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Tick microbial communities within enriched extracts of *Amblyomma maculatum*

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Abstract

Our objective of this study was to explore the bacterial microbiome in fresh or fresh-frozen adult *Amblyomma maculatum* (Gulf Coast ticks) using extracts enriched for microbial DNA. We collected 100 questing adult *A. maculatum*, surface disinfected them, and extracted DNA from individual ticks collected the same day or after storage at -80°C . Because only extracts with microbial DNA concentrations above 2 ng/ μL were considered suitable for individual analysis, we expected fewer samples to meet these requirements. Of individual ticks extracted, 48 extracts met this minimum concentration. We pooled 20 additional extracts that had lower concentrations to obtain seven additional pools that met the minimum DNA concentration. Libraries created from these 55 samples were sequenced using an Illumina MiSeq platform, and data sets were analyzed using QIIME to identify relative abundance of microorganisms by phylum down to genus levels. Proteobacteria were in greatest abundance, followed by Actinobacteria, Firmicutes, and Bacteroidetes, at levels between 1.9% and 6.4% average relative abundance. Consistent with the *Francisella*-like endosymbiont known to be present in *A. maculatum*, the genus *Francisella* was detected at highest relative abundance (72.9%; SE 0.02%) for all samples. Among the top ten genera identified (relative abundance 0.5%) were potential extraction kit contaminants, *Sphingomonas* and *Methylobacterium*, the soil bacterium *Actinomyces*, and the known *A. maculatum*-associated genus, *Rickettsia*. Four samples had *Rickettsia* at greater than 1% relative abundance, while nine additional samples had *Rickettsia* at low (0.01 to 0.04%) relative abundance. In this study, we used the entire microbe-enriched DNA extract for whole ticks for microbiome analysis. A direct comparison of the microbiome in microbe-enriched DNA and total

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genomic DNA extracts from halves of the same tick would be useful to determine the utility of this extraction method in this system. We anticipate that future tick microbiome studies will be valuable to explore the influence of microbial diversity on pathogen maintenance and transmission, and to evaluate niche-specific microbiomes within individual tick tissues.

Keywords

Ixodidae; Mississippi; microbiome

1. Introduction

Like vertebrates, invertebrates carry a microscopic community of diverse organisms that may include bacteria, protozoa, and viruses. Arthropod vectors are unique among invertebrates because their microbial community may include pathogens that are transmissible to a host, most commonly via blood feeding. Interest in these microbial communities, or microbiomes, of tick vectors has recently gained momentum (Narasimhan and Fikrig, 2015). To begin to address current gaps in knowledge, investigators have characterized the microbiomes of ticks relative to rearing conditions (lab versus wild), sex, life stage, and geographical range, compared the microbiome within specific tissues, and evaluated tick microbial communities in relation to pathogen presence and after antibiotic treatment (Budachetri et al., 2014; Budachetri et al., 2016; Clayton et al., 2015; Trout Fryxell and DeBruyn, 2016; Van Treuren et al., 2015; Williams-Newkirk et al., 2014). While tick microbial communities may include a diverse array of microorganisms, studies in general have focused on bacteria, with next-generation sequencing tools utilizing the 16S rRNA gene to identify bacterial populations. Still, limitations exist, sample treatment varies, and the technology continues to evolve. Certainly the question of what is the optimal extraction method for microbiome studies is one that already has seen debate in the area of gut microbiomes in humans (Mackenzie et al., 2015). However, methods relied upon in the gut microbiome field generally use commercial kits for soil or stool samples, and specific protocols to enrich for microbial DNA, as opposed to using total genomic DNA extraction kits.

The Gulf Coast tick, *Amblyomma maculatum* (Acari:Ixodidae), is a vector of increasing medical and veterinary importance, with a geographic range that extends beyond the Gulf Coast to the Atlantic Coast and inland to states including Kansas and Kentucky (Paddock and Goddard, 2015; Teel et al., 2010). Budachetri et al. (2014) examined the microbiome of *A. maculatum* using genomic DNA extracts of salivary glands and midgut tissues collected from field and laboratory-reared tick populations after 8 days of vertebrate host infestation. Bacterial 16S rRNA sequences obtained using 454 pyrosequencing demonstrated that Proteobacteria constituted the majority of the bacterial communities from both tissues in the field and laboratory sources of ticks, and not surprisingly, *Francisella* and *Rickettsia* were the most common genera in salivary gland and midgut tissues, respectively, of field-collected ticks (Budachetri et al., 2014). Other approaches to studying the tick microbiome include the use of genomic DNA extracted from unfed field-collected *Amblyomma americanum* tick halves for bacterial 16S rRNA sequencing using an Illumina MiSeq

