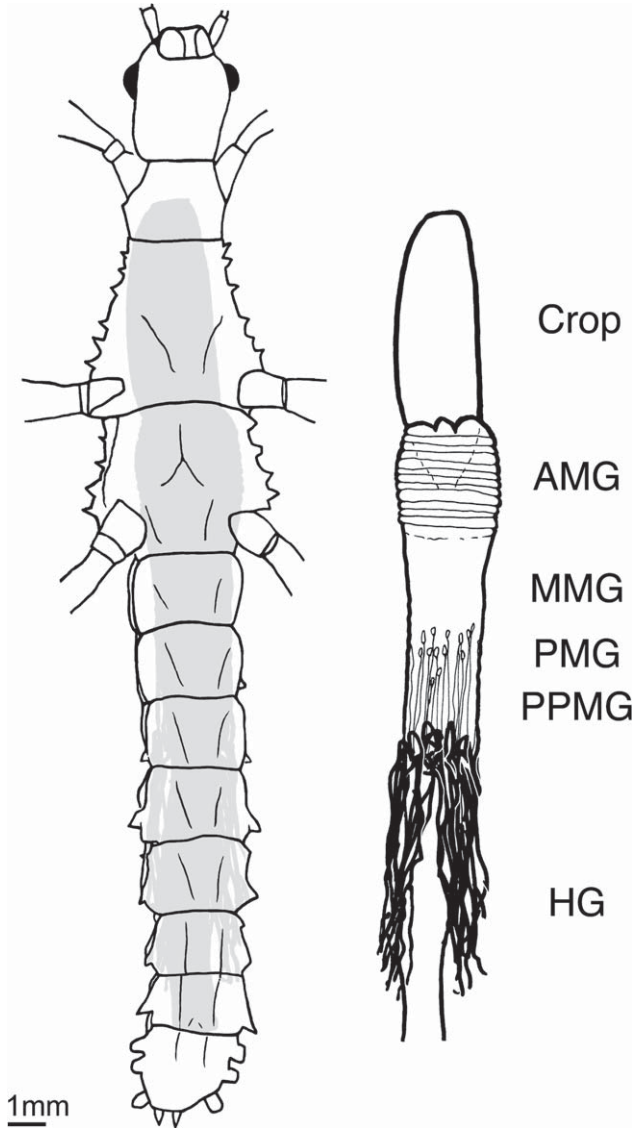
Fig. 1. Illustration of the typical Phasmatodea digestive system. Based on a third instar *A. asperrimus*. Sections are the crop, anterior midgut (AMG), middle midgut (MMG), posterior midgut (PMG) marked with the appendices of the midgut, post posterior midgut (PPMG), and hindgut (HG). The Malpighian tubules originate at the junction between the PPMG and HG.

observed moving from the ampules into the midgut, suggesting a direction of flow. Toluidine Blue O (CI# 52040) was also a useful stain, but at 1% solution the phasmids were killed instantly and the gut contents were overstained purple. Diluting the stain 100-fold kept phasmids alive and produced a metachromatic effect: the appendices turn blue, but the malpighian tubules are blue to purple to pink (Fig. 9). This effect is more noticeable 2-days post staining.

