

ORIGINAL ARTICLE

A bacterial filter protects and structures the gut microbiome of an insect

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Associations with symbionts within the gut lumen of hosts are particularly prone to disruption due to the constant influx of ingested food and non-symbiotic microbes, yet we know little about how partner fidelity is maintained. Here we describe for the first time the existence of a gut morphological filter capable of protecting an animal gut microbiome from disruption. The proventriculus, a valve located between the crop and midgut of insects, functions as a micro-pore filter in the Sonoran Desert turtle ant (*Cephalotes rohweri*), blocking the entry of bacteria and particles $\geq 0.2 \mu\text{m}$ into the midgut and hindgut while allowing passage of dissolved nutrients. Initial establishment of symbiotic gut bacteria occurs within the first few hours after pupation via oral–rectal trophallaxis, before the proventricular filter develops. *Cephalotes* ants are remarkable for having maintained a consistent core gut microbiome over evolutionary time and this partner fidelity is likely enabled by the proventricular filtering mechanism. In addition, the structure and function of the cephalotine proventriculus offers a new perspective on organismal resistance to pathogenic microbes, structuring of gut microbial communities, and development and maintenance of host–microbe fidelity both during the animal life cycle and over evolutionary time.

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Introduction

Nutritional mutualisms between animals and microbes are widespread (Backhed *et al.*, 2005; Hongoh, 2011), often taking place in the alimentary canal where microbes can have an important role in food digestion (McFall-Ngai *et al.*, 2013). Maintenance of fidelity between mutualistic partners seems straightforward for obligate endosymbionts that are cosseted inside special cells or organs associated with the gut (Moran *et al.*, 2008). In contrast, microbes in the lumen of the one-way bilaterian gut generally face a downstream flow of ingested content that may flush away resident bacteria (for example, Nyholm and McFall-Ngai, 2004; Blum *et al.*, 2013) or introduce non-symbiotic and pathogenic microorganisms that can be harmful for both the host and the resident bacterial community (for example, Nelson *et al.*, 2012; Jones *et al.*, 2013; Cariveau *et al.*, 2014). With this

consistent downstream flow of ingested food and microorganisms, how does the host maintain partner fidelity with its beneficial microbes?

In general, the composition of gut microbiomes is known to be structured through diet (Muegge *et al.*, 2011), gut physiology (Kwong and Moran, 2015) and compartmentalization (Engel and Moran, 2013), avoidance of parasites through hygienic behavior (Cremer and Sixt, 2009), physical barriers (for example, peritrophic matrix (Hegedus *et al.*, 2009)) and innate immune systems (Nyholm and Graf, 2012). Here we report on a novel means of host manipulation of gut microbiota: an anatomical filter capable of protecting the host and their microbiota from disturbance by non-symbiotic microbes and likely involved in promoting high specificity between host and symbiotic microbiota over evolutionary time.

Turtle ants in the genus *Cephalotes* (118 species) consume a mostly herbivorous diet (Russell *et al.*, 2009) supplemented by pollen, bird feces and vertebrate urine (Baroni Urbani and de Andrade, 1997; Powell, 2008). *Cephalotes* hosts 16–20 core bacterial strains (Hu *et al.*, 2014; Sanders *et al.*, 2014) that are present in large numbers in the midgut and hindgut lumen (Roche and Wheeler, 1997) and likely

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have an important role in host nutrition (Russell *et al.*, 2009; Anderson *et al.*, 2012). Microbial communities in *Cephalotes* are highly similar among nestmates, within and between species (Hu *et al.*, 2014), and have codiversified with their hosts indicating a history of vertical transmission at the colony level (Sanders *et al.*, 2014). Such vertical transmission of microbes is usually associated with obligate intracellular symbionts (Moran *et al.*, 2008; Engel and Moran, 2013) rather than inhabitants of the digestive tract, where horizontal acquisition of microbes with ingested food is frequently observed (Pernice *et al.*, 2014). This level of core gut microbiota stability over evolutionary time is quite unusual, and the mechanism enabling this stability is unclear (Hu *et al.*, 2014; Sanders *et al.*, 2014).

We investigated the function of the proventriculus, a valve in the gut of *Cephalotes rohweri*, to determine whether it could serve as the mechanism for maintaining gut microbiota partner fidelity. The proventriculus separates the crop and midgut in insects and exhibits a remarkably divergent shape in *Cephalotes* ants, the function of which has been speculated on (Baroni Urbani and de Andrade, 1997; Roche and Wheeler, 1997) but remains unknown. A symbiont sorting mechanism has been recently described in the bean bug *Riptortus pedestris*—a gut constriction that is only permeable to the passage of their gut symbiont, an environmentally acquired bacterium in the genus *Burkholderia* (Ohbayashi *et al.*, 2015). Unlike *R. pedestris*, *Cephalotes* gut symbionts are maternally inherited and consistently found across different species of *Cephalotes*. A second distinction is that while *Burkholderia* is hosted in a special, isolated portion of the gut in *R. pedestris*, the symbiont community of turtle ants is found in the lumen throughout the alimentary canal and in constant exposure to ingested food. In host-gut symbiont systems, ingested, non-symbiotic microorganisms may cause detrimental changes in the community structure of the resident bacteria, resulting in lower immune response and development of diseases in the host (Sansone, 2004; Stecher *et al.*, 2013). The *Cephalotes* proventriculus has previously been demonstrated to filter larger solid particles (> 12 µm, (Roche and Wheeler, 1997)) and we hypothesize that it may also prevent particles as small as bacteria from transiting the gut. To test this hypothesis, we investigated the morphology of the proventriculus, its porosity and its association with the microbiome structure within the alimentary canal of *Cephalotes*.