platform (Trout Fryxell and DeBruyn, 2016) and using DNA extracts from surface-disinfected or untreated field-collected *A. americanum* with 454 pyrosequencing (Williams-Newkirk et al., 2014). Approaches may vary in part due to the question addressed, with additional variation possible due to differences in available technology or other resources.

In this study, we used extracts enriched for microbial DNA, and bacterial 16S rRNA sequencing using an Illumina MiSeq platform, to evaluate the microbiome of unfed, field-collected *A. maculatum*. Sequencing based on MiSeq technology has been used in a variety of applications and provides comparable coverage to other commercial sequencing systems (Metzker, 2010; Glenn, 2011; Quail et al. 2012). Because the ability to use archived ticks is desirable for long-term field studies and allows flexibility in general, we extracted samples after storage at -80°C , as well as fresh samples extracted on the day of collection. Of 100 ticks collected, we analyzed 48 individual tick extracts and seven pools (20 ticks) by Illumina MiSeq. Based on identified taxa, we confirmed presence of some of the previously reported genera, absence of others, and additional genera associated with other ixodid ticks. This study differed from previous microbiome studies of *A. maculatum* in its use of whole ticks and DNA extracts enriched for microbial DNA. However, due to the concentration of microbe-enriched DNA extracted, and the requirements for performing Illumina MiSeq, we were unable to extract genomic DNA from half of the same tick for comparison. Thus, we did not assess whether the use of enriched extracts provides a more comprehensive snapshot of bacterial abundance. Still, the utility of this approach in future studies may be of value when exploring interactions among pathogens and other tick-associated bacteria in tick populations.

2. Materials and Methods

2.1. Collection of *A. maculatum*

Adult ticks were collected by flagging or dragging vegetation with a 1 m² piece of muslin cloth on six different dates from May 4, 2016 to June 22, 2016 from sites in Starkville, a city in Oktibbeha County, Mississippi. Once collected, ticks were morphologically identified (Goddard and Layton, 2006) and *A. maculatum* were surface disinfected with 5% household bleach then rinsed in water prior to placement in individual 1.5 mL microfuge tubes. Ticks were then placed in a -80°C freezer (within 4 hours of collection) until DNA extraction 8 to 16 days later (“frozen”) or immediately extracted that day (“fresh”).

2.2. DNA extraction of whole *A. maculatum*

To enrich for microbial DNA in our extracts, we used the protocol for isolating DNA from stool for pathogen detection in the QIAamp Fast DNA Stool Mini Kit (found in kit handbook, page 20–22; Qiagen Inc., Valencia, CA). This protocol includes an additional heating step (70°C) for lysis of microorganisms including bacteria and other parasites. We additionally modified the protocol to use whole tick bodies, rather than stool material. Previously cleaned ticks were placed in 250 μL InhibitEX Buffer and cut multiple times with a scalpel blade (no. 11) prior to vortexing for 1 min and heating at 95°C for 10 min to lyse microorganisms, then the remainder of the manufacturer’s protocol followed. In addition, 30

μL of molecular biology grade water was used for initial elution and then transferred back onto the column for a final centrifugation at $20,000 \times g$ to increase DNA concentration.

We used a Qubit® 3.0 Fluorometer and Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA) to measure DNA concentration in our extracts. We selected this approach to more accurately measure DNA as a low concentration was expected using the selected extraction protocol for microbial DNA. The Nanodrop 1000 and 2000 Spectrophotometers, also available to us, have a claimed detection limit of 2 ng/μL, and are unreliable at low concentrations. A concentration at or above 2 ng/μL was required for sequencing library preparation. Extracts with concentrations below the minimum were pooled with like samples of the same sex, storage method, and collection date to meet minimum concentration, then subjected to clean-up and concentration using a Zymo Research DNA Clean and Concentrator (Zymo Research, Irvine CA) and concentrations re-assayed using the Qubit 3.0 Fluorometer as above.

