EFFECT OF PARENTAL CARE ON THE VERTICAL TRANSMISSION OF ENTERIC BACTERIA IN *NICROPHORUS DEFODIENS*

by

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ABSTRACT

Author: Miller, Christopher, J. MS
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Title: Effect of Parental Care on the Vertical Transmission of Enteric Bacteria in *Nicrophorus defodiens*
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Parental care has evolved promote fitness gains. Burying beetles engage in extensive pre-hatching and post-hatching parental care providing several avenues to transmit bacteria to their offspring. One aspect of pre-hatching parental care consists of preserving a small vertebrate carcass via oral and anal secretions, allowing the carcass to be used as a reproductive resource. Post-hatching parental care consists in large part of parental regurgitations of the preserved carcass. We sought to determine if pre-hatching parental care resulted in bacterial transmission from adults to carcasses via anal secretions. We then sought to determine if lab-rearing conditions affected the digestive tract bacterial communities of F1 and F2 generation adults. We finally sought to determine if carcasses and post-hatching parental care in the form of parental regurgitations resulted into bacterial transmission to larvae. Using High-Throughput Illumina MiSeq, we were able to characterize bacterial communities of adult and larval digestive tracts, anal secretions, and unprepared and prepared carcasses. Our results show that bacterial communities of adults are dissimilar from anal secretions and prepared carcasses. We then show that lab-rearing conditions do not significantly alter digestive tract bacterial communities of F1 and F2 generation adults relative to wild caught adults. We proceed to show that larvae receiving parental regurgitations have digestive tract bacterial communities similar to their parents whereas larvae that do not receive parental regurgitations have dissimilar digestive tract bacterial communities from their parents. We further show that bacterial communities of prepared carcasses are dissimilar from all larvae. Our evidence suggests that anal secretions to preserve carcasses for the reproductive bout and have no influence on bacterial transmission to neither carcasses nor larvae. Our evidence also suggests that parental regurgitations influence bacterial transmission to offspring.

INTRODUCTION

Microbes provide beneficial and sometimes essential functions for their hosts. For example, they may aid in development (Baendle *et al.,* 2003), assist in digestive processes and nutrient acquisition (Backhead, 2005), act as an innate defensive barrier against pathogens (Ferrari *et al.,* 2007), and prime the host immune system (Macdonald & Monteleone, 2005). Some host-microbial relationships are responsible for creating or preserving a food source. For example, *Littoraria irrorata* (sea snails) defecate on wounded sea grass in order to preferentially culture Ascomycetes, an important fungal food source (Sillman, 2003). *Acromyrex octospinosus* (leafcutter ants) cultivate fungal gardens in or around their colonies as a food source (Quinlan & Cherret, 1978). These ants will also secrete antimicrobial-producing bacteria onto their fungal cultures in order to preserve and protect their resource from other microbial competitors (Currie *et al.,* 1999). *Philanthus traingulum* (European beewolves) provide honeybees for developing larvae using venom with paralytic (Strohm *et al.,* 2001) and antimicrobial activity that prevents fungal growth and carcass decomposition (Strohm *et al.,* 2001).

The beneficial nature of host-microbial relationship makes it imperative that organisms are capable of transmitting their microbes to subsequent generations. There are a number of ways in which insects vertically transmit their bacteria. Obligate enteric bacteria, such as *Blochmannia* in carpenter ants and *Buchnera* in aphids, are transmitted through the female germline (Baumann, 2005; Koga *et al.,* 2012). Bacteria may be indirectly vertically transmitted; for example, coprophagous insects including Isoptera (termites) and Hemiptera (true bugs) shed their bacteria into feces, which are ingested by offspring (Bourtzis & Miller, 2006). Other social insects such as *Bombus terrestris* (bumble bees) transfer bacteria to offspring via trophallaxis and/or smearing bacteria-containing secretions onto a food reservoir that is consumed by offspring (Koch & Schmid-Hempel, 2011). Fire bugs (Pirrhocoridae) produce microbe-containing secretions that are applied to and absorbed by eggs allowing for microbial acquisition (Kaltenpoth *et al.,* 2009).

Recent research with burying beetles (*Nicrophorus* spp.) has explored the role of microbes found in their anal secretions. These microbes are placed on small vertebrate carcasses, which is the burying beetle's food resource for their larval offspring (Duarte *et al.,* 2017; Shukla *et al.,* 2018). When a burying beetle discovers a carcass, it is buried underground, stripped of fur or feathers, rolled into a ball, and covered with oral and anal secretions (Trumbo, 1992; Scott; 1998,

Arce *et al.,* 2012; Trumbo *et al.,* 2016). Oral and anal secretions contain antimicrobial molecules that preserve the carcass by eliminating decomposing microbes (Arce *et al.*, 2012; Arce *et al.,* 2013). For example, *Nicrophorus* secretions can control the carcass microbiome by promoting the growth of *Yarrowia,* an oleaginous fungal yeast, and inhibiting growth of other fungal species (Shukla *et al.,* 2017; Vogel *et al.,* 2017). These secretions also contain a variety of bacterial and fungal species (Shukla *et al.,* 2017; Vogel *et al.,* 2017). Controlling the carcass microbiome in this way provides larvae prolonged and easy access to carcass nutrients, which in turn can increase their survivorship (Shukla *et al.,* 2018; Vogel *et al.,* 2017).

Once larvae have hatched from their eggs and arrive on the prepared carcass, parents engage in post-hatching parental care, which consists in large part of carcass regurgitations (Eggert, Reinking, & Müller, 1998; Capodeanu-Nägler *et al.,* 2016). Post-hatching parental care results in faster growth rates and greater survivorship for developing larvae (Eggert, Reinking, & Müller, 1998). Some burying beetles, such as *N. orbicollis* and *N. americanus*, depend on carcass regurgitations (Capodeanu-Nägler *et al.,* 2016). Other species, such as *N. vespilloides* and *N. defodiens*, are less dependent on parental regurgitations and can survive without them, however larval growth rates and survivorship are negatively impacted (Eggert, Reinking, & Müller, 1998; Capodeanu-Nägler *et al.,* 2016). Pre-hatching and post-hatching parental care provide several avenues for *Nicrophorus spp.* to transmit bacteria to their offspring and in this study, we explore how both pre-hatching and post-hatching parental care influence bacterial transmission to larvae in *N. defodiens.*

Evidence indicating transmission of fungi through the anal secretions of *Nicrophorus* spp. onto the carcass has been established (Shukla *et al.*, 2017; Shukla *et al.*, 2018). In addition, similar bacterial species are found in the adult beetle digestive tracts, their anal secretions, and the prepared carcass (Duarte *et al.,* 2017; Vogel *et al.,* 2017). However, it is not known if *Nicrophorus* secretions are a medium for microbial transmission. In this study, we test two alternative hypotheses regarding the transfer of adult *Nicrophorus defodiens* digestive tract bacteria on to the prepared carcass via anal secretions: adaptive bacterial transmission (ABT) vs. non-adaptive bacterial transmission (NABT) hypotheses. The ABT hypothesis suggests that anal secretions deposit adult digestive tract bacteria onto the carcass in order to control the microbial community of the prepared carcass. Here digestive tract bacteria dominate secretions and carcasses, and act as a mode of preservation and/or as a potential route of microbial transmission to larvae. The NABT

hypothesis suggests the role of anal secretions is to preserve the carcass through antimicrobial compounds and promoting the growth of *Yarrowia*. In this later case, adult digestive tract bacteria do not dominate secretions or carcasses, and any bacteria that are transmitted to a carcass are a non-adaptive byproduct of the carcass preservation process mediated by the secretions.

To test these two hypotheses, we performed high-throughput sequencing of the bacterial communities found in adult digestive tracts, in their anal secretions, and on prepared carcasses. For the purpose of comparison, we sequenced bacterial communities found on unprepared carcasses to gain insight into the degree that which secretions/bacteria shape prepared carcass bacterial communities. The ABT hypothesis predicts that (1) anal secretions contain bacterial communities that are similar to that of adult beetle digestive tracts; (2) prepared carcasses possess bacterial communities that are similar to those found in anal secretions; and (3) bacterial communities in adult digestive tracts are similar to those on prepared carcasses (Figure 1). The NABT hypothesis predicts (1) bacterial communities found within anal secretions can be similar or different from those found in adult digestive tracts; (2) bacterial communities found in anal secretions are different from those found on prepared carcasses; and (3) bacterial communities in adult digestive tracts are different from those found on prepared carcasses (Figure 1).

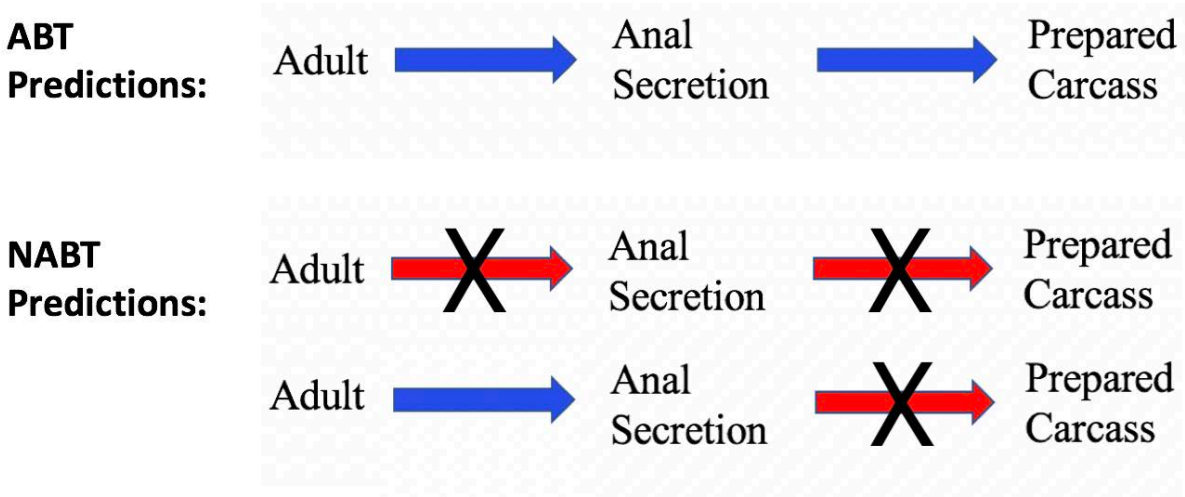

Figure 1. Predictions for hypotheses**.**
Outline of predictions for A) the adaptive bacterial transmission (ABT) hypothesis and B) the non-adaptive bacterial transmission (NABT) hypothesis. Green arrows indicate bacterial transmission is occurring and bacterial communities between sample types are highly similar. Red arrows indicate bacterial transmission is not occurring and bacterial communities between sample types are essentially distinct

Two potential pathways of vertically transmitting digestive tract bacteria to larvae are directly through regurgitations or indirectly through oral and anal secretions applied to a carcass. In this study, we evaluated these two pathways using the burying beetle *N. defodiens*, a species that can survive in the absence of parental regurgitations. In addition, we explored how lab-rearing conditions impact the digestive tract bacterial communities of F1 and F2 generation adults relative to wild caught adults and determined if there was a change in bacterial communities when non-reproductive adults transition to the reproductive adult stage. To approach these objectives, we used a culture-independent means of characterizing bacterial communities. Our study builds on the previous work by Wang & Rozen (2017), who used culture-based methodology to address the role of parental regurgitations in microbial transmission. Due to the limitations of culture-based methodologies (Davis, 2014; Demarco & Burnham, 2014; Douterelo *et al.,* 2014), a culture-independent approach was utilized to better address this question. We took this approach by characterizing bacterial communities using Illumina high-throughput sequencing of the 16s rRNA gene.

