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Gut microbiota of dung beetles correspond to dietary specializations of adults and larvae

SHANTANU P. SHUKLA,* JON G. SANDERS,* MARCUS J. BYRNE† and NAOMI E. PIERCE*
*Department of Organismic and Evolutionary Biology, Harvard University, 26 Oxford Street, Cambridge, 02138, MA, USA,
†School of Animal, Plant, and Environmental Sciences, University of Witwatersrand, Wits 2050, South Africa

Abstract

Vertebrate dung is central to the dung beetle life cycle, constituting food for adults and a protective and nutritive refuge for their offspring. Adult dung beetles have soft mandibles and feed primarily on nutritionally rich dung particles, while larvae have sclerotized mandibles and consume coarser dung particles with a higher C/N ratio. Here, using the dung beetles *Euoniticellus intermedius* and *E. triangulatus*, we show that these morphological adaptations in mandibular structure are also correlated with differences in basic gut structure and gut bacterial communities between dung beetle life stages. Metagenome functional predictions based on 16S rDNA characterization further indicated that larval gut communities are enriched in genes involved in cellulose degradation and nitrogen fixation compared to adult guts. Larval gut communities are more similar to female gut communities than they are to those of males, and bacteria present in maternally provisioned brood balls and maternal 'gifts' (secretions deposited in the brood ball along with the egg) are also more similar to larval gut communities than to those of males. Maternal secretions and maternally provisioned brood balls, as well as dung, were important factors shaping the larval gut community. Differences between gut microbiota in the adults and larvae are likely to contribute to differences in nutrient assimilation from ingested dung at different life history stages.

Keywords: 16S rRNA, C/N ratio, digestion, *Euoniticellus*, microbiome, symbiosis

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Introduction

One reason for the great success of holometabolous insects (those insects that undergo complete metamorphosis) is that the larvae and adults of the Holometabola typically do not compete with each other for resources (Truman & Riddiford 1999). Each life stage is specialized for a different task. For example, lepidopteran caterpillars spend the majority of their time feeding on leaves, while adults are primed for mating and laying eggs. Larvae and adult holometabolous insects rarely feed on the same food source, or adults may not feed at all (Grimaldi & Engel 2005; Price *et al*. 2011). Insect life stages may therefore differ not only differ in their diets, but also in the morphological and physiological mechanisms used to process these diets (Applebaum 1985; Price *et al*. 2011).

However, clear separation of different life history stages is not always the case, and a good example of this is seen in the coprophagous dung beetles, a highly successful group of insects where both larvae and adults consume and recycle vertebrate dung. Species of 'true' dung beetles belonging to the subfamily Scarabaeinae utilize dung either as dwellers, tunnellers or rollers, depending on whether the ovipositing female stays in the dung pat, tunnels under it or rolls away a dung ball. On closer scrutiny, however, it has become apparent that the different life history stages of dung beetles also specialize on different components of the dung that they utilize. Herbivore dung is a mixture of undigested plant fragments, intestinal secretions (including mucus and dead epithelial cells), bacteria, fungi and other undigested material (Holter & Scholtz 2007).

Correspondence: Shantanu P. Shukla, Department of Entomology and Research Group Insect Symbiosis, Max Planck Institute for Chemical Ecology, Hans Knoell Str. 8, Jena 07745 Germany, Fax: +49 (0)3641 57 1502; E-mail: sshukla@ice.mpg.de

Adult beetles use their highly specialized, soft mandibles to consume the smaller and more nutritious dung particles. By feeding on dung particles ranging between 10 and 100 μm in diameter, adult beetles concentrate nutrients by threefold to fivefold and maximize the assimilable nitrogen by avoiding dung particles with a higher C/N ratios (Holter & Scholtz 2007) and filtering out large plant fragments present in dung (Holter *et al.* 2002). In contrast, dung beetle larvae have retained ancestral, heavily sclerotized mandibles that they use to chew the coarser and drier plant fibres in the dung of their natal brood ball (Byrne *et al.* 2013). Thus, despite dung being the source of nutrition for both adults and larvae, the larvae ingest a diet that is comparatively rich in refractory plant biopolymers (including cellulose, hemicellulose, lignin, xylan and pectin) with a relatively higher C/N ratio.

Although such dietary specializations in dung beetle life stages are well known, little research has investigated whether the gut microbiota of the adults and larvae are similarly specialized, and the potential role of the microbiota remains contentious (Holter 2016). Microorganisms have been suggested to be important mutualistic partners that facilitate the digestion of dung (Goidanich & Malan 1962; Halffter & Edmonds 1982; Hanski & Cambefort 1991; Halffter 1997; Price *et al.* 2011; Hernández *et al.* 2015). Bacterial counts in dung beetle larval guts are known to be higher than in their food, indicating possible proliferation of certain bacteria capable of metabolizing cellulose and pectin inside the larval gut (Goidanich & Malan 1962). An increase in amino acid concentration in the dung beetle pupal shell compared to the brood ball and dung has been interpreted as conversion of organic nitrogen in the dung into amino acids by the gut bacteria (Rougon *et al.* 1990). Adult and larval dung beetles have also been predicted to host different bacterial communities in their guts (Halffter & Matthews 1971; Rougon *et al.* 1990), adapted to support the type of dung upon which the life stages have evolved to specialize. Estes *et al.* (2013) used sterile rearing and a combination of molecular and culture-based techniques to analyse the transmission of the microbiota of the dung beetle, *Onthophagus taurus*. This study provided strong evidence that the female parent transmits specific microbiota to her offspring via the maternal brood chamber, and supported the hypothesis that gut microbiota are important for development. However, the study did not investigate whether the adult and larval gut microbiota showed signs of specialization, or the relative contribution of male and female gut microbiota to that of their offspring.

Dung beetle larvae, but not adults, have been suggested to host a specialized microbial gut community due to the high fibre content in their diet (Halffter & Matthews 1971; Rougon *et al.* 1990). However, it remains unclear whether larval gut microbiota produce digestive enzymes that aid dung digestion, or whether larvae survive on easily digestible microbial biomass present in the dung, bypassing the need for microbial enzymes (Holter 2016). Microbial biomass within dung (originating from the mammalian gut) may provide larvae with essential nutrients including amino acids, and a high rate of dung ingestion by the larvae may still provide sufficient nutrition for larval growth, despite lower assimilation of nutrients (Holter 2016).

However, as the dung of the brood ball ages, the way in which the larva continues to derive nutrition from the coarser, drier portions of dung that are rich in plant fibre is unclear. The presence of a dilated, compartmentalized hindgut (resembling a fermentation chamber) in many species of dung beetle larvae that disappears in the adult stage (Halffter & Matthews 1971) has fuelled the suspicion that cellulose-fermenting bacteria may play a vital role in larval digestion. It is also unclear, at a more fundamental level, whether dung beetle larvae and adults differ in their gut microbiota. Microbiota are known to differ between insect life stages (Vasanthakumar *et al.* 2008) and between diverse diets (Colman *et al.* 2012), and any documentation of such differences in the context of dung beetles could provide insight into the role of larval and adult gut microbiota in digestion, their routes of acquisition and transmission in larval stages, and the potential contribution of parents and dung as a source of mutualistic microbiota.

