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Review of the gross anatomy and microbiology of the Phasmatodea digestive tract

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Abstract

The sparse descriptions of the stick insect (Phasmatodea) digestive system as reported/provided in the literature are highly contradictory. This paper describes the digestive systems of several families of Phasmatodea (Timematidae, Heteropterygidae, Diapheromeridae, Pseudophasmatidae, and Phasmatidae) plus the gut microbiome of these and one other (Phylliidae) to both verify past findings and provide a general description of the Phasmatodea alimentary canal. The constrictions imposed by this anatomy on phasmid gut microbiology, its connections to recently released Phasmatodea transcriptomes, and how it differs from the anatomy of related orders in the Polyneoptera are discussed.

All Phasmatodea have ridged proventriculi lined or covered with small spines. Anterior projections of the midgut, sometimes described as gastric caeca, are only found in Euphasmatodea and often obscure the proventriculus. We define the cardia as the complex of foregut and midgut tissue where the type II peritrophic matrix is produced. Appendices of the midgut are an autapomorphy for Phasmatodea, but *Timema* have fewer and larger appendices relative to body size. We suggest caeca-like projections and the loss of large, proventricular teeth are apomorphies of Euphasmatodea. We identify a possible facultative symbiosis in *Eucalyptus*-feeding species that requires further study.

Key words

Alimentary canal, digestion, microbiome, phasmids, proventriculus, symbioses

Introduction

The order Phasmatodea (leaf and stick insects) consists of approximately 3000 known species often characterized by an elongated body cavity and procryptic camouflage (Azevedo *et al.* 2013). The relatively short and stout Timematidae are considered the sister group to the Euphasmatodea (Whiting *et al.* 2003, Terry & Whiting 2005, Friedemann *et al.* 2012, Gottardo *et al.* 2012, Tilgner *et al.* 1999). Recent works suggest Phasmatodea are a monophyletic group based on morphological (Tilgner *et al.* 1999, Friedemann *et al.* 2012, Beutel *et al.* 2013), mtDNA (Plazzi *et al.* 2011, Bradler *et al.* 2014), nuclear genomic (Buckley *et al.* 2009, Bradler *et al.* 2014), and transcriptomic (Misof *et al.* 2014) datasets. Autapomorphies include prothoracic defense glands and small, pyriform appendages on the posterior midgut (Tilgner *et al.* 1999, Beutel *et al.* 2013) recently demonstrated as having a unique excretory function (Shelomi & Kimsey 2014). The sister order to the Phasmatodea is most likely Embiidina (webspinners) forming a "Eukinolabia" clade, based on external morphology (Wipfler *et al.* 2011, Friedemann *et al.* 2012, Beutel *et al.* 2013), rDNA (Flook & Rowell 1998, Terry & Whiting 2005), expressed sequence tags (Letsch *et al.* 2012) and transcriptomics (Misof *et al.* 2014). Future phylogenetic analyses are still required to resolve the relationships of the Polyneopteran orders (Trautwein *et al.* 2012): in particular, comparisons of internal features are lacking due to the inconsistent anatomical studies.

Literature on phasmid digestive system anatomy is contradictory (Table 1). Common areas of contention are the existence of a proventriculus and gastric caeca. Some works claimed phasmids have one or the other (Heymons 1897, Cameron 1912, Caarels 2011), or neither (Chopard 1949), though never both, and some descriptions are incomplete (Bordas 1897, Clark 1976). Reports differ on whether or not the inner surface of the proventriculus is toothed, and on what counts as teeth: one study claimed that absence of proventricular dentition is an autapomorphy of the Euphasmatodea (Tilgner *et al.* 1999), based on a distinction between "spinules" and "teeth" (Judd 1948). Those claiming caeca exist all agree they are reduced in size, relative to those of Orthoptera for example, but it remains unclear whether they are differentiated from the midgut tissue. Descriptions and names of the "appendices of the midgut," the Phasmatodea-specific tubules arising from pear-shaped ampules on the posterior midgut, also vary, such as whether or not they are tracheated (Savage 1962). One author referred to them as "gastric caeca" (Azevedo *et al.* 2013), another as "Malpighian tubules" (Savage 1962). Unhelpful is the fact that these studies are often limited to one to three species each, and do not cover the diversity of Phasmatodea.

Studying the Phasmatodea digestive system is important not only for potential use in systematics, but also in the evolution of their diet: Unlike other Polyneoptera, phasmids are exclusively folivorous (Calderón-Cortés *et al.* 2012, Azevedo *et al.* 2013, Beutel *et al.* 2013). A long-standing question in phasmids is whether or not their digestion is symbiont-dependent. While cellulolytic gut symbionts would certainly facilitate digestion of the phasmids' leafy diets (Watanabe & Tokuda 2010), the acidic and moderately oxidizing phasmid digestive tract is not conducive to fermentative digestion (Caarels 2011), and its straight and simple shape suggests it is more likely to be inhabited by transient microbes passing with the diet (Douglas & Beard 1996, Lacey *et al.* 2007) than symbionts (Dillon & Dillon 2004, Watanabe & Tokuda 2010). Recent transcriptomic assays confirmed that Phasmatodea produce their own plant cell wall degrading enzymes (Shelomi *et al.* 2014a) in certain sections

Table 1. Review of previous Phasmatodea anatomical studies, including terms given for the midgut appendices. Arranged by first author. Species listed are given their currently accepted names. A dash (-) indicates the article does not mention said organ. PMG = posterior midgut. Prov. = proventriculus. Valve = oesophageal valve at end of foregut.

Author	Species	Caeca	Prov.	Toothed?	Valve	PMG Tubules + Name Used
Azevedo *et al.* 2013	*Cladomorphus phyllinus* Gray	-	Yes	Yes	-	Yes, "gastric caeca-like"
Beadle 1972	*Carausius morosus* Sinéty	-	-	-	Yes	Yes, "conical tubular organs"
Bordas 1897	*Acanthoderus spinosus* Gray, *Hermarchus pythonius* Westwood, *Sipyloidea erechtheus* Westwood	-	Yes	Yes	Yes	Yes, "glandes coniques… appendice filiforme"
Caarels 2011	*Extatosoma tiaratum* Macleay	No	Yes	No	-	-
Cameron 1912	*Bacillus rossi* Rossi	Yes	No	-	Yes	Yes, "conical tubular organs"
Chopard 1949	Order Phasmatodea	No	No	-	Yes	Yes, "small glands"
Clark 1976	*Extatosoma tiaratum* Macleay	-	Yes	-	-	Yes, "papillae"
de Sinéty 1901	*Carausius morosus* Sinéty	Yes	-	-	Yes	Yes, "Appendices de l'intestin moyen"
Heymons 1897	*Bacillus rossi* Rossi	Yes	No	-	Yes	Yes, "Anhänge des Mitteldarmes"
Judd 1948	Order Phasmatodea	-	Yes	Yes	Yes	-
Marshall & Severin 1906	*Diapheromera femorata* Say	No	No	-	Yes	Yes, "Anhänge des Mitteldarmes"
Monteiro *et al.* 2014	*Cladomorphus phyllinus* Gray	No	Yes	-	-	Yes, "appendices"
Ramsay 1955	*Carausius morosus* Sinéty	-	-	-	-	Yes, "appendices"
Rutschke *et al.* 1976	*Carausius morosus* Sinéty	No	-	-	-	Yes, "Glandulae piriformes"
Savage 1962	*Carausius morosus* Sinéty	-	-	-	-	Yes, "appendices" are "true Malpighian tubes"
Tilgner *et al.* 1999	*Timema cristinae* Vickery	No	Yes	Yes (Spinules plus six teeth)	No	Yes, "pyriform, filament bearing processes"

of the gut, theoretically enabling symbiont independent digestion of compounds like cellulose (Shelomi *et al.* 2013), but this finding is controversial (Moran *et al.* 2008, Caarels 2011) and does not address other potential functions of the microbiome, such as in defending against plant secondary toxins (Boone *et al.* 2013).