We characterized the bacterial communities in the digestive tracts of laboratory-reared adults, larvae either receiving or not receiving parental regurgitations, and on prepared carcasses. We compared the bacterial communities of adults to prepared carcasses, adults to their larvae, and larvae to the carcasses on which they were raised. If regurgitations influence the transmission of digestive tract bacteria, we expect larvae receiving parental regurgitations to have digestive tract bacterial communities similar to their parents; and larvae not receiving parental regurgitations to have digestive tract bacterial communities dissimilar to their parents. If prepared carcasses influence the transmission of digestive bacteria to offspring, we expect prepared carcasses to have bacterial communities similar to those of adults; and all larvae to have digestive tract bacterial communities similar to those of prepared carcasses.

In addition, we explored the effect of laboratory-rearing conditions on wild caught digestive tract bacterial communities and if there is a transition in digestive tract bacterial communities when adults shift from the non-reproductive to reproductive stage. We did this by characterizing digestive tract bacterial communities of wild caught non-reproductive adults, F1 & F2 laboratory-reared non-reproductive adults, and F1 & F2 laboratory-reared reproductive adults. If laboratory-rearing conditions affect digestive tract bacterial communities of wild caught adults, we expect digestive tract bacterial communities of wild caught non-reproductive adult and

laboratory-reared non-reproductive adults to be dissimilar. If there is a transition in digestive tract bacterial communities when adults shift from the non-reproductive to reproductive stage, we expect digestive tract bacterial communities of non-reproductive and reproductive adults to be dissimilar.

METHODS

Beetle Collection, Population Maintenance, Carcass Sampling

Baited pitfall traps were used to collect *N. defodiens* during June and August of 2017 in Big Falls, Wisconsin. Captured beetles were used to establish the laboratory population used in our study. The laboratory population was kept individually in small plastic containers (7 x 7 x 5 cm) with moist paper towels. All beetles were kept in an environmental chamber set to 20^{O}C with a 14:10 hour light:dark cycle (Krishnan *et al.*, 2014) and fed chicken liver twice weekly (Creighton *et al.*, 2014). Upon reaching sexual maturity, roughly 15 - 25 days after eclosion, mating pairs were placed in large, sterilized plastic containers (18 x 15 x 10 cm) with a freshly thawed 15-20 g mouse carcass on top of 4 – 5 inches of freshly autoclaved commercial top soil.

To characterize carcass bacterial communities, unprepared carcasses were sampled immediately after thawing by swabbing all external surfaces with a sterile cotton swab that was dipped in sterile PBS. Forty-eight hours after beginning the reproductive bout, prepared carcasses were swabbed on all external surfaces and within feeding holes using a sterile cotton swab that was dipped in sterile PBS. Prepared carcasses were defined as having all hair removed and having an observational presence of anal secretions (Duarte *et al.*, 2017). Cotton swab tips were placed in 1.5 mL Eppendorf tubes and stored in pure glycerol at -80^{O}C until needed for DNA extraction (Metherel & Stark, 2015).

Anal Secretion Collection

To characterize bacterial communities found in anal secretions, females were removed from brood chambers 48 hours after initiation of a reproductive bout. Due to the beetles' posterior being dragged across the carcass and soil, beetle posteriors' were surface sterilized with 70% isopropanol wipes to eliminate external contamination. Beetles were air dried and anal secretions were collected by gently pressing a sterile capillary tube directly onto the beetle's posterior. Anal secretions were transferred to a .5 mL Eppendorf tube and diluted 1:5 with sterile PBS and stored at -80^{O}C until needed for DNA extraction (Kaltenpoth & Steiger, 2013).

Treatment Set Up

To evaluate the effect on lab-rearing conditions on wild-caught beetles, three male and three female wild caught adults were immediately surface sterilized and dissected upon arrival to the laboratory. Wild caught adults were not exposed to a small vertebrate carcass for at least 48 hours and were therefore considered non-reproductive. F1 and F2 generation non-reproductive adult digestive tracts were also sampled. Four F1 and three F2 males and females were individually placed in brood chambers with freshly autoclaved commercial top soil and a small portion of chicken liver. After 48 hours, beetles were removed from their brood chambers and immediately surface sterilized and dissected.

To evaluate the effect of post-hatching parental care on digestive tract bacterial transmission, three experimental treatment groups were setup. In the biparental care (BPC) treatment group, both parents remained with their larvae for the duration of the reproductive bout (7 – 9 days). Immediately prior to larval dispersal, both parents and three larvae were removed from brood chambers, immediately surface sterilized, and dissected. In the maternal care only (MC) treatment group, the male parent was removed from the brood chamber prior to larval arrival on the carcass, immediately surface sterilized, and dissected. The female parent remained with larvae for the remainder of the reproductive bout (5 – 7 days) and just prior to larval dispersal, the female parent and three larvae were removed from the brood chamber, immediately surface sterilized, and dissected. In the no-parental care (NPC) treatment group, both parents were removed from the brood chamber prior to larval arrival on the carcass, immediately surface sterilized, and dissected. This eliminated any possibility of post-hatching parental care within this treatment group. To aid in larval survival, sterile fine point scissors were used to grind up carcass tissue inside of the feeding hole at 24-hour intervals for three days. Prior to larval dispersal, three larvae were removed from the brood chamber, immediately surface sterilized, and dissected. All reproductive adults used in this study were either F1 or F2 generation beetles.

Dissections

All beetles and larvae were dissected immediately upon removal from the brood chamber in order to minimize the effect of sudden environmental changes on digestive tract bacterial communities (Krishnan *et al.,* 2014). Beetles and/or larvae were surface sterilized by rinsing in

70% ethanol twice and again with sterilized diH_20 eliminating contamination from the soil, carcass / liver, or beetle exoskeleton. Specimens were euthanized by decapitation using sterilize fine point scissors. Body cavities were opened by creating an incision at anterior end and cutting down the side of the beetle towards the posterior using sterile fine point scissors. Sterile insect pins were used to pin open body cavities and the complete digestive tract was removed with sterile fine point forceps. Digestive tracts were placed in .5 mL Eppendorf tubes, submerged in pure glycerol, and stored at -80^{O}C until needed for DNA extraction (Metherel & Stark, 2015).

DNA Extractions and High-Throughput DNA Sequencing

DNA was extracted from cotton swab tips using a DNeasy Power Soil DNA Extraction Kit (Qiagen, Venlo, Netherlands). Cotton swab tips were added directly to the beaded tube and the protocol from the manufacturer was followed. DNA from anal secretion samples was extracted using a DNeasy Blood and Tissue DNA Extraction Kit (Qiagen, Venlo, Netherlands) following manufacturer protocol. DNA was extracted from *N. defodiens*' digestive tracts using the same DNeasy Power Soil DNA extraction kit. The manufacturer's protocol was followed with the addition of a 10-minute heating step at 75^{O}C prior to vortexing the beaded tube with solution A. The additional heating step aided in degradation of intestinal and stomach tissue that might otherwise not have been degraded as a result of mechanical force from vortexing. Pure DNA extracts were sent to the University of Colorado – Boulder for High Throughput Illumina MiSeq following previously described methods (Carini *et al.*, 2016).

Bioinformatic Processing

Barcoded sequences were imported into Quantitative Insights into Microbial Ecology (QIIME) v. 2.4. Multiplexed single-end reads were demultiplexed and chimeric sequences were removed and samples were subsequently denoised via the Deblur pipeline (Caporaso *et al.*, 2010; Callahan *et al.*, 2016). Samples were rarefied to 5,000 sequence reads per sample and operational taxonomic units (OTUs) were assigned using the Greengenes 13_8 reference database with an 88% sequence similarity (Callahan *et al.*, 2016; Bokulich *et al.*, 2018). Taxa bar plots were generated using the q2-feature-classifier plugin (Bokulich *et al.*, 2018). Representative sequences from each OTU were aligned and masked using mafft (Katoh & Standley, 2013) and a rooted

phylogenetic tree was created in FastTree (Price, Dehal, & Arkin, 2009). Alpha- and beta-diversity analyses were carried out with the q2-diversity plugin (Caporaso *et al.*, 2010).

Statistical Analysis and Source Tracking

Statistical analysis was carried out using QIIME v.2.4 and using the R version 3.4.3 software package (https://www.r-project.org/). Kruskal-Wallis analysis was performed in QIIME v.2.4 and was used to compute alpha-diversity indices (Chao1, Evenness, Faith's Phylogenetic Diversity, Observed OTUs [standard richness], and Shannon-Weiner). Bray-Curtis dissimilarity distance matrices were utilized for Analysis of Similarities (ANOSIM) with 999 free permutations. ANOSIM produces an R^2 value ranging from -1 – 1 where values closer to 0 represent a more similar relationships and values further from 0 represent more dissimilar relationships (Price *et al.*, 2017). Permutational multivariate analysis of variance (PERMANOVA) using 999 free permutations was performed in R version 3.4.3. Post-hoc PERMANOVA pairwise analyses were conducted with Bonferroni corrections. PERMANOVA produces a pseudo-F value that when closer to 0 represents a more similar communities and when further from 0, a more dissimilar relationship.

Source tracker is an R software package that utilizes a Bayesian approach to analyze bacterial communities to determine the percentage of bacteria of a given sink that are derived from a given source (Knights *et al.*, 2011). Source Tracker assumes that a given bacterial community is a mixture of bacterial communities from either known or unknown sources and proceeds to estimate the percentage of a given bacterial community that is derived of other bacterial sources (Flores *et al.*, 2011; Knights *et al.*, 2011). If a given bacterial community contains taxa that do not match taxa from a source environment, those bacteria are assigned to an "unknown" source (Flores *et al.*, 2011; Knights *et al.*, 2011).

RESULTS

Bacterial Transmission to Prepared Carcasses via Anal Secretions

High Throughput Sequencing

Quality filtering and chimera removal resulted in 677,802 high quality sequence reads, with each of the 50 samples having 13,557 (± 4,348) bacterial sequences on average. Two extraction-blank samples contained less than 150 sequences each, suggesting minimal contamination. These sequence types were removed from all samples prior to subsequent analyses. All samples were rarefied to a sampling depth of 5,000 sequences. Rarefaction analysis (Fig 2) showed the collector's curve saturated at a sampling depth of ca. 4,500 sequences, suggesting that rarefaction at a depth of 5,000 sequences captured the full breadth of bacterial diversity in all samples. Rarefaction did not lead to subsequent loss of any samples. Clustering recovered an assembly of 605 distinct OTUs; however, OTUs not represented by 50 sequences or more were removed from subsequent analyses due to their potential to be sequencing artifacts (Brown *et al.*, 2005).