2.3. Library Preparation and Sequencing Using Illumina MiSeq Platform

Sequencing libraries were constructed based on the V4 region of the bacterial 16S rRNA gene using 10 ng total DNA from individual or pooled extracts. Amplification conditions and sequencing from were performed as previously described by Kozich et al, specifically in the supplementary files for that publication (Kozich et al., 2013).

2.4. Sequence data processing

Raw sequencing data were processed using a Quantitative Insights into Microbial Ecology (QIIME) pipeline (1.9.0) (Caporaso et al., 2010). De-multiplexed sequences were assembled (multiple_join_paired_ends.py) for operational taxonomic units (OTUs) table construction. The OTUs with 97% identity were assigned taxonomy based on the Greengene database (13_5). After removing chimera OTUs and low reads number samples from table, we calculated relative abundances across increasing taxonomic levels from phylum to genus, and alpha and beta diversity were generated.

3. Results

We visited four sites in Starkville, MS from May 4, 2016 to June 22, 2016, and collected *A. maculatum* ticks in two sites. One of these sites yielded 96 ticks over six visits, and the other site yielded four ticks on a single visit, June 2 (Table 1). Sixty-eight ticks were used for sequencing. Of 25 ticks that were extracted on the same day as collection (fresh), six individual extracts (24%) had sufficient DNA to be used for library preparation and microbiome analysis and two (females) had sufficient DNA after pooling (1 pool). All of the four ticks collected on June 2 were extracted fresh but none had sufficient DNA for sequencing, thus all analyzed ticks were from one location. Of the 75 ticks that were extracted after a period of 8 to 16 days at -80°C storage (frozen), 42 individual extracts (56%) had sufficient DNA for library preparation and 18 had sufficient DNA after pooling in groups of 2 to 4 (6 pools). Samples with insufficient DNA concentrations ($n = 19$; average concentration 0.68 ng/μL), and with no like samples for pooling, as well as samples ($n = 13$) that were pooled but still did not meet a 2ng/μL minimum concentration, were not

submitted. The average DNA concentration in extracts submitted for sequencing was 6.58 ng/μL for individual samples and 2.88 ng/μL for pooled samples, with female samples having a higher DNA concentration (8.28 ng/μL) than male samples (3.18 ng/μL).

Forty-eight individual and seven pooled tick extracts collected within two late months from one location in Oktibbeha County were used for sequencing. While the microclimate may have changed over the course of the seven weeks that adult *A. maculatum* were collected, we did not have sufficient numbers from each date of collection to evaluate temporal differences in microbiome composition. With one collection site for all submitted extracts, we eliminated any variations in site location. By using an extraction protocol that enriched for microbial DNA, we minimized host (tick) DNA in the sample. Thus, DNA concentrations, determined using a Qubit® Fluorometer, more accurately reflected microbial DNA in the whole tick. We sought to compare the microbiome of *A. maculatum* extracted on the day of collection (fresh) compared to those extracted after a period of storage at -80°C (frozen) in order to evaluate concordance and the utility of using archived samples. However, sample sizes to fully assess this were low as only seven fresh samples met the minimum DNA concentration after extraction, while the remainder were frozen.

The total number of raw sequence reads obtained from 55 independent samples was 16,465 to 117,083 reads passing the quality filter in an Illumina BaseSpace®, and used for analyses in the QIIME pipeline with a normalized number (16,000). Alpha and beta diversity analyses were performed on QIIME (1.9.0). To estimate within-community diversity (alpha diversity), rarefaction curves were generated for average observed OTUs (Fig. 1A) and average chao (Fig. 1B) for the two treatments, and by tick sex (Fig. 1C, D). No significant differences in alpha diversity were observed between the two treatments (Fig. 2A) or male and female ticks (Fig. 2B). In beta diversity analysis, we generated weighted (Fig 3A, B) and unweighted (Fig 3C, D) principal coordinate analysis (PCoA) plots for individual samples.