In this study we describe the digestive systems of species widely spread across the Phasmatodea phylogenetic tree (Fig. 1). Our goals were to resolve the confusion in the literature, determine whether or not a common digestive system ground pattern exists for the order (especially relative to other Polyneoptera), and identify species differences in the number and placement of the midgut appendices. In addition, we cultured and stored aerobic and anaerobic gut microbes of the phasmids, to identify possible phylogenetic or dietary patterns in microbiota composition, formulate functional hypotheses, and provide microbial strains for future assays testing these hypotheses.

Materials and methods

The following Phasmatodea species were used: *Diapherodes gigantea* Gmelin, *Eurycantha calcarata* Lucas, *Extatosoma tiaratum* Macleay, *Medauroidea extradentata* Brunner, and *Ramulus nematodes* De Haan (Phasmatidae), plus *Sipyloidea sipylus* Westwood (Diapheromeridae), *Aretaon asperrimus* Redtenbacher (Heteropterygidae), *Peruphasma schultei* Conle & Hennemann and *Anisomorpha buprestoides* Stoll (Pseudophasmatidae), and *Phyllium siccifolium* L (Phylliidae). The latter two were only available for microbial assays. Phasmids were reared at room temperature in the Bohart Museum of Entomology, University of California, Davis, on an *ad libitum* diet of privet (*Ligustrum* sp.) for *Pe. schultei*, *Eucalyptus citriodora* for *Ex. tiaratum* and *D. gigantea*, live oak (*Quercus* sp.) for *Eu. calcarata*, and of rose (*Rosa* sp.) or blackberry (*Rubus* sp.) for all other species. Also examined were wild-caught *Timema* sp. Scudder (Timematidae) found on *Ceanothus* sp. from Gates Canyon, Vacaville, CA, USA (38.387811 -122.077398, 464m). All insects were maintained and used as per the University of California, Davis' Institutional Animal Care and Use Committee guidelines (http://safetyservices.ucdavis.edu/ps/a/IACUC).

For anatomical studies, insects were preserved and dissected in 70% ethanol. Photos were taken from Nikon SMZ2B stereomicroscopes (Chiyoda, Japan), and drawings produced with a *camera lucida* and Adobe Photoshop® CS3 for image processing. For scanning electron microscopy, dissected tissues were preserved in Karnovsky's fixative at 4°C for at least 24 hours, mounted on stubs, sputter coated in gold with a Pelco Auto Sputter Coater SC-7 (Ted Pella Inc., Redding, CA), and viewed using a Philips XL30 TMP Scanning Electron Microscope (F.E.I. Company, Hillsboro, OR) with iTEM Software (Olympus Soft Imaging Solutions GmbH). The pH of the gut contents of fresh *Ar. asperrimus* specimens were measured using Hydrion® pH paper (Micro Essential Laboratory Inc., Brooklyn, NY) and with an AMANI-650 micro pH electrode (Warner Instruments, Hamden, CT), both of which gave comparable results.

To culture microbes, phasmids were anaesthetized on ice and surface sterilized in two changes of 70% ethanol and a rinse in sterile deionized water. Insects were dissected on parafilm with flame-sterilized forceps and scissors in a biosafety cabinet. Gut sections for large insects or whole guts for small ones were macerated in 1mL of sterile PBS (phosphate buffered saline, pH 7.4) with a micropestle, and samples streaked aseptically onto two plates each of 1/10 Tryptic Soy Agar (Remel, Lenexa, KS) with 1% cyclohexamide for bacteria or Rose Bengal Chloramphenicol Agar (BD, Franklin Lakes, NJ) for fungi. One pair of plates per sample was incubated in an anaerobic chamber. All plates were incubated at room temperature. Plates were similarly prepared from samples of leaves collected in a sterile Whirl-Pak® bag directly from the plant and macerated in sterile PBS to isolate surface microbes, swabs from the insects' rear-

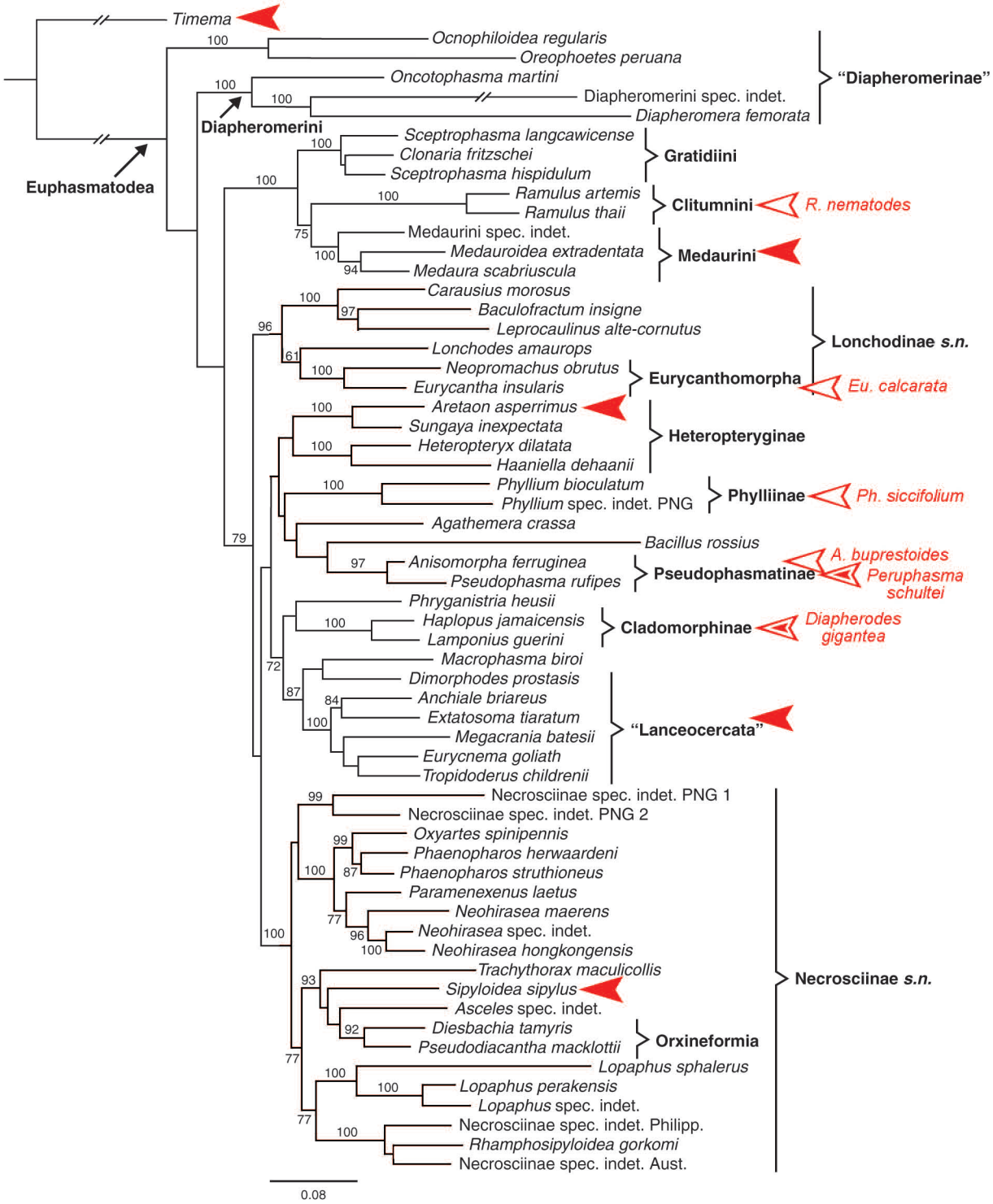

Fig. 1. Location of species used on the Phasmatodea phylogenetic tree *sensu* Bradler. Maximum likelihood tree based on Fig. 2 of Bradler *et al.* (2014), p210, with bootstrap values given. Solid arrows are species included in the tree and this study. Hollow arrows with names are species in the same genus. Double arrows with names point to the subfamily for genera not in the tree.