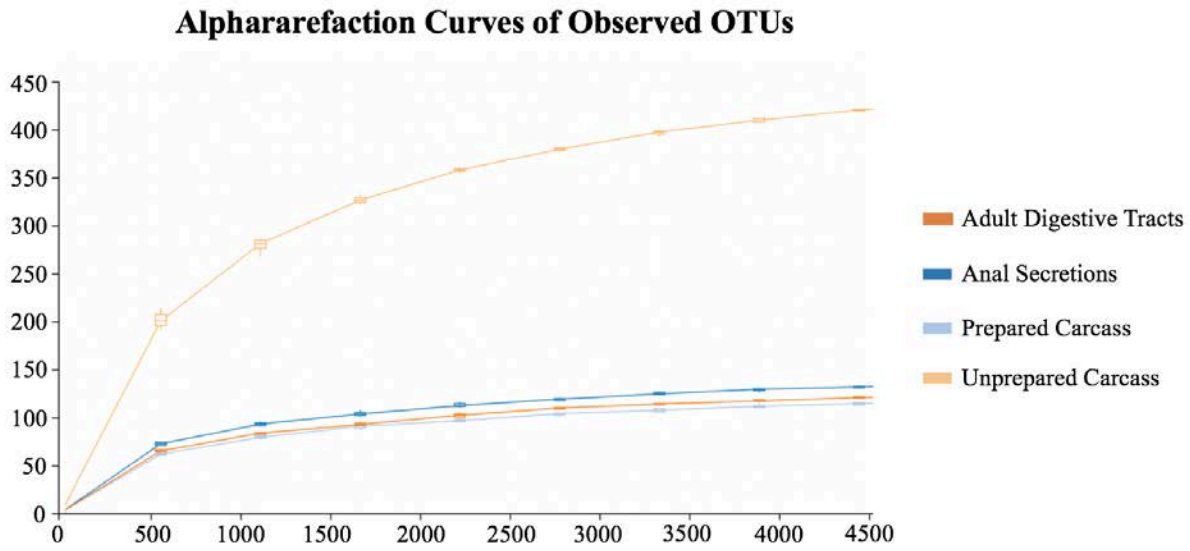

Figure 2. Alphararefaction of observed OTUs.
Observed OTUs found in adult digestive tracts (n=20), their anal secretions (n=10), on unprepared carcasses (n=10), and on prepared carcasses (n=10). Rarefaction curves show the total number of unique OTUs per sample type.

Taxonomic Assignments

Each sampling type was dominated by two classes of bacteria (Fig 3). The dominant classes for adult digestive tracts were *Gammaproteobacteria* (41% of all sequences) and *Clostridia* (28%). The *Gammaproteobacteria* (45%) and *Betaproteobacteria* (16%) dominated prepared carcasses, while the most dominant classes for unprepared carcasses were *Gammaproteobacteria* (26%) and *Clostridia* (13%). Dominance in the anal secretions differed slightly from the other sample types, with the dominant classes in this sample type being *Bacilli* (30%) and *Clostridia* (28%).

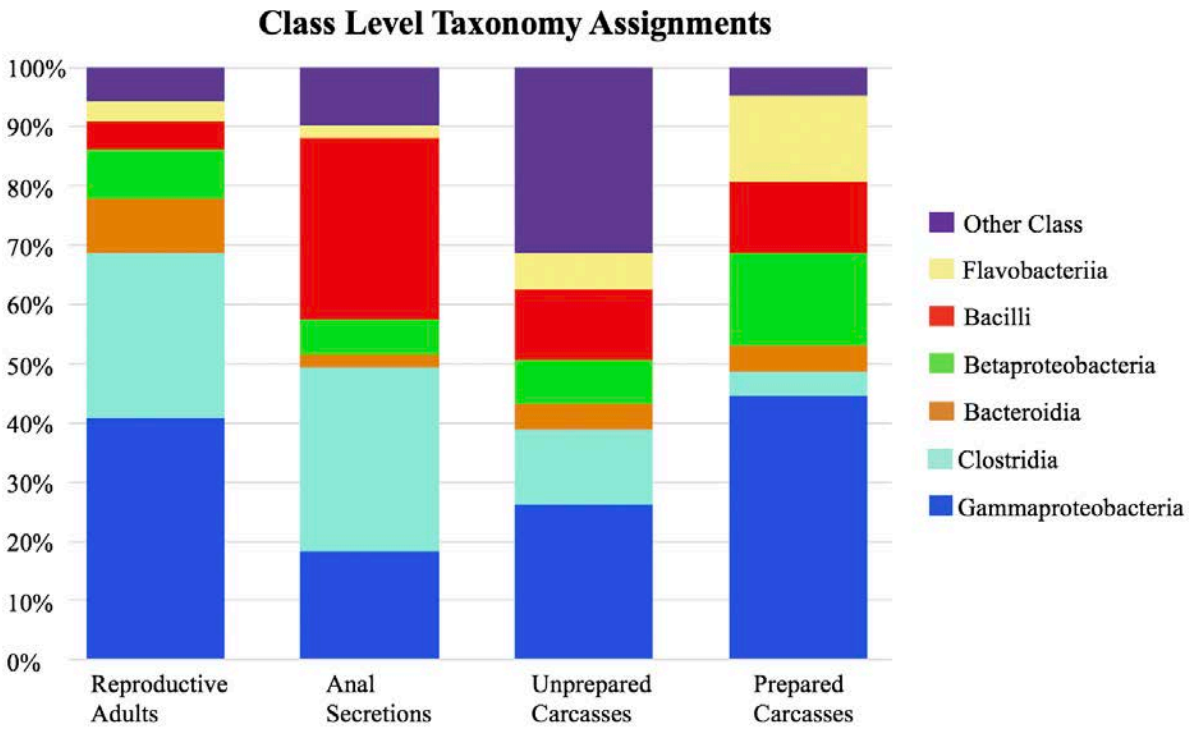

Figure 3. Class level taxonomic assignments.
Bar lengths describe the average percentage of each bacterial class found within sample types. All classes not specifically identified were assigned to Other including unidentified bacterial classes.

Relative abundances of prevalent class- and genus-level bacterial groups differed among all sample types (Table 1). Adult digestive tracts had three bacterial groups with a relative abundance greater than 10%: *Wohlfahrtiimonas* (13.3%), unidentified Gammaproteobacteria (10.4%), and *Clostridium* (10.2%, Table 1). Anal secretions also had three bacterial groups with relative abundances greater than 10%: unidentified *Planococcus* (13.3%), unidentified *Tissierella* (10.4%), and unidentified *Lactobacillus* (10.0%). Unprepared carcasses, which exhibited the greatest overall diversity, had only one bacterial group, *Acinetobacter* (10.5%), with a relative abundance greater than 10%, while the prepared carcasses had two, *Acinetobacter* (24.8%) and *Vitreoscilla* (14.3%).

Table 1. Percentage of common taxa found across sample types.
Taxonomic assignments of the most common bacterial genera/class found in each sample type. A relative abundance threshold of > 5% of all sequences was used here for taxa considered to be 'common'; however, values lower than 5% are seen for some sample types in the table for comparative purposes. Bolded percentages represent the three most abundant bacterial groups within a given sample type, and the bottom row is the total percentage of bacteria listed present within a given sample type. Acronyms are as follows: DT – Adult digestive tract, AS – Anal secretions, UC – Unprepared carcasses, PC – Prepared carcasses.

Taxon	**Sample Type**			
	DT	**AS**	**UC**	**PC**
Wohlfahrtiimonas	**13.3**	5.7	**2.7**	5.9
Unidentified Gammaproteobacteria	**10.4**	1.0	0.1	<0.1
Clostridium	**10.2**	2.1	<0.01	<0.01
Unidentified *Tissierella*	7.4	**10.4**	0.4	0.3
Acinetobacter	6.8	9.0	**10.5**	**24.8**
Vitreoscilla	5.6	4.3	**3.9**	**14.3**
Dysgonomonas	5.1	0.7	0.6	2.1
Unidentified *Planococcus*	0.8	**13.3**	1.8	**9.2**
Unidentified *Lactobacillus*	2.4	**10.0**	0.1	0.6
Unidentified *Ruminococcus*	2.4	6.2	0.3	0.1
Peptoniphilus	1.4	5.1	0.6	2.7
Myroides	1.3	1.0	2.1	8.9
Total Representation	**69.5**	**68.8**	**23.2**	**69.1**

Alpha and Beta Diversity

Alpha diversity measures for all sample types are provided in Table 2. Unprepared carcasses had the highest average richness values, with 386 OTUs, while after preparation, carcass diversity dropped considerably to just 71 OTUs, on average. Adult digestive tracts and their anal secretions had a similar average richness values, with 86 and 80 OTUs recovered from these sample types, respectively. Shannon-Weiner index values (Fig 4, Table 2), Chao1, Evenness, and Faith's Phylogenetic Diversity showed similar trends (Table 3). ANOSIM analysis indicated that differences in adult digestive tract bacterial communities between the sexes were not significant (R statistic = -0.062; p = 0.77); however, bacterial communities among all sample types were

significantly different overall (R statistic = 0.610; p = 0.001). When individually compared, difference of bacterial communities was significant between adult digestive tracts vs. their anal secretions (R statistic = 0.553; p = 0.001), anal secretions vs. prepared carcasses (R statistic = 0.779; p = 0.001), and adult digestive tracts vs. prepared carcasses (R statistic = 0.588, p = 0.001). Bacterial communities found on unprepared carcasses were also significantly different from those on prepared carcasses (R statistic = 0.458; p = 0.031). Post hoc pairwise PERMANOVA with Bonferroni corrections confirmed that bacterial communities between all sample types were significantly different (Table 4).