Adult and larval guts can be considered as dissimilar micro-ecosystems, differing in the gut physicochemical conditions (such as anoxicity, pH) due to presence/absence of a compartmentalized hindgut, and the type of nutrients entering the system due to modifications in mandibular structure. Dietary specialization due to physicochemical differences arising from such ontogenetic morphological adaptations could support and select for specialized microbial gut colonizers between dung beetle life stages. The current study aims to describe and compare the microbiota in larvae and adults of the tunnelling dung beetle species, *Euoniticellus intermedius* (Reiche) and *E. triangulatus* (Harold) (Scarabaeidae: Scarabaeinae: Oniticellini). A sexually mature *Euoniticellus* female tunnels below a dung pat, dragging down dung to make balls (brood balls). She lays a single egg inside each of the brood balls. The ball provides the entire food supply for a single larva's development. With each egg, the female also deposits a maternal secretion, or 'maternal gift', which acts as an anchor for the egg (Fig. 1), is consumed immediately by the larva upon hatching and is known to be important

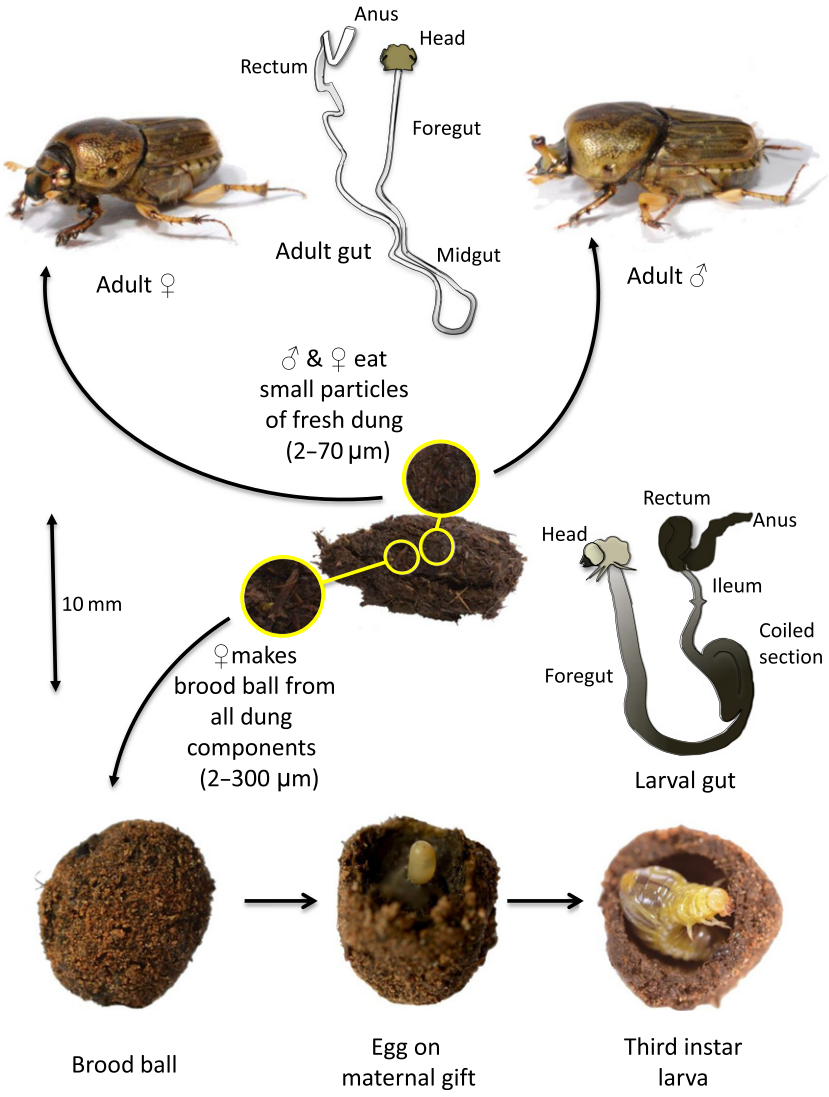

Fig. 1 Dung usage by the life stages of *Euoniticellus intermedius*. Adult males and females feed on filtered dung particles and have a noncompartmentalized gut. Adult females use all dung components (including larger dung particles and plant fibre therein) to make the brood ball. Each egg is laid in the brood ball on top of an inner layer consisting of maternal secretions called the maternal gift. Dung beetle larvae feed on the fibre-rich dung particles in the brood ball to complete development and have a compartmentalized gut containing a pouch-like section in their hindgut. [Colour figure can be viewed at wileyonlinelibrary.com]

for early instar larval survival (Byrne *et al.* 2013). The larva pupates in a faecal shell and eventually emerges as an adult to repeat the cycle (Fig. 1).

We explored three questions in the current study. First, we examined whether differences in the feeding ecology between adult and larval dung beetles are reflected in differences in their bacterial gut communities. Second, as larvae face limitations in the availability of utilizable carbon and nitrogen relative to adults, we tested the hypothesis that estimated functional gene abundances in larval and adult gut microbiota would reflect differences in their capacities for cellulose digestion and nitrogen metabolism. Finally, we investigated whether secretions in the maternal gift could facilitate symbiont transmission from mother to offspring (Halffter 1997; Byrne *et al.* 2013; Estes *et al.* 2013).

Materials and methods

Rearing and collection of beetles

Adult *Euoniticellus intermedius* and *E. triangulatus* were collected near Johannesburg in South Africa, brought to the laboratory and reared as previously described (Pomfret & Knell 2008; Byrne *et al.* 2013). Beetles were reared in the laboratory for a year (about six generations) prior to sampling for this study, during which time males and females were paired randomly. Briefly, adults were provisioned with moist soil (heat sanitized at 60 °C for 2 days) and cow dung (collected fresh and stored at −14 °C for several weeks). Pairs of beetles were reared at 25 °C at ambient humidity (approximate range of 30–70%) and a 12:12 h light-dark cycle. Randomly harvested brood balls (from separate parents)

were opened to collect eggs, samples of 0.10 to 0.20 g of the brood ball and the maternal gift. This was carried out before the eggs had hatched, approximately 1 or 2 days after they were laid. Third instar larvae were collected approximately 3 weeks after initial brood ball construction (Fig. 1). All individuals in the study received the same dung stock, and hence, the dung consumed by adults was from the same source that was used by those beetles to construct dung balls on which the larvae fed.

Similar quantities of dung were sampled from the same dung used to feed the beetles (referred to as 'untreated dung' in this study; Fig. 1). Untreated dung was collected from a dairy farm at a different location from where adults were collected, and mixed, frozen and thawed, before being presented to the beetles, and sampled for DNA extraction. Whole guts (from proventriculus to rectum) of larvae and adults were dissected and preserved in 100% ethanol. All stages were reared in captivity, and all samples were collected independently, such that adults used in this study were not parents of the larvae analysed. Eggs, adults and larvae were not surface sterilized or washed before dissection. Genomic DNA was extracted from the following samples: brood balls, maternal gifts, eggs, larval guts, adult female guts, adult female whole-body samples for *E. intermedius* and *E. triangulatus*, as well as adult male guts and whole-body samples for *E. intermedius*. 'Whole-body samples' refer to the tissue remaining after removal of the guts, and these were included in the study to account for the possibility of mutualistic bacteria being present on the cuticle or in extra-intestinal tissue in the beetles. Due to larger sample sizes and availability of males, data presented here are primarily for *E. intermedius* (unless otherwise mentioned), with comparisons between the two species whenever possible.