ing cages to identify potential environmental contaminants, and phasmid eggs to check for vertical microbe transmission as seen in certain Hemiptera (Prado *et al.* 2009).

To identify isolated microbe colonies to species, PCR was used to amplify the 16S rRNA gene with the 27F and 1492R primers for bacteria and the D1/D2 domain sequence of 26S rRNA gene using the NL1 forward and NL4 reverse primers for fungi (Golomb *et al.* 2013). PCR products were purified using a QIAquick PCR Purification kit (Qiagen, Valencia, CA) and products sequenced using the forward primers at the UC Davis DNA Sequencing Facility (http://dnaseq.ucdavis.edu/, Davis, CA) on a 3730 Capillary Electrophoresis Genetic Analyzer with BigDye Terminator v3.1 Cycle Sequencing chemistry (Applied Biosystems, Foster City, CA). Gene sequences were identified by comparing them to the NCBI database using

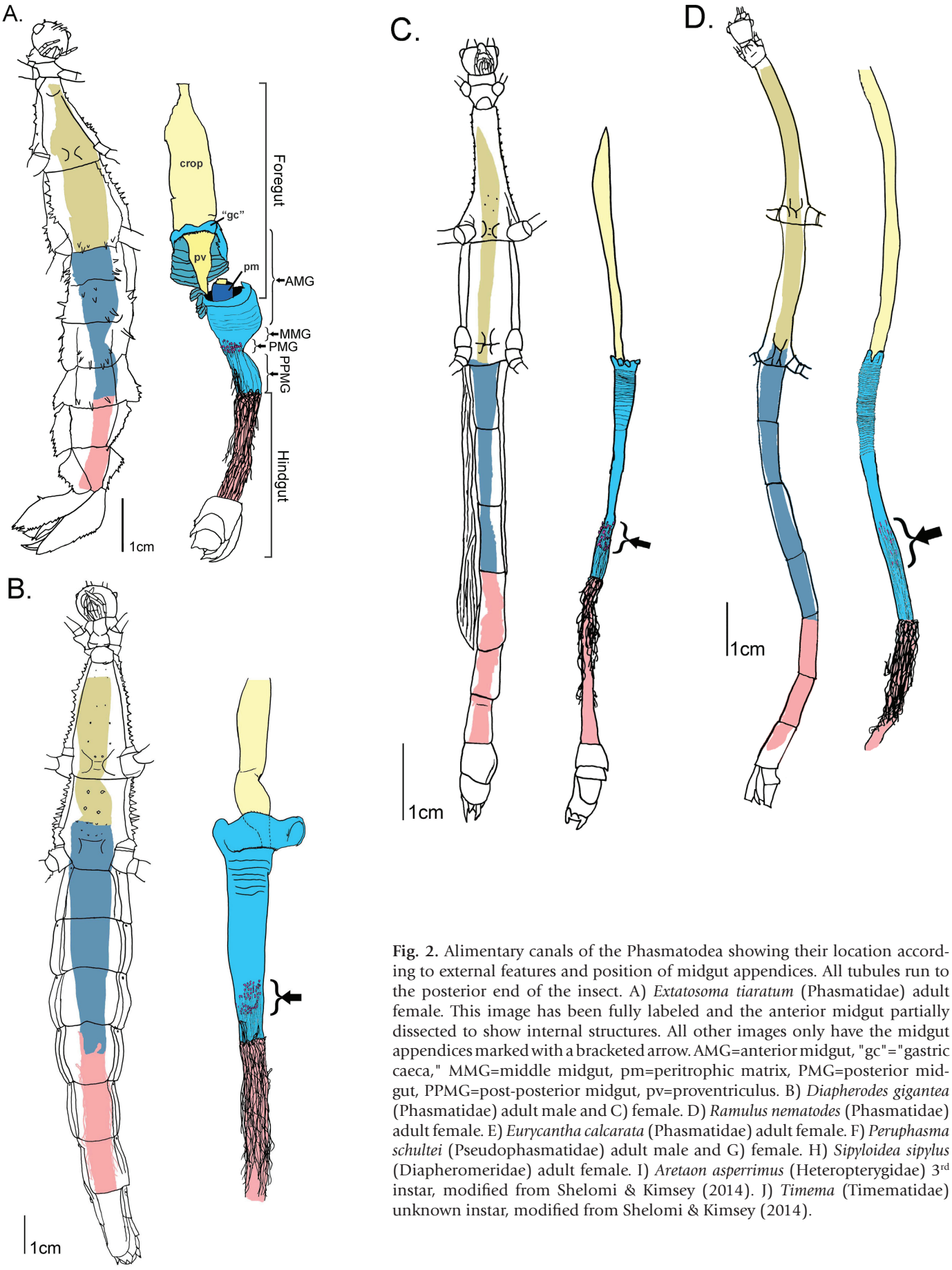

Fig. 2. Alimentary canals of the Phasmatodea showing their location according to external features and position of midgut appendices. All tubules run to the posterior end of the insect. A) *Extatosoma tiaratum* (Phasmatidae) adult female. This image has been fully labeled and the anterior midgut partially dissected to show internal structures. All other images only have the midgut appendices marked with a bracketed arrow. AMG=anterior midgut, "gc"="gastric caeca," MMG=middle midgut, pm=peritrophic matrix, PMG=posterior midgut, PPMG=post-posterior midgut, pv=proventriculus. B) *Diapherodes gigantea* (Phasmatidae) adult male and C) female. D) *Ramulus nematodes* (Phasmatidae) adult female. E) *Eurycantha calcarata* (Phasmatidae) adult female. F) *Peruphasma schultei* (Pseudophasmatidae) adult male and G) female. H) *Sipyloidea sipylus* (Diapheromeridae) adult female. I) *Aretaon asperrimus* (Heteropterygidae) 3rd instar, modified from Shelomi & Kimsey (2014). J) *Timema* (Timematidae) unknown instar, modified from Shelomi & Kimsey (2014).