The relative abundance of bacterial phyla within the analyzed samples demonstrated a preponderance of Proteobacteria (86.5%), Actinobacteria (6.4%), Firmicutes (2.0%), and Bacteroidetes (1.9%); other phyla rarely encountered, with an average abundance between 0.1% and 1% included Chlorflexi (0.4%), Planctomycetes (0.9%), and Acidobacteria (0.1%) (Fig 4A). The most abundant genera encountered in samples were, on average, *Francisella* (72.9%), *Sphingomonas* (4.5%), *Methylobacterium* (2.5%), an unidentified genus of Nocardioideae (1.2%) and an unidentified genus of Sphingomonadaceae (1.3%) (Fig. 4B, Fig. 5). There were 85 genera with average relative abundances between 0.05% and 1.0%; other genera rarely found are listed in the supplementary table (Table S1). Four of the 55 samples had *Rickettsia* in present, one individual male (14% relative abundance) and three pooled samples of males, with *Rickettsia* found at 1%, 4%, and 14% relative abundance. We also detected *Ehrlichia* in low relative abundance (0.05%) from one individual tick extract, while *Anaplasma* and *Borrelia* were not detected in any of the 55 samples.

4. Discussion

Published studies evaluating the microbiome of ixodid tick tissues or whole bodies have generally used genomic DNA, extracted using various commercial kits and with various levels of prior cleaning (Budachetri et al., 2014; Budachetri et al., 2016; Carpi et al., 2011; Clay et al., 2008; Gall et al., 2016; Menchaca et al., 2013; Rynkiewicz et al., 2015; Trout Fryxell and DeBruyn, 2016; Van Treuren et al., 2015; Williams-Newkirk et al., 2014; Zolnik et al., 2016). In this study, we provide an analysis of the *A. maculatum* whole tick microbiome using extracts enriched for microbial DNA. The microbiome in genomic DNA from *A. maculatum* whole tick bodies has not yet been published. However, other studies using whole ticks include rodent-attached *Dermacentor variabilis* and *Ixodes scapularis* ticks that were placed in 70% ethanol prior to genomic DNA extraction (Rynkiewicz et al., 2015), and whole *Amblyomma americanum* pretreated with 10% bleach and 70% ethanol prior to extraction (Williams-Newkirk et al., 2014). Both studies identified a preponderance of Proteobacteria, as well as common endosymbionts expected in those ticks species (Rounds et al., 2012).

For comparison to published microbiome data specific for *A. maculatum*, we focused on the taxa previously identified in salivary gland and gut tissues (Budachetri et al., 2014). Similar to Budachetri and colleagues, *A. maculatum* appears to be universally colonized with *Francisella*-like bacteria and *Coxiella* is absent; though, unlike that study, we did not detect *Wolbachia* (Budachetri et al., 2014). The distribution of the *Francisella* endosymbiont is not ubiquitous within tick tissues, and may depend on feeding status and environment. For example, *Francisella* was most common in both salivary gland and gut tissues of laboratory-reared ticks, but was not detected in the saliva of field-collected ticks; *Wolbachia* was detected only in salivary glands of field-collected ticks (Budachetri et al., 2014). Whether *Wolbachia* could have been found in dissected salivary glands and was simply below detection limits here is unknown. Both studies used Mississippi ticks, though slight geographical differences within the state may also play a role. Our study would not be the first to find inconsistencies in the detection of *Wolbachia* from *Amblyomma* ticks. Williams-Newkirk et al. (2014) reported *Wolbachia* in *Amblyomma americanum*, however, to our knowledge, this genus was not reported in *A. americanum* from other microbiome studies, including Clay et al. (2008) and Trout-Fryxell and DeBruyn (2016). The significance of the finding by Budachetri et al. (2014) is unclear considering the family, Anaplasmataceae was not one of the abundant families detected in that study. Interestingly, Zhang et al. (2014), detected *Wolbachia* endosymbionts by PCR using *Wolbachia*-specific primers and found in *A. americanum* females (not males) and pooled nymphs from Maryland. As the infection rates were low, it is possible that detection may have been missed with the small sample sizes used in our study. Further studies that include confirmation with specific PCR would be useful to resolve the role of *Wolbachia* in *Amblyomma* spp. in the United States.

