Kansas Biological Survey (KBS)

Rev 1

Standard Operating Procedures and Protocols for Mosquito Taxonomic Identification

18 October 2016

Table of Contents

1.0 Traceability of Analysis

Macroinvertebrate Diagnostics Laboratory has sorted, processed, identified, and archived macroinvertebrate specimens from ~2000 aquatic, terrestrial, and fossorial samples from 26 countries throughout the Americas, Asia, Africa, and tropical Pacific Islands over the past decade. Laboratory head Dr. D. Christopher Rogers's research expertise encompasses mosquitoes, as well as freshwater and terrestrial crustacean taxonomy and systematics. He has identified more than 2 million mosquitoes (to generic or species level) for federal and state environmental projects and in his research. Dr. Rogers identified mosquitoes for the state of California for three years, and now runs the mosquito surveillance program for the state of Kansas, where he has identified mosquitoes for the last five years.
Dr. Rogers's curriculum vita is attached.

Dr. David Goldhammer is our dipterist. He specializes in midges in the family Chironomidae, and currently is our primary diagnostician regarding mosquitoes. 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).

Entomology at KU is written into the 1866 founding documents of the university, with a current established permanent staff of 3 faculty-curators, two collection managers, seven research scientists and graduate and undergraduate students. This infrastructure of people, space and systematics facilities ensures the timely delivery of all grant and contract obligations.

The Macroinvertebrate Diagnostics Laboratory has a standard taxonomic voucher collection of mosquitoes from across the USA. KU Entomology also has extensive mosquito collections from all over the world, which may be used in direct comparison determinations if questions arise in the keys.

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

2.0 Procedure

2.1 Identify and Group Specimen Procedure

KBS 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; see Figure 2). Some samples may have additional numbers or characters appended to the sampleID from an older sampleID format. NEON Inc. will provide electronic documentation translating older sampleID formats to the current sampleID format. Samples have been frozen by NEON Inc. and stored in a -80°C freezer prior to shipment to KBS.

- Vials of mosquitoes will be shipped to KBS on dry ice with a hard-copy shipping manifest. Prior to delivery, KBS will also receive an electronic copy of the shipping manifest with tracking information. Upon receipt of each package, KBS 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. KBS will return this electronic manifest to a BOX folder provided by NEON Inc. to confirm receipt of the package and all samples (see Figure 3). KBS must notify the NEON Inc. Contract Officer and Technical Representative of any problems with the shipment within 48 hours of receipt, and;

    - While in KBS'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, KBS 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.

- Consolidate all the mosquitoes from one trap into a single batch of mosquitoes and weigh and record the weight of the total contents of this trap in grams (g) prior to sorting mosquitoes from the trap (enter as 'total Weight' on the sorting datasheet; see Figure 3). NEON Inc. requires that scales have a precision of one milligram.

- A random selection of up to 500 mg of insects within a trap shall be removed from the total mosquito catch; KBS shall select a subsample as close to 500 mg as possible. If the contents of the whole trap are less than 500 mg, then the subsample will comprise the entire contents of the trap. Record the weight of this subsample in grams (enter as 'subsample Weight' on the sorting datasheet). Any remaining mosquitoes that are not part of the 500 mg subsample shall be returned to their original vial(s) and stored at -

80°C until NEON Inc. has received and approved identification data and provided information on disposition of samples.

- Sort the 500 mg subsample into mosquitoes and nonmosquito (by-catch) organisms. All mosquitoes will be counted and the number of mosquitoes of each sex and species will be recorded in the identification datasheet (described below). Weigh and record the weight of the by-catch organisms for this subsample (enter as 'bycatch Weight on the sorting datasheet). The bycatch may then be discarded.

- KBS will record the number of individual mosquitoes (enter as 'individual Count' on the identification datasheet) of each species (enter as 'scientific Name' on the identification datasheet) and sex (enter as 'sex on the identification datasheet) present in each subsample. Afterward, KBS will sort mosquitoes from each 500 mg 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.

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

- KBS must return sample data using the sample ID provided. Data are to be returned on the NEON datasheet (Attachment 2) according to the Required Turnaround Time in Exhibit B. The NEON database links data based on sample ID information and as a result it is imperative that this sample ID be correct. KBS 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 KBS, 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

For 2016 samples only, KBS will select voucher specimens to point/pin. KBS will receive instructions from NEON Inc. to remove leg from certain specimens after NEON Inc. reviews the identification data submitted by KBS. For each specimen specified by NEON Inc., KBS will remove one leg from the voucher. This leg will be designated for barcoding. KBS 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 KBS'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. KBS 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 KBS. KBS may then apply these labels to the specimen in lieu of generating them independently.

- Voucher specimens will be stored Room temperature in Schmidt boxes (or equivalent) with adequate preservation measures (e.g., moth crystals).

- Based on the individual ID information reported in the identification returned by KBS, the NEON Inc. Technical Representative will notify KBS at the end of the sampling season (typically November) which specimens require leg removal for DNA barcoding. KBS 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 shipped at room temperature to an archive facility chosen by NEON Inc. within 21 days of sample receipt.

- KBS will create a shipping manifest that lists all samples included in the shipment. KBS 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. KBS will also provide an electronic version of the shipping manifest to NEON Inc. in a .csv file.

2.3 Pooled Sample Procedure

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

- KBS 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 KBS. Pooling instructions will be available once all samples have been identified.

- After all samples are identified, KBS will upload the sorting datasheet and identification datasheet (as csv files) to a web-application2 provided by NEON Inc. KBS must upload all data pertaining to each bout (do not upload partial bouts). Uploads of partial bouts (e.g., only half of bout 7 from OSBS is returned, with many records missing) will result in erroneous instructions. KBS will upload data for all sites in one sorting data file and one identification data file.

- The web application will check the datasheet for common errors (other errors may be present); if errors are detected, KBS 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 KBS 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, KBS 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 label) according to the instructions in the pooling datasheets (see Figure 5). If KBS must deviate from the instructions for any reason, then KBS will contact the NEON Inc. Technical Representative for further instruction. Pools shall be stored as indicated in Table 5. Final specimen vials and labels must be suited for cryo-storage (safe to -80°C). The following vial sizes should be used when storing various quantities of mosquitoes:

    - 2 mL snap cap centrifuge tubes for the pathogen testing and for archival of species with only one or two specimens per bout.
    - 2 mL cryovials for species with 3-50 specimens per bout
    - 5 mL cryovials for species with 51-200 specimens per bout
    - 15 mL cryovials for 201-500 specimens per bout

- After the pooling datasheet instructions are implemented, KBS will upload all 4 datasheets into the NEON database. 2014 samples will use the web-application. Due to

updates to the web-application system, 2016 samples may instead be required to be uploaded to a BOX cloud-based data repository folder. More detailed instructions will be provided upon contract award by NEON.

- Upon direction from the NEON Inc. Technical Representative, KBS 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. Technical Representative 14 days from completing identifications.

3.0 QAQC Protocols

KBS 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, KBS 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. KBS will reconcile count data in final identification datasheet. KBS 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. KBS 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, KBS 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. KBS 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” KBS will indicate that QC was performed. KBS 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).