Appendices did not stain with these compounds in phasmids stained post mortem. This suggests the staining of appendix tissues is due to active transport of the stains into the appendices. Brilliant green, carminic acid (CI# 75470), crystal vio-
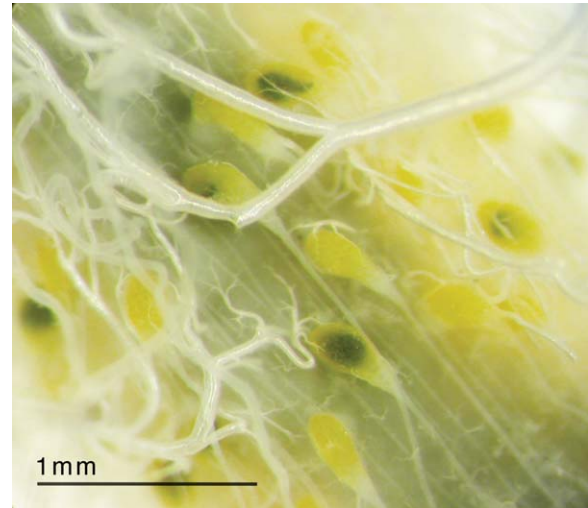
Fig. 2. Appendices of the *Peruphasma schultei* posterior midgut. Insect was dissected in saline, unstained. The yellow ampules of the appendices are visible along with their long, uncolored tubules, as are the silvery tracheoles. Note the black substances inside certain ampules, of an unknown function and composition. Scale bar = 1 mm.

let, eosin B, India ink (CI# 77266), picric acid (CI# 10305), and toluidine blue are toxic and often kill the phasmids within the 30 min. Absorbed dyes would be mostly cleared from the Malpighian tubules and present in a band of color in the hindgut within hours or days. Some dyes persisted in the appendices for weeks, however, concentrating in the proximal portion of the ampules. A test with nonelective stain neutral red (CI# 50040) after 2 weeks showed a strong red band of color in the midgut starting at the point where the appendices begin, long after the stain had been cleared from the midgut wall tissue (Fig. 10). Dissection showed grains of dye along the midgut luminal wall: it is unclear whether the dye entered the
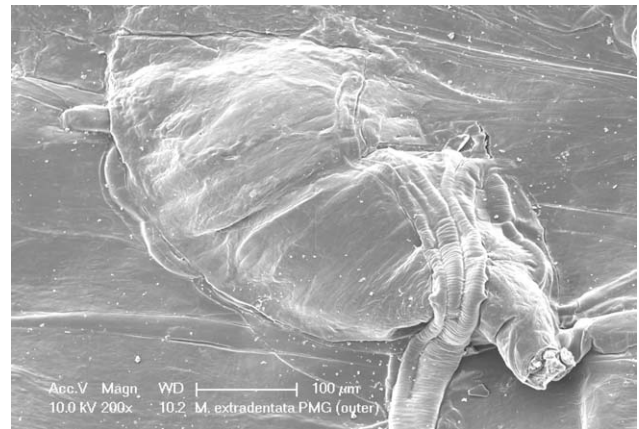

Fig. 3. Scanning electron micrograph of a midgut appendix ampule in *M. extradentata*. Note the tracheal tubes on the bottom right of the ampule, and the longitudinal muscles running horizontally above and below the ampule (whose opening to the midgut is likely in between these muscles). Scale bar = 0.1 mm.
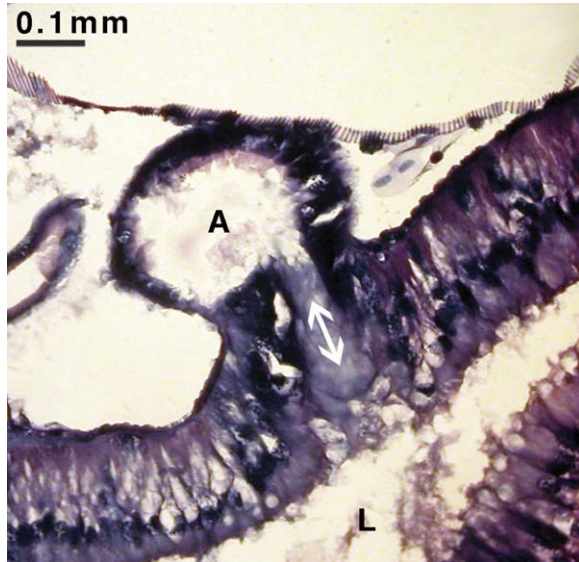
Fig. 4. Micrograph of a *Peruphasma schultei* posterior midgut appendix. Tissue embedded in Paraplast®, stained in Heidenhaim's haematoxylin and eosin. Note the pore (white arrows) between the ampule of the appendix of the midgut (A) and the midgut lumen (L). Scale bar = 0.1 mm.

midgut from the ampule pores or via passive diffusion. Similarly, after 6 days, new methylene blue N can only be found in the ampules of the appendices as a dark purple that can be seen also lining the channel between ampule and midgut (Fig. 7B).