Materials and methods

Colonies used for experiments

Cephalotes rohweri colonies were collected from Sonoran Desert scrub habitat at Tucson Mountain Park, Tucson, AZ, USA (permit from Pima County Natural Resources, Parks and Recreation) in the spring

and summer of 2012 and 2013. Colonies live in cavities bored by beetles into the branches of the tree *Cercidium microphylla*. During warm months, workers forage on the branches and leaves and rarely visit the ground. The average distance between collected colonies was 0.5 miles and trees were spaced widely in the habitat, ensuring that we used separate colonies. Colonies were maintained in the laboratory in nests consisting of a wood cutout between two sheets of plexiglass inside a flouon-painted acrylic box, reared at ~ 25 °C, provided with water in cotton-stoppered test tubes, and fed a diet of freeze-killed, bisected cockroaches and 20% honey water.

Imaging

All specimens used for scanning electron microscopy were dissected from freshly killed ants and dehydrated in an ethanol series followed by critical point drying. We then microdissected the dry specimens and mounted them on stubs with conductive carbon adhesive tape, followed by sputter coating with platinum. All samples were imaged with a Hitachi S-4800 Type II Field Emission Scanning Electron Microscope (Hitachi High Technologies America Inc., Pleasanton, CA, USA). The specimen in Figure 1c was treated with 10% KOH for 24 h, and then washed in Milli-Q (Millipore Corporation, EMD Millipore, Merck KGaA, Darmstadt, Germany) water for 24 h before dehydration to remove all tissue except the cuticular layer comprising the upper surface of the proventriculus and wall of the crop.

Gut specimens for transmission electron microscopy (TEM) imaging were dissected from ants and fixed in 4% formaldehyde, 0.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.0) for 8 h at 4 °C, rinsed in buffer and fixed in 2% osmium tetroxide for 30 min. Specimens were again rinsed and then dehydrated in an ethanol series followed by acetone, infiltrated in a series of Spurr's resin/acetone mixtures before being embedded in Spurr's. Ultrathin sections were cut with a diamond blade and ultramicrotome, placed on formvar film grids, stained with uranyl acetate and lead citrate, and imaged with a Philips CM-12 TEM (FEI, Hillsboro, OR, USA). Light microscopy specimens were embedded as above for TEM, but with chlorazol black and 1% methylene blue as stains. Images in Figures 1b and e and Figures 4a and d were colorized in Adobe Photoshop CS6 (Adobe Systems Incorporated, San Jose, CA, USA) to indicate the different parts of the structure. The illustration in Figure 1a was drawn by MCL based on scanning electron microscope, TEM, confocal and light microscopy data. Original, uncolored micrographs are provided for all data in Supplementary Figures S1–S3.

Sequencing and analysis of gut microbiome

We sequenced ants from field-collected colonies, but kept in laboratory conditions for 2 months (colonies

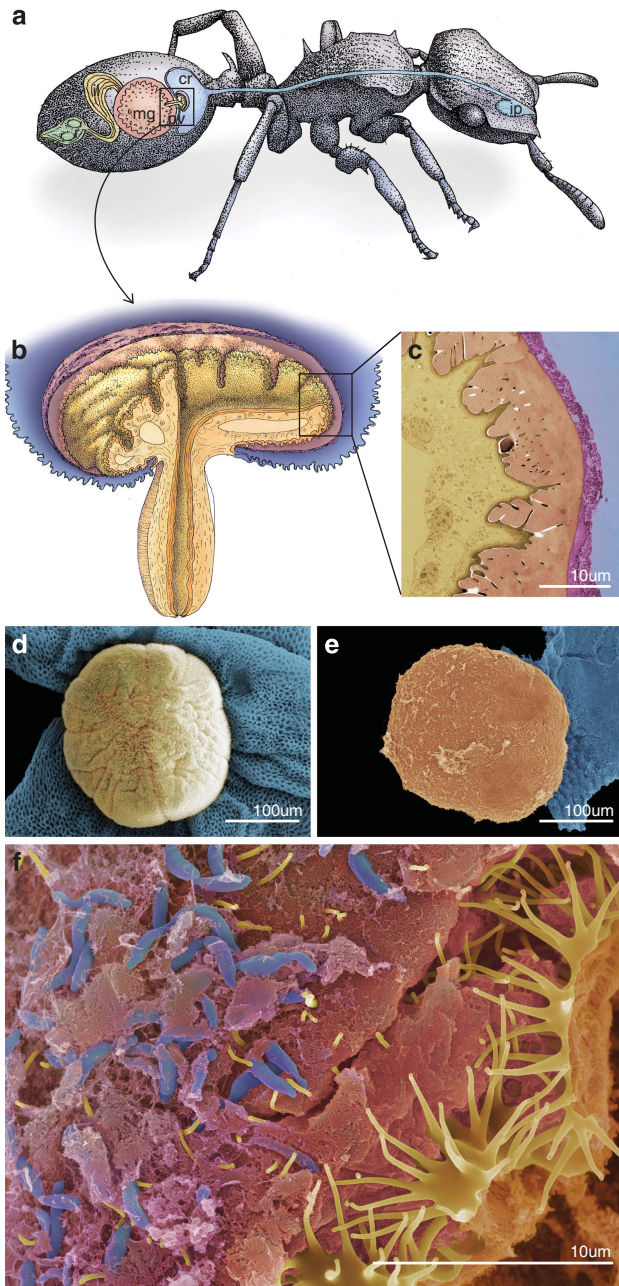


Figure 1 Morphology of the *C. rohweri* proventriculus. Panels show (a) the location of the proventriculus relative to other parts of the gut, (b) cutaway diagram showing the channels within the proventriculus, (c) TEM cross-section through the proventriculus and filtering layer, (d) Scanning electron microscope (SEM) top view of the proventriculus with the filtering layer removed from the cuticular spines, (e) SEM top view of the proventriculus with the filtering layer in place and (f) SEM of a cross-section of the proventriculus and associated layer showing cuticular spines, filtering layer and bacteria on surface. In panels b–f the cuticular and underlying cellular parts of the proventriculus are colored yellow, the filtering layer is red, bacteria and detritus on the surface of the filtering layer are purple and the crop lumen is blue. Illustration and SEM colorization by MCL.