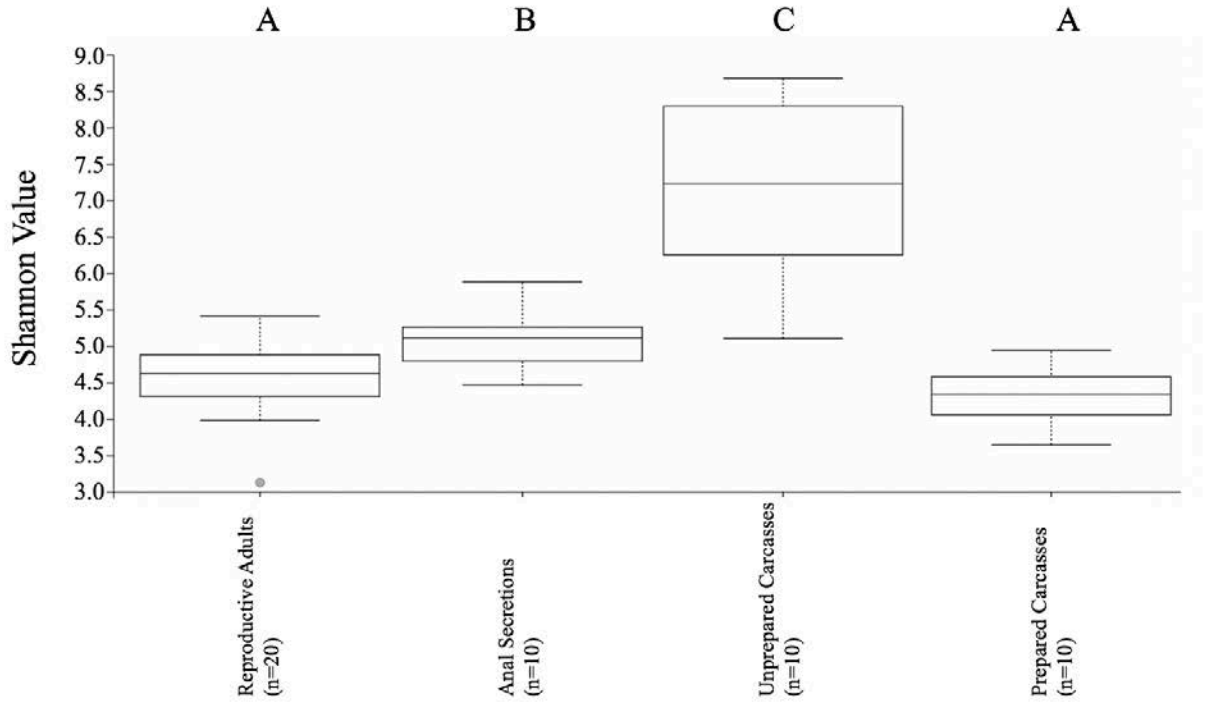

Figure 4. Shannon-Weiner box blots for sample types.
Statistical analysis comparing Shannon-Weiner values was done using pairwise Kruskal-Wallis analysis. Letters designate significantly different groups ($p < 0.05$).

Table 2. Average standard richness and Shannon-Wiener index values within sample types. Standard richness and Shannon-Wiener Index values are provided with standard error. Acronyms are as follows: DT – Adult digestive tracts, AS – Anal secretions, UC – Unprepared carcasses, PC – Prepared carcasses.

	Sample Type			
	DT	**AS**	**UC**	**PC**
Standard Richness	86 ± 8	80 ± 11	386 ± 23	71 ± 7
Shannon-Wiener Index	4.65 ± 0.09	5.10 ± 0.13	7.38 ± 0.39	4.47 ± 0.11

Table 3. Alpha diversity indices.
Alpha diversity metrics for Chao1, Evenness, and Faith's Phylogenetic Diversity (Faith's PD). Letters correspond to bacterial communities of specific sample types: A. Reproductive adult digestive tracts and prepared carcasses; B. Anal secretions; C. Unprepared Carcasses. Significance was calculated using pairwise Kruskal-Wallis tests in QIIME v.2.4.

Alpha Diversity Metric	**Comparison**	**P-value**
Chao1	A & B	$p < 0.050$
Chao1	A & C	$p < 0.001$
Chao1	B & C	$p = 0.028$
Evenness	A & B	$p < 0.050$
Evenness	A & C	$p < 0.001$
Evenness	B & C	$p = 0.001$
Faith's PD	A & B	$p < 0.050$
Faith's PD	A & C	$p < 0.001$
Faith's PD	B & C	$p = 0.001$

Table 4. Pairwise PERMANOVA with Bonferroni Corrections.
Bacterial communities in samples were compared in R.3.4.3 using pairwise PERMANOVA analysis with Bonferroni corrections. Pseudo-F values (PF) and P-values (*p*) are provided. Sample types were given acronyms for simplicity: DT – Adult digestive tract, AS – Anal secretions, UC – Unprepared carcass, PC – Prepared carcass.

	DT	**AS**	**UC**
AS	PF = 55.318 *p* = .001	---	---
UC	PF = 30.554 *p* = .001	PF = 14.993 *p* = .001	---
PC	PF = 5.350 *p* = .002	PF = 14.467 *p* = .001	PF = 12.699 *p* = .001

Source Tracking

Source tracking showed that just over 25% of bacteria found in anal secretion bacterial communities were derived from adult digestive tracts (Fig 5A). Bacterial communities found on the exterior of prepared carcasses were mostly derived from other sources (e.g., within the carcass or from the surrounding soil, Fig 5B). Less than 20% of prepared carcass bacterial communities were derived from known sources, with the largest percentage coming from anal secretions and smaller percentages from adult digestive tracts and unprepared carcasses (Fig 5B).

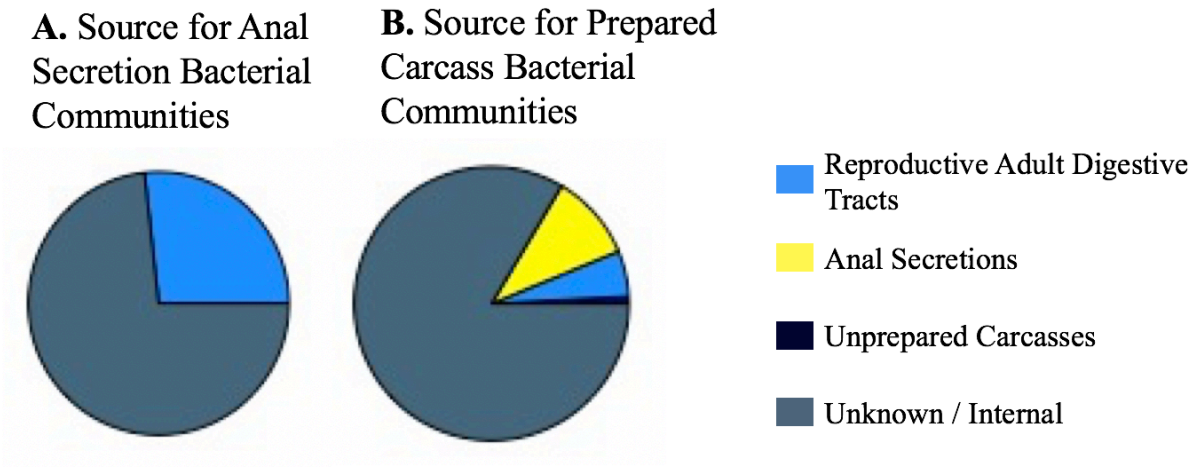

Figure 5. Source tracking for anal secretion and prepared carcasses.
Source tracker data determining the percentage of a given bacterial community that was derived from particular sources. A. The percentage of bacteria found in anal secretion derived from adult digestive tracts (n = 10). B. The percentage of bacteria found on prepared carcasses derived from adult digestive tracts, their anal secretions, and unprepared carcasses (n=10).

Bacterial Transmission to Larvae

High Throughput Sequencing

Quality filtering and chimera removal resulted in 1,752,765 high quality sequence reads, with each of the 119 samples having 14,730 (± 6,156) bacterial sequences on average. Two blank samples contained less than 200 sequences each, suggesting minimal contamination. These sequences were removed from all sample types prior to subsequent analysis. Samples were rarefied to a sampling depth of 5,000 sequences resulting in the loss of one larval sample. This sample was not included in subsequent analysis and is not reflected in reported sequence totals. Rarefaction analysis resulted in a collector's curve saturating at a sampling depth of ca. 4,500 sequences (Figure 6), suggesting that rarefaction at a sampling depth of 5,000-sequences was sufficient to encompass maximum bacterial diversity. Clustering led to a total of 295 distinct OTUs; however OTUs not represented by 50 sequences or more were removed from subsequent analyses due to their potential to be sequencing artifacts (Brown *et al.*, 2015)

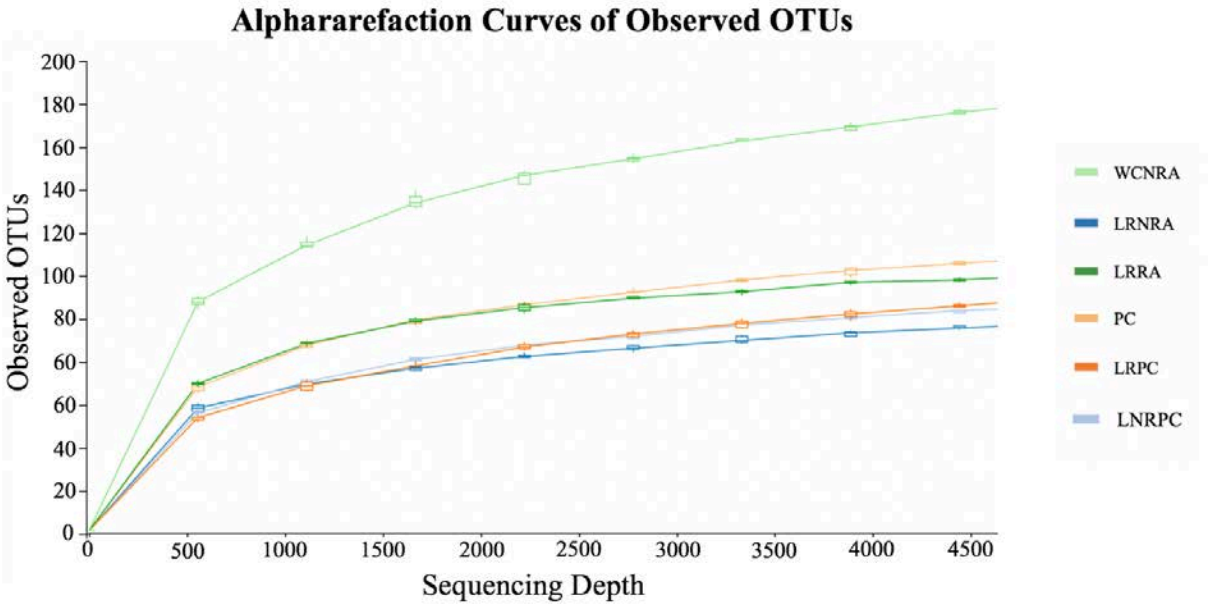

Figure 6. Alphararefaction of observed OTUs.
Observed OTUs found in each sample type. Rarefaction curves show the total number of unique OTUs per sample type. Acronyms and sample sizes are as follows: WCNRA – Wild caught non-reproductive adults (n=6), LRNRA – Laboratory-reared nonreproductive adults (n=14), LRRA – Laboratory-reared reproductive adults (n=36), LRPC – Larvae receiving parental care (n=35), LNRPC – Larvae not receiving parental care (n=18)

Taxonomic Assignments

Each sampling type was dominated by two classes of bacteria. Gammaproteobacteria represented the majority class in all sample types (Figure 7). The second most dominant class varied among sample types. Clostridia was the second most dominant class found in digestive tract bacterial communities of wild caught non-reproductive adults (26%), laboratory-reared non-reproductive adults (34%), laboratory-reared reproductive adults (25%), and larvae receiving parental regurgitations (16%, Figure 7). Bacteroidia was the second most dominant class for larvae not receiving parental regurgitations (16%) and Betaproteobacteria was the second most dominant class on prepared carcasses (16%, Figure 7). Wild caught non-reproductive adults, laboratory-reared non-reproductive adults, and larvae receiving post-hatching parental care digestive tracts had a third bacterial class with a relative abundance ≥ 10% (Bacteroidia, 17%, 14%, and 10%, respectively, Figure 7). Prepared carcass bacterial communities contained Flavobacteriia with a relative abundance above 10% (12%, Figure 7).

