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INVERTEBRATE MICROBIOLOGY

Parental Care Alters the Egg Microbiome of Maritime Earwigs

Jordan A. Greer[1,2] · Andrea Swei[1] · Vance T. Vredenburg[1] · Andrew G. Zink[1]

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Abstract

Recruitment of beneficial microbes to protect offspring, often reducing the energetic costs of care, is now recognized as an important component of parental care in many animals. Studies on earwigs (order Dermaptera) have revealed that removal of females from egg tending increases mortality of eggs due to fungal infections, possibly caused by changes in the bacterial microbiome on the egg surface. We used a controlled female-removal experiment to evaluate whether female nest attendance in the maritime earwig, *Anisolabis maritima*, influences the bacterial microbiome on the egg surface. Further, we analyzed the microbiomes of mothers and their eggs to determine if there are a core set of bacteria transferred to eggs through female care. Microbiomes were analyzed using 16S rRNA bacterial DNA sequencing, revealing that bacterial operational taxonomic unit (OTU) richness and diversity were both significantly higher for female attended versus unattended eggs. The core microbiome of adult females contained bacteria which have the potential to carry anti-fungal characteristics; these bacteria were found in higher presence and relative abundance on eggs where females were allowed to provide care. These results demonstrate that female egg attendance significantly impacts the bacterial microbiome of *A. maritima* eggs, and identifies specific bacteria within the egg microbiome that should be investigated further for beneficial anti-fungal properties in this system.

Keywords Microbiome · Vertical transmission · *Anisolabis maritima* · Parental care · Eggs · Earwigs

Introduction

Parental care can be defined as any parent behavior that increases the survival and/or reproductive potential of offspring [1]. While parental care functions to benefit offspring, it involves fitness trade-offs; parents must balance their energetic investment into thier current brood against the loss of resources that could otherwise be reserved for future reproduction [2, 3]. To circumvent the costs of parental care, many animals have evolved strategies to transfer some or all of these costs to other individuals (often termed alloparents). Strategies include cooperative breeding [4–6], conspecific brood parasitism [7–10], and mutualisms with other species that directly protect offspring [11, 12]. In particular, interspecific mutualisms with bacteria have been shown to effectively facilitate offspring care [13]. In such mutualisms, attending parents reduce the costs of care by vertically transmitting bacteria that directly aid in offspring development [14, 15]. In particular, these bacteria can function to protect thier hosts from predators and pathogens, such as infections from fungus [16, 17]. In this study, we contribute to the emerging evidence that microbes play an essential role in parental care; in particular, we examine the role of maternally transferred bacteria in the maritime earwig, *Anisolabis maritima*. We experimentally manipulated female egg clutch attendance to investigate the impact of *A. maritima* maternal care on egg microbiome structure. We also evaluated whether a common core set of bacteria were consistently transferred from parent to offspring among all clutches, and tested whether core bacteria had potential benefits for clutch protection.

Microbiome research is becoming increasingly important as new molecular techniques reveal that bacteria acquired directly from their environment, through social interactions, and/or from parental inheritance play essential roles in an organism's health and survival [18–21]. The specific roles that microbes play in an organism's life history are deeply varied,

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✉ Jordan A. Greer
jag1@uchicago.edu

[1] Department of Biology, San Francisco State University, 1600 Holloway Ave., San Francisco, CA 94132, USA

[2] Committee on Evolutionary Biology, University of Chicago, 5801 S Ellis Ave, Chicago, IL 60637, USA

including hyena kin recognition, increased vector competency in ticks, and regulation of immune response in mouse models [22–24]. Among insects in particular, the pathways—intracellular and/or extracellular—by which parents transmit symbiotic microbes to their offspring vary extensively and represent a multitude of evolutionary strategies attending adults use to inoculate their offspring with microbes [14, 15, 25–30]. Vertical transmission of microbes can support offspring development while reducing the energetic costs of care incurred by the mother. These symbiotic bacteria often produce specific compounds necessary for offspring survival, relinquishing the need for adults to metabolize these costly compounds themselves [31].

We utilized an insect system with well-documented extended maternal care to assess whether beneficial bacteria are transmitted to offspring as a function of prolonged egg attendance. The nest-tending behaviors of the maritime earwig, *A. maritima* [32], have been previously shown to greatly enhance hatching success [33]. While a primary function of female clutch attendance in *A. maritima* is to defend eggs from conspecific cannibalism [34], prior research indicates that cleaning of and salivation onto the egg surface decreases fungal growth on the eggs themselves [33]. In addition, similar cleaning behaviors have been observed to increase hatching success in the European earwig, *Forficula auricularia*, but only when eggs were directly exposed to fungal spores [35]. Boos et al. [35] posit that chemical hydrocarbons placed on the egg surface by attendant earwig females may protect eggs by making the shell surface unsuitable for fungal growth, but their lack of support for this hypothesis leaves the exact mechanism behind fungal protection of earwig eggs by mothers unexplained. Further, these authors suggest that vertically transmitted bacteria with anti-fungal properties are likely the main contributor to protective egg function. If mutualistic bacteria are transferred from earwig mothers to eggs, we expect that such transfer occurs during egg cleaning; a common behavior seen among nest-attending earwig females during egg incubation prior to hatching [33, 36, 37].

To investigate the potential roles of vertically transmitted bacteria in the *A. maritima* egg clutch, we experimentally evaluated the microbiome structure of eggs that are tended or unattended by adult females. Since we anticipate that egg cleaning and salivation are the primary mechanisms for vertical microbe transmission, we also evaluated the microbiomes of the internal head cavity of adult females alongside their tended eggs. Previous experiments indicating that female egg attendance reduces fungal growth have suggested that anti-fungal bacteria sequestered within the head of female earwigs (most likely residing in the salivary glands) may be an important component of egg protection [33, 35, 38]. Through DNA extraction and bacterial *16S rRNA* DNA sequencing, we identified bacteria found in common between the head cavity of earwig females and the egg surface of their attended versus unattended clutches over time. For the core bacteria showing a clear pattern of female inoculation (head and egg correspondence) across all clutches, we identified these bacteria to the species level and then determined if these bacteria have known anti-fungal properties through a literature survey.

Materials and Methods

Earwig Collection and Housing

One hundred *A. maritima* earwigs were collected on June 6th, 2016 from Harbor Point in Richardson's Bay, Marin County, California (37° 53′ 01.6″ N 122° 30′ 40.1″ W). All animals were collected along the shoreline and found primarily under rocks and woody debris. Adult females were identified by size, number of abdominal segments, and shape of forceps as this species exhibits a distinct sexual dimorphism in forceps curvature [34]. Animals were handled with gloves and placed into individual vials and carried in a cooler for transport. They were then taken to San Francisco State University and held in captivity for the duration of the experiment. To limit contamination by transient bacteria, each earwig was placed in a separate, covered Petri dish (100 mm diameter × 15 mm depth), which contained a mixture of 25 mL autoclaved Petco brand terrarium sand and 10 mL double-distilled water. Insects were fed on autoclaved "Trader Joe's Premium Ocean Catch" cat food ad libitum. Dishes were sprayed periodically with ddH_20 to maintain moisture. To encourage egg laying, each dish was equipped with an opaque plastic square resting atop the center of the clear lid to give earwigs the illusion of a cover item; previous work has shown that this species prefers dark shelters [39]. Dishes were monitored every 2 days, at which point food was replaced. On each of these monitoring days, data on female mortality, presence of eggs, and presence of fungal hyphae on eggs were recorded for each nesting chamber.