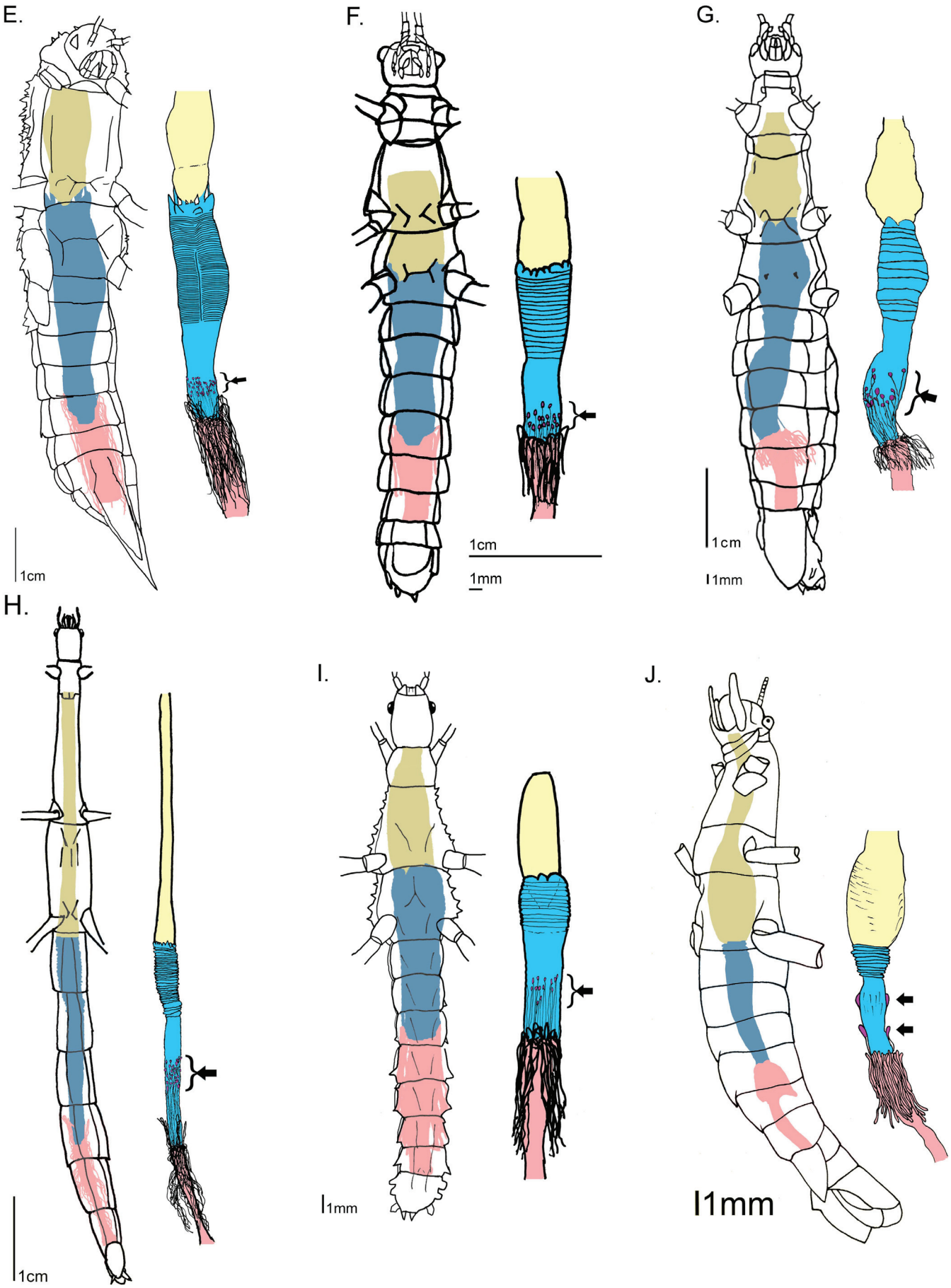

Table 2. pH of the *Aretaon asperrimus* digestive tract. See methods. Values given are for mean pH ± standard deviation, with ranges in parentheses, based on readings taken at the exact middle of each segment. AMG=anterior midgut. HG=hindgut. MMG=middle midgut. PMG=posterior midgut. PPMG=post posterior midgut.

Sex	n	Crop	AMG	MMG	PMG	PPMG	HG	Hemolymph
M	10	3.97±0.5 (3.5-5)	4.74±1.2 (3.75-7)	5.31±0.9 (4-7)	8.61±0.3 (8-9.25)	8.06±0.1 (8-8.13)	6.84±1.1 (5-8.88)	7.18±0.3 (6.88-7.75)
F	13	3.86±0.6 (3-5)	4.36±1 (3.5-7)	5.18±1.2 (3.5-7.25)	8.57±0.4 (7.5-9.13)	8.65±0.5 (7.63-9.2)	6.5±0.8 (5-7.25)	6.91±0.1 (6.6-7)

BLAST (Altschul *et al.* 1990). As cultured microbes can be used for later bioprospecting assays of strains for industrial applications, such as biodegradation, biofuels, or pharmaceuticals (Lacey *et al.* 2007, Poulsen *et al.* 2011, Sitepu *et al.* 2013), isolated bacterial and fungal colonies were preserved in 20% glycerol at -80°C and are publicly available from the Phaff Yeast Culture Collection (phaffcollection.ucdavis.edu).

Results

1. Generality of phasmid gut morphology.— Of the eleven species available, anatomical studies were performed on nine, all but two (*R. nematodes* and *M. extradentata*) from different subfamilies *sensu* Bradler *et al.* (2014) (Fig. 1). Though insufficient numbers of all species were available for quantitative comparative analyses, we were still able to address the contradictions in the Phasmatodea literature, as they are primarily qualitative. All species examined (Figs 2a-j) had a similar digestive tract. The oesophagus continues without demarcation into an expandable, transparent crop lined with cuticle and filled with large, green fragments of shredded leaves. The midgut starts within or just before or after the 3rd thoracic segment. Within the midgut lumen, particulate matter is much smaller and oxidized brown. The midgut can be clearly divided into four parts. The "anterior midgut" is pleated externally and internally except for along a single dorso-longitudinal furrow. The number of pleats varies with species but does not correlate with body size: female *Eu. calcarata* had the most with more than fifty, while female *D. gigantea* had the least with fewer than ten. The pleating gradually decreases then disappears, becoming a smooth-surfaced "middle midgut," whose length can be greater or less than that of the anterior midgut depending on the species or gender. The "posterior midgut," defined as the region studded irregularly with the appendices (elaborated on later), is followed by a smooth "post posterior midgut." The undifferentiated hindgut starts within the 4th or 5th abdominal segment, marked by the origin of the Malpighian tubules. These number in the dozens in all species, with excretory and calciferous tubules as described in Savage (1962).

Comprehensive measurements of pH (Table 2) were only done on *Ar. asperrimus*, however other species showed the same patterns both in our preliminary work and in other literature (Caarels 2011,