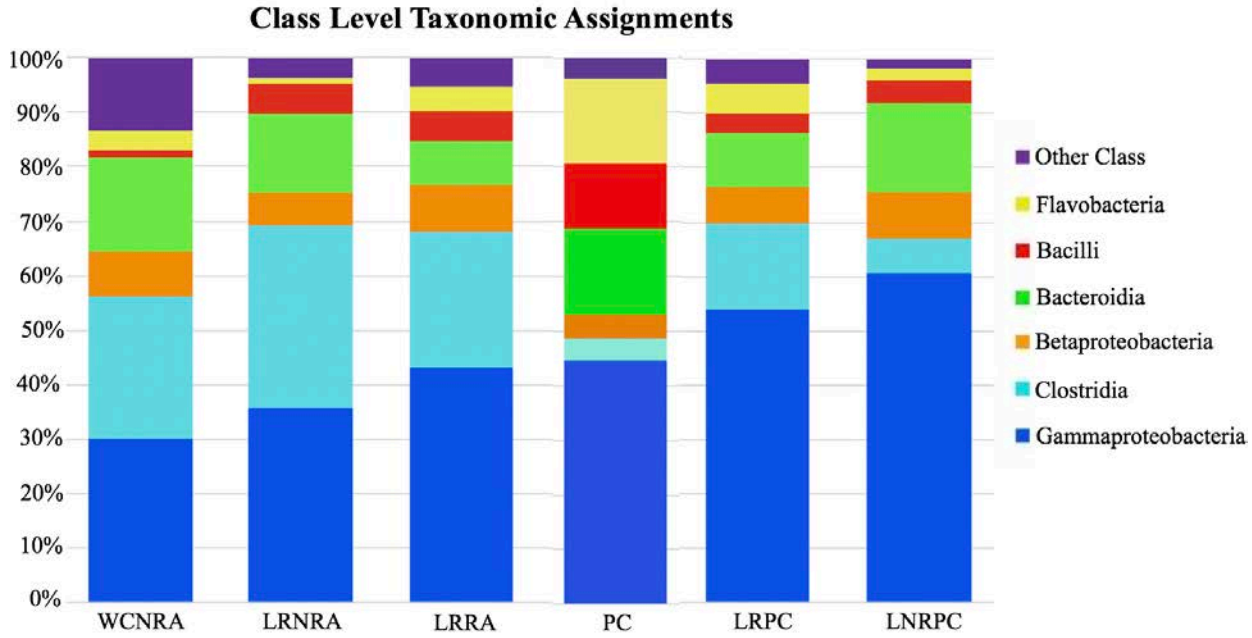

Figure 7. Class level taxonomic assignments.
Bar lengths describe the average percentage of each bacterial class found within sample types. All classes not specifically identified were assigned to Other including unidentified bacterial classes. Acronyms are as follows: WCNRA – Wild caught non-reproductive adults, LRNRA – Laboratory-reared nonreproductive adults, LRRA – Laboratory-reared reproductive adults, LRPC – Larvae receiving parental care, LNRPC – Larvae not receiving parental care.

Relative abundances of prevalent class- and genus-level bacterial groups differed among all sample types (Table 5). Wild caught non-reproductive adults had one lower level taxonomic bacterial group above 10% relative abundance, unidentified Gammaproteobacteria (Table 5). Wild caught non-reproductive adults were the only sample type to contain measurable levels of *Acheoplasma* (Table 5). Laboratory-reared non-reproductive adults and laboratory-reared reproductive adults shared two bacterial groups with a relative abundance above 10%, *Clostridium* and *Wohlfahrtiimonas* (Table 5). Laboratory-reared non-reproductive adults also had a third bacterial group nearing a relative abundance of 10%, *Dysgonomonas* (Table 5). All larvae had high relative abundances of *Ignatzschineria* and *Proteus* (Table 5). Larvae not receiving parental regurgitaions had a third bacterial group with a relative abundance above 10%, *Dysgonomonas* (Table 5). Prepared carcasses had two bacterial groups with relative abundances above 10%, *Vitreoscilla* and *Acinetobacter*. Four bacterial genera (*Myroides*, *Chryseobacterium*, *Leucobacter*, and *Paracoccus*) were found in low relative abundances in both lab-reared reproductive adults and larvae receiving parental regurgitations (< 5.0%) but were completely absent or almost immeasurable in larvae not receiving parental regurgitations (Table 6).

Table 5. Percentage of common taxa found across sample types.
Taxonomic assignments of the most common bacterial genera/class found in each sample type. A relative abundance threshold of > 5% of all sequences was used here for taxa considered to be 'common'; however, values lower than 5% are seen for some sample types in the table for comparative purposes. Bolded percentages represent the three most abundant bacterial groups within a given sample type, and the bottom row is the total percentage of bacteria listed present within a given sample type. Acronyms are as follows: WCNRA – Wild caught non-reproductive adults, LRNRA – Laboratory-reared nonreproductive adults, LRRA – Laboratory-reared reproductive adults, LRPC – Larvae receiving parental care, LNRPC – Larvae not receiving parental care.

Taxon	**Sample Type**					
	WCNA	LRNRA	LRRA	PC	LRPC	LNRPC
Unidentified Gammaproteobacteria	**10.8%**	7.8%	**9.3%**	<0.1%	2.1%	0.5%
Dysgonomonas	**7.2%**	**9.9%**	4.0%	2.1%	5.5%	**12.8%**
Unidentified Bacteroidales	**6.4%**	1.4%	1.5%	0.2%	0.4%	0.9%
Unidentified Ruminococcaceae	6.3%	3.6%	1.9%	0.1%	0.7%	0.1%
Acheoplasma	5.6%	0.0%	0.0%	0.0%	0.0%	0.0%
Unidentified Lachnospiraceae	5.4%	2.7%	0.7%	<0.1%	0.2%	0.0%
Clostridium	5.1%	**16.4%**	**11.2%**	<0.1%	2.0%	<0.1%
Wohlfahrtiimonas	3.2%	**16.0%**	**13.8%**	5.9%	**5.8%**	8.0%
Tissierellaceae	1.3%	5.9%	5.8%	0.3%	5.4%	2.6%
Acinetobacter	2.4%	2.0%	7.1%	**24.8%**	2.6%	1.7%
Vitreoscilla	4.7%	2.1%	6.5%	**14.3%**	5.4%	8.2%
Planococcaceae	<0.1%	0.2%	0.9%	**9.2%**	0.8%	1.5%
Myroides	0.2%	0.6%	4.0%	8.9%	4.1%	<0.1%
Ignatzschineria	<0.1%	<0.1%	3.5%	0.3%	**21.0%**	**27.2%**
Proteus	<0.1%	<0.1%	3.5%	0.9%	**13.7%**	**17.4%**
Total Representation	58.9%	68.8%	74.7%	67.3%	69.7%	81.1%

Table 6. Percentage of lowly abundant bacteria.
Bacteria found in low relative abundance in the digestive tracts of lab-reared reproductive adults and larvae. Acronyms are as follows: LRRA – Lab-reared reproductive adult digestive tracts, LRPC – Larvae receiving parental regurgitations digestive tracts, and LNRPC – Larvae not receiving parental regurgitations digestive tracts.

Taxon	**Sample Type**		
	LRRA	LRPC	LNRPC
Myroides	4.0%	4.1%	<0.01%
Chryseobacterium	1.4%	1.3%	0.0%
Leucobacter	1.1%	1.4%	0.0%
Paracoccus	1.1%	1.3%	<0.01%

Alpha and Beta Diversity

Alpha diversity measures for all sample types are provided in Table 7. Wild caught non-reproductive adults had the highest average richness values, with 108 OTUs. Laboratory-reared reproductive adults and larvae receiving parental regurgitations showed a similar standard richness, with 87 and 82 OTUs respectively. Laboratory-reared non-reproductive adults and prepared carcasses also showed similar richness values, 71 for each. Larvae not receiving parental regurgitations had the lowest standard richness, with 58 OTUs. Similar to standard richness, wild caught non-reproductive adults had the greatest Shannon-Weiner index value (Figure 8, Table 7), which was significantly higher than all other sample types (Kruskal-Wallis, $p < 0.01$, Figure 8). Laboratory-reared adults and prepared carcasses did not statistically differ in their Shannon-Weiner index values (Figure 8, Table 7). Laboratory-reared adults and prepared carcasses had a significantly higher Shannon-Weiner index value compared to all larvae (Kruskal-Wallis, $p < 0.01$, Figure 8, Table 7). Larvae receiving parental regurgitations did not significantly differ from larvae not receiving parental regurgitations (Kruskal-Wallis, $p = 0.74$, Figure 8, Table 7). Chao1, Evenness, and Faith's Phylogenetic Diversity Index showed similar trends (Table 8).

Table 7. Average standard richness and Shannon-Wiener index values within sample types. Standard richness and Shannon-Wiener Index values are provided with standard error. Acronyms are as follows: WCNRA – Wild caught non-reproductive adults, LRNRA – Laboratory-reared nonreproductive adults, LRRA – Laboratory-reared reproductive adults, LRPC – Larvae receiving parental care, LNRPC – Larvae not receiving parental care.

	Sample Type					
	WCNRA	LRNRA	LRRA	PC	LRPC	LNRPC
Standard Richness	108	71	87	71	82	58
Shannon-Weiner Index	5.65 ± 0.05	4.28 ± 0.13	4.51 ± 0.09	4.47 ± 0.11	3.81 ± 0.09	3.84 ± 0.07

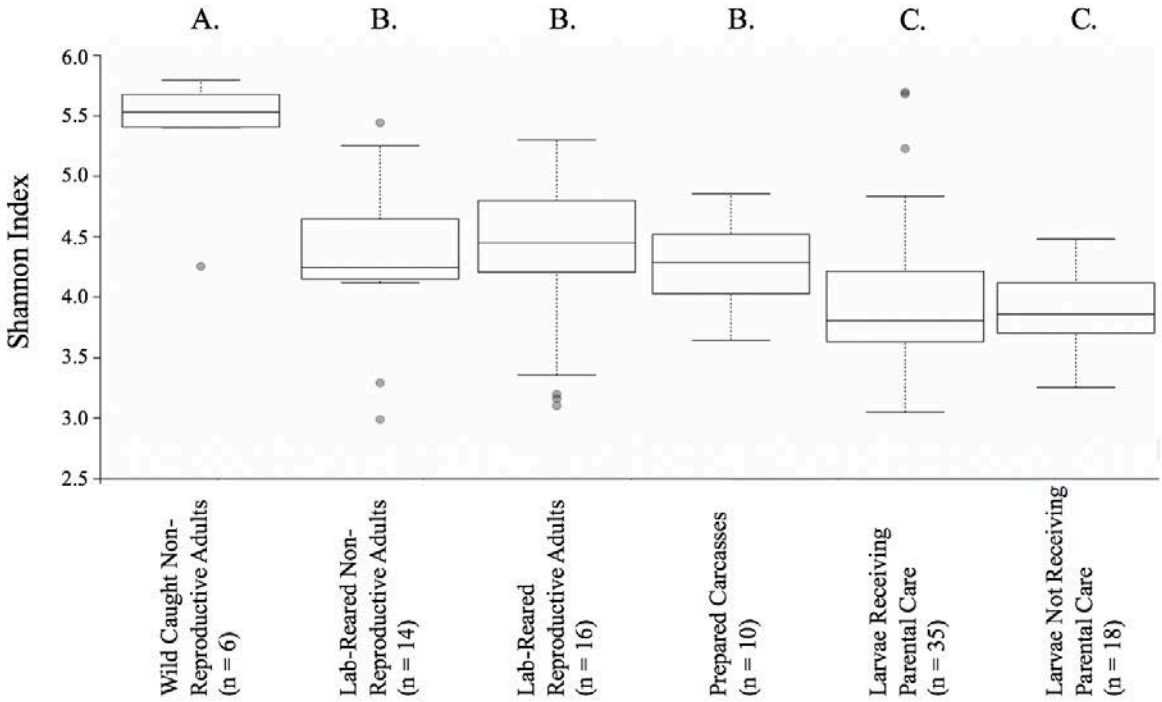

Figure 8. Shannon-Weiner box blots for sample types.
Statistical analysis comparing Shannon-Weiner values was done using pairwise Kruskal-Wallis analysis. Letters designate significantly different groups ($p < 0.05$).