Bacterial community characterization

Bacterial DNA was extracted and purified from the samples using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, USA) following the modified PowerSoil protocol of Rubin *et al.* (2014). Pure DNA was quantified using a Qubit dsDNA HS assay kit (Life Technologies) using a Qubit Fluorometer (Invitrogen). Amplification and sequencing were performed at Argonne National Laboratory, Lemont, IL, USA, using the 515f (GTGCCAGCMGCCGCGGTAA) and Golay barcoded 806R (GGACTACHVGGGTWTCTAAT) primers (Caporaso *et al.* 2012) amplifying the V4 region of the 16S rRNA gene and sequenced using the Illumina Miseq paired end sequencing platform. A DNA extraction blank processed and sequenced identically as other samples but containing only the DNA extraction kit reagents (negative control) yielded no 16S amplicon sequences, probably due to very low DNA content (<0.05 ng/mL). To rule out the possibility that our samples contained low biomass and we were simply amplifying contaminants, we performed qPCR analysis (Rubin *et al.* 2014) on three randomly selected samples: one female gut and larval gut sample (both *E. triangulatus*), and one untreated dung sample. The estimated 16S copy number in these samples was 1.26×10^5, 5.12×10^4 and 3.8×10^7 gene/μL, respectively, compatible with the levels of microbial load recommended to obtain robust amplicon sequencing results (Salter *et al.* 2014).

Paired Illumina reads were merged in QIIME (Caporaso *et al.* 2010b) using the 'fastq-join' method (Aronesty 2011) and demultiplexed using default parameters in QIIME version 1.8.0. High-quality reads were processed for quality filtering, chimera removal and Operational Taxonomic Unit (OTU) picking using the USEARCH (Edgar 2010) pipeline implemented in QIIME. Chimeras were identified using de novo and reference-based chimera checking ('GOLD' database version microbiomeutil-r20110519) by UCHIME (Edgar *et al.* 2011) within the pipeline, and the 'union' of the sets of nonchimeric tagged reads were retained. OTUs were clustered at 97% identity. A set of representative OTU sequences was picked using the 'longest' parameter. Taxonomy was assigned using the RDP classifier (Wang *et al.* 2007), with the minimum confidence to record an assignment set to 0.8 using the GREENGENES reference database version 13.8 (DeSantis *et al.* 2006). OTU tables were generated in QIIME and OTUs with abundance <0.005% of the total data set were removed as an additional level of quality filtering (Bokulich *et al.* 2013; Navas-Molina *et al.* 2013). Filtered representative sequences were aligned using PYNAST (Caporaso *et al.* 2010a), and a phylogenetic tree was constructed using the default 'fasttree' method (Price *et al.* 2010) in QIIME.

Diversity analyses

Alpha diversity measures such as observed OTUs and Faith's Phylogenetic Diversity (Faith 1992) were calculated in QIIME and abundance-based coverage estimator (ACE, Appendix S1, Supporting information), and a nonparametric estimate for species richness (Chao *et al.* 2005) was calculated in EstimateS (Colwell 2013). Beta diversity measures: unweighted and weighted UniFrac distance matrices (Lozupone & Knight 2005) were calculated in QIIME using a subsampled OTU table (11 000 sequences per sample). Chao's estimator for Chao's Jaccard index (Chao *et al.* 2005) (used here to measure similarity between within-group replicates, see Appendix S1, Supporting information) was calculated

using default parameters in EstimateS. Pielou's evenness index was calculated (Magurran 1988). Permutation-based *t*-tests with 999 Monte Carlo permutations and FDR (false discovery rate)-corrected *P*-values were used to compare: (i) Phylogenetic Diversity (PD) in QIIME and (ii) UniFrac distances in R version 3.1.0 (R Core Team 2014) using the package Deducer (Fellows 2012). Jackknifed principal coordinate analysis (PCoA) was performed on UniFrac distances using the QIIME workflow. Venn diagrams were generated using 'VENNY' version 2.1.0 (Oliveros 2007).

We used bipartite network analysis to evaluate the extent of specialization of larval and adult beetle guts in terms of the bacterial community they hosted, using the package 'bipartite'(Dormann *et al.* 2008) in R. OTU tables were pooled for *E. intermedius* male, female and larval guts, respectively, collapsed at the family level and subsampled at 11 000 sequences per group for the analysis. To statistically evaluate network specialization, we compared the OTU matrix against a null model comprising a randomized OTU interaction matrix using the Patefield algorithm. We used the standardized specialization index d' (modified Shannon diversity index, ranging between 0 and 1, signifying greater specialization with increasing values of the index) to calculate specialization for male, female and larval guts, and H2' (standardized two-dimensional Shannon entropy) to evaluate specialization for the entire interaction matrix (Blüthgen *et al.* 2006; Dormann 2011).

To predict putative functional capacities of the bacterial assemblages in *E. intermedius*, we compared predicted relative abundances of KEGG Orthologs based on evenly rarefied 16S rRNA gene amplicon sequences using PICRUST version 1.0.0 (Langille *et al.* 2013). Comparisons were made between larval, male and female gut communities; and between untreated dung and maternal gifts. We hypothesized that the larval gut community should have higher abundances of genes involved in cellulose digestion and nitrogen metabolism compared to adult guts due to the higher C/N ratio in larval food compared to adult food. To identify bacterial contribution to plant polysaccharide breakdown, we used all predicted KO groups associated with glycoside hydrolase enzymes (GH enzymes that hydrolyse O- and S- glycosyl compounds, primarily responsible for degradation of complex polysaccharides such as cellulose) having the enzyme class 3.2.1, from the PICRUST output (Table S3, Supporting information). For nitrogen metabolism, we considered two possible scenarios by which gut bacteria could provide nitrogen to hosts: first, by the nitrogenase activity of nitrogen-fixing bacteria in insect guts that convert atmospheric nitrogen to ammonia (Benemann 1973; Brune 2014);and second, by the recycling of uric acid that is a waste product of insect nitrogen metabolism. Uric acid is transported through the Malpighian tubules to the hindgut, where hindgut uricolytic bacteria convert uric acid to ammonia, which can eventually be assimilated by the host tissue (Potrikus & Breznak 1981; Brune 2014).

For comparison, KEGG Orthologs were selected based on those that constitute the KEGG nitrogen fixation module 'M00175' (http://www.genome.jp/dbget-bin/www_bget?M00175; Table S3, Supporting information), and those involved in the conversion of uric acid to urea (Table S3, Supporting information) that constitute the KEGG purine metabolism pathway 'ec00230' (http://www.genome.jp/dbget-bin/www_bget?ec00230). Relative abundances of KEGG Orthologs in larval guts were compared to male and female gut communities using a *t*-test via Monte Carlo permutation (FDR-corrected *P*-values) using the package 'Deducer' (Fellows 2012) in R. Similar pairwise comparisons were performed between predicted gene abundances of maternal gift and untreated dung samples. The Nearest Sequenced Taxon Index (NSTI) values for PICRUST predictions ranged from 0.009 to 0.166 (mean $\pm$ SD = 0.07 $\pm$ 0.04; Table S2, Supporting information) reflecting good availability of reference genomes on which metagenome function predictions were based – comparable to those observed in human gut samples (0.03 $\pm$ 0.02 SD) (Langille *et al.* 2013).

Results

Gut morphology

Larvae and adults of *Euoniticellus intermedius* showed several differences in their gut morphology. The larval gut was characteristically a smooth walled, large diameter tube with an apparent compartment in the hindgut that folds and is held together to form a pouch-like section (Fig. 1), ending in a bulbous and enlarged rectum. The adult gut was narrower, without any pouch-like compartmentalization in the hindgut. Instead, the tubular gut was a uniform width from the foregut to the hindgut which then widened gradually into a thick-walled rectum.