1a, 6a and 7a) in addition to colonies freshly collected from the field (colonies 1b, 2b, 3b and 4b). DNA was extracted from each of the following compartments of the alimentary canal

(‘gut compartments’ from hereafter): the crop, proventriculus, midgut, ileum and rectum. DNA was also extracted from a rinsed leg (control), surface-sterilized larva and scrapings from the nest interior surface. Minor workers were dissected under sterile conditions: each ant was chilled in a sterile petri dish at 0 °C for 5 min, and repeatedly rinsed vigorously in sterile Milli-Q water. The gaster was opened by insertion of sterile ultra-fine forceps (Dumont #5SF) between tergites 1 and 2, such that tergite 1 was lifted up and away from the body in a way that prevented the exterior surface from contacting the interior of the gaster at any time. The whole intact gut was then lifted from the interior of the gaster and placed in sterile phosphate buffer with a separate pair of freshly sterilized forceps. The crop, midgut, ileum and rectum were then separated and placed in separate tubes, again with freshly sterilized forceps for each separation. Specimens were discarded when any portion of the gut ruptured, touched another portion, contacted the outside surface of the exoskeleton, or was otherwise thought likely to have been contaminated. All tools were flame sterilized with 100% ethanol between every change in position during the dissections, and tools were cleaned via sterilization and sonication between dissections. To acquire sufficient DNA for sequencing, we pooled each gut compartment from five workers for each colony. Sequences are available under accession number SRP068471.

We ground each sample with sterile pestles in enzymatic lysis buffer and extracted DNA using the Qiagen DNAeasy Blood and Tissue (Qiagen Inc., Valencia, CA, USA) extraction kit following the pretreatment protocol for Gram-positive bacteria with an increase of the pretreatment lysozyme incubation period to 24 h. Samples were screened for bacterial DNA using a universal 16S ribosomal RNA primer (Tet199F/1513R) and DNA in each sample was quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Extraction yielded enough bacterial DNA to attempt pyrosequencing (>3 ng µl⁻¹) for most, but not all samples (Supplementary Table S2).

DNA was sent to Research and Testing Laboratories, Lubbock, TX, USA for Roche 454 FLX-Titanium pyrosequencing (Roche Applied Science, Indianapolis, IN, USA), using the 28F-519R bacterial assay for the V1-V3 variable regions of the 16S rDNA gene (Dowd *et al.*, 2008). Sequence data was processed using Mothur v.1.33.0 (Schloss *et al.*, 2009) following the standard operating procedure for 454 generated data (http://www.mothur.org/wiki/454_SOP). We discarded sequences with fewer than 200 bp or more than two mismatches to the primer, aligned them to the SILVA database (Pruesse *et al.*, 2007; Schloss, 2009) and removed chimeras using UCHIME (Edgar *et al.*, 2011). The remaining sequences were matched against the Mothur 16S rRNA reference (RDP) database, and mitochondrial,

chloroplast, archaea, eukaryote and unknown sequences were removed.

Sequences were grouped into operational taxonomic units with at least 97% similarity, and representative sequences were classified both by matching against the Mothur RDP reference database and by nucleotide BLAST against the NCBI database. Statistical comparisons were conducted on data subsampled to 1685 sequences (the lowest number of sequences yielded by a gut sample). To investigate the similarity of bacterial communities along the alimentary canal, we compared samples using principal coordinates analyzes and hierarchical analyses with Jaccard distances as a measure of diversity within a gut compartment and Ward minimal variance criterion for sample similarity clustering. To investigate what variables best explained variation in the bacterial communities, we conducted PermanovaG (Chen *et al.*, 2012). All analyzes were done in R (Team RDC, 2008) and Mega v. 6.0 (Tamura *et al.*, 2013). The following R packages were used: Vegan (Oksanen *et al.*, 2015), Ape (Paradis *et al.*, 2004), GUniFrac (Chen *et al.*, 2012) and RColorBrewer (Neuwirth, 2014).

Fluorescent bead experiments

To determine the size of particles capable of passing the proventriculus, adult ants were removed from lab-reared colonies and placed in closed petri dishes, where they were provided with a 100- μ l droplet of 20% sucrose in Milli-Q water, 0.1% methylene blue dye and 0.2% yellow-green fluorescent latex microsphere beads (Fluoresbrite, Polysciences Inc., Warrington, PA, USA). We separately tested particle sizes of 6, 2, 0.5 and 0.2 μ m. Twenty-four hours after feeding, each ant was immobilized by cooling at 0 °C for 5 min, rinsed in Milli-Q water and dissected under sterile conditions similar to those described above. Because contamination by beads from the exoskeleton or ruptured gut could lead to false positives, great care was taken to flame-sterilize the dissecting tools and surfaces during every repositioning, and dissection tools were frequently examined under a fluorescence microscope for bead contamination. Ants were discarded if any portion of the gut ruptured, if contact between the gut and outside of the ant occurred, if the blue dye had not advanced to the rectum after 24 h (indicating that the food had passed all the way through the gut), or if they had numerous beads visibly adhered to their exoskeleton due to contact with the food droplet (posing a high risk of contamination by beads during dissection). Each portion of the freshly dissected gut (infrabuccal pocket of the mouth, crop, midgut, ileum of hindgut and rectum) was placed under a separate cover slip in phosphate buffer and immediately examined for the presence of beads. To ensure that contact with the gut contents did not diminish the brightness of the yellow-green fluorescent dye, we incubated 0.5 μ m beads with midgut and hindgut

contents at 27 °C for 24 h, confirming that brightness of the microspheres did not decrease ($n=3$). To ensure that the blue dye and sugar solution did not diminish the brightness of the yellow-green fluorescent spheres, we also incubated a sample of the mixture at 27 °C for 24 h and confirmed that the brightness did not differ from a fresh sample of the spheres.