If ethyl green or other stains are unavailable, ethanol can also be used to differentiate the appendices from the rest of the insect, as it decolorizes these and the Malpighian tubules to white
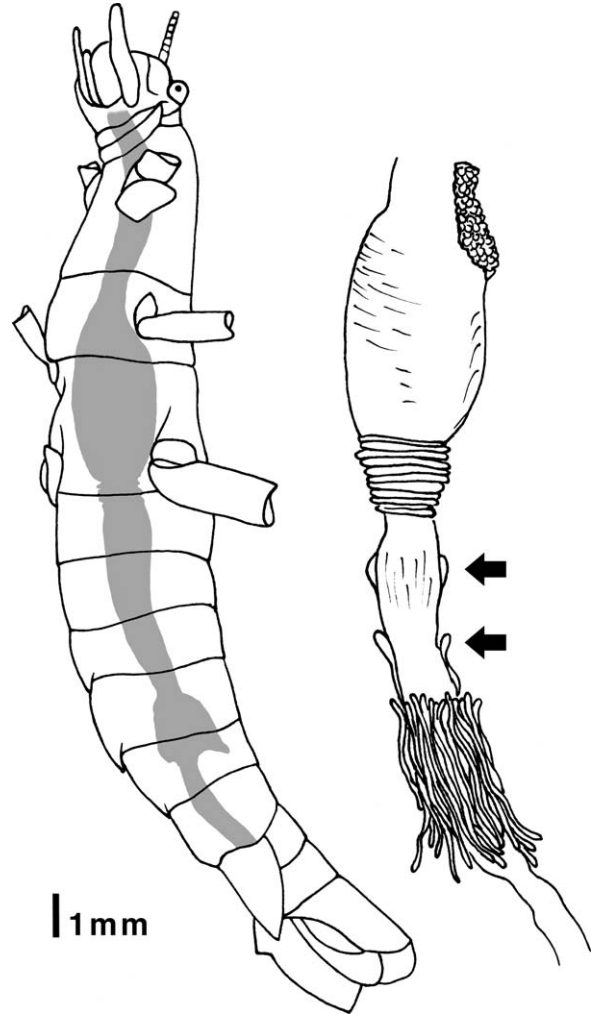

Fig. 6. Internal and external anatomy of *Timema*. Note the two pairs of appendices (arrows). Scale bar = 1 mm.

while leaving the midgut beige. Also of note: Celestine blue (CI# 51050) stained the superior and inferior Malpighian tubules violet and light blue, respectively, Janus Green B stains the proximal ends of the Malpighian tubules pink and the distal ends blue and turns purple after 2 days, and trypan blue, methyl blue (CI# 42780), and aniline blue (actually a mix of methyl blue and water blue, CI# 42755) stained the gut wall blue and Malpighian tubule contents violet. Last, to highlight the tubules in ethanol-preserved specimens, staining the tissue in 1% Chlorazol Black E (CI# 30235) for 5–10 s and differentiating in ethanol outlines the tubules, appendices, and midgut longitudinal muscles in black, as it does when used vitally.