Diversity analysis

Following demultiplexing, quality filtering and chimera removal, 3 472 568 reads were retained for the 33 samples for *E. intermedius*, 23 samples for *E. triangulatus* and five untreated dung samples (Table S1, Supporting information). These clustered into 6302 OTUs that reduced to 1300 OTUs after applying the 0.005% abundance cut-off. Rarefaction curves ('observed OTUs') showed a saturating number of OTUs, indicating

adequate sampling of 16S rDNA sequences for all the samples (Fig. S1, Supporting information). Rarefaction curves and abundance-based coverage estimator (ACE) of species richness indicated highest species richness in untreated dung and brood balls, and lowest species richness in *E. intermedius* male guts (Table 1, Fig. S1, Supporting information). Larval and female guts in *E. intermedius* had similar measures of ACE (species richness). Larval and female gut communities also had higher species evenness than male gut communities. Within-group similarities were higher for *E. intermedius* female and larval gut communities (Chao's abundance-based Jaccard similarity index) – 0.87 and 0.92, respectively, compared to 0.63 in male gut communities, indicating more consistent diversity in bacterial communities in female and larval guts (Table 1).

Differences in larval and adult bacterial gut communities

The most abundant OTUs (average relative abundance ± SD between replicates) in *E. intermedius* larval guts were *Dysgonomonas* sp. (0.15 ± 0.005), an unassigned genus belonging to Comamonadaceae (0.10 ± 0.088), and *Paracoccus* sp. (0.07 ± 0.040). The most abundant OTUs in adult female guts were members of the order Entomoplasmatales (0.15 ± 0.130), *Dysgonomonas* sp. (0.10 ± 0.113) and members of Clostridiaceae (0.09 ± 0.047). In adult male guts, the most abundant OTUs were likewise members of the order Entomoplasmatales (0.60 ± 0.410), *Dysgonomonas* sp. (0.07 ± 0.163) and Enterobacteriaceae (0.03 ± 0.068). A total of 12 OTUs were identified as belonging to Entomoplasmatales, and all 12 were present in both males and female guts of both species. In *E. intermedius*, members of Entomoplasmatales, Porphyromonadaceae, Enterobacteriaceae, Clostridiales and Moraxellaceae had a cumulative relative abundance of 48.4% in adult females and 71.0% in adult males, but only 25.8% in *E. intermedius* larval guts (pooled for each group, Fig. 2). By comparison, Porphyromonadaceae, Comamonadaceae, Rhodobacteraceae, Sphingobacteriaceae and Pseudomonadaceae cumulatively constituted 63.3% of sequence reads in larval guts, but cumulatively constituted only 17.8% and 16.3% *in E. intermedius* adult female and male guts, respectively (Fig. 2).

Bacterial communities of *E. intermedius* adult and larval beetle guts showed a clear pattern of specialization based on unweighted UniFrac distances (principal coordinate analysis PCoA, Fig. 3a). A similar separation between adult (female) and larval gut communities was also seen when *E. intermedius* and *E. triangulatus* life stages were plotted together (Fig. S3, Supporting information), indicating consistency of results in both species. Larval guts and female gut communities in *E. intermedius* separated in PCoA space despite both having similar Phylogenetic Diversity measures (PD, a measure of alpha diversity that sums the length of all

Table 1 Comparison of OTU diversity estimates for 16S rRNA gene amplicons of untreated dung and various body components of the dung beetles *Euoniticellus intermedius* and *E. triangulatus*, and their immature stages. ACE = average values for abundance-based coverage estimator for species richness and its standard deviation (SD) calculated between rarefaction runs. Evenness was calculated as [Shannon's index/ln (number of OTUs)]. Chaos' abundance-based Jaccard similarity index and its SD (calculated for all pairs within a group) are also reported

Sample name	Sample size	ACE (SD)	Species evenness	Chao's abundance-based Jaccard Index (SD)
E. intermedius				
Brood ball	5	1734.12 (191.8)	0.65	0.90 (0.05)
Maternal gift	4	1433.9 (140.85)	0.60	0.93 (0.01)
Egg	4	1195.3 (338.3)	0.67	0.75 (0.14)
Female body	5	1025.3 (137.4)	0.57	0.90 (0.02)
Female gut	5	1106.7 (163.5)	0.59	0.87 (0.04)
Male body	2	945.3 (12.66)	0.61	0.86 (0.00)
Male gut	5	405.5 (216.1)	0.30	0.63 (0.27)
Larval gut	3	1148.1 (8.5)	0.66	0.92 (0.01)
E. triangulatus				
Brood ball	5	1736.31 (77.9)	0.65	0.93 (0.02)
Maternal gift	1	1589.7 (0)	0.66	–
Egg	5	1079.5 (377.5)	0.68	0.83 (0.06)
Female body	5	890.9 (230.5)	0.50	0.85 (0.07)
Female gut	5	602.8 (312.7)	0.34	0.69 (0.27)
Larval gut	2	1036.4 (90.2)	0.67	0.71 (0)
Untreated dung	5	1894.3 (138.0)	0.68	0.75 (0.26)

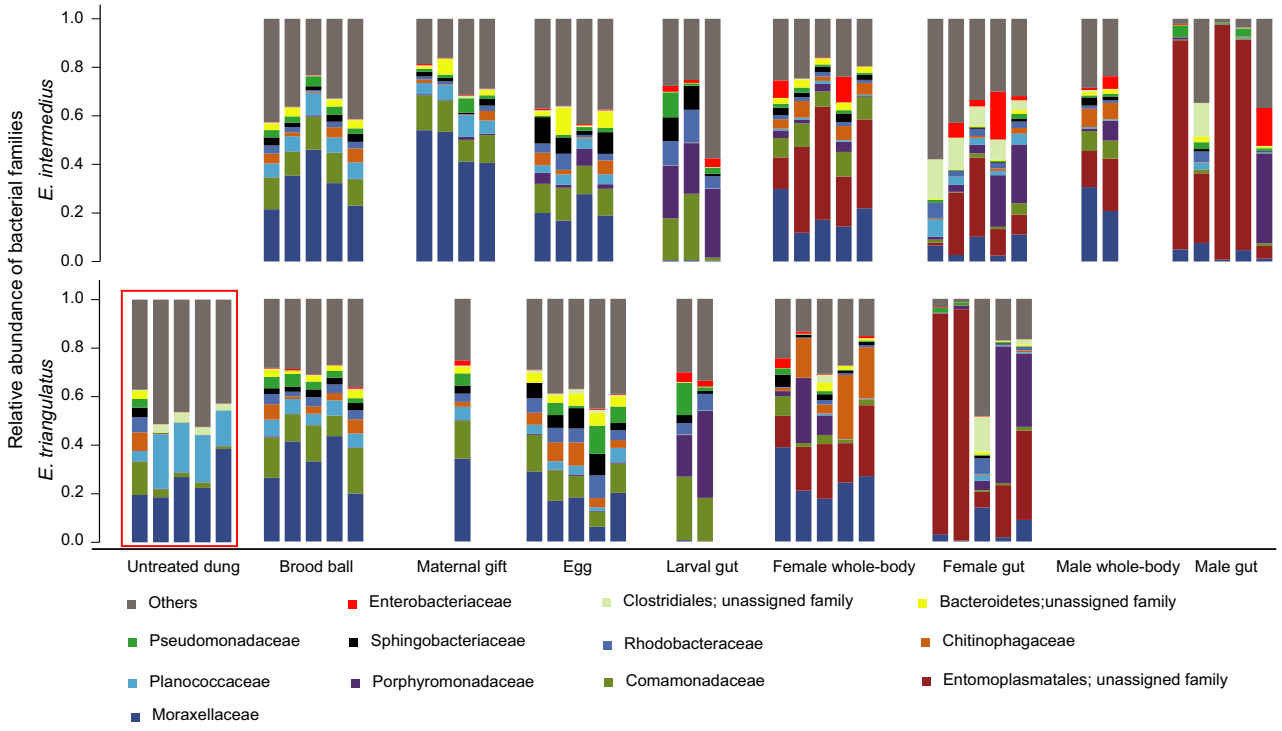

Fig. 2 Taxonomic composition of microbial communities associated with dung beetles. Relative abundances of the ten most abundant OTUs are plotted at the family level for a total of 61 samples for *Euoniticellus intermedius* (upper panel) and *E. triangulatus* (lower panel), including beetle life stages, brood ball, maternal gift and untreated dung. Untreated dung samples though plotted along *E. triangulatus* samples were common to both species and not associated with *E. triangulatus* samples alone (indicated by a coloured boundary around untreated dung). [Colour figure can be viewed at wileyonlinelibrary.com]