Experimental Design

When eggs were first observed in a nesting chamber (presumably on the first day of oviposition), the female was randomly assigned to a mother absent or mother present group; at this stage, all females were allowed to remain with their eggs for an additional 2 days. This allows for sufficient time for oviposition to complete in this species [21, 33]. We were careful to assign the very next dish presenting eggs to the opposite of the two treatments (absent/present). This allowed both treatments to be evenly distributed across time (the first clutch appeared on June 8th, 2016 and the last observed clutch appeared on July 20th, 2016). At 48 h after the initial oviposition, females assigned to the mother absent groups were

removed and immediately frozen with liquid nitrogen and stored in −20 °C freezer within 1.5 mL polypropylene microcentrifuge tubes for later DNA extraction. The total number of eggs in each clutch was recorded after this initial 48 h period, and then moved to a freshly prepared Petri dish with sterilized sand (eggs were only moved, allowing them to retain the microbiome acquired on the shell surface from the first 2 days).

In preliminary trials, we observed that unattended eggs had an immediate increase in fungal growth shortly after female removal, making the clutches unusable for experiments. We could not apply fungicide to eggs for concern that it would artificially alter the bacterial microbiome. Because unattended eggs are rapidly overtaken by fungal growth, and because we were interested in comparing the microbiome of unattended versus attended clutches over the full 21 days of egg development, we moved unattended eggs to freshly prepared Petri dishes 48 h after initial recorded oviposition, as described above. This effectively prevented the growth of fungus on unattended eggs for the duration of the experiment. Immediate fungal growth did not occur on female attended eggs—likely due to the female's nest cleaning behavior. Previous work had shown that exaggerated nest disturbance leads to whole clutch cannibalism by the females [33] and as such we did not replace the sand in the mother present group in the same manner as the unattended egg group.

While bacteria from the mother present eggs may have accumulated in the soil during the first 2 days, both treatments (mother present and mother absent) should have identical initial egg surface microbiomes by the end of the 48-h oviposition period. This gives eggs in both treatments equal potential to further contaminate the surrounding soil during the remaining 16 days of the study. Nevertheless, the aim of this study was to examine if female presence directly influenced the microbiome of eggs over time (from day 2 onward) via direct manipulation during egg cleaning, given an equal initial egg microbiome in both treatments.

While females in the mother absent group were removed immediately after the 48-h oviposition period, females in the mother present group were allowed to remain with their egg clutches for an additional 16 days, at which time they were frozen and stored in the same manner as females in the mother absent group. Egg samples for microbiome analysis (described below) were taken from clutches on days 0, 8, and 16 after the initial 48-h oviposition period, regardless of treatment group (Fig. 1). With this design, there were no differences expected in clutch microbiomes between the two treatments on day 0, as females in both groups had remained with

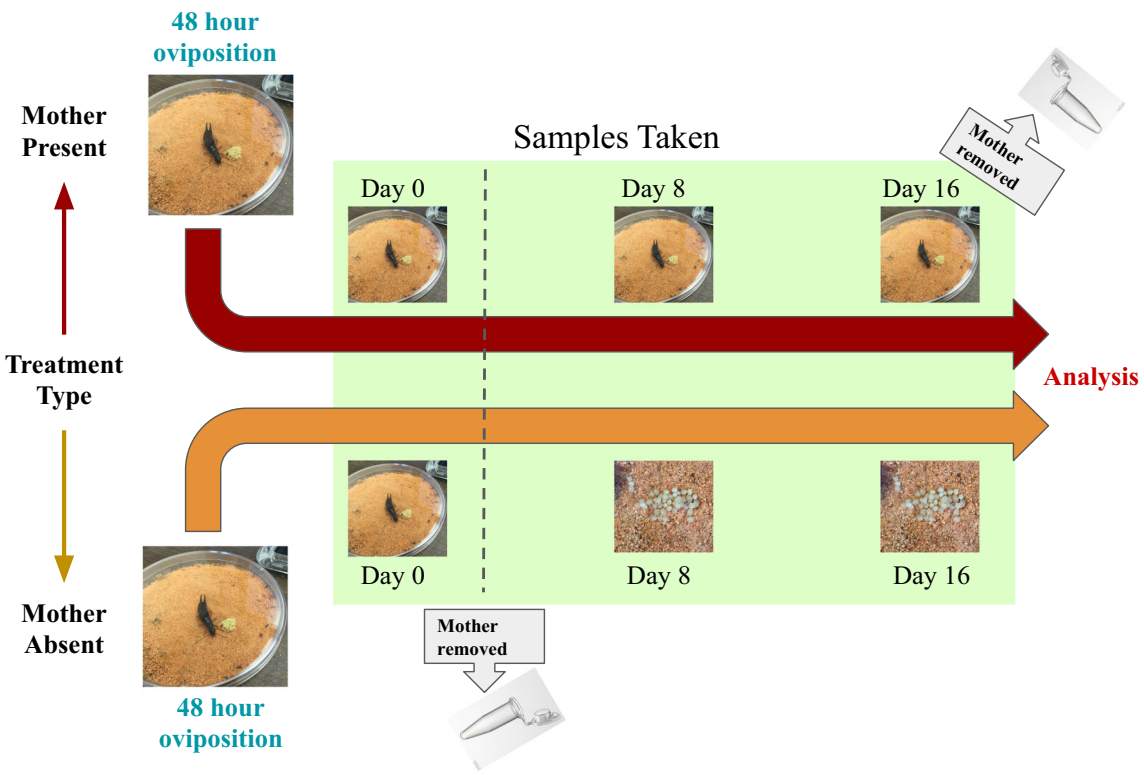


Fig. 1 Experimental design of the lab-rearing phase. Egg samples were taken on days 0, 8, and 16 after initial 48-h oviposition period. Egg samples taken to the right of the dashed line (days 8 and 16) are experimentally different across treatments (females removed from mother absent group). In the mother absent group, females were removed on day 0 (48 h after observed oviposition). In the mother present group, females were removed at day 16

their eggs for 48 h prior to egg sampling (note that day 0 egg sampling in the mother absent group occurred before eggs were transferred to a new container). These time points were chosen because eggs begin hatching roughly 21 days after initial egg laying in this species; thus, experimentally sampling across the 18 days since eggs were initially oviposited allowed for a near full assessment of the entire egg incubation period [33]. Egg samples consisted of the removal of roughly 4–10 eggs at each time point for any given clutch (Online Resource 1, Table S1). In the majority of cases, we took the same number of eggs from a particular dish on each of the sampling days (days 0, 8, and 16) to limit bacterial community variation collected based upon sample size (more details in Online Resource 1, Tables S1–S3). During egg sample collection in the mother present group, females were briefly placed into a sterilized vial, and returned to the same dish with their nest once egg collection was completed. Eggs were collected with tweezers which were heat sterilized between cages. After collection, eggs were immediately flash frozen using liquid nitrogen, and stored/frozen in the same manner as adults. Due to fungal growth, some clutches were lost from the experiment over time; these sample sizes for each stage can be found in the Online Resource 1, Figure S1.

DNA Extraction

DNA was extracted from both the frozen adults and eggs using Qiagen Blood and Tissue Kit (Qiagen, Valencia, CA) following the prescribed protocol. Prior to extraction, adults were thoroughly surface sterilized by adding 500 uL of 3% hydrogen peroxide into their containment vial, and vortexing for 30s. This liquid was replaced with 500 uL of 70% ethanol and vortexed for 30s, followed by two final washes with 500 uL de-ionized H_20. This procedure ensured that there were no transient bacteria on the body surface of earwigs that could contaminate the head sample. After sterilization, the earwig was removed from its vial and placed on a Kimwipe to dry briefly. The head was then removed with sterilized dissection scissors, bisected with a scalpel, and placed in a new 1.5-mL centrifuge tube for extraction. Only the head was analyzed, which allowed for more precise analysis of the cleaning behavior as the mode of bacterial transmission.

We were only interested in bacteria directly on the egg surface, so egg samples from the various dishes/time points were individually digested in lysis buffer and proteinase K solution for 30 min. As stated by Qiagen DNeasy Blood and Tissue Kit protocol, this allowed enough time to lyse gram-positive bacteria, and did so without causing the eggs to lose their integrity or rupture. We added 20 mg/mL of lysozyme to the proteinase K incubation step for both eggs and adults; this allowed for increased potential to retrieve gram-positive bacteria. The remainder of the procedure followed Qiagen manufacturer guidelines for DNA extraction.

