Benthic Aquatic Research (BAR)

Standard Operating Procedures and Protocols for Mosquito Taxonomic Identification

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1.0 Traceability of Analysis

Dr. David S. Goldhammer is a dipterist. He specializes in midges in the family Chironomidae, and currently is the primary diagnostician regarding mosquitoes. He was lead taxonomist for the Kansas Biological Survey for the mosquito surveillance program from 2016 to 2019 and for the NEON mosquito taxonomic identification program from 2016 to 2023. Dr. Goldhammer's curriculum vita is attached.

Mosquito identification will be based on the most current references, with Darsie & Ward (2005) as the primary, defining text for all NEON Domains, except Puerto Rico. If NEON mosquito samples are provided from Puerto Rico then other appropriate references will be used, such as Fox (1953). Additional publications may be referenced as listed below.

- Andreadis, T. G., M. C. Thomas, and J. J. Shepard. 2005. Identification guide to the mosquitoes of Connecticut. Connecticut Agricultural Experiment Station. 966.
- Burkett-Cadena, N.D. 2013. Mosquitoes of the southeastern United States. The University of Alabama Press, Tuscaloosa, Alabama. 188pp.
- Carpenter, S.J. and W.J. LaCasse. 1974. Mosquitoes of North America (North of Mexico). University of California Press.
- Harbach, R.E. and K.L. Knight. 1980. Taxonomists' glossary of mosquito anatomy.
- Harrison, B. A., B.D. Byrd, C.B. Sither, and P.B. Witt. 2016. The mosquitoes of the mid-Atlantic region: an identification guide. Western Carolina University. Cullowhee, North Carolina, USA.
- Stanuszek, W.W. 2013. A dichotomous key to commonly encountered mosquitoes in Saginaw County, Michigan.
- Thielman, A.C. and F.F. Hunter. 2007. A photographic key to adult female mosquito species of Canada (Diptera: Culicidae). Canadian Journal of Arthropod Identification No. 4.
- Tulloch, G. S. 1937. The Mosquitoes of Puerto Rico. Journal of Agriculture of the University of Puerto Rico 21:137-167.

All identifications and QC will be conducted by Dr. Goldhammer and Dr. Rogers. No other individuals will be conducting any identifications.

2.0 Procedure

2.1 Identify and Group Specimen Procedure

BAR is required to identify and group specimens from bouts at NEON sites to species and sex. Specifically:

- Mosquito specimens collected by Neon Inc. have been transferred from each trap into one or more vials (typically 15mL or 50mL tubes, BD Falcon or similar). Each vial is labeled with a sampleID in the following format: plotID.collectDate.collectTime (e.g., OSBS_001.20160601.0725). Samples have been frozen by NEON Inc. and stored in a -80°C freezer prior to shipment to BAR.

- Vials of mosquitoes will be shipped to BAR on dry ice with a hard-copy shipping manifest. Prior to delivery, BAR will also receive an electronic copy of the shipping manifest with tracking information. Upon receipt of each package, BAR will:

    - Open the container and ensure all samples listed on the manifest have been received and are in good condition (e.g., not broken or missing; labels are legible);

    - Record date received and sample condition on the electronic shipping manifest provided by NEON Inc. BAR will return this electronic manifest to a NEON data portal provided by NEON Inc. to confirm receipt of the package and all samples. BAR must notify the NEON Inc. Contract Officer and Technical Representative of any problems with the shipment within 48 hours of receipt, and;

    - While in BAR's custody, samples must be stored at -80˚C.

- During processing, mosquitoes shall be handled on a chill table and not allowed to thaw in order to preserve samples for subsequent pathogen testing. If a chill table is not available, BAR will use dry ice to keep the material properly chilled so that arbovirus (if any) may be detected in the mosquitoes. NEON Inc. must approve any alternatives to a chill table.

- All mosquitoes from one trap are consolidated into a single batch of mosquitoes and weighed and recorded to the nearest 0.01 grams (g) prior to mosquito sorting from the trap (entered as 'total Weight' on the sorting datasheet).

- The sample is then visually assessed if it is likely to contain either 1) 200 or fewer mosquitoes or 2) 200 or greater mosquitoes. If the complete sample is estimated to contain 200 or fewer mosquitoes, then the subsample comprises the entire trap contents. The weight of this subsample in grams is the recorded (entered as 'subsampleWeight' on the sorting datasheet). All Culicidae are identified from the sample and the weight of any unidentified non-culicid bycatch is recorded as the 'bycatchWeight'.