Gut compartments of ants fed with 6, 2 and 0.5 μ m beads were examined and photographed using a Nikon Eclipse E600 fluorescence microscope (Nikon Instruments Inc., Melville, NY, USA) using $\times 20$ and $\times 40$ dry lenses with $\times 10$ eyepieces ($\times 200$ and $\times 400$ magnification) under fluorescein isothiocyanate lighting. The number and location of beads in each gut compartment were noted and photographs of each gut compartment were taken using a Diagnostic Instruments RT Color Spot Microscope Camera 2.2.1 (Sterling Heights, MI, USA). The smallest beads we tested, 0.2 μ m, were not bright enough to view under the fluorescence microscope using dry optics. We therefore examined the gut compartments of 20 ants fed 0.2 μ m fluorescent beads using a Zeiss 510 Meta Laser scanning confocal microscope on an Axioimager Z1 with a $\times 40$ oil plan fluor NA 1.3 lens (Carl Zeiss Microscopy LLC, Thornwood, NY, USA), under which the 0.2- μ m beads appeared clearly. Each gut compartment was first searched for the presence of beads visually using the multi-track line scan for the fluorescein isothiocyanate and Rhodamine filters at $\times 400$, under which beads appeared bright bluish green and autofluorescent structures such as chitin and spherocrystals (Bution and Caetano, 2010) appeared yellow-green, making them easy to distinguish. Each slide was separately examined by MCL or PAPR, who dissected the ants, and PJ, who had no prior expectation of the location of beads or of the gut compartment being viewed. MCL/PAPR and PJ separately noted the number and location of beads present in each slide. We then acquired confocal images of a representative region of each gut compartment using the 488-nm line on the Ar laser and the 543-nm green HeNe laser. In the cases where beads were found on midgut, ileum or rectum slides, we used image z-stacks to determine whether the beads were on the outside surface of the organ (likely due to contamination during dissection), inside the lumen of the organ (likely due to passage through the proventriculus) or in the buffer outside the organ (likely due to contamination during dissection and mounting).

Video analysis of oral-rectal trophallaxis, formation of filter layer

Two colony subsamples consisting of 8–10 mature workers, 3–5 larvae and 3–5 pupae were video recorded from the time a pupa began to move its legs. A new adult (callow) was considered to have emerged from the pupa when it stood up and walked for the first time, and the time from emergence to

the first instance of oral–rectal trophallaxis was calculated from the video. We chose to use the terminology oral–rectal trophallaxis because it clearly describes the contact between the ants. Previous authors have used a number of terms including abdominal trophallaxis (Wilson, 1976) and anal trophallaxis (Sanders *et al.*, 2014).

Results and discussion

Structure of the proventriculus

The hymenopteran proventriculus is derived from the foregut crop and is similarly lined with cuticle (Eisner, 1957). Although the proventriculus of most Myrmicine ants is comprised of a simple tube and sphincter (Eisner, 1957), the genus *Cephalotes* has evolved a novel proventricular structure consisting of a large, flattened bulb covered in small cuticular spikes (Figure 1). Our electron and confocal microscopy data revealed that the proventricular surface facing the interior of the crop is coated with a thick, non-cellular mucilaginous layer that is held in place by the spiky surface, leaving no obvious opening through the valve to the midgut (Figures 1c–e). This acellular layer is similar in some ways to the peritrophic matrix—an envelope of chitin fibers and glycoproteins produced in the midgut of many invertebrates (Hegedus *et al.*, 2009)—but it is produced in the foregut and remains adhered to the proventriculus rather than passing to the midgut. Liquid moving from the crop to the midgut must pass through this layer before entering the channels within the proventriculus that converge to a single tube entering the midgut (Figures 1b and d). TEM data revealed that the tube to the midgut is ringed with muscle, suggesting that liquid is pulled through the layer by pumping action from below. The sclerotized upper surface of the valve, however, is rigid and lacks associated musculature, with no mechanism by which it could move or open.

Our micrographic evidence further indicated that although bacteria are typically associated with the crop wall and the surface of the proventricular layer facing the lumen of the crop (Figure 1d, Supplementary Figure S1), they are absent within the proventricular layer and connecting channels to the midgut. These data may suggest that bacteria are unable to pass through the proventricular layer.

Gut microbe distribution in relation to the proventriculus

To determine whether the proventriculus has a role in structuring the gut microbiome, we investigated (1) whether bacterial communities differ upstream (crop) and downstream (midgut) of the proventriculus, and (2) whether the proventricular surface hosts a distinct bacterial community from the crop. Using 454 amplicon pyrosequencing, we sequenced the crop, proventricular surface, midgut, hindgut ileum

and rectum of field-collected workers from seven spatially segregated colonies. We found no effect of colony source (four sequenced from freshly collected colonies vs three kept in the lab for 2 months) on variation within colonies (PermanovaG, $F=1.457$, $P=0.234$), allowing us to analyze all samples together.