Amplification and Sequencing

All samples were PCR amplified targeting the V3–V4 region of the bacterial 16S rRNA gene using Illumina primers (Illumina, San Diego, CA). PCR reactions were comprised 12.5 μL KAPA HiFi HotStart Ready Mix, 10 uM forward primer, 10 uM reverse primer, 8 μL UV radiated PCR grade H_20, and 2.5 μL of sample extract. Each sample was amplified in triplicate to limit PCR bias, and all were subsequently pooled. The cycling regime was as follows: initial denaturation at 95 °C for 3 min; followed by 20 cycles of 95 °C for 30s, 57 °C for 30s, 72 °C for 30s, and then 72 °C for 5 min. Index PCR was then used to dual barcode each amplified sample with unique tags. These barcodes were provided through the Illumina Nextera Index Kit. Similar to amplicon PCR, 25 μL PCR reactions was used comprised 12.5 uL KAPA HiFi HotStart Ready Mix, 1 uM of barcode 1, 1 μM of barcode 2, 5 μL UV irradiated PCR grade H_20, and 2.5 μL of amplicon PCR product. The cycling regime was same as the initial PCR, except 12 cycles were used unless gel visualization determined that more cycles were needed. Samples were purified after both the initial and index PCRs using Sera-mag speedbeads [40].

After amplification, Qubit was used for initial quantification of DNA, and samples were then diluted. qPCR (KAPA Library Quantification Kit, KAPA Biosystems) was then used to give a more precise quantification. Each qPCR reaction had a volume of 10 μL, comprised 6 μL KAPA SYBR Fast qPCR Master Mix, 2 μL of ddH20, and 2 μL of DNA template. The cycle regime was as follows: 95 °C for 5 min; 35 cycles of 95 °C for 30s; and 60 °C for 45 s; with a final dissociation step. Equimolar concentrations of 4 nM DNA diluted with 10 mM Tris-Cl and 0.01% Tween-20 buffer were created for each sample. All samples were then pooled for sequencing on an Illumina 300 bp, paired-end Miseq v3 (Illumina, Inc., San Diego, CA). Fifteen percent PhiX was added to the Miseq run to accommodate low-diversity libraries. A total of 202 samples were loaded on the Miseq, 49 samples of *A. maritima* adult females (present females = 21, absent females = 28), 150 total samples of eggs, plus 1 adult male, and 2 negative controls.

Sequence data were rarefied to a sequencing depth of 6230 reads and used for all subsequent alpha diversity metrics. Normalization via cumulative sum scaling (CSS) was also run on the quality-filtered sequence data, and results were used for all relative abundance-based beta diversity analyses [41]. CSS has been found to be a more robust method for assessing group similarity when dealing with relative abundance changes among samples with largely similar microbial communities and therefore was deemed a more robust for analysis of these data [42].

Bioinformatics Methods

The pipeline Quantitative Insights into Microbial Ecology (QIIME 1.9.1) was used for demultiplexing of reads, OTU picking, and taxonomic assignment [43]. Only sequences with a phred quality score of Q20 or higher were retained (minimum of 99% base call accuracy). Chimeric sequences were removed prior to OTU picking using USEARCH version 6.1 [44]. The open reference OTU picking strategy was used via the "uclust" method for sequence clustering [45].

Adult and egg samples containing less than 100 reads were removed from the analysis. Since no standardized mock community was included in the Miseq run, OTUs representing less than .0025% of all reads were filtered out from the data; this is a slightly more conservative estimate than that recommended by Bokulich et al. [46]. After quality filtering was complete, the number of reads per experimental sample ranged from 2919 to 84,992 per sample. The SILVA database was used for assignment of OTUs to specific sequences at a 97% similarity threshold (https://www.arb-silva.de/). Sequence alignment was conducted through the use of PyNAST [47]. To standardize sampling effort, sequences were rarefied to a depth of 6230 reads which reduced the sample size from 199 to 194 in the analysis. OTUs with a relative abundance across all samples above .05% within the negative controls were also removed. Complete removal of OTUs found within negative controls may otherwise remove relevant bacteria from the analysis, as some of these OTUs may represent low-level cross sample contamination [48, 49].

Fungal Analysis

To determine the possible impact of fungal growth on the clutch microbiome, the presence of egg fungal growth was noted on mother absent clutches (days 8 and 16), and the microbiome structure of clutches with and without fungus was compared. We ran a weighted Unifrac PERMANOVA to determine if eggs collected with fungal growth impacted our beta diversity results. If a clutch showed fungal growth on day 8 or day 16 in the mother absent group, both sampling dates were considered to have gained a fungal infection and included in the analysis. Note that this was primarily a classification of day 8 (where fungus was missed) for the same clutch where fungus visually emerged on day 16 (fungus never "disappeared" from days 8 to 16). We included these prior day 8 samples into the fungus-infected groups as a conservative measure with the assumption that fungal spores must have been present on the same clutch at day 8.

Statistical Analysis

All statistical analyses were conducted and visualized through QIIME v.1.9.1 and R Studio. Principal R packages used were Vegan and Phyloseq. To assess how the bacterial community structure changes over time in the mother absent and mother present clutches, differences in the microbiome were evaluated across eight categories: present females, absent females, eggs day 0 (present and absent), eggs day 8 (present and absent), and eggs day 16 (present and absent). Alpha and beta diversity metrics were assessed for all six egg sample groups, as well as for the core microbiomes of the adults (mother present/absent). We also examined the extent to which this core adult microbiome was reflected in eggs collected during the various treatments.

Alpha diversity metrics were calculated in QIIME, then analyzed and plotted in R using rarefied sequence data (R Core Team 2016). OTU richness and Shannon's diversity were calculated for each treatment group. OTU richness tells us the number of unique OTUs present within samples, while Shannon's diversity is a composite of both OTU abundance and their evenness. Group differences in OTU richness and Shannon's diversity were evaluated with a general linear mixed effects model, followed by an estimation of the marginal means and pairwise comparisons across treatments. As we removed eggs from the same clutch on multiple days, we controlled for clutch as a random effect in our model. Adults were compared using a Shapiro-Wilk test of normality, followed by an ANOVA.

Beta diversity was measured through weighted Unifrac principal coordinates analysis (PCoA). In weighted Unifrac plots, each sample is placed in vector space according to its community compositions which allows us to compare differences in relative abundance between groups [50]. Distance matrices for weighted Unifrac were calculated in QIIME using CSS data and plotted using the Phyloseq package in R. Pairwise PERMANOVA was used to calculate significant differences between groups.

Core OTUs, defined as OTUs consistently found across individual samples, were determined in QIIME at thresholds of both 100 and 98% presence across all sampled adults. Analyzing core OTUs is useful as it limits our analyses only to bacteria that are ubiquitous throughout the adult females, and therefore have a strong possibility of being biologically relevant to *A. maritima* females, generally [18]. These biologically relevant bacteria could then be passed down to clutches through maternal care as a means of guarding them from pathogens. We expected that these core bacteria would have a higher abundance and prevalence in mother present egg clutches compared with mother absent egg clutches, since removed females are not able to re-inoculate their eggs through continued cleaning, as opposed to females that were allowed to remain. Core bacteria OTUs showing a pattern of vertical transmission broadly across present/absent treatments were then analyzed for significant differences in relative abundances using a GLMM controlling for clutch and sample date, followed by post hoc pairwise comparisons. Core OTU

sequences were run through BLAST to gain an understanding of the potential function of these bacterial groups.

