AMCARF Project Status Report

This report will be used to communicate the progress, successes, and challenges of your AMCARF Funded project. The report will be used by the Review Committee to assess overall productivity of the project and its continued alignment with the AMCARF objectives. It will also be used to make future prioritizations of research and development efforts.

The report should not exceed 8 pages of text. Please include any additional attachments with key figures that may highlight critical findings. Use the following headings to build your report:

Individual filling out this form:

Email:

Report Type:

✓	**Report Type**	**Report Deadline**
	Progress Report for all 2018 awardees (including multi-year)	Monday, July 16, 2018
	Progress Report for 2018 awardees of multi-year projects requesting an additional year of funding	Monday, September 17, 2018
X	**Annual Report** for all 2018 awardees (including multi-year)	Friday, December 14, 2018

Project Title: Assessing the arboviral landscape via metagenomic analyses of sentinel FTA cards

AMCARF project number:

Project Cost: $42,500

Project Leader: Dana C. Price, Ph.D (Rutgers University, Center for Vector Biology)

Collaborators: Andrea M. Egizi, Ph.D (Monmouth County Mosquito Control, New Jersey), Brian Johnson, Ph.D (QIMR Berghofer Research Institute, Australia) and Nicole E. Wagner (Rutgers University Dept. of Biochemistry and Microbioogy [AMCARF funded @ 8% effort; Lab Technician])

Project Objectives: (Bullets as stated in the approved proposal. Add new or modified objectives.)

- To augment the CDC box gravid trap, CDC standard light trap, and Biogents BG-Sentinel trap to house a collection and feeding chamber that presents honey-soaked FTA feeding and excreta collecting cards.
- To trap a diverse selection of NJ mosquito habitat with a focus on known vector species e.g., *Culex pipiens* spp, *Aedes albopictus* and WNV and Eastern equine encephalitis virus (EEE).
- To extract and elute metagenomic DNA and total RNA (including D/RNA viruses) bound to the FTA cards, and to prepare and sequence Illumina next-generation sequencing libraries from both sources.
- To informatically assemble the metagenome-derived shotgun DNA and RNA sequence data generated in Aim 3 to contigs/scaffolds, and to classify and annotate metagenomic contigs, transcripts and viruses identified within the assembly.
- To corroborate the results of NGS-mediated pathogen screening of FTA cards (results of Aim 4) with quantitative PCR assays using captured mosquitoes from the same trap**.**

Total Project Progress:

Key Research Accomplishments: (Bulleted list of accomplishments from this project)

Note: The status report submitted as part of our request for a 1-year no-cost extension of this project has been included at the end of this document and summarizes project status through October 2018.

- Modification of box gravid trap, wooden resting box, and design of updraft CO2-baited trap to accommodate collection chamber with mounted FTA collection card. Additional objectives included modification of the BGS trap, however mortality of BGS collections remained extremely high due to desiccation. This is a common problem, and modifications proposed by Timmins et al. (2018 *J. Med Entomol*) are very similar to what we have employed for next season.
- We have trapped ca. 36 mosquito pools thus far (6 sites x 3 traps/site x 2 trap nights each) and were successful in designing an efficient delivery system for the honey-soaked FTA cards (via 35mm slide film mount) as well as collecting mosquito excreta (using a second, horizontal, slide film mount or alternatively lining the bottom of a cardboard soup container for extended exposure. As reported in the October No-Cost-Extension request (attached) however, we discovered that FTA reagent impregnated on the cards is particularly toxic to mosquito species, with > 90% mortality witnessed after only 24hrs of exposure. This prevented us from processing saliva and excreta from the same mosquitoes. Twelve pools were selected for sequencing and each of two (saliva, excreta) cards were extracted using a custom protocol.
- We were successful at designing lab protocols to extract DNA and RNA from the FTA cards using magnetic bead technology coupled with whole-genome and whole-transcriptome amplification. This template was used to prepare Illumina sequencing libraries, and we have thus far sequenced three pools (six libraries) with an additional six pools (twelve libraries) in the queue for the first week of January.
- Illumina MiSeq sequencing generated ca. 3.5Gbp of short-read data (~ 25 million total reads) combined for these six libraries. The data were assembled to metagenome contigs and additionally binned as raw reads; both assemblies and raw reads were annotated.
- The metagenome data analysis is currently ongoing, and we have quite interesting and novel results to report. Focusing on three excreta RNAseq libraries for which we have concentrated the bulk of our early analyses, we find that FTA capture of mosquito excreta is indeed a valuable tool to assess the mosquito microbiome. We have identified many genes, genome fragments and likely full viral genomes from multiple viruses, bacteria, microbial eukaryotes and host mosquito species within our samples.