In our study, it was not entirely surprising that we detected the genus *Rickettsia* at a relative abundance at or above 1% in four extracts, or that three of these were pooled extracts. Interestingly, the relative abundance of *Rickettsia* in the single individual sample was one of the highest, at 14%. Seven of nine additional individual samples with a relative *Rickettsia* abundance between 0.01 to 0.04% were also individual samples. *Rickettsia* was not detected

(0.0% abundance) in any ticks extracted “fresh,” however only six “fresh” tick extracts had sufficient DNA to be used for sequencing. It is unclear what *Rickettsia* species this may be, however, rickettsial levels in whole, unfed ticks from this area were higher by quantitative PCR for the pathogenic species, *R. parkeri*, than for a sympatric *Rickettsia* of unknown pathogenicity, “*Candidatus Rickettsia andeanae*” (Lee et al., 2017). Within the same northern area of Mississippi, the known pathogen, *R. parkeri*, is commonly detected in unfed ticks at an infection rate of about 19% to 30%; whereas “*Candidatus R. andeanae*” is typically found at lower infection rates, although rates vary annually (Ferrari et al., 2012; Lee et al., 2017). If the *Rickettsia* identified at low relative abundance in ticks here was detectable by our QPCR assay, infection rates would be approximately 17% for individual samples (8/48), and a minimum infection rate of 25% (5/20) from pooled samples. Other genera that include species of medical or veterinary importance, *Anaplasma* and *Borrelia*, were not detected here. However, we did find *Ehrlichia* in low relative abundance (0.05%) from an individual fresh sample. Loftis et al. (2016) found *Ehrlichia* spp., including the Panola Mountain *Ehrlichia* sp. (PME), in *A. maculatum*, but not samples collected in Mississippi. It is unclear whether this is PME; ongoing screening in our lab of *A. maculatum* attached to cattle has not revealed PME by conventional or QPCR, thus far (Varela-Stokes, unpublished results). Unfortunately, our QIIME analysis was unable to discriminate species in this study and we did not have extracts remaining to further process samples for species identification.

We found broad range in abundance of *Francisella* for individual tick samples (27.5% to 96.8%) with pooled samples falling within this range. The individual sample with lowest abundance of *Francisella*, also had the highest abundance of *Sphingomonas* among 55 samples analyzed, and we often observed a reciprocal relationship between *Francisella* and *Sphingomonas* relative abundance. While *Sphingomonas* was not previously identified in midgut or salivary gland tissues of fed *A. maculatum* (Budachetri et al., 2014) or from midguts of four sampled *A. tuberculatum* (Budachetri et al., 2016), *Sphingomonas* was identified in whole *A. americanum* and *I. scapularis* (Benson et al., 2004; Trout Fryxell and DeBruyn, 2016; Zolnik et al., 2016). It is unclear whether the *Sphingomonas* includes species that are soil contaminants which may have adhered to the surface of extracted ticks, as suggested (Benson et al., 2004), or species that may also be transmitted to the host (Zhang et al., 2014). Considering we cleaned the surface of ticks with 5% household bleach (minimum 2500 ppm sodium chlorite), we suspect most of the surface contaminants were removed. However, simple momentary immersion was not likely enough to destroy all contaminating bacteria or their DNA. Still, contaminating DNA present in the extraction kits can vary among commercial kits and should be considered when determining the significance of identified taxa. Contaminants from common kits, including the one used in this study, are largely Proteobacteria such as *Sphingomonas*, *Methylobacterium*, *Pseudomonas*, *Kocuria*, *Escherichia* and *Bacillus*. In this study, we did not include a negative control for analysis. Thus, we were limited in evaluating kit contaminants. However, three of the top 50 genera identified in our samples (at or above 0.1% average relative abundance), were among the 92 genera identified based on Salter et al. (2014) as commercial kit contaminants. The QIAamp Fast DNA Stool Mini Kit, and stool pathogen detection protocol, that was used here, was included in the study by Salter et al. (2014). Based on

these comparisons, we believe some of the genera identified may have been environmental or kit contaminants. Experimental validation of microorganisms identified at a rare abundance is necessary to confirm their presence as members of the microbial community prior to determining biological significance, if any. Future studies must specifically evaluate contributions of valid microbiota on the tick surface that were acquired from the environment, and eliminate microbial contaminants that are present in commercial kits (including in eluates such as water).