the branches for the OTU phylogenetic tree within a sample; *t*-test with Monte Carlo permutations, $P > 0.05$; Fig. 3b). In addition to specialization of adult and larval stages, adult male and female gut communities showed no overlap (PCoA on unweighted UniFrac distances, Fig. 3a). The specialization index d', indicating the specialization of beetle guts in hosting unique OTUs, was highest for *E. intermedius* larval guts ($d' = 0.57$), followed by male guts ($d' = 0.28$) and female guts ($d' = 0.21$). The entire OTU-gut network (male, female and larval guts) had a H_2' value of 0.35 and was significantly different from a matrix in which the OTU associations were randomly assigned (null model *t*-test, $P < 0.05$).

PCoA analysis based on unweighted UniFrac distances showed an overlap of bacterial communities between untreated dung, brood ball and the maternal gift, such that the brood ball samples were situated between untreated dung and female gut, with an overlap between brood ball and maternal gift (Fig. 4a). The

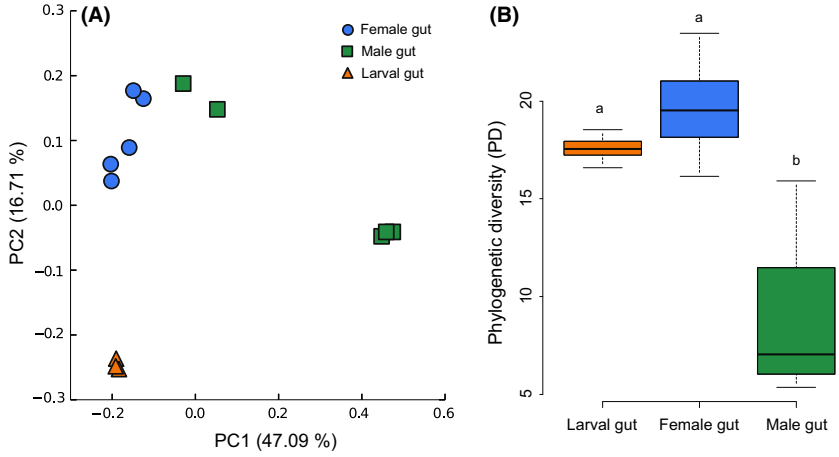

Fig. 3 Differences in bacterial communities in adult male and female guts, and larval guts in *Euoniticellus intermedius* (A) principal coordinate analysis (PCoA) based on unweighted UniFrac distances show no overlap between adults and larval dung beetle guts, or between male and female gut communities. (B) Larval and female gut communities had high Faith's Phylogenetic Diversity (PD) measures that were significantly higher than those for male gut communities (*t*-test with Monte Carlo permutations, $P < 0.05$) [Colour figure can be viewed at wileyonlinelibrary.com].

larval gut community had greater similarity to the female gut community, eggs, the brood ball and the maternal gift than to untreated dung and male gut communities (unweighted UniFrac distance comparison using *t*-test with Monte Carlo permutations, FDR-corrected *P*-values < 0.05, Fig. 4c), and shared more OTUs with the female gut, the brood ball and the maternal gift than with untreated dung and male guts (ANOVA, Tukey's post hoc test, $P < 0.05$, Fig. S6, Supporting information). Quantitatively, based on weighted UniFrac distances (which takes into account relative abundances of each OTU in addition to whether it is shared between pairs of samples), larval gut communities were as similar to female gut communities as they were to untreated dung and brood balls, and were furthest from male guts (*t*-test with Monte Carlo permutations on weighted UniFrac distances, FDR-corrected *P*-values < 0.05, Fig. 4d). In *E. triangulatus*, larval gut samples overlapped with egg samples, and female gut samples, and unweighted larval gut–female gut UniFrac distances were not different from larval gut–untreated dung distances (Figs S4 and S5, Supporting information), most likely due to the high heterogeneity in female gut communities (Fig. S8, Supporting information), or reduced sample sizes. However, larval guts in the two species *E. intermedius* and *E. triangulatus* were more similar to each other than they were to adult females of their own species (*t*-test with Monte Carlo permutations on unweighted UniFrac distance metrics, FDR-corrected *P*-values < 0.05, Fig. 4b), indicating a consistent larval gut community in both the dung beetle species.

The most abundant OTUs in *E. intermedius* male whole-body and female whole-body samples were the Entomoplasmatales and *Acinetobacter* sp. (together constituting about 44% and 50% of the reads in males and females, respectively, Fig. S2, Supporting information); 108 OTUs belonging to the genus *Acinetobacter* and 12 OTUs belonging to the order Entomoplasmatales. The most abundant OTU in *E. intermedius* eggs was *Acinetobacter* sp. (constituting about 20% of the reads). However, the Entomoplasmatales constituted <0.001% reads, and *Acinetobacter* constituted only about 0.5% of the reads in larval guts. The maternal gift in *E. intermedius* had high alpha diversity and relatively high species evenness (Table 1) as well as high within-group Jaccard similarity index, indicating a bacterially rich and consistent community. The maternal gift had a high proportion of reads associated with the families Moraxellaceae, Comamonadaceae and Planococcaceae (Fig. 2). OTUs with the highest relative abundance in the maternal gift were *Acinetobacter*, Comamonadaceae, *Acholeplasma* and *Solibacillus*. OTUs with the highest relative abundance in the brood ball were *Acinetobacter* (32.8% in *E. triangulatus* and 31.0% in *E. intermedius*), Comamonadaceae (12.1% in *E. triangulatus* and 10.3% in *E. intermedius*), Alteromonadales (0.01% in *E. triangulatus* and 4.4% in *E. intermedius*) and *Solibacillus* (3.4% in *E. triangulatus* and 4.2% in *E. intermedius*) (Fig. S2, Supporting information).

Functional prediction of larval and adult gut communities

We found 328 level 3 KEGG Orthology (KO) groups predicted in *E. intermedius* and *E. triangulatus* larvae and adult gut samples (PICRUST analysis), and the predicted gene abundance in *E. intermedius* larval guts was more strongly correlated with predictions from female guts ($r = 0.97$) than male guts ($r = 0.86$). Metagenomic predictions (KEGG level 3) between *E. intermedius* and *E. triangulatus* larval guts ($r = 0.99$) showed strong correlations between functional profiles, suggesting that the metabolic predictions described here may well hold true for *E. triangulatus* larvae in general. PICRUST analysis predicted a total of 67 KEGG Orthologs identified as glycoside hydrolase (GH) enzymes belonging to the enzyme family 3.2.1 (Table S3, Supporting information). These enzymes included (but were not limited to) those involved in the breakdown of cellulose, xylan and pectin.