Further details in Reportable Outcomes, below.

Reportable Outcomes:

Our modified collection chamber design allowed us to sample saliva and excreta from multiple wild-caught mosquito pools. We have created twelve Illumina shotgun sequencing libraries from isolated RNA and DNA corresponding to six pools, and have sequenced six such libraries. Informatic analyses are underway, and we have annotated both assembled metagenome contigs as well as raw read data from three excreta-card pools (11, 14 and 17 herein). Mosquitoes from each pool were allowed to feed and excrete in the lab, such as to provide part of a 'baseline' data series that is free from environmental contaminants such as trap by-catch. Data such as these will illustrate the utility of our protocols to characterize the mosquito microbiome using only FTA sequencing prior to moving cards to the field. Results are summarized below; further manual quality-control remains; this generally involves examining

taxonomic distribution of all genes encoded on the contig and examining short-read mappings to rule out chimeric contigs. Our initial sequencing runs were performed using 300-cycle Illumina kits in a 150bp x 150bp paired-end run, i.e., 150bp of each end of the DNA amplicon is sequenced. It is likely that a single-end 300bp or 500bp fragment will provide stronger taxonomic assignment for future runs. Among the most interesting observations from our initial sequencing include not only the identification of a broad range of RNA and DNA viruses (some with complete genomes recovered), but also potentially undescribed species of the rickettsial parasites *Anaplasma* and *Rickettsi*a, the spirochaete *Borrelia*, and eukaryotic parasites *Leptomonas*, *Ascogregarina*, and *Plasmodium.* We will continue with targeted efforts to assemble as much genomic material from these species as possible. These three pools tested negative for West Nile virus via Q-PCR and thus we are not yet able to judge the efficacy of FTA card shotgun sequencing for this aim, however we have many samples remaining to process.

Pool 11: Gravid trap; *Cx. spp (47)* w/ *Ae. albopictus* (5), *Ae. vexans (1)*, *An. punctipennis (1)* (Excreta; August 2018)
RNA: 8.9 million reads 12,421 annotated transcripts
DNA: 8.8 million reads 15,425 annotated contigs

Notable findings:

- RNA reads mostly eukaryotic (77%) – bacteria (21%) – viruses (2%)
- DNA reads mostly viral (80%) – bacteria (13%) – eukaryotic (8%)

- RNA viruses: Culex flavivirus, Alphamesonivirus (Houston virus), Biggievirus_Mos11 / Culex Biggie-like virus, Acyrthosiphon pisum virus, Rosy apple aphid virus

- DNA viruses: Overwhelming representation of *Culex* circovirus-like virus (ssDNA virus), with Avipox virus - Avian pox pathogen (dsDNA virus) also present. *Ralstonia* and *Burkholderia* phage present, likely complete genomes. Complete genome of a Gemygorvirus (Mallard-associated gemygorvirus 1-like).

- Bacteria: Proteobacteria are abundant w/ *Erwinia*, *Pseudomonas* and *Burkholderia* top genera. *Serratia* and *Wolbachia* mosquito symbionts also present.

- Undetermined species of *Borrelia* and *Anaplasma.*

- Genomic DNA predominantly ascomycote Fungi (Teratosphaeriaceae and Cladosporiaceae); mosquito DNA classified to 3 bins: *Culex pipiens* complex, *Anopheles* and *Aedes*. This matches the species composition per manual ID, with power to reveal even a single *An. punctipennis* mosquito present in the pool.

- A trypanosomatid *Leptomonas*-like apicomplexan parasite.