Results

Microbiome Sequencing

Prior to quality filtering, the initial output OTU table contained 7.7 million reads across 15,906 OTUs. Due to our filtering conditions, no OTUs were retained that were found in less than three samples. After quality filtering was complete, 199 samples (adults and eggs total) out of 202 were retained with approximately 6.5 million reads across 657 unique OTUs. Rarefaction curves (Online Resource 3, Figure S3) were used to retain 193 samples at the rarefaction depth of 6230 (adults ($n = 44$): present females ($n = 17$), absent females ($n = 27$), eggs ($n = 149$): present day 0 ($n = 25$), absent day 0 ($n = 27$), present day 8 ($n = 26$), absent day 8 ($n = 27$), present day 16 ($n = 23$), absent day 16 ($n = 21$)).

Alpha Diversity

At the start of our experiment (day 0), experimental groups had similar OTU richness and Shannon diversity ($p = 0.99$), a result we expected given our design; females of both treatments remained with eggs until this sampling day (Fig. 2). Analysis of OTU richness revealed that the experimental removal of females in the mother absent group on day 0 resulted in a significant reduction in OTU richness in subsequent clutches (days 8 and 16) for this category (Fig. 2b). In these mother absent clutches, there was a marked decrease in OTU richness between day 0 and day 8 ($p < 0.001$), but not between day 8 and day 16 ($p = 0.94$), suggesting that by day 8, the drop in OTU richness had stabilized at a lower value. Because of this drop by day 8, there was a significant overall difference between the OTU richness of day 0 and day 16 in mother absent clutches ($p < 0.001$). In the mother present clutches, OTU richness of the eggs was stable and did not change from day 0 to day 8 ($p = 0.93$). However, richness did increase from day 8 to day 16 ($p < 0.05$) and from the start of experiment on day 0 to the end of the 16-day guarding period ($p < 0.005$) in the present treatments. However, the most drastic and informative differences in OTU richness were when directly comparing the mother present and mother absent clutches for day 8 ($p < 0.001$) and day 16 ($p < 0.001$) respectively; in both cases, the mother present eggs showed significantly higher OTU richness relative to mother absent eggs. Importantly, the OTU richness of the adults collected from the mother present and mother absent groups did not differ ($p = 0.93$, Fig. 2a).

Analyses of Shannon diversity index revealed a pattern very similar to results of OTU richness (Fig. 2c–d). In the mother absent clutches, there was a significant decrease in diversity from day 0 to day 8 ($p < 0.005$) and from day 0 to day 16 ($p < 0.05$), while day 8 and day 16 were not different from one another ($p = 0.99$). The mother present clutches were not significantly different from day 0 to day 8 ($p = 0.80$) or from day 8 to day 16 ($p = 0.07$), but there was a significant increase in diversity between day 0 and day 16 ($p < 0.005$). When comparing across the mother present and mother absent treatments, the day 8 clutches ($p < 0.001$) and the day 16 clutches ($p < 0.001$) were significantly different, again showing a significant decrease in bacterial diversity when a female was removed from her eggs (Fig. 2d). As with richness, Shannon diversity of the adults in the mother present and mother absent groups did not differ significantly from one another ($p = 0.25$, Fig. 2c). Raw values of both richness and Shannon diversity can be found in Online Resource 3, Table S4.

Beta Diversity

Weighted Unifrac analysis (in QIIME) identified significant differences among the egg and adult microbiome samples collected overall (PERMANOVA $F = 26.04$, df = 192, $p = 0.001$, Fig. 3). When pooling both experimental egg time points (days 8 and 16) into single mother present and mother absent groups, the beta diversity of mother present and mother absent clutches was significantly different from one another ($p < 0.005$, Fig. 3a) and both treatment groups were distinct from the eggs collected on day 0 (mother absent clutches $p < 0.005$; mother present clutches $p < 0.01$, Fig. 3a).

When the absent and present treatments were separated into their respective sampling regimes, the beta diversity of the egg microbiomes differed significantly from absent day 0 to day 8 ($p < 0.005$) but not from absent day 8 to day 16 ($p = 0.83$, Fig. 3b). However, there were significant differences from absent day 0 to day 16 ($p < 0.005$). The mother present clutches, in contrast, displayed no significant changes in the egg microbiome community structure from present day 0 to day 8 ($p = 0.74$), from present day 8 to day 16 ($p = 0.89$), or between present day 0 and day 16 ($p = 0.06$). When directly comparing across the mother absent and mother present clutches, both the day 8 ($p < 0.005$) and day 16 ($p < 0.005$) time points revealed a significant difference in beta diversity (Fig. 3b). From the above results, our weighted Unifrac analyses show that the totality of mother present clutches was more tightly nested within the initial microbiome of day 0, while the mother absent clutches became more divergent after females were removed.

When pooled together, adults clustered on their own in vector space relative to all egg samples ($p < 0.01$), demonstrating their unique bacterial community structure (Fig. 3a). In spite of this, separated mother present adults and mother absent adults were significantly different from one another

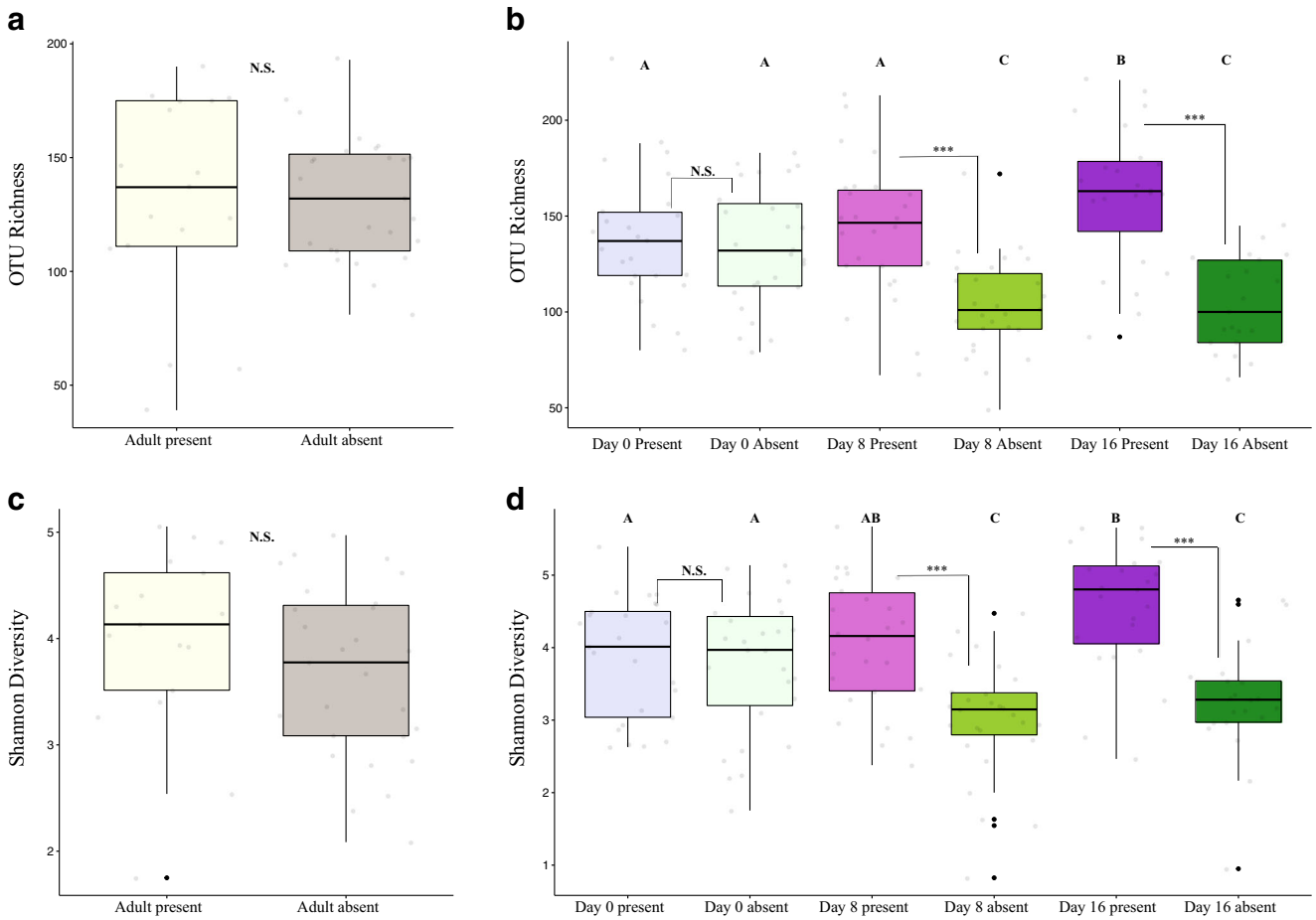


Fig. 2 Alpha diversity boxplots. OTU richness and Shannon diversity plots across various sampling regimes. **a** and **c** Results from the internal head capsule of attendant females. **b** and **d** Comparisons of clutches across treatments. Letters above treatments denote significant differences between groups. Lines within each boxplot indicate the median and whiskers display the range. Direct comparisons between the same day of the present and absent groups with significance values are depicted. Grey dots represent data points and black dots represent outliers. $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$, N.S. = no significant difference