DISCUSSION

The gut morphologies of the phasmids we analyzed are similar to those described in Bordas
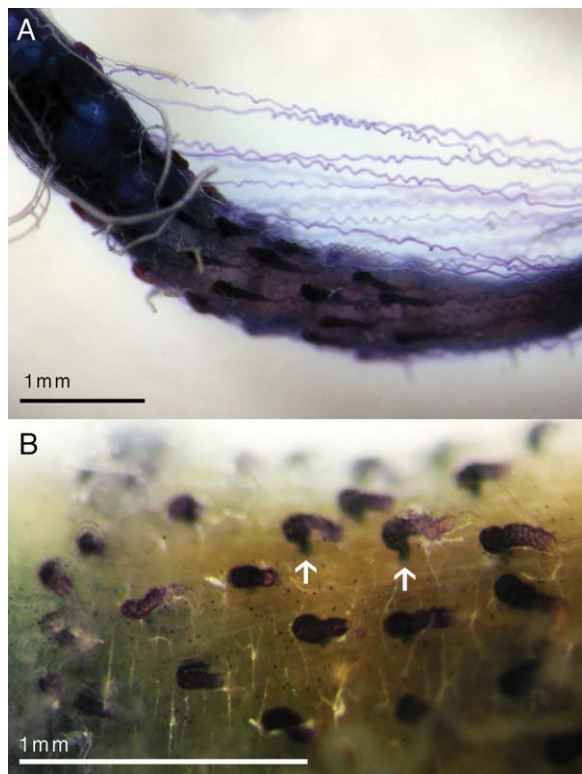

Fig. 5. Fluorescence micrograph of the appendices of the midgut of *Medauroidea extradentata*. Photo taken via a Nikon TE-2000U. A central tracheal trunk is visible with tracheoles connecting to two the ampules (A) of two midgut appendices. Also visible are the tubules (T) leading away from the ampules. Scale bar = 0.1 mm.

Fig. 7. Micrographs of phasmid posterior midguts after vital staining with 1% new methylene blue N. (**A**) Gut of adult *R. nematodes* dissected 30 min after staining, showing purple coloration throughout the midgut tissue and appendices. (**B**) Gut of nymph *A. asperrimus* dissected 6 days after staining. The stain is now concentrated in the appendix ampule and can be seen in the channel between the appendices and midgut lumen (white arrows). Scale bars = 1 mm.

(1897) for the three species *Hermarchus pythonius*, *Acanthoderus spinosus*, and *Sipyloidea erechtheus* (Phasmatidae); and de Sinéty (1901) for *C. morosus* (Phasmatidae). The appendices of the midgut are tracheated and terminated at the posterior end of the insect, in contrast to Savage's (1962) claims that they are untracheated and terminate shortly after the midgut-hindgut junction.

Constrained by its distinctive shape, the phasmid gut cannot harbor modifications such as twists, curves, large diverticulae, and enlargements as in other insects. Thus, phasmids have evolved unique physiological modifications, producing a straight gut that is compartmentalized into different functions and morphologies along its length. The foregut's contents are green, relatively large strips of leaves as ingested by the insect, which are later shredded into small pieces for midgut digestion by the toothed proventriculus. Phasmid gastric caecae are rudimentary, in contrast to the large, branched gastric caecae of the Acrididae (Billingsley and Lehane, 1996) and Gryllidae (Çakici and Ergen, 2012). The folding of the anterior midgut likely serves to increase midgut
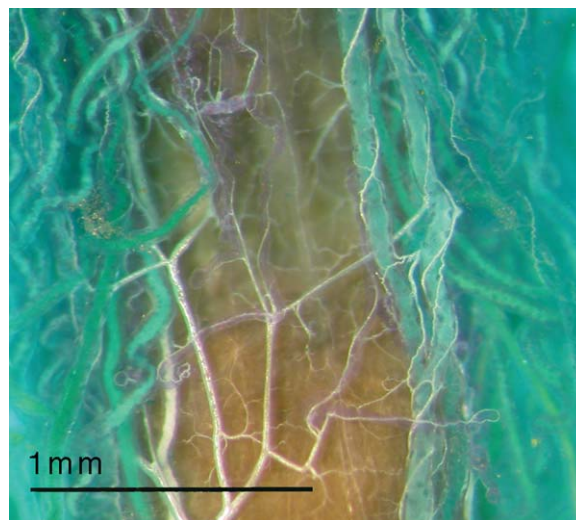