Pool 14A: Gravid trap; *Cx. Spp* (33) w/ *Ae. albopictus* (12), *Ae. vexans* (5), *An. punctipennis* (2) (Excreta; 10/07/2018)
RNA: 7.2 million reads 30,737 annotated transcripts
DNA: 6.2 million reads 39,053 annotated contigs

Notable findings:

- RNA reads mostly eukaryotic (78%) – viruses (12%) – bacteria (10%)
- DNA reads mostly viral (51%) – bacteria (32%) – eukaryotic (17%)

- RNA viruses: There is a broad diversity of ssRNA viruses: Quaranjavirus (Wuhan Mosquito Virus 6), Flavivirus (Culex flavivirus), Nodavirus (Culex Biggie-like virus, Culex Hubei-like virus), Picornavirales (Yongsan picorna-like virus 2), Tombusviridae (Culex-associated Tombus-like virus), ShiM-2016 clade (Hubei diptera virus 15, Hubei mosquito virus 4, Hubei tetragnatha maxillosa virus 8, Hubei tombus-like virus 28, Hubei tombus-like virus 30, Sanxia tombus-like virus 8, Wenzhou tombus-like virus 11, Wuhan insect virus 35, Zhejiang mosquito virus 1), Unclassified (Biggievirus_Mos11, Culex pipiens associated Tunisia virus, Culex negev-like virus 3)

- DNA viruses: As in pool 11, there is heavy representation of Culex circovirus-like virus (ssDNA virus), with *Ralstonia* and *Burkholderia* phage also present.

- Bacteria: The proteobacterial genus *Arcobacter* is highly represented, with *Gilliamella, Escherichia* and *Erwinia* the remaining top genera. *Serratia* and *Wolbachia* mosquito symbiont also present.

- Undetermined species of *Rickettsia*.

- Genomic DNA predominantly mosquito in origin with *Culex*, *Aedes* and *Anopheles* genera represented.

- A trypanosomatid *Leishmania*/*Leptomonas*-like apicomplexan parasite at greater representation than pool 11.

- An apicomplexan *Ascogregarina* parasite, previously reported from *Ae. albopictus* in the tropics and southern United States, and evidence that the parasite has followed its mosquito host to the near-northern extent of its range.

Pool 17: CO2 trap; *Ae. albopictus (22)* w/ *Ae. vexans (5)*, *Cx. pipiens (1)* (Excreta; 09/21/18)
RNA reads: 5.8 million 20,697 annotated transcripts
DNA reads: 7.1 million 31,360 annotated contigs

Notable findings:
- RNA reads mostly bacterial (62%) – eukaryotic (30%) – viruses (7.5%)
- DNA reads mostly bacterial (73%) – eukaryotic (26%) – viruses (6%)

- RNA Viruses: High abundance of Nodaviridae (Culex mosquito virus-1), with Picornavirales (Bundaberg bee virus, Yongsan picorna-like virus 2) and ShiM-2016 clade (Hubei diptera virus 15, Sanxia tombus-like virus 30, Wuhan insect virus) and Unclassified (Culicine-associated Z virus, Marsac virus)

- Absence of circovirus suggests this virus is specific to Culex species, or perhaps transmitted upon feeding and thus more prevalent in gravid mosquito excreta.

- DNA Viruses: Predominantly Parvoviridae (Lone star tick desnovirus 1). Remaining RNA viruses are phage of various commensal bacteria (*Burkholderia* phage, *Xanthomonas* phage, *Erwinia* phage, *Enterobacter* phage.

- Bacteria: Proteobacteria dominant with *Pantoea*, *Klebsiella*, *Enterobacter* and *Lonsdalea* the top genera. *Wolbachia* symbionts. Undetermined species of *Borrelia* pending further identification; this genus is not commonly reported from mosquitoes, and little is currently known regarding the relationship.

- Undetermined species of *Plasmodium*. Further work will place this taxon (and others of interest e.g., *Borrelia*, in a larger phylogeny to illustrate shared ancestry with currently known species)

Progress Assessment:

The mortality associated with the FTA reagent necessitated a second trapping season for the project. We do not expect to abandon any key portions of the project, however completing our objectives is predicated on trapping in Summer 2019.

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Refine FTA extraction protocols (DP,AE) **100% completed**	X	X	X	X								
Modify light, gravid and BGS traps (DP,BJ) **75% completed; BGS trap remains**	X	X	X	X	X							
Identify WNV/EEE study sites (AE, BJ) **100% completed**	X	X	X	X	X							
Trap placement / testing (team) **75% completed; BGS trap remains**				X	X	X						
Active trapping (team) **Season 2 will replace FTA with filter paper for saliva collections in Summer 2019**							X	X				
Lab extractions / sequencing (DP,AE) **Extractions completed for 6 of 15 samples in season 1 submitted for sequencing; remaining collections in summer 2019**							X	X				
Bioinformatic analyses (DP) **Analysis pipeline has been refined to an easily repeatable state; awaiting further samples**							X	X	X	X		
qPCR validation (AE)											X	X
Draft manuscript / AMCA 2019 (team) **Data within this report + six libraries scheduled to be sequenced in early January will be presented at AMCA 2019**										X	X	X