Larval guts had significantly higher relative abundances of predicted GH enzymes, and KEGG Orthologs involved in nitrogen fixation than adult male and female gut communities (separate permutation *t*-test, FDR-corrected $P < 0.05$, Fig. 5a). However, there was no difference between the relative abundances of KEGG Orthologs involved in uric acid metabolism (K07127, K01466, K01477, K00365) between larvae and adults (permutation *t*-test, FDR-corrected $P > 0.05$, Fig. 5a). Untreated dung had higher predicted relative abundances of GH enzymes and nitrogen fixation proteins compared to the maternal gift, but they had similar relative abundance of predicted KO groups involved in uric acid metabolism (separate permutation *t*-test, FDR-corrected $P > 0.05$, Fig. 5b).

Discussion

The dietary specialization of dung beetle life stages involves key ontogenetic traits that enable larvae and adults to feed on different dung types. In this study, we report that morphological adaptations in mandibular and hindgut structure between life stages are also reflected in the gut microbiota in the dung beetles *Euoniticellus intermedius* and *E. triangulatus*. Morphological specialization of the adults and larvae, in combination with differences in the portion of dung that is consumed, could lead to differing physicochemical conditions in the gut, creating gut micro-ecosystems that support and select for

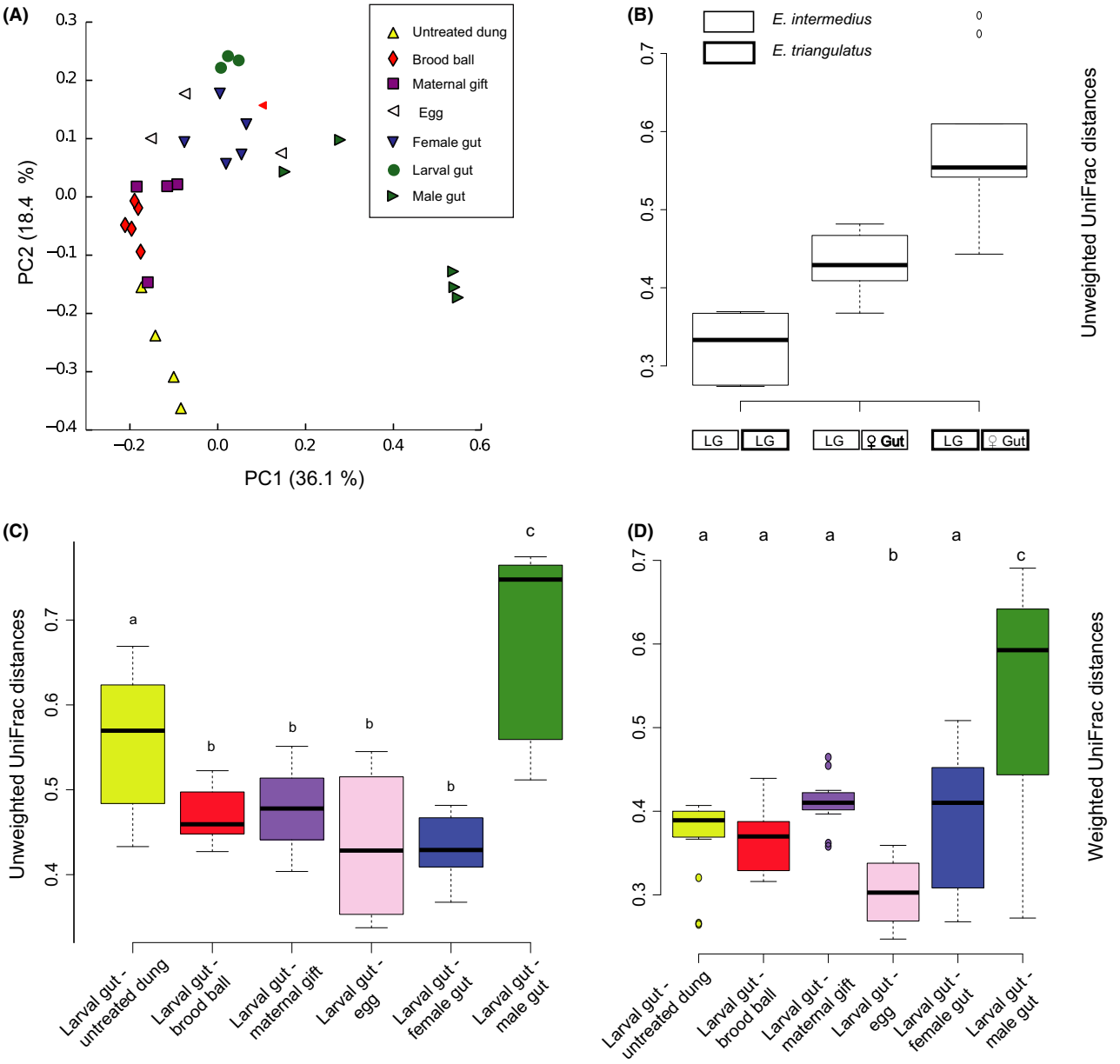

Fig. 4 Similarities in bacterial communities between *Euoniticellus intermedius* life stages, dung, brood ball and the maternal gift. (A) Principal coordinate analysis (PCoA) plots based on unweighted UniFrac distances show overlap between untreated dung, brood ball and maternal gift. (B) Larval guts (LG) of *E. intermedius* were more similar to larval guts of *Euoniticellus triangulatus* than to the females of their own species, indicating a consistent bacterial community in both the species. All results based on permutation *t*-test with Monte Carlo simulations, FDR-corrected $P < 0.05$). (C) Comparison of unweighted UniFrac distances for *E. intermedius*. Larval guts were more similar to female guts, brood ball and maternal gift than to untreated dung despite the brood ball originating from untreated dung, suggesting the role of the maternal gift in affecting the communities in the brood ball and larval gut. Larval gut communities were farthest from male guts. (D) Comparison of weighted UniFrac distances for *E. intermedius*. Larval gut communities had greater similarity to untreated dung, the brood ball and female guts than to male gut communities. [Colour figure can be viewed at wileyonlinelibrary.com]

different gut communities. Larvae consume a cellulose-rich and relatively nitrogen-poor diet. Cow dung, for example, contains up to 30% cellulose, 28% hemicellulose and 2% pectin (Hindrichsen *et al.* 2006), and larval diet consists primarily of coarse dung with a high C/N ratio; a limitation that adult beetles circumvent by feeding on filtered nutritious dung particles (Holter & Scholtz 2007). Mandibles that are sclerotized and toothed in the larvae become soft and membranous during metamorphosis to the adult stage (Simonnet & Moczek 2011). As a consequence, the two life stages differ in their diets (Fig. 1). The dominant food source for adult and larval dung