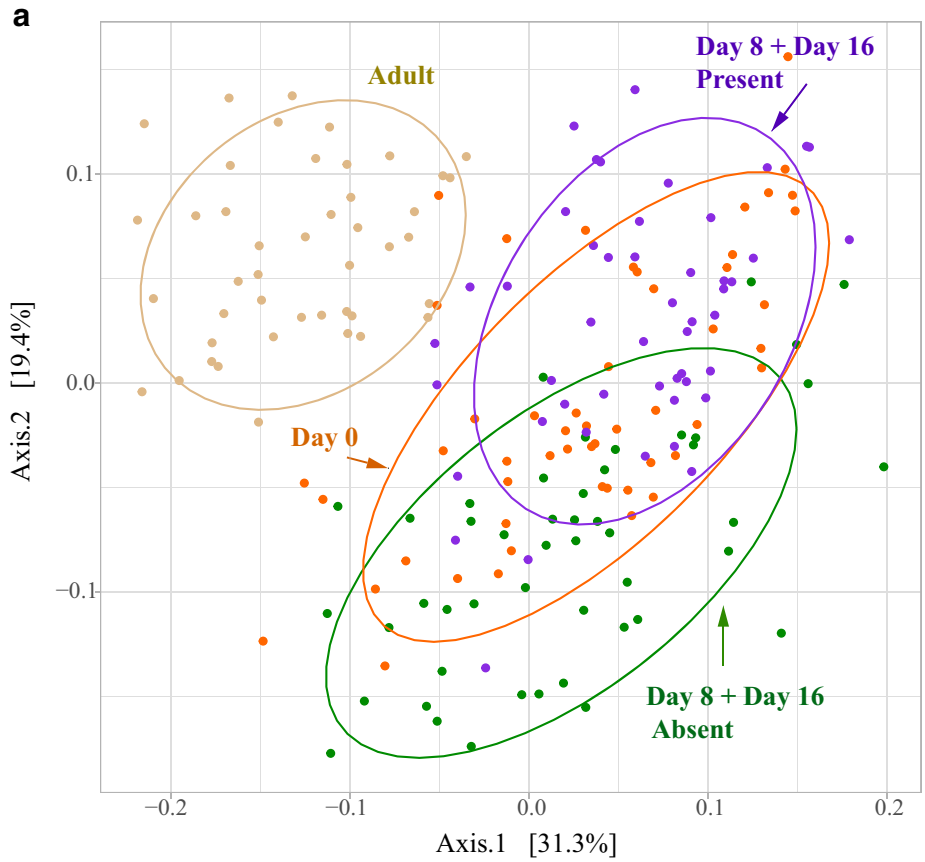
($p < 0.005$, Fig. 3b). This likely reflects a changing microbiome over the subsequent 16 days in the maternal head capsule of females that remained relative to females that did not, given that treatments were randomly assigned and equally distributed across initiation dates. This could be one additional contributing factor to the differences in egg microbiomes between treatments.

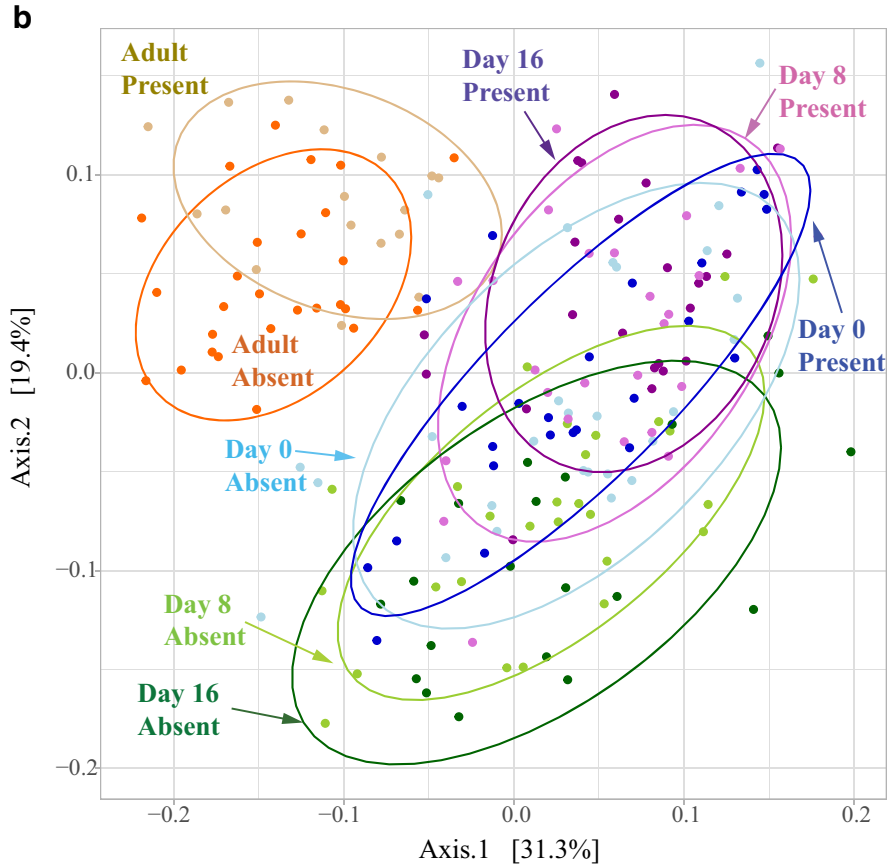
Community structure was composed of four principal Phyla: Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (Fig. 4a). Bacteroidetes dominated all experimental groups, irrespective of treatment or adult/eggs, accounting for 60–75% of community structure. Firmicutes, on the other hand, had the largest range, representing less than 2% of the absent day 16 community structure but comprising more than 12% of reads in adult females. Acinetobacter and Pseudomonas (Phylum Proteobacteria) and Sphingobacterium (Phylum Bacteroidetes) were the most abundant genera across all groups, comprising roughly 20–35% of all reads across the various time points (Fig. 4b). However, over 30 genera were present above the 1% relative abundance threshold, and at least 13 more were identified but unable to be classified by SILVA.

Fungal Analysis

A total of 13 clutches displayed fungal development prior to or on day 16 (mother present = 3, mother absent = 10). Unless significant fungal growth caused eggs to rupture or dissolve, samples were still taken for microbiome analysis. A one-tailed Chi-squared test with a Yates correction revealed that there were significantly fewer clutches with fungal growth in the female present treatment ($p < 0.05$); a one-tailed test was justified as Miller and Zink [33] had already shown that female removal enhances fungal presence on eggs in this species.