Plans for the following year:

Our major aims for the following year involve (1) Informatic analysis of remaining sequence data generated during winter 2018/2019, performed as in Reportable Outcomes above, (2) testing of modified BGS traps, with the aim to reduce dessication while housing FTA cards, and (3) testing efficacy of filer paper as a substitute for the toxic FTA cards in regards to saliva collection; this will allow us to link salivary and excreta microbiomes from the same mosquitoes and assess whether either method is more suitable for future arbovirus surveillance, i.e., "excreta is easy to collect; do we need to test saliva cards at all?"

Upon completion of this research, we expect to publish multiple manuscripts detailing our findings. Additionally, we plan to design and test an optimized microbiome sampling trap/chamber with a removable base containing a hydrophobic screen that allows excreta to pass through prior to being funneled to an FTA filter paper square for easy collection.

Conclusion:

The FTA sampling methods employed here will be invaluable for screening vector populations in a timely and cost-effective manner, while also keeping abreast of emerging vectors and facilitating design of rapid assays such as quantitative PCR. The preliminary results here have already identified novel viruses and parasites within mosquito excreta that are not commonly reported and for which the full association remains unknown. Further testing of saliva cards will assess the possibility of transmission.

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October 2, 2018

To: The American Mosquito Control Association Science & Technology Committee

I would like to thank the American Mosquito Control Association for the generous support of our proposed Research Fund application "Assessing the arboviral landscape via shotgun metagenome sequencing of sentinel FTA cards." We look forward to presenting our results at the 2019 annual meeting in Orlando.

I am writing to request a 1-year no-cost extension (no additional funds requested) of our award, currently set to end on Jan 14th, 2019, to Jan 14th, 2020. Reasons for this request are the following:

(1) We have found that the FTA reagent, widely used to preserve nucleic acids – including those from mosquito expectorate and excreta – is toxic to mosquitoes.

Our objectives are to perform shotgun metagenome sequencing of saliva and excreta captured on honey-soaked FTA cards offered to field mosquito populations. This approach requires that the mosquitoes feed on the cards for a period of time during which they also excrete onto a second card positioned below (Fig. 1). However, we observed very high mortality (> 90% after only 24hrs under laboratory conditions) among mosquitoes that fed on honey-soaked FTA cards. This had not been reported in the literature prior to our project start – however was just recently witnessed in the lab setting by Brugman et al. (2018):

> "Survival of both species of Anopheles mosquitoes provided with glucose on both types of FTA cards was significantly reduced compared to those given glucose on cotton wool (Fig. 5). The effect was significant for both *An. stephensi* (Log-Rank statistic X2df = 2 = 82.88, $p < 0.0001$) and *An. coluzzii* (Log-Rank statistic X2df = 2 = 119.4, $p < 0.0001$) suggesting mortality from sugar feeding on FTA cards is independent of Anopheline mosquito species. Furthermore, the greatest mortality occurs within the first few days of feeding on both classic and indicating FTA cards and by day 5 less than 10% of mosquitoes were alive."

We found that when using honey-soaked FTA cards, mosquitoes did not feed to repletion, taking only small amounts of honey and often dying within 24 hrs. Figure 2 depicts dead mosquitoes accumulating on an excreta card, indicating the mortality took place during or immediately after feeding. While there is evidence in the literature that arbovirus can be detected on FTA cards after mosquito probing alone, this development hinders our ability to compare the detection sensitivity of expectorate cards vs. excreta cards vs. mosquito testing, one of the aims of our project. The small amounts of honey taken did not allow sufficient excreta collection from trapped mosquitoes, and limited our ability to recognize fed vs. unfed mosquitoes for confirmatory qPCR testing.