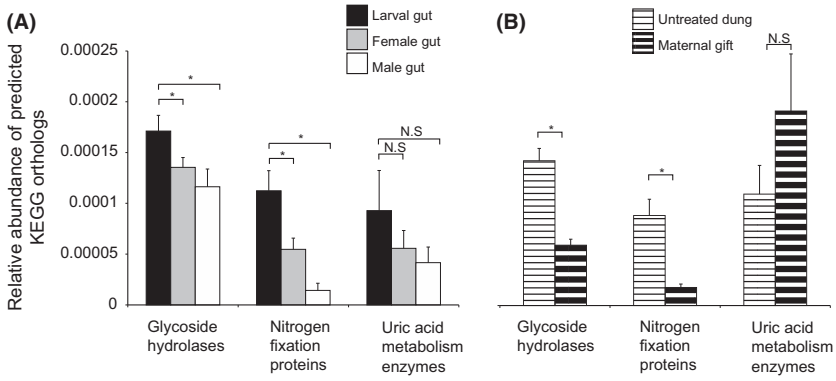

Fig. 5 Comparison of predicted KEGG Ortholog counts. (A) Relative abundances of Glycoside Hydrolase (GH) enzymes, and nitrogen fixation proteins were significantly higher in *Euoniticellus intermedius* larvae as compared to adult male and female guts. Predicted relative abundances of enzymes involved in uric acid metabolism did not differ between larval guts, male guts and female guts. (B) Predicted relative abundances of GH enzymes and nitrogen fixation proteins were significantly higher in untreated dung as compared to *E. intermedius* maternal gift. There was no difference in abundances of enzymes involved in uric acid metabolism between the two groups. Results based on pairwise *t*-test with Monte Carlo permutation (999 permutations, FDR-corrected *P* values). Bar plot shows average values with standard error plotted as whiskers. $*P < 0.05$; N.S, nonsignificant.

beetles differs both in age (fresh, liquid vs. older, drier dung) and in quality (smaller vs. larger dung particles). Larval guts have thus been predicted to host specialized gut bacteria that contribute to the breakdown of cellulose-rich substrates, whereas adult dung beetles may not need to establish their own 'enteric cultures' (Halffter & Matthews 1971; Rougon *et al.* 1990). In this study, we found that the bacterial communities of *E. intermedius* larval guts were similar to those of adult female guts in terms of alpha diversity, but were higher than the species-poor, inconsistent bacterial communities of adult male guts. Both male and female guts showed a high relative abundance of Entomoplasmatales. The Entomoplasmatales (arthropod-associated Mollicutes) identified in this study, as well as in army ants (Funaro *et al.* 2011), were more abundant in adults compared to larval or egg stages, indicating adult-specific roles. Based on their occurrence in other insects, these roles could be to function either as parasites (Jiggins *et al.* 2000) or as protective symbionts against parasites (Jaenike *et al.* 2010), or as pathogens (Clark 1977). Larval guts had a higher number of OTUs associated with Porphyromonadaceae, Comamonadaceae, Rhodobacteraceae and Sphingobacteriaceae. The Porphyromonadaceae are anaerobic bacteria that can ferment carbohydrates; Comamonadaceae are aerobic bacteria capable of utilizing a wide range of organic acids including amino acids and are present in diverse environments including other insect guts; whereas Rhodobacteraceae are primarily symbiotic with marine organisms that also form biofilms attached to living and nonliving surfaces; and the Sphingobacteriaceae are commonly found in soil and compost and are capable of degradation of several important biomolecules (Rosenberg *et al.* 2014a,b). We suggest that differences in gut microbiota between the life stages may be supported by differences in their gut structure, such that *E. intermedius* larvae may further provide suitable growth conditions for cellulolytic bacteria. *Euoniticellus* larvae appear to have a compartmentalized hindgut that is absent in the long, tubular adult gut (Fig. 1), possibly aiding the growth of anaerobic fermenting bacteria (*viz. Dysgonomonas* sp. *Paracoccus* sp. that had high representation of reads in larval guts and are known to grow under anaerobic conditions).

Greater similarity between the gut communities of larvae and females compared to males of *E. intermedius* indicated a possible maternal route of bacterial transmission through the brood ball and the maternal gift. The overlap of bacterial OTUs between untreated dung, the maternal gift and the brood ball (Fig. 4a) and the greater similarity of larval gut communities with female rather than male guts also supported such a route of microbial transmission. A similar separation between samples was found using an unfiltered data set, without applying the 0.005% abundance-based cut-off (data not shown), indicating that these results were robust to removal of low abundance OTUs. As larvae–brood ball unweighted UniFrac distances were not different from larvae–maternal gift distances, but were significantly different from larvae – untreated dung distances, and as the maternal gift is deposited in the brood ball, we infer that the maternal gift contains bacteria that inoculate the brood ball community, making it significantly different from untreated dung. As brood balls were sampled prior to emergence of larvae, we rule out larval interaction or larval faeces as a potential source of alteration of the brood ball community. Moreover, as it is the females that prepare the brood ball and deposit

the maternal gift inside it, our results suggest that this preparation primes the brood ball with maternally transmitted bacteria that later colonize the larval guts. The maternal gift in *Euoniticellus* (as in other dung beetles) is thought to be faecal in origin (Halffter & Edmonds 1982) and thus likely to originate from the female gut, providing a possible explanation as to how female secretions can mediate transmission of bacteria to the offspring.

Female transmission of microbiota through the brood ball has also been shown in *Onthophagus* dung beetles (Estes *et al.* 2013), although the contribution of dung, and the differences in gut communities between life stages has not been shown for *Onthophagus* and other dung beetles. The bacterial community associated with *E. intermedius* and *E. triangulatus* eggs was highly similar to that of the female gut, the maternal gift and the brood ball (Figs 2, 4a, 4c, S4 and S5, Supporting information), and the eggs did not appear to contain any specialized or distinctive bacteria. However, as the eggs were not washed or surface sterilized, it is possible that bacteria present in female secretions (such as the maternal gift) or from the hindgut are coated on the eggs. The egg surface is an important route for vertically transmitted symbionts (Fukatsu & Hosokawa 2002; Kaltenpoth *et al.* 2009), and further experiments will be required to test the role of the maternal gift, if any, in transmitting microbiota to the egg surface during oviposition or when they are anchored on the maternal gift.

Male gut contribution to the larval gut community is possibly insignificant (Fig. 4), a scenario that corresponds well with the biology of *Euoniticellus* beetles, where only female beetles prepare and provision the brood balls, and males generally guard the tunnels against competing males. Although there was separation of male gut samples from female and larval gut samples based on UniFrac distances, we observed considerable heterogeneity within the bacterial communities of *E. intermedius* males and also of *E. triangulatus* females (Fig. S8, Supporting information). In *E. intermedius*, two male gut samples separated from the remaining male gut samples, leading to a large variation within male gut communities (Fig. 4c,d). These two male guts were characterized by lower relative abundance of Entomoplasmatales, and higher prevalence of members belonging to Porphyromonadaceae, Clostridiales and Enterobacteriaceae. The similarity of these two male gut samples with female gut samples may also contribute to larger variation in larvae-male gut UniFrac distances, as reflected in large range (whiskers) of observed values in Fig. 4a,b. Such heterogeneity could arise due to differences in age, presence of transient commensals or pathogens, or be due to interaction with females prior to sample collection.

The *E. intermedius* larval gut was also highly similar to untreated dung, which is the source of food for the developing larvae. Owing to its high cellulose content, the dung pat may facilitate higher selective proliferation of cellulolytic bacteria originating from the ruminant gut or from the soil and may already be enriched in cellulolytic bacteria before reaching the larval gut. We found that untreated dung contained bacteria that could produce cellulolytic enzymes *viz.* Comamonadaceae, Alteromonadales, Porphyromonadaceae (albeit some of them at low relative abundances) and that were also present in the *E. intermedius* and *E. triangulatus* larval gut (Figs S2, S6 and S7, Supporting information). As larval guts showed the highest extent of specialization in terms of hosting unique OTUs (bipartite network analyses) and many of the bacteria present in larval guts were absent in male and female guts, these additional bacteria could have been acquired from dung (Figs S6 and S7, Supporting information). The *E. intermedius* maternal gift was predicted to be relatively deficient in cellulolytic and nitrogen-fixing bacteria compared to untreated dung (Fig. 5b), and if larvae do require microbial assistance in the digestion of dung, the untreated dung could be a better source of cellulolytic or nitrogen recycling bacteria. Experimental removal of the maternal gift from the brood ball leads to high mortality in *E. intermedius* larvae (Byrne *et al.* 2013), suggesting an important role of the maternal gift in larval development. However, amongst the larvae that do survive, experimental removal of the maternal gift does not reduce larval development time or body size of emerging beetles (Byrne *et al.* 2013), suggesting that the maternal gift may primarily function as a nutritional meal, or that larvae could compensate missing symbionts by acquiring them from dung.