Fig. 8. Micrograph of the hindgut of *Ramulus artemis* vitally stained with 1% ethyl green for 90 min. The Malpighian tubules (stained bright green) have been pulled back to show the tubules of the appendices (stained purple), which run alongside the hindgut wall (unstained). Note the silvery tracheoles leading to the appendices. Scale bar = 1 mm.

surface area and slow down the passage of ingested material, both of which increase the amount of food digested and absorbed. None of the
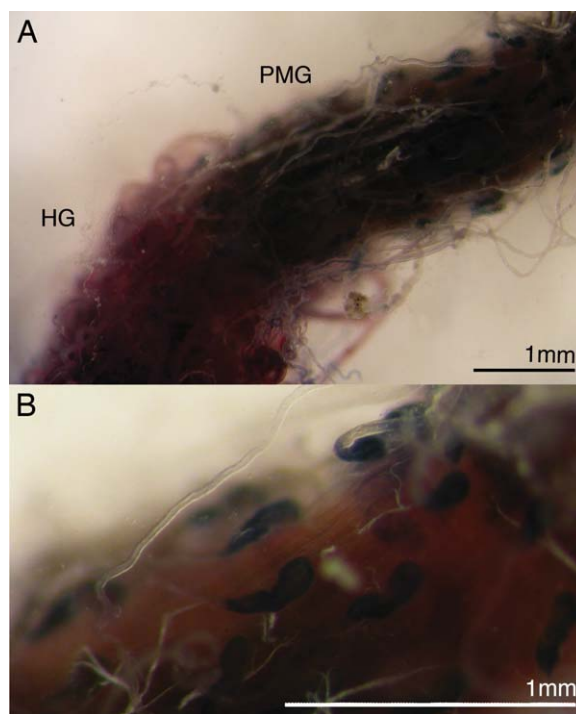