As a result, we were obliged to spend several weeks during peak mosquito season isolating the cause of the mortality (e.g. FTA cards vs. sterile honey vs. food dye etc.) and adjusting our methods to accommodate this circumstance. We were able to conclude the season with three sample collection protocols (each constituting ~1/3 of our total samples):

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a.) FTA card saliva + excreta collections – our initial protocol; these samples are likely to provide data from saliva cards only, due to mortality.
b.) Remove FTA cards after 8h and replace with honey-soaked plain filter paper to generate additional excreta – these samples will generate both saliva + excreta data types, however each is likely to be generated from different mosquito cohorts as only surviving mosquitoes would feed on the filter paper and excrete (an example of excreta cards produced in this manner is shown in Fig. 3).
c.) Provide non-sterile honey-soaked cotton for generation of as much excreta as possible – the plain filter paper used in (b) above dries out after a period of time, so use of honey-soaked cotton prolongs the period of time for excreta collection without having to replace it, making it more suitable for field deployment. While this strategy will only provide data from excreta cards, new research by Ramirez et al. (2018) found that excreta was actually a better indicator of arboviral positivity than saliva for Australian strains of Ross River virus and West Nile virus (Kunjin strain).

(2) Our work depends on field mosquito collections made during WNV transmission season in the Northeastern USA.

This predicates much of the project be built around a short but critical collection window. While we plan to revise our collection strategy as detailed below, we must wait until July 2019 to continue fieldwork. As of September 28, 2018, we have collected more pools (35) than originally proposed (20), however as detailed above ca. 2/3 of these did not generate saliva and excreta from the *same* mosquitoes (due to FTA-associated mortality) for comparison regarding recovery and efficacy. Therefore we are requesting this no-cost extension to allow for a second field season of data collection.

Modifications for 2019

We are presently extracting nucleic acids from cards collected during the current field season, and will sequence and analyze samples first from the "FTA+filter paper" and "excreta only" protocols (b. and c. above, respectively) over the winter months to determine which approach is superior for metagenomic detection going forward. We also plan to evaluate a fourth approach: (d) utilize an untreated filter paper to offer the sterile honey solution in place of the saliva-collecting FTA card. Grubaugh et al. (2015) used sucrose-soaked filter paper to collect WNV isolates from experimentally infected mosquitoes in the lab for future sequencing. Although lacking nucleotide preservation reagents and RNAse inhibitors, it is likely that a short collection interval (e.g., within 12 hours) would prevent samples from degrading. Once these methods have been optimized we fully expect to be able to sequence an additional 12-15 pools in summer 2019 from additional sites without need for additional funds,as PIs are performing the fieldwork and reagents purchased in bulk will remain available. We will be happy to present a follow-up talk at the AMCA 2020 meeting on the further results of the project.

Thank you for your time and consideration.

Sincerely,

Dana C. Price, Ph.D.
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Department of Plant Biology
Center for Vector Biology

References cited:

Brugman VA, Kristan M, Gibbins MP, Angrisano F, Sala KA, Dessens JT, Blagborough AM, Walker T. 2018. Detection of malaria sporozoites expelled during mosquito sugar feeding. *Sci Rep*. 8:7545.

Ramírez AL, Hall-Mendelin S, Doggett SL, Hewitson GR, McMahon JL, Ritchie SA, van den Hurk AF. 2018. Mosquito excreta: A sample type with many potential applications for the investigation of Ross River virus and West Nile virus ecology. *PLoS Negl Trop Dis*. 12: e0006771

Grubaugh ND, Fauver JR, Rückert C, Weger-Lucarelli J, Garcia-Luna S, Murrieta RA, Gendernalik A, Smith DR, Brackney DE, Ebel GD. 2017. Mosquitoes Transmit Unique West Nile Virus Populations during Each Feeding Episode. *Cell Rep*. 19:709-718.