We found striking partitioning of microbial communities between gut compartments, with highly similar communities occurring in all seven colonies (Figure 2). Examining the effect of location within the gut on microbial composition (blocked by colony), we found that gut compartments harbored significantly different communities of bacteria, with more variation explained by gut location than colony identity (PermanovaG, colony $F=1.779$, $P=0.065$, gutpart $F=12.729$, $P=0.001$). Midgut samples yielded high numbers of reads for a single bacterial strain in the Oplitales clade (Verrucomicrobia; Figure 2), suggesting that this section primarily hosts an Oplitales. The same strain was present at lower numbers throughout the gut.

Although the midgut, ileum and rectum communities were nearly identical across all colonies, the crop and proventricular surface showed more variation. We found that the surface of the proventriculus did not host a distinct bacterial community from that of the crop wall and crop contents (Figure 3), although the crop community as a whole was distinct from the rest of the gut. The crop tended to be dominated by several strains of Rhizobiales, a group of bacteria that has been hypothesized to fix nitrogen (Russell *et al.*, 2009) and to be involved in the digestion of pollen (Hu *et al.*, 2014). The exception was Colony 4b, in which the crop and proventricular samples contained predominantly *Lactobacillus sp.* Workers from Colony 4b were collected when gathering extrafloral nectar from a cactus (*Cylindropuntia acanthocarpa*) near the host tree, while workers from all other colonies were foraging exclusively on the host tree. Ingestion of bacteria in the nectar is one possible explanation for the differing crop microbiota of this colony.

Supporting our filter hypothesis, bacteria associated with the nest environment and foregut were absent or present at only very low numbers in the midgut, hindgut and rectum of all colonies examined (Figures 2 and 4). For example, the six most numerous operational taxonomic units of the crop and proventriculus (excluding operational taxonomic unit 1 Oplitales from calculations) accounted for >90% of sequences obtained from these organs, yet accounted for only 0.6% of sequences from the midgut and hindgut samples. Similarly, only 4 of the 40 most numerous larval and nest environment operational taxonomic units (90% of larval and nest reads) were present in the midgut and hindgut, comprising only 0.008%. Most impressively, although 14 317 *Lactobacillus sp.* sequences were found in the crop and proventriculus samples for colony 4b, not a single read was

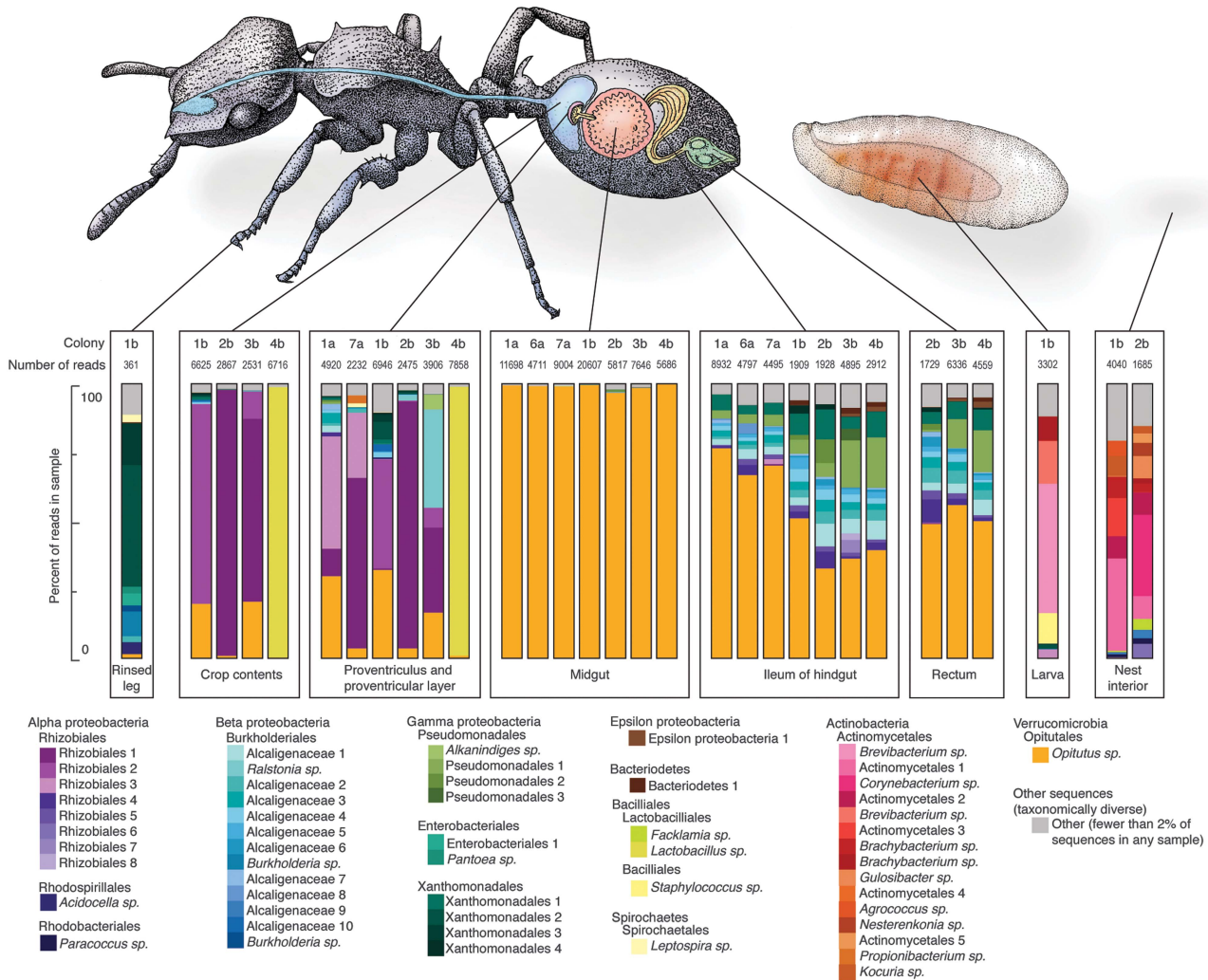


Figure 2 The microbial communities found in different gut compartments (crop, proventriculus, midgut, ileum and rectum), the larva and the nest interior. The percentage of sequences in each sample is shown for 97% operational taxonomic units found in the crop contents, proventriculus and associated layer, and midgut for seven field-caught colonies, as well as control (rinsed leg), larval and nest interior sequences for samples that could be sequenced (Supplementary Table S2). Illustration by MCL.

recovered from the midgut, ileum and rectum samples of this colony (Figure 4).