Whatever the function of the maternal gift, our results support the hypothesis that the maternal gift influences the microbial community of the brood ball and the larval gut, making the dung ball similar to the bacterial community of the female gut (Fig. 4), and thus mediating the transmission of a maternal bacterial community. It is possible that the females transmit cellulolytic or nitrogen-supplementing bacteria in addition to the ones that the larvae acquire from dung. Alternatively, the bacteria associated with the maternal gift may provide larvae with other functions such as detoxification, production of amino acids or vitamins, development of the immune system, gut tissue development or resistance against antagonistic gut colonizers, as has been found in other insects (Engel & Moran 2013). Finally, they could also be involved in the production of antimicrobials to attenuate growth of pathogens and saprophytes in the brood ball, thereby preventing entomopathogenic fungal attacks during larval development.

The diverse and complex bacterial community in *E. intermedius* larval gut suggests that if larvae depend on microbial dung digestion, such a role is most likely fulfilled by a diverse bacterial community, similar to the gut community of termites (Brune 2014), where carbohydrate and nitrogen metabolism is carried out by a consortium of gut bacteria. *Comamonas* sp. found in the *Euoniticellus* larval gut and the maternal gift have a poor ability to utilize carbohydrates, but are known to utilize aromatic compounds, nitrates and organic acids (Ma *et al.* 2009), and also occur in termites (Kuhnigk *et al.* 1994), phytophagous beetles (Montagna *et al.* 2015) and in the dung beetle *Onthophagus taurus* (Estes *et al.* 2013). The genus *Dysgonomonas* had the highest relative abundance in the larval gut and are known to carry *nif* genes that can fix nitrogen under in vitro conditions (Inoue *et al.* 2015). *Dysgonomonas* is also found in fungus-growing termites, where they possibly hydrolyse cellulose due to their high β-glucosidase activity (Zhang *et al.* 2014), along with similar functions in beetles (Huang *et al.* 2012). The Alteromonadales which were abundant in the brood ball are known to produce pectin degrading enzymes (Kim *et al.* 2012). *Sphingobacterium* sp., which was present in larval guts and the maternal gift, has also been isolated from the gut of wood-boring beetle larvae (Zhou *et al.* 2009) and could degrade lignin (Rosenberg *et al.* 2014b) and xylan in dung beetles' guts.

The prevalence of a diverse bacterial community, many members of which can hydrolyse and ferment plant fibre, indicates a concerted strategy of microbial degradation of dung in larval guts, either targeting different polysaccharides, or different substrates within the breakdown of the same polymer. Functional metagenome predictions in this study support a higher prevalence of glycoside hydrolases (GH) in larval guts that may breakdown cellulose, pectin and xylan, thereby providing essential nutrients to the developing larvae. The larval gut was also predicted to host a higher abundance of nitrogen-fixing bacteria than the adults. Organic nitrogen enrichment could occur by fixing atmospheric nitrogen or via the reduction in nitrate to ammonia by denitrifying bacteria such as *Paracoccus*, and *Pseudomonas* that were also abundant in the larval gut. Predicted gene abundances (Fig. 5) did not support the possibility that larval gut communities metabolize uric acid at a higher rate than adult guts; however, this prediction does not rule out the possibility that uric acid recycling is equally important in adults and larvae. Given the inherent limitations of metagenome predictions based on 16S sequences (Langille *et al.* 2013), our data only suggest metabolic potential for enzymatic activity within the gut microbiota, and empirical studies will be necessary to demonstrate which, if any, microbial nitrogen enrichment pathways are important for the larval gut community.

The diverse and complex bacterial community associated with the *Euoniticellus* dung beetles reported here throws new light not only on how dung beetle life stages may specialize on different dietary components of dung, but also on the possible role of the microbiota in enabling the adaptive radiation of dung beetles into a novel and highly specialized niche like dung, which presents a novel challenge of obtaining nutrients from others' waste materials. In this study, we set out to answer three questions to understand the microbial ecology of *E. intermedius*. We conclude that first adult and larval stages differ in their gut microbiota in a way that reflects the differences in their diets. Second, based on the prevalent microbial communities in adult and larval guts, we show that larvae were predicted to host a higher proportion of bacteria involved in cellulose digestion and nitrogen metabolism that may help overcome the high C/N ratio in their diets. Third, the composition of the bacterial community in the maternal gift is similar to that of the brood ball and the larval guts, indicating that females may use maternal secretions to transmit a bacterial community to their offspring, and that the presence of nutritionally important gut bacteria may facilitate development in a nutritionally challenging substrate such as dung. Further comparative studies of dung beetle-symbiont interactions, from other species utilizing other dung and types of detritus, promise novel insights into the evolutionary ecology of dung digestion and utilization.

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N.E.P. and M.J.B. conceived the study during a chance meeting in South Africa in 2014, and S.P.S. made it possible to pursue the research. M.J.B. contributed samples; N.E.P. contributed reagents; S.P.S. designed the sampling strategy for the molecular work and performed data analysis. J.G.S. advised and assisted in data analysis; S.P.S., J.G.S., M.J.B. and N.E.P. wrote the study. All authors approved the final manuscript version and declare no conflict of interest.

Data accessibility

All chimera-checked 16S rRNA gene sequences of representative OTUs have been deposited with NCBI (Accession nos KX459698–KX460822). The de-replicated, quality filtered Illumina MiSeq data set file, metadata mapping file and the final OTU table are deposited at Dryad (doi:10.5061/dryad.vq744).

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Supplemental methods detailing calculation of alpha and beta diversity metrics in EstimateS; and functional metagenome prediction using PICRUST.

Table S1 Summary of 16S rDNA read counts for *Euoniticellus intermedius*, *E. triangulatus*, and five untreated dung samples.

Table S2 Nearest Sequenced Taxon Index (NSTI) scores produced from PICRUST output for *Euoniticellus intermedius* larvae and adult gut, maternal gift and untreated dung.

Table S3 List of KEGG Orthologs produced through PICRUST analysis used for comparison between *Euoniticellus intermedius* communities.

Figure S1 Rarefaction curves for 'observed OTUs' for *Euoniticellus intermedius*, *E. triangulatus* and untreated dung samples.

Figure S2 Relative abundances of OTUs for *Euoniticellus intermedius*, *E. triangulatus* and untreated dung samples plotted as a heat map.

Figure S3 Principal coordinate analysis plot for adult and larval gut communities in *Euoniticellus intermedius* and *E. triangulatus*.

Figure S4 Principal coordinate analysis plot for unweighted UniFrac distances for untreated dung and *Euoniticellus triangulatus* samples.

Figure S5 Unweighted UniFrac distances plotted as a boxplot for *Euoniticellus triangulatus* samples.

Figure S6 Bar plot showing average number of OTUs shared between *Euoniticellus intermedius* larvae and adult guts, maternal gift, brood ball and untreated dung.

Figure S7 Venn diagram showing overlap of OTUs between *Euoniticellus intermedius* female gut, larval gut and untreated dung.

Figure S8 Comparison of within-group unweighted UniFrac distances for *Euoniticellus intermedius* and *E. triangulatus* female and larval gut communities.