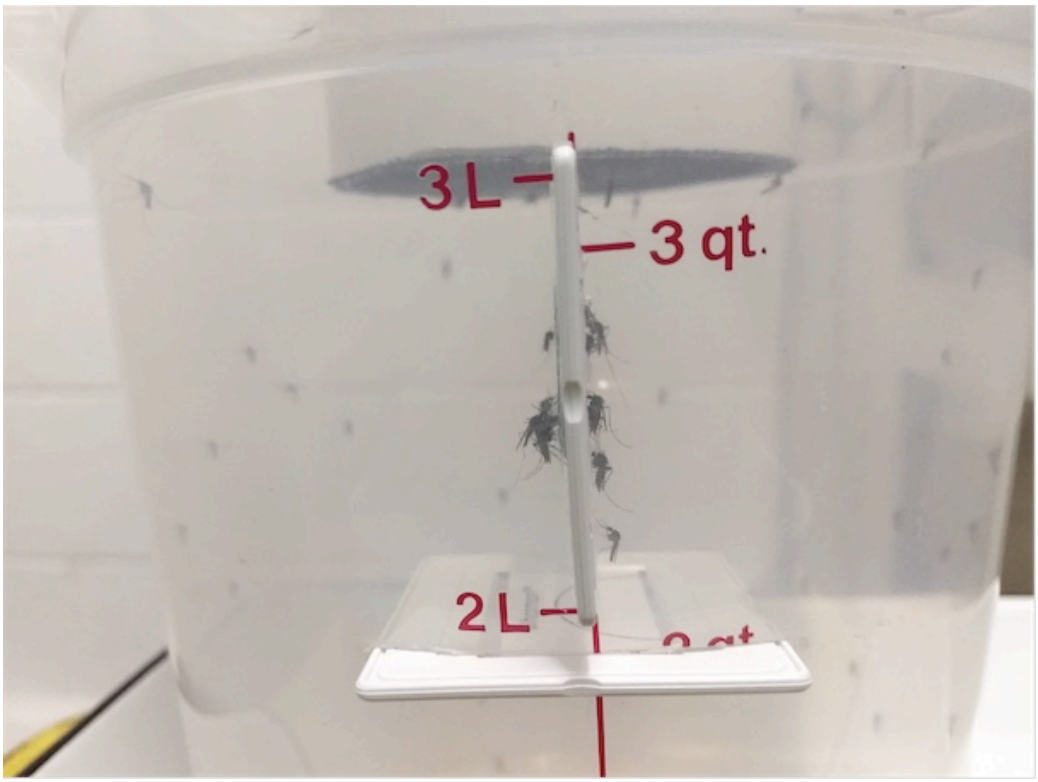

Figure 1. Updraft host-seeking mosquito trap constructed for this study, with associated saliva (vertical) and excreta (horizontal) collecting FTA card housing.

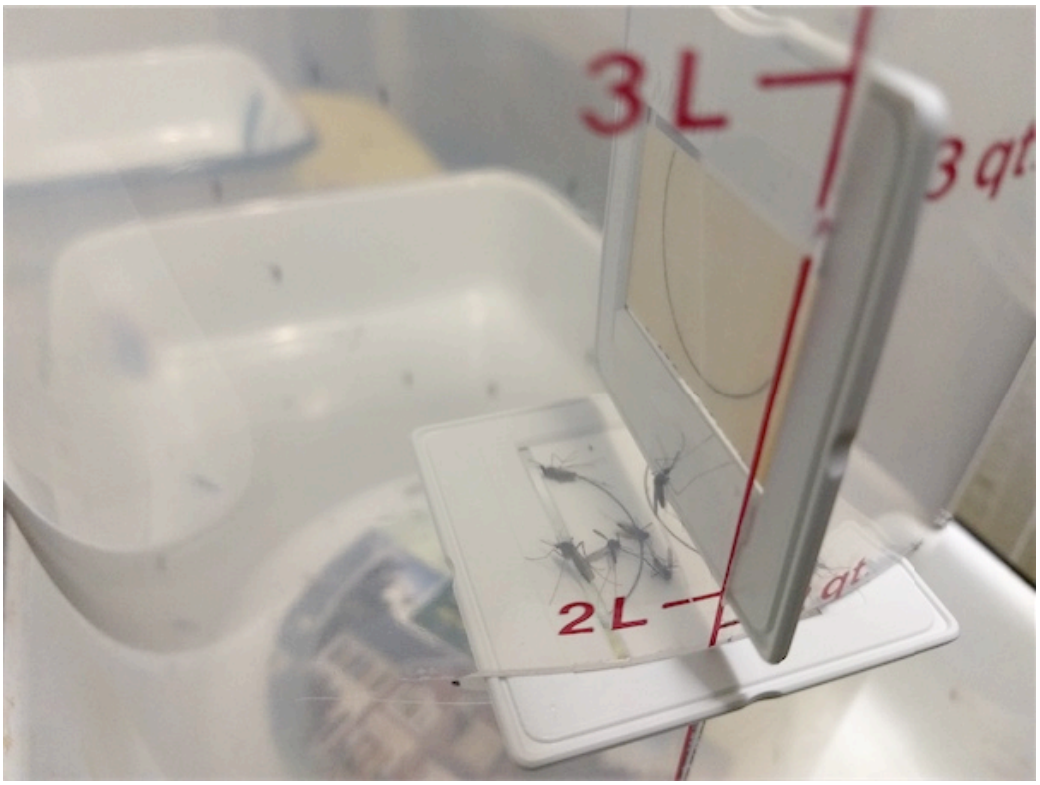

Figure 2. Deceased mosquitoes resulting from FTA card feeding.