Infected mother absent time point samples ($n = 21$) within the 10 clutches that displayed fungus growth were significantly different from the remaining fungus-


a
Adult
Day 8 + Day 16
Present
Day 0
Day 8 + Day 16
Absent
0.1
0.0
−0.1
−0.2
−0.1
0.0
0.1
0.2
Axis.2 [19.4%]
Axis.1 [31.3%]


b
Adult
Present
Day 16
Present
Day 8
Present
Adult
Absent
Day 0
Present
Day 0
Absent
Day 8
Absent
Day 16
Absent
0.1
0.0
−0.1
−0.2
−0.2
−0.1
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0.1
0.2
Axis.2 [19.4%]
Axis.1 [31.3%]

◀ **Fig. 3** Beta diversity PCoA plots. **a** Weighted Unifrac PCoA depicting females in both treatments (gold), day 0 eggs from both treatments (red), and the remaining time points (days 8 and 16) concatenated into their treatment type (absent = green, present = purple). **b** Weighted Unifrac PCoA similar to **a**, but now present and absent groups have been separated into their respective time points (absent day 16 = dark green, absent day 8 = light green, absent day 0 = light blue, present day 16 = purple, present day 8 = pink, present day 0 = dark blue). Adults separated into present (gold) and absent (red) groups. PC1 represents 31.5% of the variation and PC2 represents 19.3% of the variation in all graphs and each is displayed with 85% confidence ellipses.

free mother absent time point samples ($n = 27$, $p < 0.01$), but the effect size was small compared with the other treatment types ($F = 20.82$, df = 192, $p < 0.001$, Online Resource 2, Figure S2). Despite this difference, mother absent egg samples without fungus remained significantly different from mother present samples ($p < 0.005$). The sample size of eggs infected with fungus in the mother present group was low ($n = 5$), so they were not analyzed.

Core Microbiome

Analysis of the bacteria found in adults revealed that eight OTUs were found in 100% of females sampled, and two additional OTUs in 98% of females (Online Resource 4, Table S5). Within the egg samples, four of the core adult OTUs demonstrated prevalence between 60 and 100% in the mother present clutches by day 16; in addition, there was a 45–85% drop in prevalence by day 16 in the mother absent clutches; two of the four OTUs were from the genus *Trabulsiella*, one from the genus *Lactobacillus*, and one from the genus *Enterococcus* (Fig. 5). Relative abundance measures of these four candidate OTUs across the various time points can be seen in Fig. 6. In the *Lactobacillus* and *Enterococcus* OTUs, there was a significant decrease in the OTU relative abundance in mother absent clutches when compared with the mother present clutches (*Lactobacillus* $p < 0.05$, *Enterococcus* $p < 0.005$). In both the *Trabulsiella* OTUs, however, there was no significant difference between treatments detected (*Trabulsiella* OTU1 $p = 0.18$, *Trabulsiella* OTU2 = 0.25). For all four of the core OTUs, OTU relative abundance was not different between present and absent adults (Online Resource 4, Figure S4).

BLAST was used to assign each of the core OTUs to a species level, if possible. BLAST analysis was unable to identify specific species from the two OTUs assigned to the genus *Trabulsiella*. The closest BLAST assignment for the one genus *Enterococcus* OTU was the species *Enterococcus thailandicus* (99% identity, *E* value = 0.0). For the one genus *Lactobacillus* OTU, *Lactobacillus curvatus* (98% identity, *E* value = 0.0) was revealed as the most confident species delineation. *Enterococcus thailandicus* does not have known anti-fungal characteristics; however, the *Trabulsiella*, *Enterococcus*, and *Lactobacillus* (including *L. curvatus*) genera all contain species with known anti-fungal properties [51–54]. Further analyses were conducted on other bacteria found within the same taxonomic categories of the core OTUs to investigate potential bias of microbial transfer among taxa with a high likelihood to contain anti-fungal properties (Online Resource 4).

Discussion

Our experimental results support the hypothesis that vertical transfer of microbes may be an important function of maternal care in earwig populations. In addition, we present evidence for the transmission of a core set of microbes from parent to offspring through extended maternal care within *Anisolabis maritima*. The core microbiome of both eggs and adults included bacteria that have the potential to serve a role in protecting earwig eggs from fungal infection and subsequent egg death.

The presence of attendant mothers had profound impacts on the abundance and community structure of bacteria on the egg surface across all biodiversity analyses. The sharp declines in both OTU richness and Shannon diversity in the mother absent clutches from day 0 to day 8 suggest that female attendance acts to maintain and possibly enhance bacterial diversity over time (Fig. 2, Online Resource 3, Table S4). Our finding that genera representing < 1% of the bacterial community are collectively reduced in the mother absent clutches (Fig. 4b) supports this hypothesis; less common bacterial species may be competitively exlcuded by a common bacterium that would otherwise be selectively suppressed if females were allowed to remain with their brood. While very unlikely, more indirect colonization (e.g., from other parts of the female's body or from feedbacks with infected sand itself) cannot be completely ruled out, and are suggestive of potential areas of future research.

We observed that the structure of the bacterial community between clutches with and without attendant females diverged significantly over time. Mother present days 8 and 16 clutches overlap with eggs from present day 0 more tightly than do the absent clutches, suggesting that female presence helps maintain the microbial community found on the egg surface shortly after oviposition. In contrast, female absence appears to result in a greater microbiome divergence from absent day 0 over the remaining 16-day period (Fig. 3b). Further, these results suggest that female presence is necessary to maintain the early established microbiome on the egg surface and that females are able to create a suitable egg surface microbiome within the first 48 h of nest attendance.