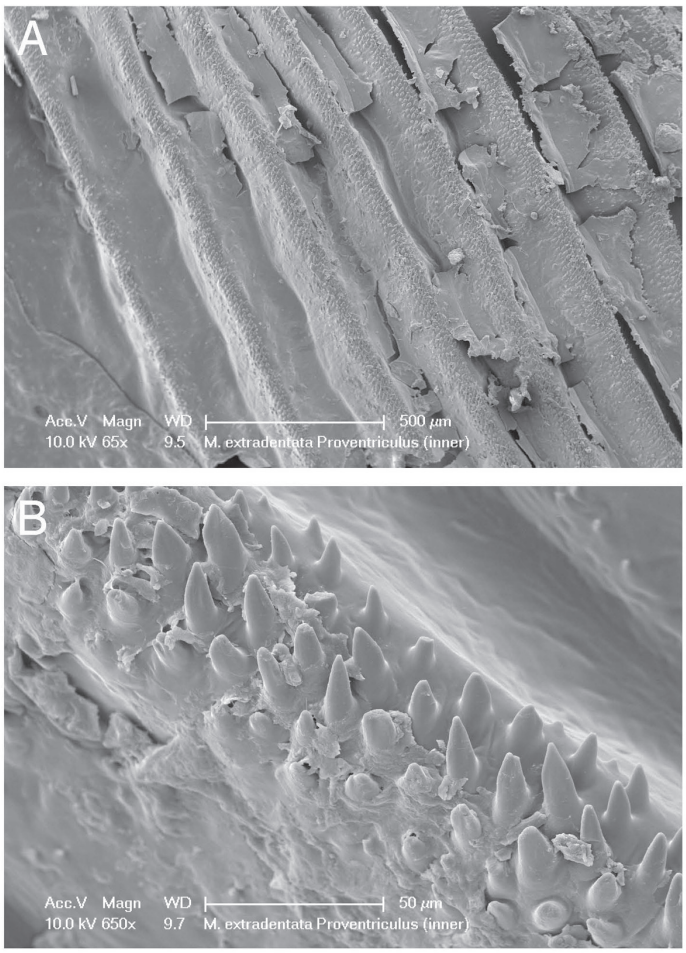

Fig. 3. Scanning electron micrograph of the inside of the *Medauroidea extradentata* (Phasmatidae) proventriculus. Note the spines on top of the ridges.

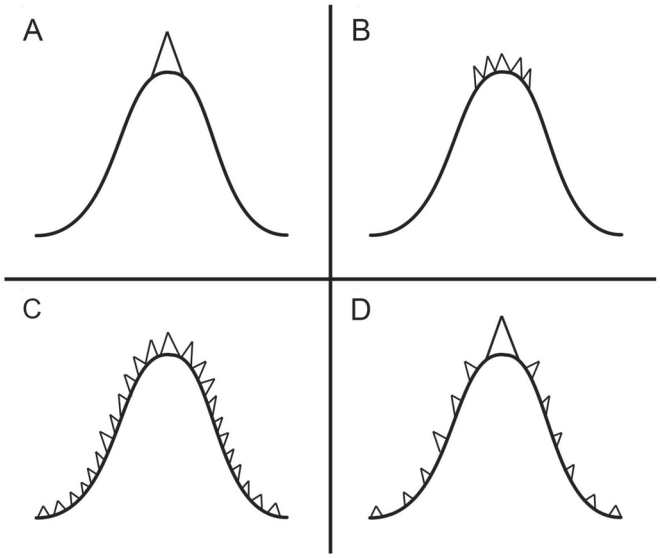

Fig. 4. The four types of Euphasmatodea proventricular dentition. A) A single row of spinules on ridge apex, as in *Peruphasma schultei* and *Aretaon asperrimus*. B) Many irregularly arranged spinules on the ridge apex, as in *Medauroidea extradentata* and *Sipyloidea sipylus*. C) Many tiny spinules all over the ridges and valleys between them, as in *Diapherodes gigantea*, *Eurycantha calcarata*, and *Extatosoma tiaratum*. D) Spinules all over ridges and valleys, with a single row of larger spinules on ridge apex, as in *Ramulus nematodes*. Not to scale.

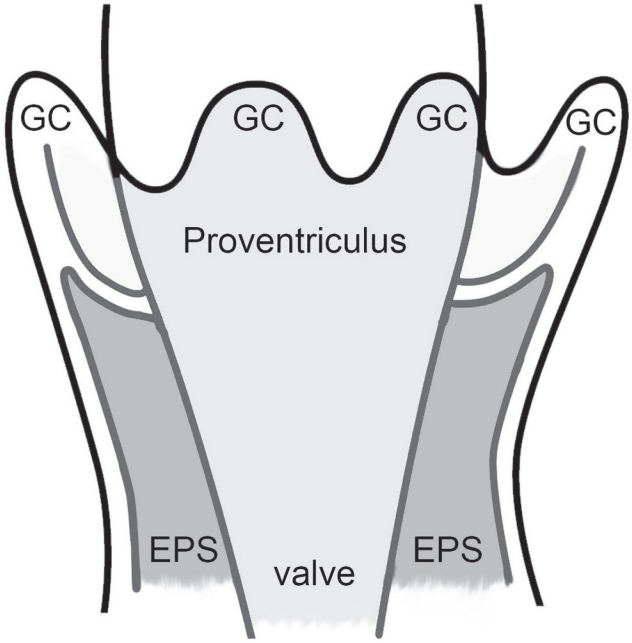

Fig. 5. Diagram of the Euphasmatodea cardia showing attachments between the foregut and midgut. The proventriculus extends into the midgut, attached via midgut tissue, and ends within the anterior midgut with a stomodeal/oesophageal valve. EPS=Exoperitrophic space, GC = "gastric caeca." Not to scale.

Monteiro *et al.* 2014). No significant differences were found between male and female insects. The foregut lumen pH was acidic (3-5) and increased along the midgut, becoming basic (7.5-9) at the appearance of the appendices and returning to acidic (5-7) at the start of the hindgut.

2. Proventriculus and gastric caeca.— In all species, the crop terminates in a muscular proventriculus, originating at the base of the second or third thoracic segment. Externally along the gut, the junction between crop and proventriculus is unmarked. In most cases, the proventriculus is also partially concealed by part of the midgut, as visualized in Fig. 2a, opening into the midgut lumen with a small valve. Internally, the proventriculus is differentiated from the crop by thirty to sixty longitudinal ridges covered in tiny, chitinous spinules (Fig. 3). This dentition varies across species (Fig. 4): some have a single row of spines along the apex of each ridge, some have several irregular "rows" of spines along the apex, and some have spines in the valleys between the ridges as well. Note that *Timema*, as reported in Tilgner *et al.* (1999), also have six large, sclerotized "teeth" in addition to the spined ridges. In nearly all cases, determining the proventriculus' existence requires dissecting away the midgut obscuring it and checking the inside wall for these ridges.

In all phasmids except *Timema*, small pouches of anterior midgut tissue, sometimes still pleated, mark the start of the anterior midgut, obscuring part of the foregut. These pouches are the "gastric caeca" occasionally mentioned in the literature: however, their tissue lacks a differentiated luminal epithelium relative to the anterior midgut, nor do they harbor large quantities of symbiotic microbes, and thus may not be considered "true" caeca (Beadle 1972, Shelomi *et al.* 2013). They are greatly reduced in size when compared to the caeca of Acrididae (Hodge 1943), never longer than 2 mm except in gigantic species such as *Eu. calcarata* or *D. gigantea*. They number in three, six, eight or twelve. A thin membrane connects the midgut to the proventriculus (Fig. 5), with the "gastric caeca" comprising