It is worth noting that the similarity between communities of each compartment is associated with their location relative to the proventriculus, that is, foregut communities tend to be more similar to each other than to the midgut and hindgut communities (Figure 3b). The significant partitioning of gut communities, along with the fact that most bacterial phylotypes from the foregut are not found in the midgut nor in the hindgut supports the idea that the proventriculus is involved in structuring and protecting the gut microbiome.

Porosity of the proventriculus

To determine the filtering capability and porosity of the proventriculus, we fed ants yellow fluorescent microspheres of one of the following sizes: 6, 2,

0.5 and 0.2 μm , given in a solution of water, sugar and blue dye. After 24 h we dissected the ants and examined the gut compartments for the presence of microspheres. In no case did we find that microspheres of any of size passed through the proventriculus, and even the smallest microspheres tested, 0.2 μm , did not pass beyond the crop of 20 ants (14 mature workers, 5 callow workers, 1 male, Supplementary Figure S4, Supplementary Table S3). Nonetheless, the dye successfully reached the midgut and hindgut, indicating that dissolved molecules of food pass through the proventricular filter. In a more detailed examination of the position of particles, we found that 0.2 μm beads were present on the surface of the filtering layer facing the crop interior but never within the proventricular channels ($n=5$, Supplementary Figure S5, Supplementary Table S4). Previous work has suggested that particles as large as 12 μm can pass the proventriculus of *C.*

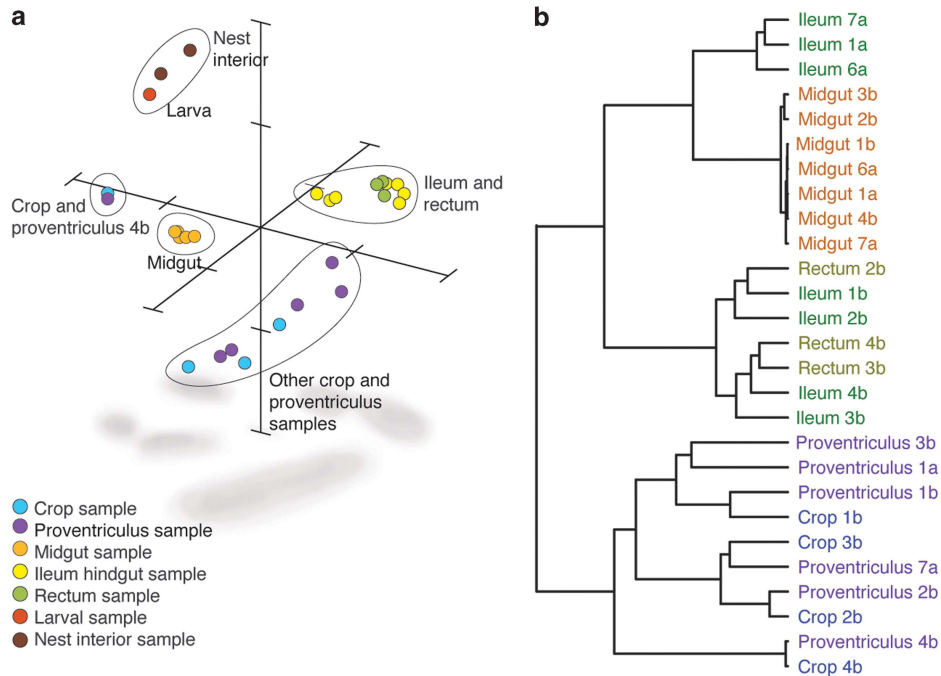


Figure 3 Principal coordinate analysis plot showing clustering among sample types (a) and (b) Hierarchical clustering of gut samples (Created with R packages Vegan (Oksanen *et al.*, 2015), Ape (Paradis *et al.*, 2004) and RColorBrewer (Neuwirth, 2014)).

rohweri (Roche and Wheeler, 1997). However, under light microscopy it is difficult to distinguish such particles from the many spherocrystals (Supplementary Figure S4) produced in the midgut (Bution and Caetano, 2010).

This remarkable filtering capacity is comparable to commonly used water purifying systems that typically use ceramic filters with 0.2 μm pore size to remove bacteria, for example, Milli-Q. Because all but the very smallest bacteria cannot pass a 0.2- μm filter (Razin and Hayflick, 2010), we conclude that the proventriculus of *Cephalotes rohweri* has the capability of excluding most ingested bacteria while allowing passage of dissolved molecules.