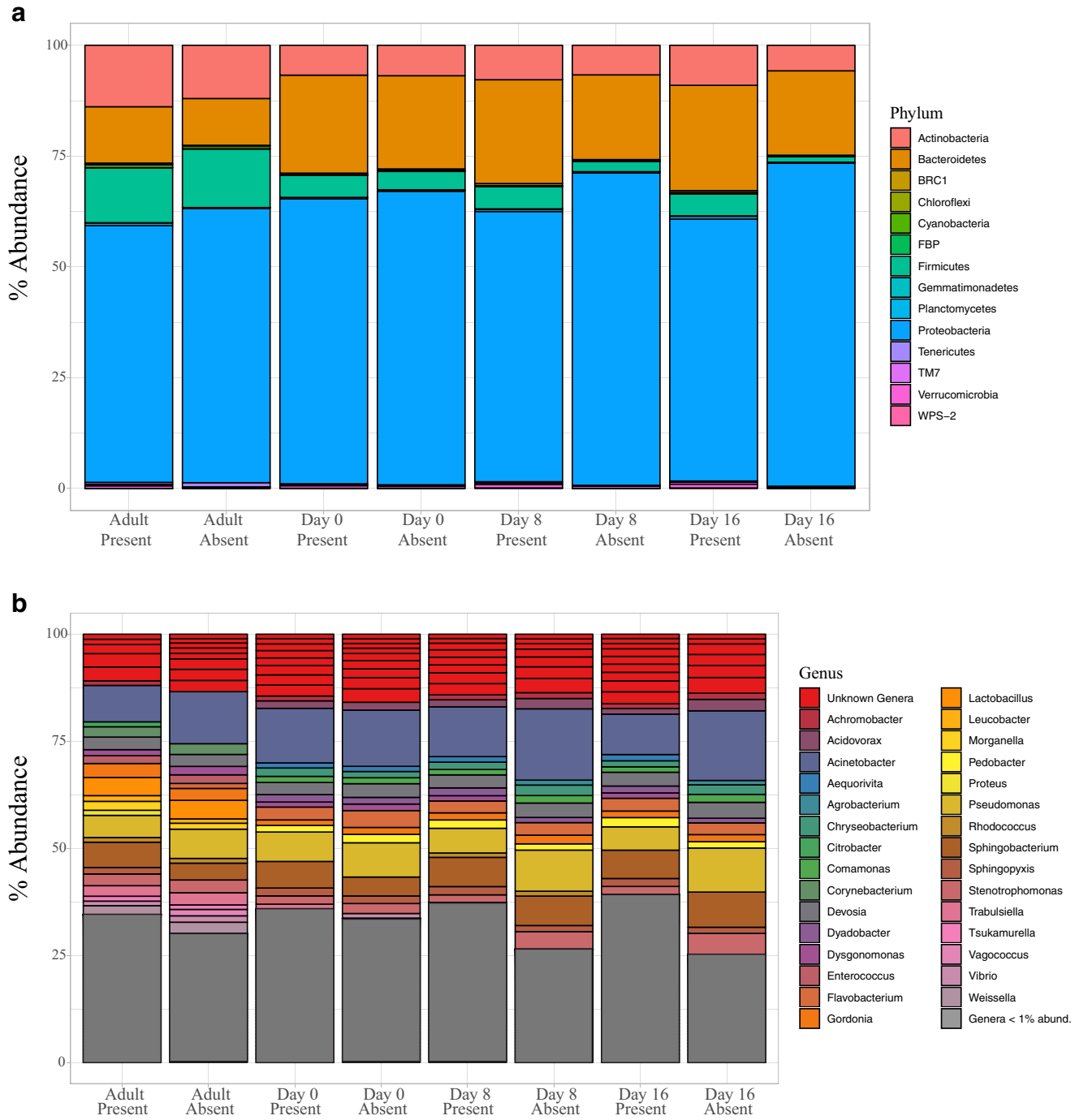

Fig. 4 Relative abundance stacked taxa plots. Plots of the various bacterial taxa found within the adult and clutch samples, with the length of a colored column representing the relative abundance of a particular group. **a** Relative abundance taxa plot of representative phyla across *Anisolabis maritima* adults and eggs from sample time points. **b** Relative abundance taxa plot at the genus level. Separate delineations within unknown genera represent separate (unidentified) taxa

Fungus was sometimes found in the absence of females despite our attempts to maintain a sterile environment. Although we did not determine the species of fungus present, we do not believe it was saprophytic for two reasons. First, once sand was exchanged in the mother absent clutches, the appearance of fungus dropped significantly. Second, when fungus emerged late in the egg development, some eggs hatched despite being infected (unpublished observations). We found that fungus-infected eggs displayed an altered microbiome in comparison with uninfected eggs (Online Resource 2). While a specific bacterial microbiome may have allowed invasion of fungus, it is also likely that the presence of

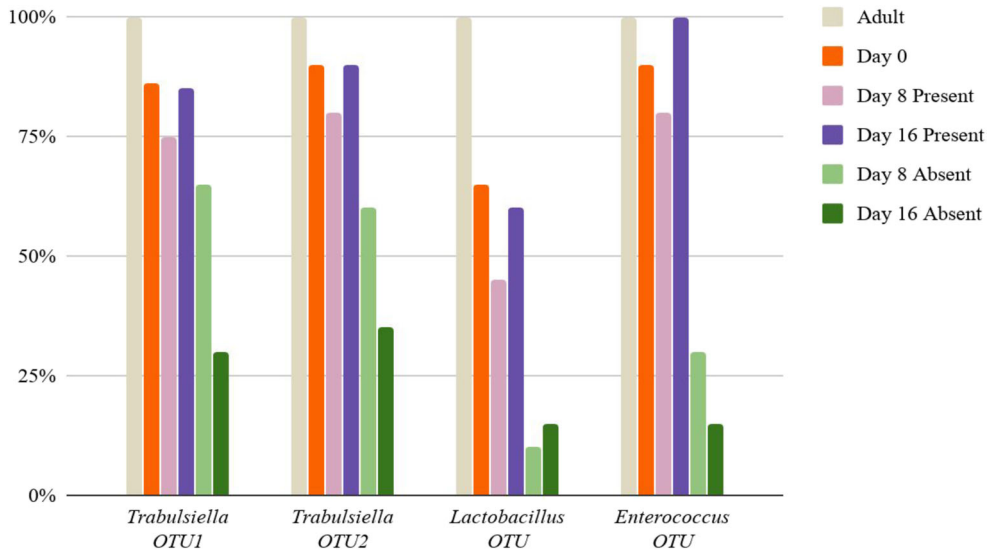

Fig. 5 Core OTU prevalence across treatments. Percent prevalence across the 4 core OTUs that show a pattern expected from vertical transmission: a maintenance of prevalence across present days and a decrease in prevalence among days where the female was removed from care

fungus further altered the bacterial microbiome through competition for resources, space, and the production of chemicals that may inhibit (or promote) bacteria growth [55]. Therefore, it is possible that these fungus-infected samples biased the overall community structure of the mother absent clutches to appear more divergent from the mother present group. However, this scenario is unlikely as restricting analysis to just uninfected eggs in the mother absent clutches showed a similar degree of divergence from mother present clutches as when infected mother absent eggs were included in the analysis ($p < 0.005$ in both cases).

Eight bacterial OTUs were present in all adults sampled, and of these, four demonstrated a potential pattern of vertical transmission as their prevalence was dramatically lower in the mother absent versus the mother present clutches (Fig. 5). The two *Trabulsiella* OTUs that fall into this category are intriguing candidates for vertical transmission of potentially beneficial bacteria from earwig mother to eggs. Although the gene region sequence did not allow us to determine exact species within this bacterial genus, prior research has found that other *Trabulsiella* species are symbiotic with termites that grow fungus such as *Odontotermes formosanus* [53, 54]. These *Trabulsiella* function in carbohydrate metabolism as well as aflatoxin degeneration; aflatoxins are poisonous carcinogens produced by certain fungi [53]. The potential for *Trabulsiella* bacteria to function in an anti-fungal capacity in the maritime earwig remains an intriguing area for future research; however, it is important to note there were no significant differences across treatments for either *Trabulsiella* OTUs (Fig. 6c, d). So while initial egg inoculation of *Trabulsiella* may serve a protective function, prolonged female presence for maintaining these bacteria may be less important.

The core *Lactobacillus* OTU, *L. curvatus*, had much more dramatic short-term changes in relative abundance compared with *Trabulsiella*, as a result of female removal (Fig. 6b). It is important to note that the transfer of *L. curvatus* to clutches in the mother present group was not ubiquitous or consistent; the bacterium was usually present in approximately half of the egg samples (Fig. 5; Online Resource 4, Table S5). In spite of this low fidelity, the anti-fungal properties of *L. curvatus* are well-established in other systems [52]. As such, maternal transmission of *L. curvatus* does present one of the several potential mechanisms for egg protection from fungal infection.

The core *Enterococcus* OTU presented the most dramatic changes in both presence (Fig. 5) and relative abundance (Fig. 6a) when comparing the mother present and mother absent clutches. The core bacterium identified, *E. thailandicus*, is a lactic acid producing bacteria; however, no clear anti-fungal properties have been identified thus far in this species [51]. However, several other *Enterococcus* species have been well-established as having anti-fungal properties, such as *Enterococcus faecalis* and *Enterococcus durans* [56, 57]. One important caveat is that this genus is typically associated with gut bacteria; the translocation from gut to mouthparts and/or salivary glands is a potential mechanism of transfer to eggs that should be further investigated in these earwigs [58].

Despite the significant prevalence and relative abundance changes in the aforementioned core OTUs (namely *L. curvatus* and *E. thailandicus*), the overall relative abundance of these bacteria in both adult and egg samples is considerably low, never exceeding 2% of all reads (Fig. 6). This result strongly suggests that there are likely to be multiple bacteria that serve as candidates for antifungal function in vertical transmission, representing a broader community of environmentally acquired bacterial species. In fact, further analysis revealed the vertical transmission pattern found among the *Lactobacillus* and *Enterococcus* OTUs is also maintained at their respective higher taxonomic levels and with greater relative abundance among egg samples (Online Resource 4, Figure S5).