Fig. 9. Micrographs of the gut of *Aretaon asperrimus* after staining with 0.01% toluidine blue O for 2 days. (**A**) The appendices of the midgut are a dark blue against an unstained midgut wall, while the Malpighian tubules are pink. (**B**) The close-up shows that the stain is concentrated in the ampules of the appendices, while the tubules are clear. PMG = posterior midgut. HG = hindgut. Scale bars = 1 mm.
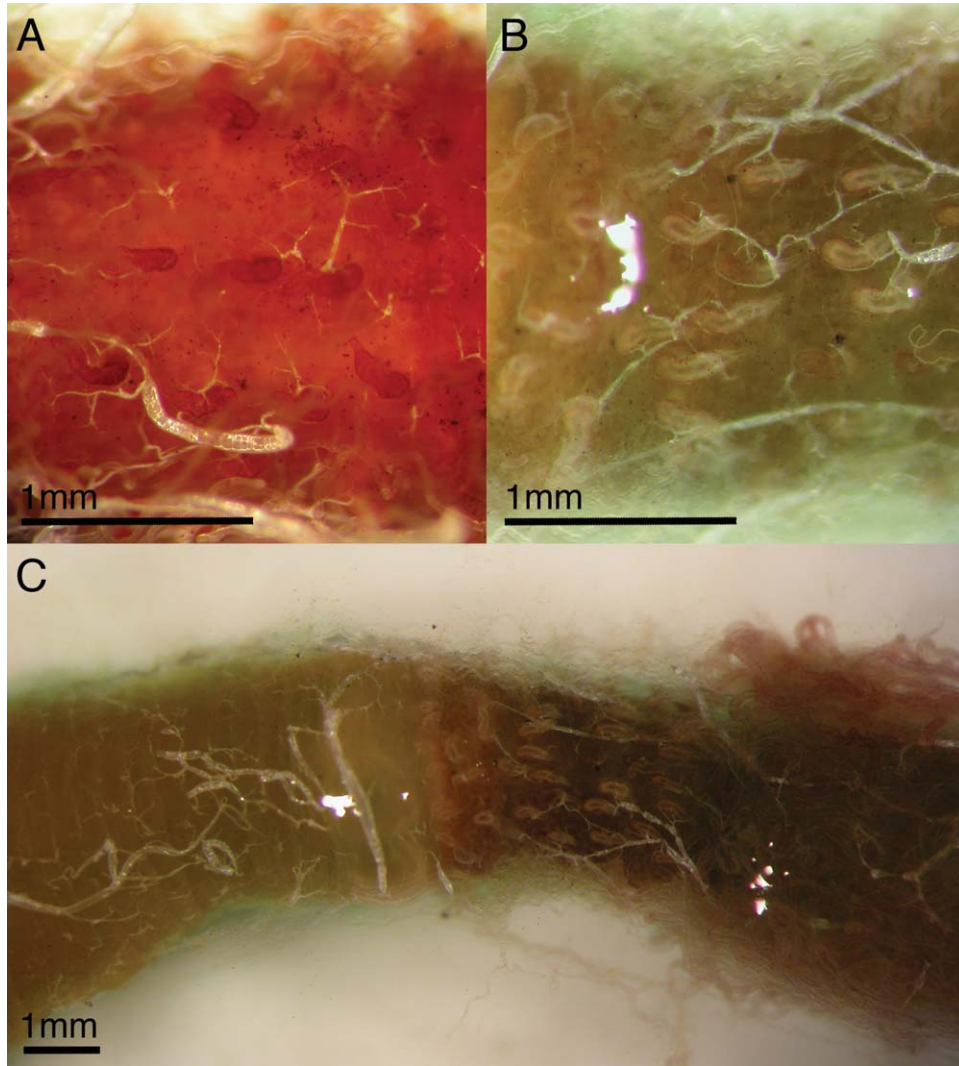
Fig. 10. Micrographs of the gut of *Aretaon asperrimus* after staining with 1% neutral red. (**A**) Posterior midgut after 1 day. The whole gut is completely red, but the stain is strongest in the edges of the appendix ampules. (**B**) Posterior midgut (different individual) after 14 days. The ampule edges are still reddish. (**C**) Same individual, gut from anterior midgut (left) to hindgut (right). The line between the middle midgut and posterior midgut is clearly demarcated by red stain in the latter. Scale bars = 1 mm.

phasmid tissues examined, including the appendices, included mycetocytes or bacteriocytes, nor did we find any other evidence of microbial symbionts as known from other insect midguts (Douglas and Beard, 1996), confirming findings from molecular biology that phasmid digestion is symbiont-independent (Shelomi et al., 2013).

The "appendices of the midgut," contrary to their name, are not vestigial. They are well-tracheated, motile, and high in mitochondria based on their affinity to Janus Green B, which stains mitochondria in living cells (Palm, 1952). Not found in any of the Phasmatodea's sister orders, they appear to have originated in the basal phasmids, the Timematidae (Kômoto et al., 2011), increasing in number in more derived families, suggesting the appendices have been evolutionarily selected for. Their appearance and staining is

unlike other insect midgut modifications, such as the protruding regenerative crypts of certain Orthoptera (Rost-Roszkowska, 2008) and adult beetles (Nardi and Bee, 2012), Orthopteran gastric caecae (Billingsley and Lehane, 1996; Çakici and Ergen, 2012), or Hemipteran bacterial crypts (Kikuchi et al., 2009); nor are they midgut pseudo-tumors as described in phasmids and other orthopteromophs (Hotmann and Dorn, 2009).