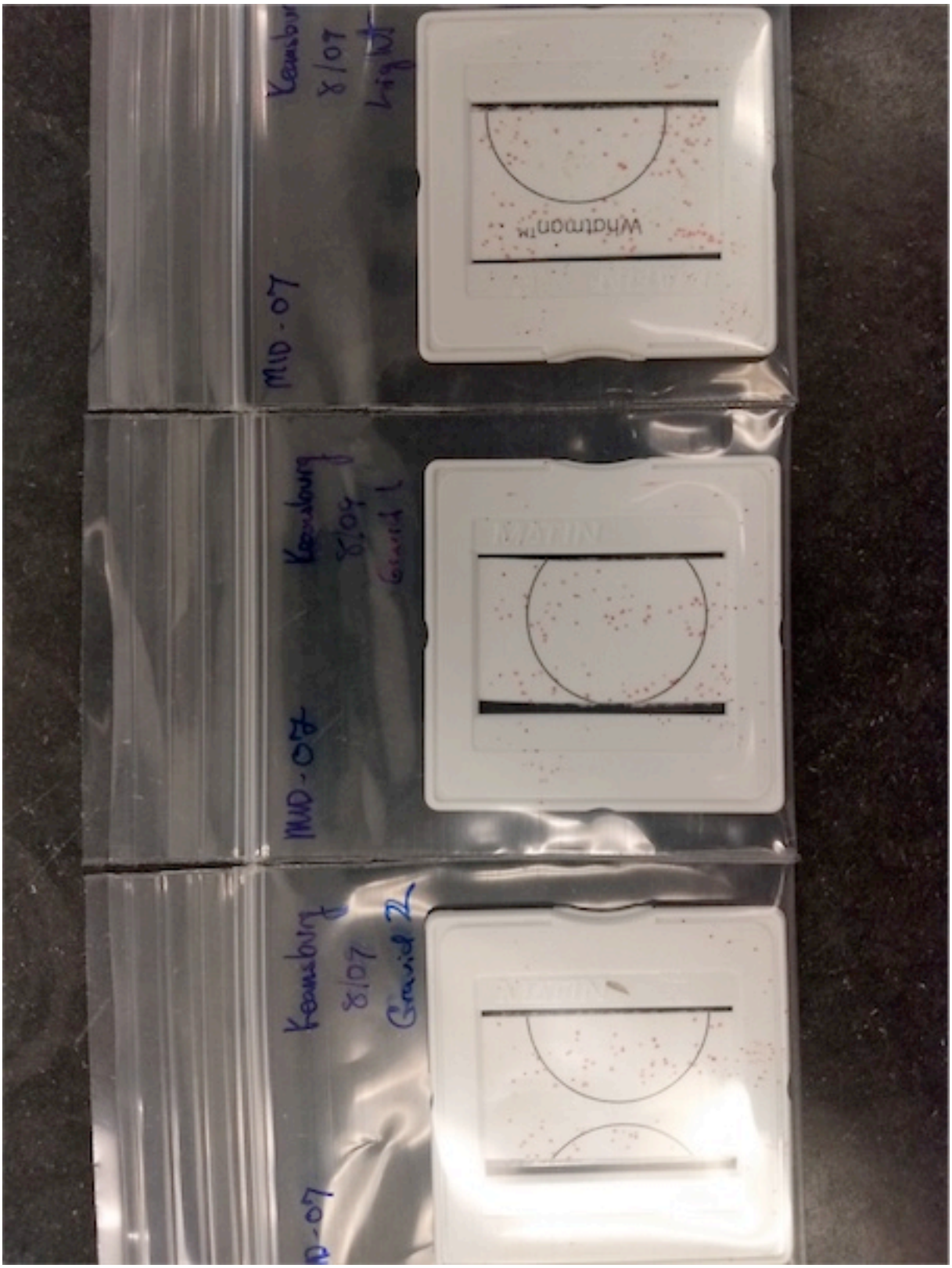

Figure 3. Excreta-collecting FTA cards with associated mosquito excreta (red dots).