- If the complete sample is estimated to contain greater than 200 mosquitoes, a randomly selected group of 200 specimens and any bycatch (non-mosquitoes) associated with those 200 are removed and weighed on the digital scale to determine subsample weight (a typical subsample of ~200 specimens will weigh approximately 500 mg, but this weight may vary with species composition and sample condition). This value is recorded in the field 'subsampleWeight'. All Culicidae are identified from the subsample. Then all unidentified bycatch from the subsample is removed and weighed on the digital scale to determine sample bycatch weight (recorded in the field called 'bycatchWeight'). Any remaining sample not part of the subsample shall be returned to the original vial(s) and stored at -80°C until Battelle has received and approved identification data and provided information on disposition of samples.

- BAR will record the number of individual mosquitoes (enter as 'individual Count' on the identification datasheet) of each species (enter as 'scientificName' on the identification datasheet) and sex (enter as 'sex on the identification datasheet) present in each subsample. Afterward, BAR will sort mosquitoes from each 200 specimen subsample into separate containers or vials, grouping mosquitos by species and sex (the 500 mg subsample from one trap may generate multiple vials). These temporary vials or containers may be any size or shape, but mosquitoes are to be maintained in groups of the same species, sex and bout (collection window). Each grouped vial shall be labeled with the scientific name, sex and bout to which the mosquitoes pertain and stored in -80C freezer until pooling instructions are available. After all samples are identified, BAR will upload the sorting datasheet and identification datasheet (as csv files) to the NEON portal provided by NEON Inc. BAR must upload all data pertaining to each bout (do not upload partial bouts). BAR will upload data for all sites in one sorting data file and one identification data file.

- BAR is required to report the primary identification reference used to make the taxonomic determination (enter as 'identificationReferences'on the identification datasheet).

- BAR must return sample data using the sample ID provided. Data are to be returned on the NEON datasheet according to the Required Turnaround Time. The NEON database links data based on sample ID information and as a result it is imperative that this sample ID be correct. BAR may not invoice for identifications performed on samples for which datasheets have been returned with inaccurate sample ID information.

- Verify certain results if NEON Inc.'s post-analytical Quality Assurance Procedure, performed within 180 calendar days of NEON Inc. receipt of data from BAR, suggests the need for verification of certain specimens.

- For Domains that request shipping materials be returned, return all NEON Inc. supplied sample shipping materials (e.g. coolers) within 15 calendar days of receiving sample shipment. Domains will provide a return shipping label.

2.2 Point and Pin specimen Procedure

BAR will select voucher specimens to point/pin. BAR will receive instructions from NEON Inc. to remove one leg from certain specimens after NEON Inc. reviews the identification data submitted by BAR. For each specimen specified by NEON Inc., BAR will remove one leg from the voucher. This leg will be designated for barcoding. BAR will send pinned/pointed specimens to a designated archive facility and the pulled legs to a barcoding facility. Specifically:

- Pull voucher specimens to pin/point and label appropriately during the sort of mosquitoes from a trap by species and sex. These specimens are not subject to cold chain requirements but can be kept cold during the process if it fits BAR's workflow. Up to 10 specimens of a species/sex combination will be pinned/pointed from each domain over the course of a sampling season. Voucher specimens should be in very good condition and selected across sampling bouts and sites for each domain from which mosquitoes are obtained. BAR shall attach locality, determination and individual ID labels to each specimen. Locality labels will include the country, state, county, locality name, spatial information about the sample (elevation, lat/lon), method of collection, collection data, collector name, 'NEON' and the sample ID. Determination labels will contain species information, identifier and year of identification. Individual ID is unique over the lifetime of the observatory and includes the information about the specimen type (i.e., 'MOS'), the domain (i.e., 'D01') and a unique six-digit number. NEON Inc. will provide a list of previously used individual ID's at the beginning of each field season. NEON Inc. will provide an application that automatically creates locality, determination, and individual ID labels from the identification data generated by BAR. BAR may then apply these labels to the specimen in lieu of generating them independently.

- Voucher specimens will be stored refrigerated in Schmidt boxes.

- Based on the individual ID information reported in the identification returned by BAR, the NEON Inc. Technical Representative will notify BAR at the end of the sampling season (typically November) which specimens require leg removal for DNA barcoding. BAR will remove one leg for each specimen selected for DNA barcoding and place the leg into a designated container (i.e., a 96-well plate) to be shipped within 48 hours of removal from the vouchered specimens to a DNA barcoding facility chosen by NEON Inc. Barcoding samples will be stored at room temperature, dry in 96-well plate until shipped to barcoding facility. Barcoding samples can be shipped at room temperature, but must be shipped to a barcoding facility within 48 hours of removal from the vouchered specimens.