Table 8. Alpha diversity indices.
Alpha diversity metrics for Chao1, Evenness, and Faith's Phylogenetic Diversity (Faith's PD). Letters correspond to bacterial communities of specific sample types: A. Wild caught non-reproductive adult digestive tracts; B. Laboratory-reared adult digestive tracts and prepared carcasses; C. Larval digestive tracts. Significance was calculated using pairwise Kruskal-Wallis tests in QIIME v.2.4.

Alpha Diversity Metric	**Comparison**	**P-value**
Chao1	A & B	$p < 0.007$
Chao1	A & C	$p < 0.006$
Chao1	B & C	$p < 0.032$
Evenness	A & B	$p < 0.050$
Evenness	A & C	$p < 0.003$
Evenness	B & C	$p < 0.001$
Faith's PD	A & B	$p < 0.003$
Faith's PD	A & C	$p < 0.050$
Faith's PD	B & C	$p < 0.02$

ANOSIM analysis indicated that there were no significant differences in the digestive tract bacterial communities between sexes for wild caught or lab-reared adults (R statistics = -0.148 and 0.022, $p = 0.50$ and 0.53, respectively). There were no significant differences between digestive tract bacterial communities of larvae receiving biparental care or larvae receiving maternal care only (R statistic = 0.035, $p = 0.37$). Digestive tract bacterial communities between parents and larvae receiving parental regurgitations were not significantly different (R statistics = -0.018, $p = 0.563$, Figure 9A). Digestive tract bacterial communities between parents and larvae not receiving parental regurgitations were significantly different (R statistic = 0.695, $p = 0.001$, Figure 9B). Digestive tract bacterial communities between larvae receiving parental regurgitations and larvae not receiving parental regurgitations were also significantly different (R statistics = -0.100, $p = 0.042$, Figure 9C). Digestive tract bacterial communities between wild caught non-reproductive adults and laboratory-reared non-reproductive adults were not significantly different (R statistic = 0.118, $p = 0.174$, Figure 10). The digestive tract bacterial communities between laboratory-reared non-reproductive and laboratory-reared reproductive adults were also not significantly different (R statistics = 0.076, $p = 0.13$, Figure 10). Bacterial communities on prepared carcasses and in the

digestive tracts of laboratory-reared reproductive adults and all larvae (R-statistic = 0.805, p = 0.001, Figure 11). Post-hoc pairwise PERMANOVA with Bonferroni corrections confirmed that all bacterial communities between all sample types were significantly different (Tables 9, 10).

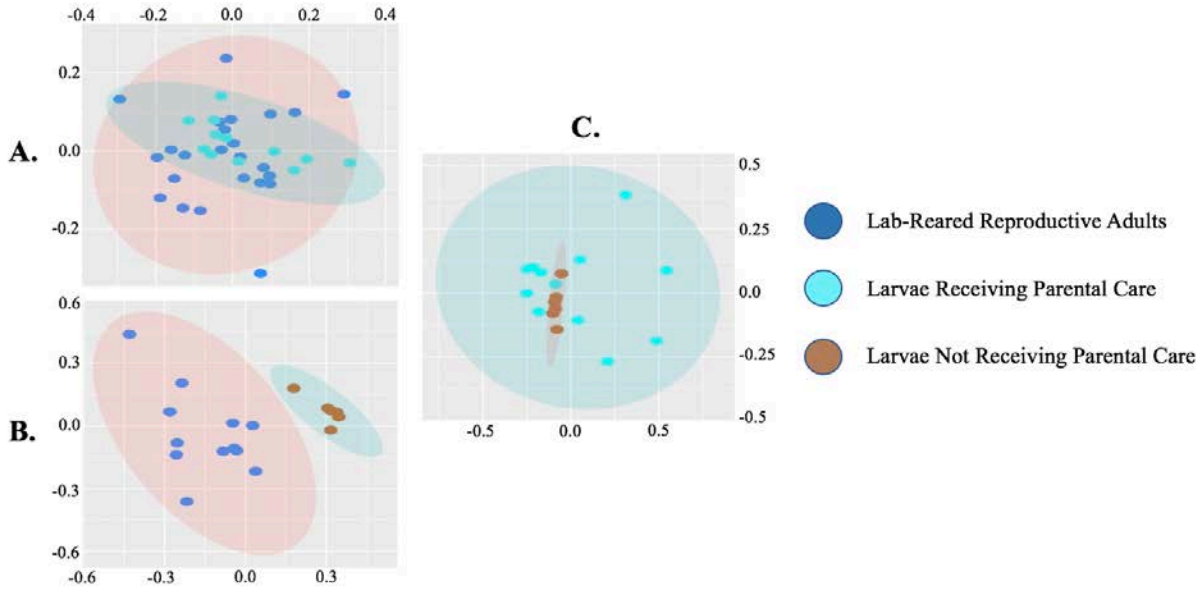

Figure 9. NMDS ordination of adults and larvae.
Nonmetric multidimensional scaling (NMDS) ordinations were generated using Bray-Curtis distance matrices in R.3.4.3 and P-values were calculated using ANOSIM. Ordinations compare A) laboratory-reared reproductive adults and their larvae receiving parental regurgitations (p = 0.563), B) laboratory-reared adults reproductive adults and their larvae not receiving parental regurgitations (p = 0.001), and C) larvae receiving and not receiving parental regurgitations (p = 0.042).

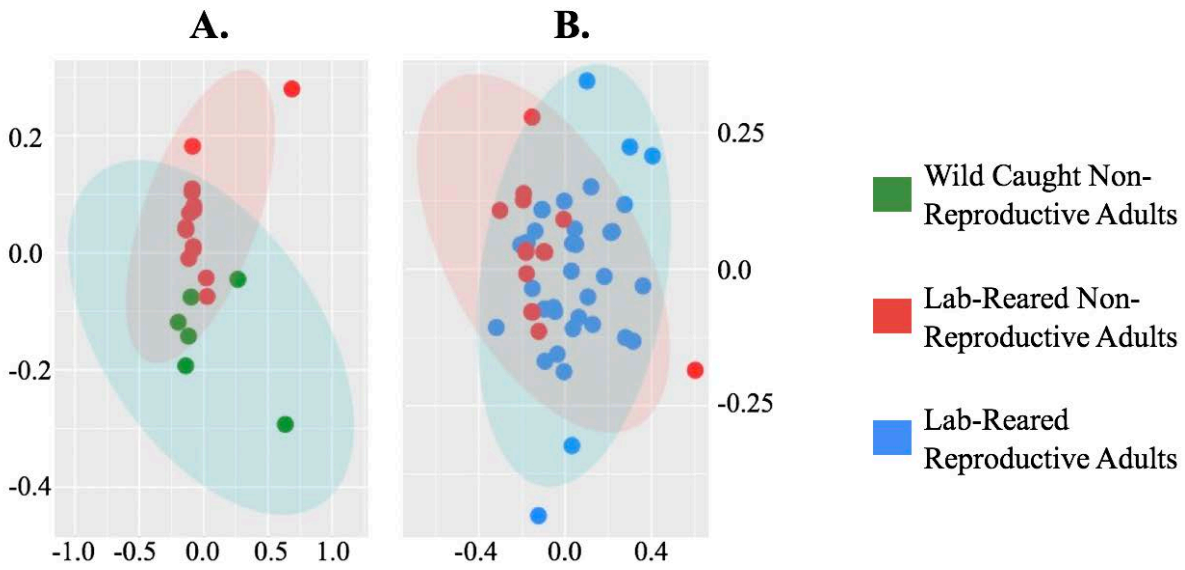

Figure 10. NMDS ordination of wild caught and laboratory-reared adults. Nonmetric multidimensional scaling ordinations were generated using Bray-Curtis distance matrices in R.3.4.3 and P-values were calculated using ANOSIM. Ordinations compare A) wild caught non-reproductive adults and laboratory reared non-reproductive adults ($p = 0.174$), and B) laboratory-reared non-reproductive adults and laboratory-reared reproductive adults ($p = 0.13$).

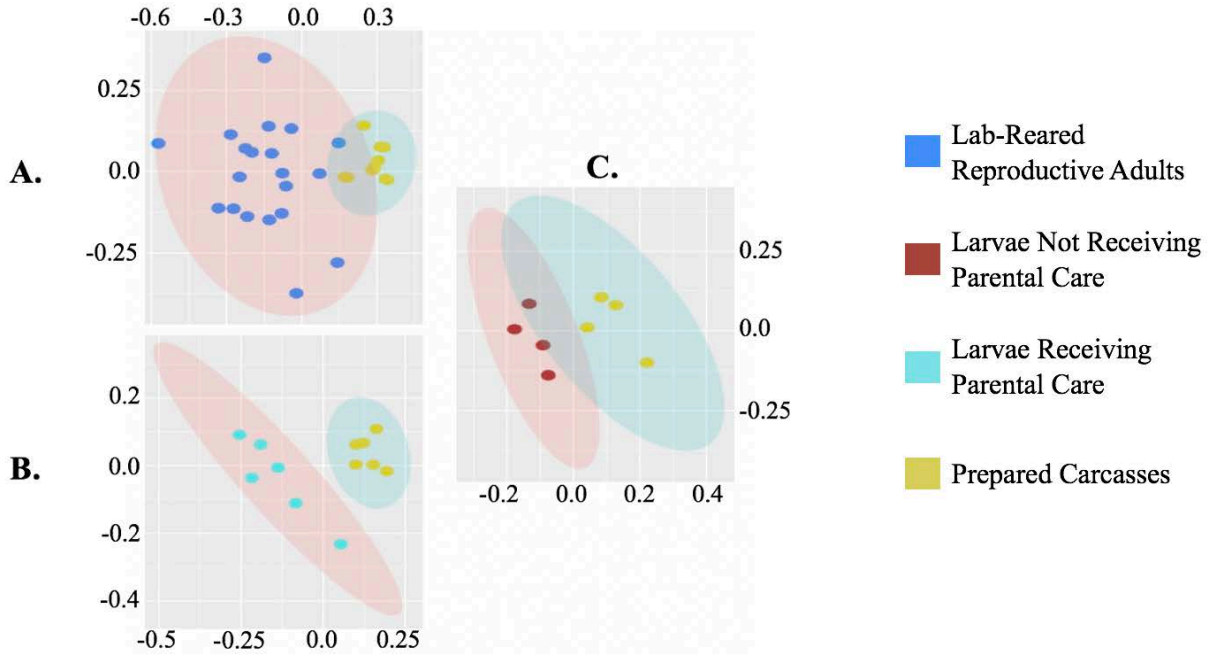

Figure 11. NMDS ordination of adults, larvae, and prepared carcasses.
Nonmetric multidimensional scaling ordinations were generated using Bray-Curtis distance matrices in R.3.4.3 and P-values were calculated using ANOSIM. Ordinations compare A) laboratory-reared reproductive adults and carcasses they prepared (p = 0.001), B) prepared carcasses and larvae receiving parental regurgitations (p = 0.003), and C) prepared carcasses and larvae not receiving parental regurgitations (p = 0.039).