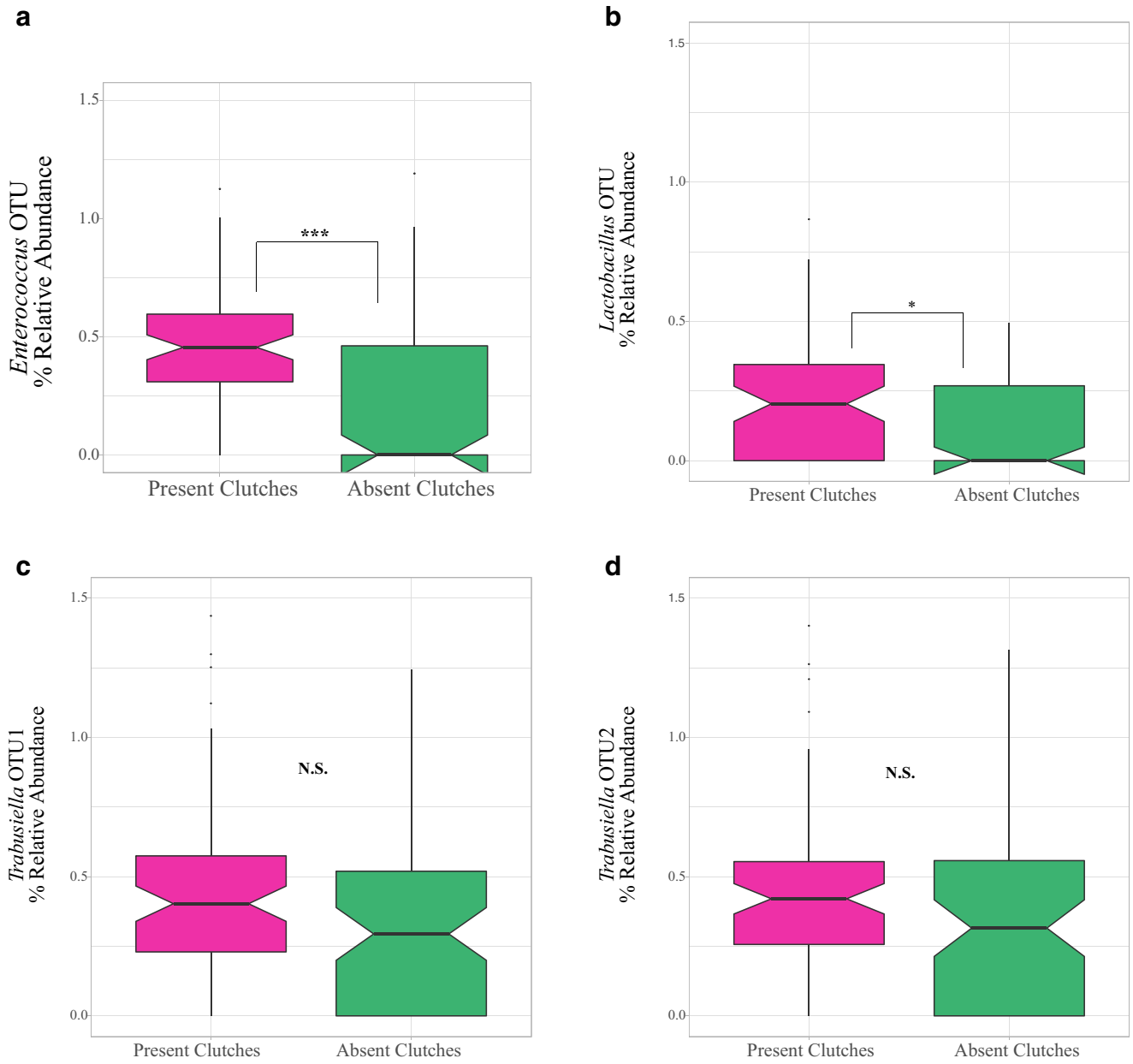

Fig. 6 Differences in relative abundance among the core OTUs showing a potential vertical transmission pattern. Comparisons of relative abundance between present/absent groups in **a** *Enterococcus*, **b** *Lactobacillus*, and **c** and **d** *Trabulsiella* OTUs. *Y* axis represents percent relative abundance in the microbiome. If boxplot waists do not overlap between groups, it demonstrates significant differences between the medians. Significant values based on GLMM analysis. $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$, N.S. = no significant difference

In contrast to a previous study on the earwig gut [59], our study focused on the earwig head capsule to address the role of mechanical egg cleaning via the mouth in nest-tending behavior. The results of our experiment cannot exclude the possibility of female ingestion of the fungal spores during mechanical cleaning rather than direct anti-fungal action by bacteria per se. Our study does not control directly for this, but it is likely that both mastication and transfer of symbiotic bacteria function simultaneously to aid in clutch survival. Future experiments could disentangle these two mechanisms by repeating this experiment with a third treatment where eggs are exposed to female microbes (e.g., through manual application of earwig head extractions), but not the live females themselves. If mechanical removal is also important, then the presence of a female (despite bacteria being present in both treatments) should have an added effect on the bacterial community by day 16. This bacterial removal through mastication by tending females could alter the overall microbiome in a way that maintains (or even increases) diversity (e.g., the intermediate disturbance hypothesis) [60, 61]. While very

unlikely, more indirect colonization (e.g., from other parts of the female's body or from feedbacks with infected sand itself) cannot be completely ruled out, and are suggestive of potential areas of future research.

If the bacteria transferred to eggs do serve an anti-fungal function for *A. maritima*, this would allow nest-attending females to reduce their energetic burdens of care (e.g., require less mechanical removal of fungal spores). Energy that would otherwise be allocated to anti-fungal metabolite production could be redistributed to other functions, including future reproduction and homeostasis. Energy conservation becomes particularly important in earwig parental care, as females are known to readily cannibalize both their eggs and/or starve nymphs under energetically strained conditions [33, 62, 63]. If the deposition of anti-fungal microbes can replace the energetically costly mechanical removal of fungi from eggs, this could both decrease the need for filial cannibalism (eating current eggs to recover energy) and increase future fecundity. Future research should address how females initially acquire these beneficial bacteria, whether it be from their environment or conspecific versus filial cannibalism [33, 34, 64, 65].

Apart from the invertebrates so far described, vertebrates, including birds and humans, demonstrate vertical transmission of beneficial microbes to offspring [13, 66]. Given the generality of microbial inoculation through parental care across taxa, we suggest that it may represent an evolutionarily stable strategy that is frequently exploited by vertebrates and invertebrates alike. By doing so, adults are better able to maximize their reproductive output, while minimizing the energetic burdens of care. As such, this work adds to our collective understanding of the widespread role of maternal care as well as the major functions of microbial associations with animal taxa. Through the use of 16S rRNA amplicon sequencing, we have uncovered a possible mechanism by which maternal care protects developing offspring in *A. maritima*. Candidate mutualistic bacteria have been identified that may serve an anti-fungal role as a result of nest attendance by brooding females. The results suggest that multiple bacteria may work in tandem to establish and maintain clutch protection, though it is reliant on constant female care to maintain appropriate bacterial loads. While we found candidate bacteria with anti-fungal characteristics, future work is needed to determine if these bacteria have a functional role in this system. As such, the culturing and isolation of these bacteria from mothers and eggs should be done to test whether these candidate bacteria produce anti-fungal compounds, which would strengthen the argument of bacterial inoculation as a protective mechanism in *A. maritima* clutches. The implications of these results suggest that parental care transfer of a microbiome to eggs allows females to conserve energy for both themselves and subsequent broods and may be a key evolutionary tactic for optimizing offspring survival.

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Author Contributions Jordan Greer designed the project, collected specimens and samples, conducted DNA-based lab work, collected and analyzed the data, and contributed to the manuscript. Andrea Swei contributed to the analysis of the microbiome data, contributed reagents and analytical tools, and contributed to the manuscript. Vance Vredenburg contributed analytical tools, helped with intellectual design, and contributed to the manuscript. Andrew Zink designed the project, collected specimens, analyzed data, and contributed to the manuscript. The first draft of the manuscript was written by Jordan Greer and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability Microbiome DNA sequence data are publicly accessible on the Sequence Read Archive (SRA) database under the BioProject accession number: PRJNA630101.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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