- Once samples have been sent for DNA barcoding, the pinned/point specimens will be stored in a refrigerator until such time as directed to be shipped at room temperature to an archive facility chosen by NEON Inc.

- BAR will create a shipping manifest that lists all samples included in the shipment. BAR will provide the archive and/or DNA barcoding facility a hard-copy of the

shipping manifest with the shipment and electronic copy of the shipping manifest when the specimens are sent. BAR will also provide an electronic version of the shipping manifest to NEON Inc. in a .csv file.

2.3 Pooled Sample Procedure

BAR shall prepare pooled samples of mosquitoes that have completed the taxonomic identification process as follows:

- BAR will have recorded sorting information (i.e., weight) and taxonomic analysis (i.e., identifications and counts) as described in Section 2.1 above. All processed mosquitoes will have been stored in a -80°C freezer in groups segregated by species/sex/bout until pooling instructions are given. NEON Inc. will decide based on the data provided from Section 2.1 whether pooled mosquitoes will be pathogen tested or sent to an archive facility and provide that information to BAR. Pooling instructions will be available once all samples have been identified.

- The web application will check the datasheet for common errors (other errors may be present); if errors are detected, BAR must remedy those errors and re-upload the document. Once the web application has an error-free copy of the sorting and identification datasheets, it will produce 4 files which BAR will download. The data will be returned as 4 csv files with UTF-8 encoding; this is the format required by the NEON database. These files are: sorting, identification, pathogen pooling, and archive pooling datasheets.

- Based on the instructions in the pooling datasheets, BAR will create pools of the size specified in the datasheet for each species/sex combination from all traps in a bout at a site (so by species/sex/bout/site) and place each pool in a new, labeled vial (or multiple vials depending on the number of mosquitoes in a pool; each vial will have a unique barcode) according to the instructions in the pooling datasheets. Final specimen vials and labels must be suited for cryo-storage (safe to -80°C). The following vial sizes boxes and labels should be used when storing various quantities of mosquitoes: This information is located in Exhibit A SOW, Appendix 1, pp. 20-22.

- After the pooling datasheet instructions are implemented, BAR will upload all datasheets into the NEON data portal.

- Upon direction from the NEON Inc. Technical Representative, BAR will pack pooled, identified mosquitoes with sufficient dry ice to keep samples frozen and ship them to a pathogen testing or archive facility as specified by the NEON Inc.

3.0 QAQC Protocols

BAR will conduct quality assurance (QA) checks, with a minimum of 3% of all subsamples recounted and reidentified by a different individual than the one who made the first counts and determinations. QA data shall be returned to NEON Inc. along with standard datasheets. The specific, standard equations are:

Equation I. Percent Difference in Enumeration (PDE):

$$PDE = 100 \text{ X } (|count_1 - count_2| / | count_1 + count_2|)$$

Equation II. Percent Taxonomic Disagreement (PTD):

$$PTD = 100 \text{ x } (1 - (agreements/n)$$

Where "***agreements*"** is the number of consistent identifications (species and sex match; if species and sex are identical, but identification qualifier differs, that is still considered an agreement), and "***N*"** is the total number of individuals in the larger of the two counts.

If PDE>0.05, BAR will use the results of the second count in the identification datasheet and explain the discrepancy in the "remarks" column in addition to the requisite language described in the reporting requirements. BAR will reconcile count data in final identification datasheet. BAR will report the QC results as a numeric value (no percent sign) in the column 'PDE' in the sorting datasheet. In the sorting datasheet "remarks" will indicate that QC was performed. BAR will use the format "QC check; PDE XX; Genus level PTD YY; Species level PTD YY" where XX is the calculated PDE value for the subsample (should match the numerical value given in the 'PDE' column).

If PTD values are >0.02 or 0.05, as applicable, BAR will use the second identification to update the scientificName and sex in the identification datasheet and explain the discrepancy in the "remarks" column in addition to the requisite language described in the reporting requirements. Acceptable explanations may include any information that affected the analysis (e.g., specimen integrity, etc.) Reconcile taxonomic data in final identification datasheet. BAR will report the QC results as a numeric value (no percent sign) in the columns 'genusPTD' genus-level PTD value) and 'speciesPTD' (specieslevel PTD value) in the sorting datasheet. In the sorting datasheet "remarks" BAR will indicate that QC was performed. BAR will use the format "QC check; PDE XX; Genus level PTD YY; Species level PTD YY" where YY is the calculated PTD value at each resolution (should match the numerical values given in the 'genusPTD' and 'speciesPTD' columns).