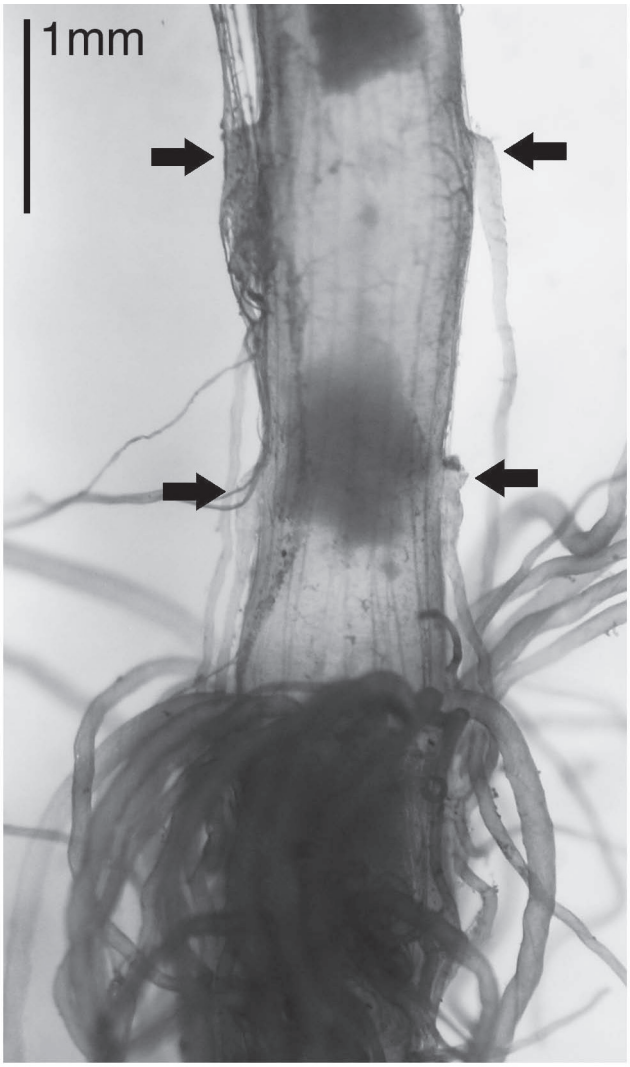

Fig. 6. Micrograph of the *Timema* sp. posterior midgut, hindgut, and excretory tubes. Tissue stained with 1% Chlorazol Black E. Arrows point to the four appendices of the midgut. The larger, darker tubules are Malpighian tubules originating at the junction between the midgut and hindgut.

most of the midgut tissue anterior to this membrane. The food bolus is enclosed within a peritrophic matrix (PM), which persists throughout the rest of the gut and into the frass (Caarels 2011). It is produced at the membrane between the proventriculus and the midgut, meaning the complex is a "cardia": a structure comprised of foregut and midgut tissue producing a type II PM as seen in some Diptera and Lepidoptera, as well as the Polyneoptera orders Dermaptera and Embiidina (Boonsriwong *et al.* 2007, Lehane 1997).

3. Appendices of the Midgut.— For Euphasmatodea, the posterior midgut always starts between the 3rd and 5th abdominal segments. The posterior midgut is usually shorter than the anterior midgut and about 46mm in length, except in longer species such as females of *D. gigantea* and *R. nematodes*. The appendix tubules are long, extending from the midgut towards the posterior end of the insect where they end blindly in the hemolymph. The ampules are less than 1mm in length and open into the exoperitrophic space of the midgut via a small pore (Shelomi & Kimsey 2014). The number of appendices varies among individuals, but correlates to some extent with gut

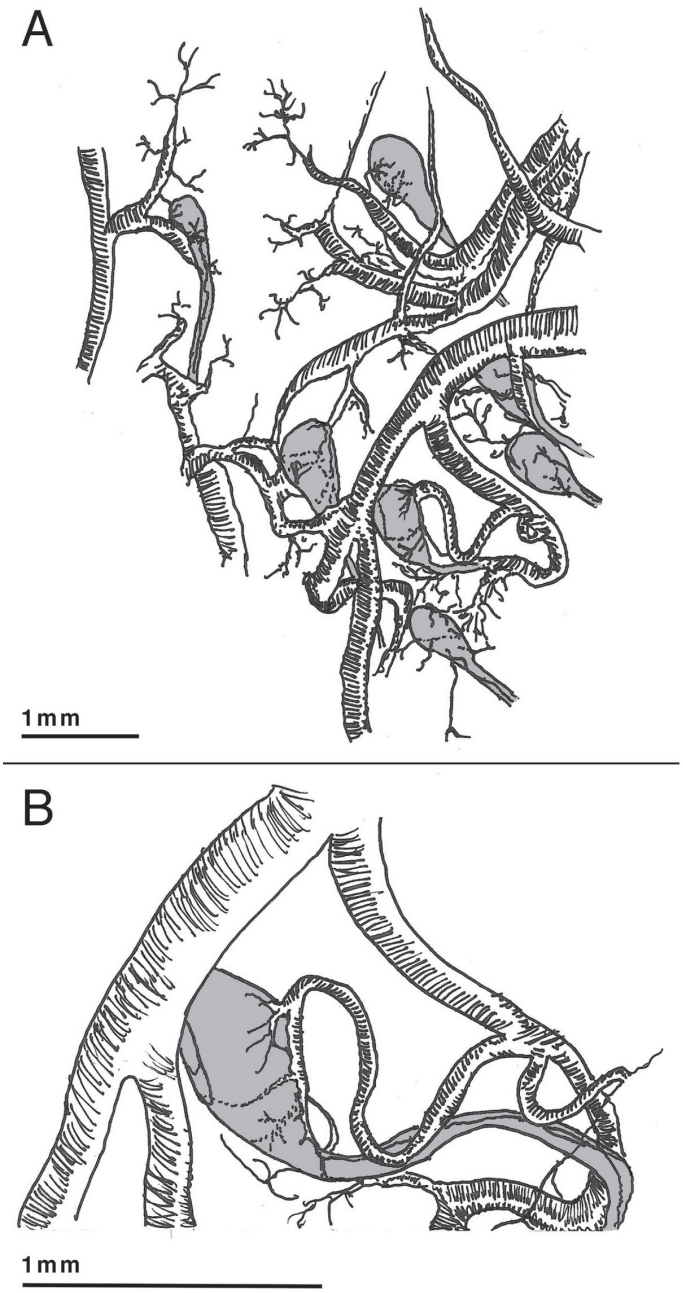

Fig. 7. Diagram of the tracheation of the appendices of the midgut. A) The large tracheal trunks that feed the posterior midgut wall branch off and feed the appendices (dark grey). B) Close-up showing how tracheoles wrap around the appendix bulbs and continue along the tubule. Several other tracheoles will attach to distal sections of the tubule along its length.

circumference: The thin males of *R. nematodes* have about twenty, *S. sipylus* and the males of *P. schultei* and *D. gigantea* have less than fifty, and the large females of *D. gigantea* and *Eu. calcarata* have nearly 100 appendices. Our record was a female *Eu. calcarata* with over 160 appendices. The exception to these patterns is *Timema*, which have only two pairs of appendices on opposite sides of the body, as reported before (Tilgner *et al.* 1999, Shelomi & Kimsey 2014). *Timema* ampules and their openings into the midgut were twice the size of those from Euphasmatodea (Fig. 6). In all cases the appendix tubules are one-third the diameter of the Malpighian tubules and motile: in Euphasmatodea they are coiled like springs, and contract and expand throughout the body cavity (Shelomi & Kimsey 2014). Ampules originate either between or on the longitudinal muscles of the midgut, which may affect the way they move within the body cavity (de Sinéty 1901). In all species, the appendices are heavily tracheated: Branches from midgut tracheal trunks surround the appendix bulbs, and multiple different tracheoles feed each appendix tubule along its length (Fig. 7). These tracheoles supply oxygen both for the muscles surrounding the tubules and responsible for their motion, and for the active excretory and other functions of the appendices themselves.

4. Culturable Microbes.— Table 3 shows a selection of microbe strains cultured from stick insects, all currently available at the Phaff collection. They are mostly aerobes and facultative anaerobes, primarily in the bacterial orders Actinomycetales, Enterobacteriales, Lactobacillales, Pseudomonadales, and Xanthomonodales. Some of the species found in the gut were also successfully cultured from the leaves the insects fed on or in their environment. No significant differences in microbial composition were found among the gut sections of single species or among different species: variations were likely idiosyncratic to the individual and/or due to variations in pH and plant allelochemical presence (Dillon & Dillon 2004).