Considering that the activity of questing adult *A. maculatum* in the South typically peaks for a short time in summer, our capacity to study various aspects of microbial composition, including geographical differences, temporal changes, and microclimatic factors, could be expanded by using archived ticks. We included a comparison of fresh and fresh-frozen (frozen the day of collection) in this study. However, we found that DNA extracts from frozen samples met the minimum DNA concentration more often than extracts from fresh samples. Thus, we had only six extracts from fresh individual ticks to analyze, as compared to 42 extracts from frozen ticks. One pooled sample was also from extracts of fresh ticks. This limitation in the number of extracts from fresh ticks may be due to the greater ease in macerating frozen ticks as compared to macerating fresh ticks. Though the sample size for fresh extracts was small, these two treatments, fresh and frozen) did not demonstrate differences in within-group diversity. In terms of alpha and beta-diversity analyses, samples were also similar regardless of treatment or sex of ticks, while the most abundance genera were also similar for pooled and unpooled samples. Like other biomes, the microbial composition of the tick biome will likely be unique in different niches, or tissues, of the tick body. Whether microbial diversity and distribution in tick tissues impact vector competency is not known, but this may become a critical factor as we move forward in understanding maintenance and transmission of tick-borne pathogens. Most investigations of tick microbiomes, to date, have relied on genomic DNA, either archived and examined as a kind of post-hoc analysis or extracted and used exclusively for the microbiome study. Based on this study alone, we cannot determine whether DNA extracts enriched for microorganisms may be an appropriate alternative to genomic DNA for tick microbiome studies. Systematically comparing this approach alongside other extraction techniques used for tick microbiome studies is required to determine whether there is a benefit in enriching for microbial DNA. This may be accomplished by comparing extracts from each half of an individual tick. However, there is clearly a cost in using enriched DNA considering lower concentrations of DNA were obtained. Thus, fewer samples will likely meet the criteria for analysis, and it may not be possible to analyze individual tick tissues unless extraction efficiencies improve. Still, we anticipate that future tick microbiome studies that include approaches to minimize contamination, take into account kit contaminants, and determine optimal methods to evaluate the microbiota of tick vectors will be valuable to the scientific community. As we continue to explore microbial diversity in ticks, we may better understand the influence of diversity on pathogen maintenance and transmission, as well as niche-specific microbiomes within individual tick tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Rarefaction curves of alpha diversity among groups. Average observed OTUs (A) and average Chao1 (B) among treatment groups of *A. maculatum*; average observed OTUs (C) and average Chao1 (D) for male and female *A. maculatum* ticks.

Figure 2.

Alpha-diversity for observed OTUs were not significantly different between the two *A. maculatum* treatments (A) or male and female *A. maculatum* (B); calculated based on a rarefaction depth of 16,000 sequences.

Figure 3.

Beta diversity among groups using weighted (A, B) and unweighted (C, D) UniFrac PCoA plots of individual samples by *A. maculatum* sex (A, C) and treatment (B, D). Red = female; blue = male (A, C); red = fresh; blue = frozen (B, D)

Figure 4.

Taxonomic summaries for phylum (A) and genus (B) levels found in *A. maculatum*, with relative abundance of all bacterial members. (A) Proteobacteria (green) constitute the most abundant phylum, with Actinobacteria (yellow), Firmicutes (orange), and Bacteroidetes (pink) following. (B) Predominant genera in order of abundance were *Francisella* (light yellow), *Spingomonas* (teal), *Methylobacterium* (grey), an unidentified genus of Nocardioideae (light blue) and an unidentified genus of Sphingomonadaceae (tan). A legend describing individual samples is provided as a separate supplementary file (Table S2).

Figure 5.

A comparison of the top ten genera (identified or family if genus uncharacterized) in pooled, unpooled, fresh and frozen samples of *A. maculatum* (A) as well as between male and female ticks (B). *Francisella* was found in greatest abundance in *A. maculatum*, regardless of group.

Table 1

Number of adult *A. maculatum* ticks collected and extracted (on the same day “fresh” or after freezing “frozen”) for each collection date. On days where tick collections were considered too low to test both storage methods, samples were stored frozen.

Collection date	Females		Males	
	Fresh	Frozen	Fresh	Frozen
4-May	0	2	0	10
13-May	1	2	2	2
19-May	0	3	0	3
26-May	3	7	3	3
2-Jun	1	0	3	0
16-Jun	3	11	3	18
22-Jun	3	5	3	9
Total	11	30	14	45