Table 9. Pairwise PERMANOVA with Bonferroni corrections.
Bacterial communities in samples were compared in R.3.4.3 using pairwise PERMANOVA analysis with Bonferroni corrections. Pseudo-F values (PF) and P-values (p) are provided. Acronyms are as follows: PC – prepared carcasses, LRRA – digestive tracts of laboratory-reared reproductive adults, LRPC – digestive tracts of larvae receiving parental care, and LNRPC – digestive tracts of larvae not receiving parental care.

	Pseudo-F	**P-value**
PC & LRRA	5.860	p = 0.001
PC & LRPC	7.260	p = 0.001
PC & LNRPC	2.631	p = 0.047

Table 10. Pairwise PERMANOVA with Bonferroni corrections.
Bacterial communities in samples were compared in R.3.4.3 using pairwise PERMANOVA analysis with Bonferroni corrections. Pseudo-F values (PF) and P-values (*p*) are provided. Sample types were given acronyms for simplicity: LRRA – digestive tracts of laboratory-reared reproductive adults, LRPC – digestive tracts of larvae receiving parental care, and LNRPC – digestive tracts of larvae not receiving parental care.

	Pseudo-F	**P-value**
LRRA & LRPC	17.327	$p = 0.001$
LRRA & LNRPC	19.102	$p = 0.001$
LRPC & LNRPC	2.53	$p = 0.049$

Source Tracking

Source tracking showed that just over 25% of the digestive tract bacterial communities of larvae receiving parental regurgitations were derived from known sources (Figure 12A). Contributions from parental digestive tracts and prepared carcasses were similar (Figure 12A). Nearly 10% of bacteria in the digestive tracts of larvae not receiving parental regurgitations were derived from their parents and prepared carcasses (Figure 12B), with nearly equal contributions from adult digestive tracts and prepared carcasses.

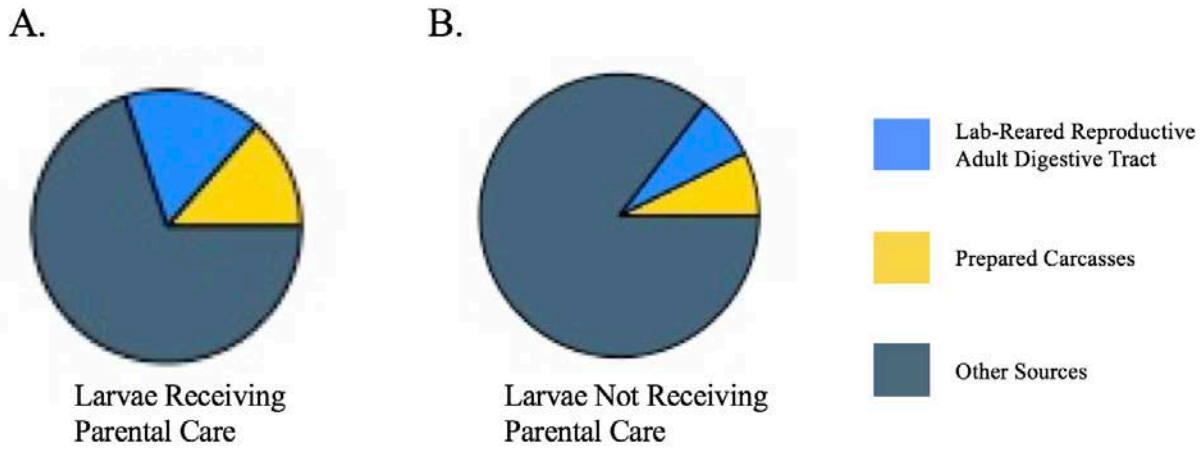

Figure 12. Source tracking for larval digestive tracts.
Source tracker data determining the percentage of a given bacterial community that was derived from particular sources. A. The percentage of bacteria found in digestive tracts of larvae receiving parental regurgitations derived from adult digestive tracts and prepared carcasses (n = 35). B. The percentage of bacteria found in digestive tracts of larvae not receiving parental regurgitations derived from adult digestive tracts and prepared carcasses (n=18)

DISCUSSION

Bacterial Transmission to Prepared Carcasses via Anal Secretions

In this study, we evaluated two competing hypotheses addressing the role of *N. defodiens* anal secretions in transmitting adult digestive tract bacterial communities to prepared carcasses. The ABT hypothesis predicts that adult digestive tract bacterial communities should be reflected in beetle anal secretions and ultimately the preserved carcasses. Conversely, the NABT hypothesis predicts that preserved carcass bacterial communities should neither reflect those of anal secretions nor adult digestive tracts. We found that bacterial communities within sample types were stable, but that adult digestive tracts and beetle anal secretions were significantly different from those found on prepared carcasses. In addition, we found that only roughly 25% of bacteria within anal secretions originated from adult digestive tracts and that an even smaller percentage of bacteria from the adult digestive tracts ultimately colonized the prepared carcasses, consistent with the NABT hypothesis (Fig 4). This the first study that specifically addresses the role of anal secretions in this process of transmission by statistically comparing bacterial communities of adult *N. defodiens* digestive tracts, their anal secretions, and to the prepared carcass using culture-independent means.

We found that there was a significant reduction in diversity between the unprepared and prepared carcasses, which is consistent with what has previously been reported (Duarte *et al.,* 2017; Shukla *et al.,* 2017; Shukla *et al.,* 2018). Many of the lower level taxonomic bacterial groups recovered in this study, such as *Myroides* and unidentified *Planococcus,* have been identified in previous studies focusing on *Nicrophorus* spp. (Kaltenpoth & Steiger, 2013; Duarte *et al.,* 2017; Shukla *et al.,* 2017; Vogel *et al.,* 2017; Wang & Rozen, 2017; Shukla *et al.,* 2018). We found an increase in *Acinetobacter, Myroides, Peptonophilus,* unidentified *Planococcus*, *Vitreoscilla,* and *Wohlfahrtiimonas* (Table 1) on prepared carcasses relative to unprepared carcasses, as well as in adult digestive tracts and anal secretions, albeit in low abundances for some taxa. However, none of these bacterial groups were dominant across all sample types and abundances varied considerably across the different sample types. The lack of sustained dominance for these taxa across the sample types suggests there is not a direct pathway of transmittance from the adult digestive tract, to the anal secretions, and then to the prepared carcasses. It is also likely that final

increases seen for these groups on the prepared carcasses were due, in part, to reduced microbial competition on prepared carcasses overall once the bacterial diversity dropped due to preservation. Furthermore, other factors such as the removal of carcass gut (Duarte *et al.,* 2017) and a controlled development of the fungal components of these communities (Shukla *et al.,* 2018), could play a role in transforming the carcass microbial communities to enhance preservation.

We found that bacterial communities within adult digestive tracts were significantly different from those found in their anal secretions, contrary to what has been previously reported (Duarte *et al.,* 2017). These conflicting reports may be the result of sampling methodology. For example, beetles drag their posteriors across prepared carcasses and soil when applying their anal secretions. As indicated by Duarte *et al,* anal secretions were collected immediately after removing beetles from the prepared carcass without sterilization (Duarte *et al.,* 2017). By sampling without removing potential contaminants, the anal secretion samples may have contained bacteria from alternative sources that could have affected subsequent analyses. In our study, beetle posteriors were surface sterilized prior to anal secretion collection, which minimized the possibility of contamination within samples as well as the effect on subsequent analyses. We also found that the adult digestive tract is not a major source of anal secretion bacteria, and this fact begs the question of the degree to which the antimicrobial compounds and/or resistances of specific secretion bacteria, or even other factors like resident fungi (e.g., *Yarrowia*), shape the anal secretion microbial communities. Continued studies will be required in this area before these dynamics can be disentangled.

Overall, our results suggest that the anal secretions, and by proxy the bacterial communities of the adult digestive tract, had little influence on the resultant structure of the prepared carcass microbial community structure. Source tracking revealed that a large portion of the final prepared carcass community was attributed to ‘other’ sources, which is postulated to be an exposure to soil during the burial process (Duarte *et al.,* 2017), or adult regurgitations. Despite the fact that the bacterial community structure transitioned considerably from adult digestive tract, to anal secretions, and on to the preserved carcass, we did recover a core group of bacteria present in all sample types. It was the change in the relative abundances of these bacterial groups that primarily drove differences observed between bacterial communities. There was a subset of core bacteria that had a substantial (greater than two-fold) increase on prepared carcasses relative to unprepared carcasses (Table 1). These included *Acinetobacter, Dysgonomonas, Myroides, Peptoniphilus,*

unidentified Planococcus, *Vitreoscilla, and Wolfahrtiimonas*. This suggests bacterial transmittance to the prepared carcass may be a more subtle process. Other authors have postulated that low abundant taxa within microbial communities can hold the potential to later become more dominant members with essential functions through processes such as microbial 'blooms' (Gilbert *et al.*, 2011) or even by microbial 'seeding' of parental microbes to control developing communities of offspring (Shade & Handelsman, 2011; Dominguez-Bello *et al.*, 2016).

Many of these potential core microbes have been isolated from digestive tracts of burying beetles or other insects, and have previously been shown to carry out several metabolic functions. For example, *Wohlfahrtiimonas* spp. have been isolated from digestive tracts of other insects including *N. vespilloides, Diptera*, and the Rocky Mountain Wood Tick (Toth *et al.*, 2008; Hall *et al.*, 2011; Duarte *et al.*, 2017; Vogel *et al.*, 2017) and they are capable of breaking down a variety of amino acids, fermenting a myriad of sugars, and reducing nitrate (Hall *et al.*, 2011). *Dysgonomonas* spp. have been isolated from carcasses prepared by burying beetles (Shukla *et al.*, 2018) as well as in the digestive tracts of other insects (Pramono *et al.*, 2014; Yang *et al.*, 2014), and they have the ability to degrade a variety of fatty acids (Kita *et al.*, 2015). *Acinetobacter* spp. are capable of producing biofilms and exogenous enzymes that degrade vertebrate tissue (Bergogne-Bérézin & Towner, 1996). *Myroides* spp. have previously been detected in the digestive tract of burying beetles and on prepared carcasses (Duarte *et al.*, 2017; Shukla *et al.*, 2017; Wang & Rozen, 2017) and are also known to produce a variety of antibacterial substances (Mammeri *et al.*, 2002; Dharne *et al.*, 2008). *Vitreoscilla*, which have previously been found on carcasses prepared by burying beetles (Shukla *et al.*, 2017; Shukla *et al.*, 2018), has the capacity to metabolize toxic chemicals (Stark *et al.*, 2012; Kumar *et al.*, 2014) and has genes that are likely involved in gastrointestinal tract colonization (Kumar *et al.*, 2014). Taken together, these data suggest roles for these potential core microbiota in eliminating microbial competitors for carcass preservation, detoxifying the carcass environment and facilitating nutrient acquisition through the breakdown of animal tissue for developing larvae, and even in assisting with re-introduction of core bacteria into the larval digestive tract.