Discussion

The span of subfamilies examined in this study, including Timematidae, suggests our results can serve as a generalization for the Phasmatodea, and are a reliable tool to address contradictions and identify and correct errors in previous literature. However, it is possible some of the species in Table 1 not included in our study were correctly described, having lost features otherwise common to the Phasmatodea. Overall gut morphologies were similar to those described in Bordas (1897) and de Sinéty (1901), and all our phasmids had proventriculi, in contrast to earlier works. These earlier authors may have overlooked the phasmid proventriculus because it is usually wholly or partially hidden by the midgut, and the border between crop and proventriculus is not clearly visible on the exterior side of the foregut. Proventriculi always contained spiny ridges, including in *Ex. tiaratum* in contrast to the findings of Caarels (2011). Similar ridges and spines were noted in Mantophasmatodea (Klass *et al.* 2002) and Embiidina (Lacombe 1971). The robust proventriculi of Phasmatodea shred the large, green leaf sections from the crop into smaller, oxidized particles, whose increased surface area would facilitate enzymatic digestion (Shelomi *et al.* 2014a,b). Dissection and analysis of the luminal side of the digestive system is necessary to view the macro- and micro-structure of the phasmid proventriculus. Different species showed different patterns of proventricular dentition (Fig. 4), which may be a morphological character of taxonomical significance for Phasmatodea as in other insects (Szinwelski *et al.* 2009).

Besides *Timema*, all phasmids had "gastric caeca" at the anterior midgut, although whether this term is appropriate is debatable as the caeca are histologically undifferentiated from the rest of the anterior midgut (Beadle 1972, Shelomi *et al.* 2013, Shelomi & Kimsey 2014). These midgut projections are connected to the cardia (Fig. 5): a region at the intersection of the foregut and anterior midgut that produces the type II peritrophic membrane. The term "cardia" has also been used to describe the proventriculus itself (King 1988), and may also be confused with the cardial / stomodeal / oesophageal valve between foregut and midgut. Lack of standardized terminology for or clear demarcation between these different regions of the alimentary canal may explain why so much literature

Table 3. Microbes isolated from the Phasmatodea digestive tract and deposited in the Phaff Yeast Culture Collection (UCDFST). Microbes with a † are of the same species as a strain (listed in the parenthesis and marked with a †) isolated from environmental samples such as the diet. Microbes with a * are likewise but of the same genus: a longer RNA sequence is needed to determine whether or not they are the same species. B=Bacteria, FF= Filamentous Fungi, Y=Yeast.

Insect	Organ	Microbes (UCDFST Strain ID)
Anisomorpha buprestoides	Whole gut	Y: *Candida palmioleophila* (10-220), *Cryptococcus flavescens* (10-219), *Rhodotorula mucilaginosa* (10-221)
Aretaon asperrimus	Whole gut	B: *Clostridium celerecrescens* (10-228), *Clostridium sphenoides* (10-230) FF: *Acremonium alternatum* (10-245)
Diapherodes gigantea	Crop	B: *Serratia marcescens* (10-259) Y: *Cryptococcus ramirezgomezianus* (10-258)
	Midgut	B: *Enterobacter cloacea*† (10-257, 11-270†)
	Hindgut	B: *Enterobacter cloacea*† (10-263, 11-270†), *Erwinia persicina* (10-256), *Serratia marcescens* (10-255)
Eurycantha calcarata	Gastric caeca	B: *Brevibacterium oceani* like (09-1475), *Kocuriapalustris*† (09-1473, 09-1392†, 09-1394†), *Lactococcus lactis lactis* (09-1471), *Ochrobactrum tritici* (09-1469), *Pseudomonas putida*† (09-1468, 09-1554†, 11-253†), *Stenotrophomonas maltophila* (09-1472)
	Midgut (anterior)	B: *Lactococcus lactis lactis* (09-1479), *Stenotrophomonas maltophila* (09-1481)
	Midgut (posterior)	B: *Acinetobacter guillouiae** (09-1482, 09-1390*, 09-1418*), *Corynebacterium variabile* (09-1485), *Kluyvera cryocrescens* (09-1467), *Kocuria palustris*† (09-1487, 09-1392†, 09-1394†), *Lactococcus lactis lactis* (09-1484)
	Midgut wall	B: *Enterobacter asburiae** (11-281, 11-276, 11-285, 11-287, 11-277, 11-270*), *Staphylococcus epidermidis* (11-286), *Stenotrophomonas maltophilia* (11-278), *Yokenella regensburgei* (11-288) Y: *Kazachstania unispora* (11-274), *Kazachstania servazzii* (11-273, 11-268, 11-272, 11-275)
	Midgut contents	B: *Acinetobacter guillouiae** (09-1462, 09-1390*, 09-1418*), *Kluyvera cryocrescens* (09-1453, 09- 1455), *Lactococcus lactis lactis* (09-1448, 09-1449, 09-1461), *Staphylococcus hominis* like (09-1464) FF: *Emericella heterothalic* (09-1439) Y: *Rhodotorula sloofiae* (09-1445)
	Hindgut contents	B: *Kluyvera cryocrescens* (09-1453, 09-1455), *Kluyvera intermedia* (09-1517), *Pectobacterium cypripedii* (09-1454) FF: *Aspergillus* sp. (09-1451), *Penicillium* sp.† (09-1450, 09-1413†, 11-250†)
	Hemolymph	B: *Dietzia* sp. (09-1541), *Kocuria palustris*† (09-1458, 09-1392†, 09-1394†), *Methylobacterium extroquens* (09-1542), *Microbacterium testaceum*† (09-1457, 09-1421†), *Stenotrophomonas maltophila* (09-1459, 09-1460), *Tsukamurella tyrosinosolvens* (09-1456)
Extatosoma tiaratum	Crop	B: *Serratia marcescens* (09-1369, 09-1370) Y: *Candida carpophila* (09-1360, 09-1362)
	Midgut	B: *Serratia marcescens* (09-1365) Y: *Cryptococcus ramirezgomezianus* (11-260, 11-264, 11-284), *Candida carpophila* (09-1363)
	Hindgut	B: *Serratia marcescens* (09-1366) Y: *Pichia guillermondii* (09-1361)
Medauroidea extradentata	Whole gut	Y: *Cryptococcus saitoi* (12-344)
Phyllium siccifolium	Midgut	B: *Serratia marcescens* (10-208)
	Hindgut	B: *Serratia marcescens* (10-203)

on Phasmatodea anatomy appears contradictory (Table 1), and complicates efforts to compare digestive tracts across orders.

In all species the appendices of the midgut were tracheated and extended deep into the posterior end of the insect, terminating blindly in the hemolymph similarly to the primary Malpighian tubules, in contrast to Savage (1962). These organs are not known in any other Polyneopteran (Hodge 1943, Bartel 1947, Walker 1949, Lacombe 1971, Klass *et al.* 2002) and are likely an autapomorphy of Phasmatodea (Beutel *et al.* 2013). *Timema* have only a few, large appendices while (considerably longer) Euphasmatodea species have dozens of smaller bulbs, suggesting the increases in the numbers of appendices are an apomorphy of Euphasmatodea and/or due to allometric differences. Evidence suggests appendices have a unique excretory role (Shelomi & Kimsey 2014). They clearly alkalinize the posterior midgut lumen (Table 2), perhaps via secretion of bicarbonate ions into the lumen by the appendices' cells, which express high levels of carbonic anhydrase as found by chemical tests (Monteiro *et al.* 2014) and transcriptomics assays (Shelomi *et al.* 2014a), or by excretion of a yet unidentified compound (Shelomi & Kimsey 2014). The prior increase in pH in the anterior midgut may be explained by compartmentalized enzymatic digestion of polysaccharides such as cellulose (Monteiro *et al.* 2014, Shelomi

et al. 2014a) or by the presence and handling of plant phenolics and tannins. The increased acidity at the midgut/hindgut junction is due to the influx of uric acid from the Malpighian tubules.