The excretion of the appendices is unlike anything in the Insecta, as they cannot be placed into the two known systems of indigo carmine and ammonium carminate excretion (Wigglesworth, 1974). Although cytological and embryological evidence suggests they are derived from Malpighian tubules, the appendices' failure to excrete the same histological stains that all other insect Malpighian tubules are capable of eliminating means

referring to them as a type of Malpighian tube is functionally inaccurate and can cause confusion. We do confirm that the appendices are excretory organs, however, as they were observed to sequester stains in their proximal ampules before gradually eliminating them into the midgut. Thus, the appendices represent a third "type" of excretory system in insects with unknown homology to other invertebrate systems, if any (Maddrell and Gardiner, 1976). This new, "noncarminic" excretory function may be performed by Malpighian tubules in other insect orders, but has been separated in phasmids (Nijhout, 1975). Although Malpighian tubules are known to excrete anionic substances, the appendices are more strongly stained with cationic dyes. The appendices thus may function to sequester cationic toxins such as alkaloids (Maddrell and Gardiner, 1976; Rheault et al., 2006).

The yellow color common to the ampules could be calcium phosphate: though a rare form for calcium to be excreted in insects, it appears to be the main form of calcium elimination in phasmids (Pantel, 1919b). Pantel (1919a) remarked on high levels of calcium phosphate in the "tubes de Malpighi" of newly hatched phasmid nymphs, though it is impossible to know if he was referring to true Malpighian tubules or the midgut appendices due to the latter being referred to as a type of the former (further evidence that a better name should be found for the appendices). Phasmid eggshells have a high quantity of calcium oxalate and/or carbonate (Moscona, 1950), and the calcium in the yolk is thought to transfer into the embryo during development (Clark, 1958). As the appendices are the first excretory tubules to appear in the embryo, their role may serve as early calcium homeostasis in the developing insect, sequestering and storing excess calcium for later excretion or utilization. The yellow compounds may also be calcium urates or other uric compounds, also alluded to by Pantel (1919b). We hypothesize that the amorphous, lipophilic concretions in the *P. schultei* ampules are ion and/or nitrogenous waste concretions (Ballan-Dufrançais, 2002), but were unable to accumulate enough material for chemical confirmation.

No physiochemical similarities exist among the stains that work on the appendices (Table 1): for example, crystal violet stains the appendices while ethyl green does not, even though they only differ in a single methyl group. This finding is not unusual, and has been reported for the Malpighian tubules and pericardial tissues as well (Lison, 1942; Wigglesworth, 1974). The consensus is that staining activity on insect excretory organs depends less on the electrochemical properties of the stains and more on their idiosyncratic reactions with the receptor, transporter, and ion channel proteins of the organs. Future research on any of the now three identified excretory systems in the Insecta should tie molecular biology to a histological foundation. Transcriptomics of the three excretory tissues (Malpighian tubules, pericardial tissue, and phasmid appendices) followed by RNAi can identify proteins such as ion channels associated with each staining/excretory type. Such techniques can be extended to other invertebrates and possibly used to address excretory and ion homeostasis systems and disorders in vertebrates. The appendices, for example, may present a new model for research into biomineralization and calcium homeostasis (Simkiss, 1977). The results of such work would also finally show the mechanisms by which vital histological staining works.

In conclusion, we present here the first evidence that the appendices of the phasmid midgut are excretory organs, whose function is wholly unlike that of the Malpighian tubules or other excretory organs of the Insecta, and which likely serve in calcium or other metal cation excretion/sequestration. Thus, we discourage referring to the appendices as Malpighian tubules or types thereof, as we feel the functional differences outweigh homology. We present evidence that the appendix tubules are tracheated and lengthy, resolving contradictions in the phasmid anatomy literature. We also provide further data on the general morphology of the Phasmatodea digestive system, including the first for the basal phasmid genus *Timema*. This article is the broadest vital staining work on insect excretion to include both acid and basic dyes. We present the toluidine blue o, ethyl green, and new methylene blue N technics for identification of phasmid appendices of the midgut (Supporting Information Fig. S1), and suggest novel ways to increase our knowledge of animal excretion, histochemistry, and biomineralization physiology.

ACKNOWLEDGMENTS

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