In this study, we sought to evaluate two competing hypotheses regarding the role of anal secretions in transmitting adult burying beetle digestive tract bacterial communities to the prepared carcass. We found that bacterial communities among adult digestive tracts, their anal secretions, and prepared carcasses all differed significantly, supporting the non-adaptive hypothesis of

bacterial transmission for *N. defodiens*. Thus, bacterial transmission from adult digestive tracts to prepared carcasses is likely a side-effect of anal secretion mediated preservation of carcasses. However, more subtle modes, such as microbial seeding of transmitting key *N. defodiens* microbiota cannot be ruled out. Further, potential core bacterial groups, including *Acinetobacter*, *Dysgonomonas*, *Myroides*, and *Wohlfahrtiimonas*, were all found to increase in relative abundance on prepared carcasses, as previous studies suggest essential functions for these bacteria in preserving the carcass, assisting with offspring nutrient acquisition, and establishing the digestive tract microbiome in the developing larvae. Further exploration will be required before core functional roles of these bacterial groups can be ascertained, or more subtle modes of bacterial transmittance from adults to larvae in *N. defodiens* can be more fully understood.

Bacterial Transmission to Larvae

In this study, we explored whether parental regurgitations in *N. defodiens* function to transmit digestive tract bacteria to offspring, if laboratory-rearing conditions effect the bacterial communities of wild caught adults, and if there is a shift in bacterial communities as adults transition from the non-reproductive to reproductive stage. We found that parents and larvae receiving parental regurgitations have similar digestive tract bacterial communities; parents and larvae not receiving parental regurgitations have dissimilar digestive tract bacterial communities; and larvae receiving parental regurgitations and larvae not receiving parental regurgitations have dissimilar digestive tract bacterial communities (Figure 9). We further found that F1 and F2 generation laboratory-reared adults and wild caught adults have similar digestive tract bacterial communities and that laboratory-reared non-reproductive and reproductive adults have similar digestive tract bacterial communities (Figures 10,11).

We found that larvae receiving parental regurgitations had digestive tract bacterial communities similar to those of their parents whereas larvae not receiving parental regurgitations had dissimilar digestive tract bacterial communities compared to those of their parents (Figure 9A, B). Larvae receiving parental regurgitations had digestive tract bacterial communities distinct from larvae that did not receive parental regurgitations (Figure 9C). Source tracking showed that larvae receiving parental regurgitations obtain roughly 13% of their digestive tract bacterial communities from parental digestive tracts whereas larvae not receiving parental regurgitations receive only roughly 4 – 5% (Figure 12). This evidence supports the hypothesis that regurgitations play a role in transmitting digestive tract bacteria from parents to offspring and agrees with conclusions previously reported (Wang & Rozen, 2017).

Larvae that received parental regurgitations had a similar number of OTUs in their digestive tract bacterial communities compared to their parents (Figure 6, Table 7). Larvae not receiving parental regurgitations had a lower number of OTUs in their digestive tract bacterial communities compared to their parents (Figure 6, Table 7). Some bacteria found in our larval samples, including *Vitreoscilla* and *Proteus,* were previously reported in larval digestive tracts in both culture-dependent and culture independent studies (Vogel *et al.*, 2017; Wang & Rozen, 2017). Other bacteria found in larval digestive tracts were previously only detected in culture-independent studies, including *Acinetobacter, Dysgonomonas, Wohlfahrtiimonas,* and *Ignatzschineria* (Vogel

et al., 2017). This suggests that culture-independent methods are likely a better tool to characterize bacterial communities in order to detect the greatest amount of bacterial diversity.

Larvae did not differ greatly in the presence of highly abundant bacterial groups, such as *Ignatzschineria* and *Proteus* (Table 5). This suggests that parental regurgitations may be playing an important role in transmitting bacteria found at lower relative abundances. Flavobacteria were found at a higher relative abundance in larvae receiving parental regurgitations when compared to larvae that did not receive parental regurgitations (Figure 7). *Myroides* was found in a relatively high abundance on prepared carcasses, in relatively low abundance in parents and larvae receiving regurgitations, but were almost immeasurable in larvae not receiving regurgitations (Table 6). *Myroides* are capable of producing antimicrobial substances and may serve in helping protect larvae from potential pathogens (Mammeri, Bellais, & Nordmann, 2002, Dharne *et al,* 2008). If larvae not receiving parental regurgitations are unable to obtain *Myroides* from parents or prepared carcasses, protective benefits of having *Myroides* in their digestive tracts is lost. *Chryseobacterium* are capable of producing cytotoxic substances and reducing nitrate which may suggest a role in eliminating pathogenic microbes and/or nutrient acquisition (Kim, 2005, Chaudhari *et al.,* 2008). Little is known about the functions of *Leucobacter* but *Paracoccus* is well known for its denitrification abilities, which may indicate a role in nitrogen acquisition for larvae (Baumann *et al.,* 1996, Stroh *et al.,* 2003). These results suggest that despite being present in relatively low abundances, these bacteria may play a significant role for developing larvae (Benjamino *et al.,* 2018).

Conflicting evidence has been reported addressing whether or not burying beetles transmit digestive tract bacteria to larvae via prepared carcasses (Duarte *et al.,* 2017, Shukla *et al.,* 2017, Vogel *et al.,* 2017, Miller *et al.,* In preparation). Here we show that prepared carcasses are likely playing a minimal role in transmitting parental digestive tract bacteria to larvae. Adults had digestive tract bacterial communities that were dissimilar from bacterial communities on prepared carcasses, which had bacterial communities dissimilar from all larvae. Some adult digestive tract bacteria are transmitted to the carcass during the preparation process in a non-adaptive manner (Miller *et al.,* In preparation) and larvae likely ingest some of those bacteria. It remains unknown if those bacteria are capable of colonizing the larval digestive tract.

Our findings comparing wild caught and laboratory-reared F1 and F2 adults suggest that our results may be representing what is naturally occurring in *N. defodiens*. These findings,

however, are contrary to what has been previously reported (Kaltenpoth & Steiger, 2013). This competing information could be the result of the authors using laboratory-reared beetles that weren't of the F1 or F2 generation. It is unclear what generation of beetles were used in the previous study and laboratory-rearing conditions could have contributed to the alterations in digestive tract bacterial communities, which was noted by the authors (Kaltenpoth & Steiger, 2013). It is also possible that diet in the laboratory or the species of beetle utilized could have resulted in the different findings. Our beetles were fed chicken liver twice weekly whereas it is unclear what previous authors fed their beetles (Kaltenpoth & Steiger, 2013). The previous authors examined digestive tract microbial communities of *N. vespilloides* whereas we looked at those of *N. defodiens.* It is possible that the different species have digestive tract bacterial communities that differ in their susceptibility to laboratory-rearing conditions. Further exploration is required to determine why these studies offer competing evidence.

Despite showing similar bacterial communities to wild caught adults, laboratory-rearing conditions still influenced the relative abundances of some bacterial groups in F1 and F2 lab-reared adults. *Clostridium* was found at a higher relative abundance in laboratory-reared non-reproductive adults but were present at lower relative abundances in wild caught non-reproductive adults (Table 5). Some *Clostridium spp.* are endemic in chickens (Fowler & Hussaini, 1975; Crawford *et al.,* 1996; Chalmers *et al.,* 2008). Our beetles were fed chicken liver, which may explain the increase in *Clostridium* in laboratory-reared beetles. Wild caught non-reproductive adults had *Acheoplasma* present in their digestive tracts but this bacteria was not detected in any samples taken from the laboratory (Table 5). Laboratory-rearing conditions likely played a role in eliminating this bacterium from laboratory-reared beetles, suggesting that this is an environmentally acquired microbe. Using food sources that more closely mimic the natural diet of burying beetles may aid in reducing the impact of laboratory-rearing conditions on beetle digestive tract bacterial communities.

We found no significant difference in the bacterial communities of non-reproductive and reproductive adults. This finding is contrary to previous reports (Duarte *et al.,* 2017, Shukla *et al.,* 2017). When burying beetles are in a non-reproductive state, their oral and anal secretions do not exhibit antimicrobial activity (Arce, Smiseth, & Rozen, 2013). When *Nicrophorus spp.* transition to the reproductive stage, their secretions begin exhibiting antimicrobial activity (Arce, Smiseth, & Rozen, 2013). Previous evidence would suggest that the antimicrobial activity of oral and anal

secretions would result in a change in digestive tract bacterial communities (Duarte *et al.,* 2017; Shukla *et al.,* 2017) but our data suggests otherwise. It is possible that *N. vespilloides* used in previous studies contain digestive tract bacteria that are more susceptible to secretion antimicrobial activity and digestive tract bacteria of *N. defodiens* are more resistant. This finding may also suggest that anal secretions do not originate in the digestive tract and that they only colocalize with digestive tract microbes at the posterior end of the hind gut. Further exploration is also required to determine the nature of all antimicrobial molecules found in oral and secretions and determine the susceptibility of bacteria to these molecules and determine where anal secretions originate.

In this study we evaluated whether parental regurgitations in burying beetles act as a method of transmitting digestive tract bacteria to larvae, if laboratory-rearing conditions affect the digestive tract bacterial communities of wild caught beetles, and if there is a shift in digestive tract bacterial communities when burying beetles transition from the non-reproductive to the reproductive stage. We found that larvae receiving parental regurgitations had digestive tract bacterial communities similar to those of their parents whereas larvae not receiving parental regurgitations had digestive tract bacterial communities dissimilar from their parents. Larval digestive tracts did not differ in the relative abundances of highly prevalent bacteria but did differ in bacteria of lower relative abundances, suggesting that parental regurgitations play a role in transmitting the latter. We further found that wild caught adults and F1 & F2 laboratory-reared adults have similar digestive tract bacterial communities and laboratory-reared non-reproductive and reproductive adults have similar bacterial communities. This suggests that F1 & F2 laboratory-reared adults can be used to demonstrate how bacteria are transmitted to larvae in the natural habitats of burying beetles. This also suggests that adults maintain a constant digestive tract bacterial community throughout their reproductive life periods. Further exploration is required to identify all bacteria that are transmitted from parents to offspring as a result of trophallaxis and determine their core functions. Further exploration is also necessary to determine why the activation of antimicrobial molecules in secretions does not affect the makeup of adult digest tract bacterial communities.

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