None of the hypothesized sister orders to the Phasmatodea have conclusively "Phasmid like" digestive tracts, especially due to absence of midgut appendices. The detritivorous Embiidina have a nearly straight gut with a single bend after the ileum, an undifferentiated midgut, and a proventriculus larger than the crop covered internally with sclerotized spines (Bartel 1947, Lacombe 1971). The carnivorous Mantophasmatodea also have a large proventriculus "armed with weak, papillose sclerites that terminate in three successive whorls of weakly sclerotized lobes" and an undifferentiated midgut (Klass *et al.* 2002). The Grylloblattodea have a large proventriculus lined with twelve "longitudinal divisions characterized by rows of flexible (unsclerotized), backwardly directed lamellae" (Walker 1949). The heavily spined and multi-ridged Phasmatodea proventriculus is thus an apomorphic trait that evolved with their obligatorily folivorous diet: breaking down and oxidizing the relatively large leaf fragments swallowed to a small enough size that the endogenous phasmid digestive enzymes (Shelomi *et al.* 2014a) can act on them. Revised, comparative internal anatomical analyses of several species each of Embiidina, Grylloblattodea, and Mantophasmatodea using modern tools such as electron microscopy or computer tomography would facilitate comparative and systematic work in Polyneoptera.

Insects that depend on microbial fermentation for digestion have diverticulae or enlarged and alkaline fermentation chambers to house the symbionts (Dillon & Dillon 2004). The demonstrated absence of such modifications or of known lignocellulolytic microbes in the phasmid gut supports symbiont independence for phasmid plant cell wall digestion, as previous works hypothesized (Watanabe & Tokuda 2010, Caarels 2011) or suggested using culturing-independent methods (Shelomi *et al.* 2013). Most phasmid gut and fecal microbes with known insect associations lack the host specificity or phylogenetic congruence seen with true symbionts (Kikuchi *et al.* 2009). For example the yeast *Candida carpophila* has been found in *Blastophaga psenes* (fig wasp), *Xestobium plumbeum* (bark beetle), *Batrocera oleae* (olive fly), and ants, as well as general habitats such as fruits, soil, and tree surfaces (Lachance *et al.* 2011). *Lactococcus lactis* is commonly found on plant surfaces and dairy environments (Salama *et al.* 1995). Other ubiquitous or cosmopolitan species found are the bacteria *Kluyvera* sp., *Lactococcus lactis* ssp. *lactis*, *Serratia marcescens*, and *Stenotrophomonas maltophilia*; and the yeasts *Cryptococcus ramirezgomezianus*, *Pichia guilliermondii*, and *Rhodotorula mucilanginosa* (Li *et al.* 2005, Zacchi & Vaughan-Martini 2002). The known ecology of these microbes suggests they were likely acquired through feeding on plant materials rather than maintained in the gut.

Even if their digestion is non-microbial, however, phasmid gut microfauna can play other, facultative roles such as preventing infection by pathogenic microbes (Mead *et al.* 1988) or reducing heat shock (Oliver *et al.* 2010). This could have impacts on conservation: the failure to rear the endangered New Zealand walking stick *Dryococelus australis* Montrouzier (Buckley *et al.* 2009, Carlile *et al.* 2009) in the San Diego and Bucharest Zoos due to development of gastrointestinal disease could be attributed to a change in gut flora (Benyó András, pers. com.). In particular, the finding of *Serratia marcescens*, a monoterpene-degrading bacteria (Boone *et al.* 2013), in all phasmids fed monoterpene-rich *Eucalyptus* suggests the microbe may function in these species to reduce the toxicity of the diet: a hypothesis that would explain how such phasmids can consume as many leaves as they do during outbreak years in Australia, where they are a significant cause of defoliation and Eucalypt dieback (Jurskis & Turner 2002). However, this bacteria species is cosmopolitan and was also found in the guts of blackberry-feeding *Phyllium*, so it is not exclusive to *Eucalyptus* feeders. Future work comparing the monoterpene-degrading ability of these different strains [Phaff Collection 09-1566, 10 203, 10-255] could show if differences exist between the same species of bacteria found in phasmids with diets varying in monoterpene content. These mutualisms are likely facultative (Moran *et al.* 2008) given that they are more strongly associated with diet or habitat than species (Thakuria *et al.* 2010, Ferrari & Vavre 2011) and that we found no evidence of vertical transmission of microbes via eggs, itself unlikely given the hatching behavior of phasmids (Severin & Severin 1911). One can theoretically test if microbes are obligate symbionts via antibiotics, however this is best done with a target microbe in mind (Visotto *et al.* 2009), and it can be difficult to determine whether reduced fitness in such experiments is due to the absence of microbes or the toxicity of the antibiotic to the insect directly (de Vries *et al.* 2004). Though these phasmids were lab-reared, and rearing conditions cause rapid changes in gut microbiota (Husseneder *et al.* 2009), we do not expect digestion in wild phasmids to be any less symbiont independent.

In summary, our analysis spanning multiple subfamilies and using scanning electron microscopy to examine finer details has resolved several inconsistencies in the Phasmatodea literature. In particular, we presume that spiny proventriculi—derived from an omnivorous ancestor and modified for herbivory— are ubiquitous or at least plesiomorphic in Phasmatodea, and "gastric caeca" plus an oesophageal valve are apomorphic for Euphasmatodea. Whether the "gastric caeca" are true caeca or merely small, undifferentiated extensions of the anterior midgut is a semantic matter, but to avoid confusion we propose referring to the entire system as the cardia (Fig. 5). We also propose "spinules" as the name for the smaller proventricular projections in Phasmatodea after Tilgner *et al.* (1999), to avoid confusion with larger, macroscopic "teeth" as in *Timema* and Blattodea (Wigglesworth 1966).

We also hypothesize that the anatomy and microbiology of the Phasmatodea gut reflect the evolutionary pressure exerted by their mimetic camouflage and diet (Azevedo *et al.* 2013). Having a body cavity so much longer than it is wide precludes the development of the large diverticulae or paunches needed for microbial activity as in Blattodea (Watanabe & Tokuda 2010) or countercurrent flow to increase enzymatic activity time as in Acrididae (Terra 1990). Instead, a spiny proventriculus physically digests the leaves prior to enzymatic digestion in the anterior midgut, whose pleats increase available surface area. Further compensating for the lack of microbes is the unusually large complement of Phasmatodea digestive enzymes, including multiple copies of cellulase and pectinase genes (Shelomi *et al.* 2014a). This synergism between physical and chemical digestion allows phasmids to survive on a purely herbivorous diet, and even thrive to the levels that cause economic or ecological damage (Jurskis & Turner 2002). Lastly, while we found no evidence for symbiont-mediated plant cell wall digestion, the role of the gut microbiota in Phasmatodea may still play a role in their survival, with potential impacts on the establishment of new species or on conservation efforts.

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