To determine whether the filtering mechanism is unique to the proventriculus of Cephalotine ants, we tested the filtering ability of the proventriculus of two other ant genera. The genus *Pogonomyrmex* belongs to the same subfamily of ants as *Cephalotes*, the Myrmicinae, but has the simple funnel-shaped organ that is typical of the subfamily (Eisner, 1957). In four *Pogonomyrmex rugosus* individuals we found that 2 μm beads easily passed through the proventriculus to the midgut (Supplementary Table S6). The genus *Camponotus* belongs to the subfamily Formicinae, which is characterized by a complex proventriculus with four hair-lined, sclerotized channels leading to a muscular pumping bulb that forces liquid through to the midgut (Eisner and Wilson, 1952). In *Camponotus fragilis*, we found that although 6 and 2 μm beads were unable to pass the proventriculus, many thousands of 0.2 μm beads passed through and were found in the lumen of the midgut (Supplementary Figure S7, Supplementary

Table S6). This result suggests that the fine filtering capability of the *Cephalotes* proventriculus is a unique highly derived trait.

Although the proventricular filter prevents adult *Cephalotes* from digesting particles, colonies are still able to use solid foods by harnessing the digestive capabilities of larvae. When we fed colonies the 0.2- μm bead mixture for 7 days ($n = 5$), we found that mature workers packed the beads into infrabuccal pellets and fed them to larvae, the guts of which were subsequently filled with beads (Supplementary Figure S7, Supplementary Table S5). Ant larvae lack the specialized gut morphology of adults but produce digestive enzymes adults lack (Hölldobler and Wilson, 1990), and are thus capable of digesting solid food. Larvae can then reciprocally provide digested liquids to feed workers (Cassill *et al.*, 2005). The colony as a whole therefore has two distinct digestive systems working in tandem.

Inoculation of the gut and formation of the filter layer
 Previous work has suggested that workers emerge from the pupa with a sterile gut (Russell *et al.*, 2009), and our imaging studies also failed to detect gut bacteria in late-stage pupae and newly eclosed workers (Supplementary Figure S8). If the proventriculus prevents the transit of bacteria through the gut and ants emerge from the pupal stage with a sterile gut, how do adult ants acquire the specialized bacteria found in the midgut and hindgut?

To find out, we video recorded the behavior of young, incompletely sclerotized (callow) workers from two colonies in the first 8 h after eclosing.

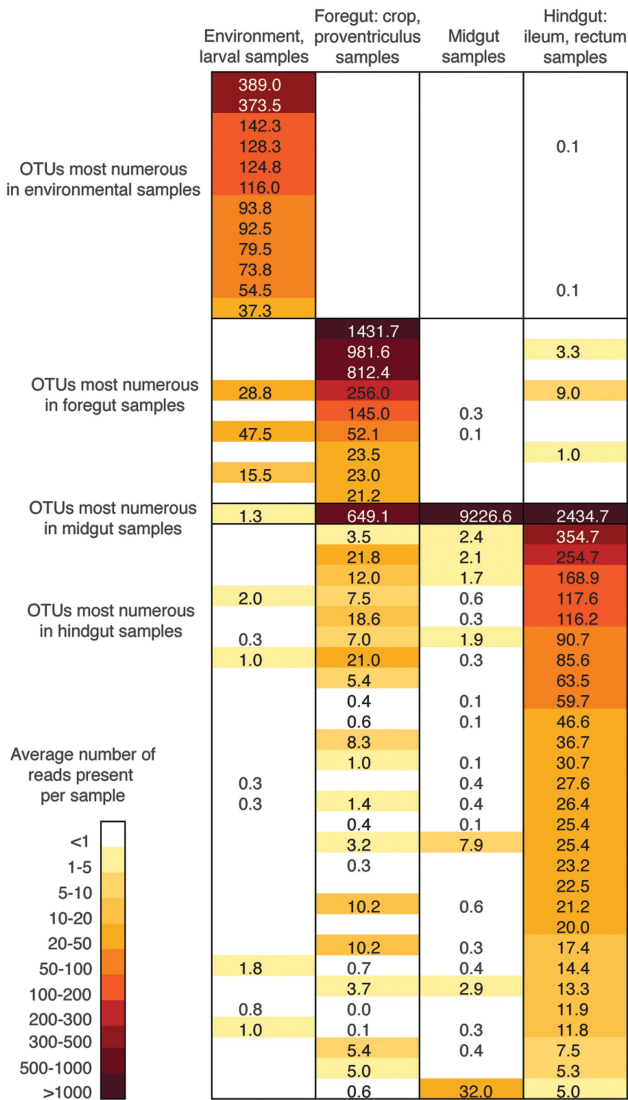


Figure 4 The average number of total reads per sample in environmental (nest and larval), foregut, midgut and hindgut samples for the 50 most numerous operational taxonomic units.

In line with previous observations (Wilson, 1976; Wheeler, 1984), we found that new workers solicit and consume rectal fluid (oral-rectal trophallaxis) from their nestmates soon after eclosion. In colony 8b, we observed that a new worker drank rectal fluid in repeated short bouts for 23 min, 6 h after eclosing (Figure 5f). Similarly, in colony 5b a callow first drank for 11 min, 3.25 h after eclosing (Figure 5e). This behavior is a potential route for microbial inoculation, provided that bacteria are able to pass through the proventriculus of new workers.

To find out whether the filtering layer is in place during this behavior, we dissected the worker from colony 5b immediately after this first bout of oral-rectal trophallaxis and prepared the proventriculus for scanning electron microscope imaging, along with ones from a pupa, a several-day-old callow and a mature forager from the same colony (Figures 5a and d). In addition, workers of different ages from

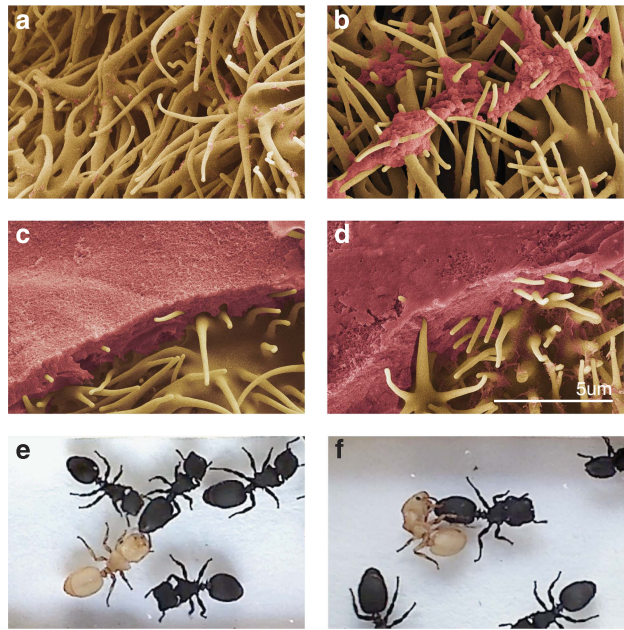


Figure 5 Scanning electron microscope images showing the development of the filtering layer before and after oral-rectal trophallaxis. In all panels the cuticular parts of the proventriculus are colored yellow and the filtering layer is red. Panels show cross-sections of the proventriculus and filtering layer from (a) a late-stage pupa, (b) a newly emerged worker that has just completed oral-rectal trophallaxis, (c) a several-day-old callow and (d) a mature worker. Panels (e) and (f) show still images from video in which callow workers (light color) participate in oral-rectal trophallaxis. The callow in e was dissected to produce the image in b.

colonies 1b, 7a, 8b and 9b were stained and examined via light microscopy to confirm that the filtering layer developed similarly across colonies. In all cases the layer was absent in late-stage pupae, first appeared in several-day-old callows and was thickest in older workers (Figure 5). The worker from colony 5b did indeed lack the layer (Figure 5b) at the time it consumed rectal secretions from a nestmate. Thus the filtering layer on the proventriculus is not formed or thickened until after the young worker adult engages in oral-rectal trophallaxis (Figure 5). New workers therefore have only a brief window of time to inoculate their gut by drinking the rectal excretions of nestmates before the proventriculus is sealed against further passage of bacteria.

Hypothetical role in immunity

The microbiota of *Cephalotes* may change when infected by pathogens such as *Spiroplasma* (Kautz et al., 2013), a bacterium that is typically acquired through the alimentary canal (Bové, 1997). It is worth noting that *Spiroplasma* is among the smallest known bacteria (Razin et al., 1998; Razin and Hayflick, 2010), smaller than the particle sizes tested here and potentially capable of infiltrating the proventriculus of *Cephalotes*. However, other microorganisms that may invade the alimentary canal of

Cephalotes are typically $>0.2\ \mu\text{m}$, for example, nematodes (Yanoviak *et al.*, 2008) and microsporidians (P. Stock, personal communication). In these cases, pathogens may use alternative strategies to overcome the proventriculus filtration system, such as infecting larvae or young workers in which the proventriculus is porous or not completely sealed. This hypothesis was raised by Yanoviak *et al.* (2008) who noticed that workers infected by a parasitic tetradonematid nematode were generally smaller than healthy workers and presented lighter cuticular pigmentation. Similarly, workers of *C. rohweri* infected with microsporidia also have lighter pigmentation (P. Stock, personal communication). Therefore, although the filtration capacity of the proventriculus may make the transit of parasites and non-symbiotic microorganisms difficult, the relative importance of its role in host immunity requires further testing.

Conclusion

We conclude that the unusual proventriculus and associated layer of *C. rohweri* is capable of excluding most if not all bacteria from entering the midgut of these ants, while still allowing dissolved nutrients to pass. To our knowledge, this is the first report of an animal organ capable of preventing ingested bacteria from transiting the gut while allowing food to pass. The recent finding of a similar mechanism in hemipterans, in which ingested microbes are sorted through a gut constriction (Ohbayashi *et al.*, 2015), suggests that microbial-filtering organs may be also found in other insects that associate with extracellular gut microbes. A microbial-filtering capability may be associated with fluid feeding, as both insect species have a liquid-based diet as adults. In the bean bug *Riptortus pedestris*, a microbial filter sequesters symbionts acquired from the environment, whereas in *Cephalotes* microbial filtering is possibly responsible for the persistent association with gut symbionts over time (Hu *et al.*, 2014) as well as the pattern of vertical transmission and coevolution between host and microbes (Sanders *et al.*, 2014). Other mechanisms of gut microbiome manipulation such as specificity through physiology and diet (Kwong and Moran, 2015) may work in concert with this novel proventricular barrier to prevent the passage and establishment of foreign microbes.

Older workers host a pool of symbionts to inoculate new adults within colonies, while newly mated queens are the vertical transmission route to new colonies. Sharing of the colony-level microbiome may be important to the functioning of many eusocial animals, and a similar pattern of oral–rectal feeding (or coprophagy) occurs in other societies including termites (Kohler *et al.*, 2012), bumblebees (Koch and Schmid-Hempel, 2011) and naked mole rats (Jarvis, 1981). For *Cephalotes*, the proventriculus

may function as part of the colony-level immune system, protecting the core gut microbiome of adults from introduction of pathogens. Processing of solids is delegated to larvae, which host an entirely different gut microbial community and act as a separate digestive system within the colony. Understanding the mechanism of microbe partitioning in this system opens up new avenues for research into host–microbe interactions and the nutritional role of the gut microbiome.

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

Research was conceived by MCL, PAPR and DEW; experiments were designed by MCL, PAPR and DEW with technical advice from PJ and AA; MCL, PAPR, PJ and AA conducted the experiments. Data were analyzed by MCL and PAPR. Manuscript was written by MCL with assistance from PAPR, all authors edited the final version of the manuscript. Figures were created by MCL.

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