TOS PROTOCOL AND PROCEDURE: MOSQUITO SAMPLING

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See configuration management system for approval history.

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Change Record

REVISION	DATE	ECO #	DESCRIPTION OF CHANGE
-	04/27/2011	ECO-00159	Initial Draft Release
A_DRAFT	10/03/2011	ECO-00280	Update to new document numbers and template
B_DRAFT	07/30/2012	ECO-00442	Adjusted for known issues from 2011 prototype and revised for Domain 3 specific information
C_DRAFT	02/24/2014	ECO-01139	Draft release. Will be finalized in next rev.
D	03/27/2014	ECO-01672	Production release, template change, and other changes as detailed in Appendix C

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1 DESCRIPTION

1.1 Purpose

The primary purpose of this document is to provide change controlled version of Observatory protocols and procedures for Plot Establishment. This document provides the content for training and field-based materials for NEON staff and contractors. Content changes (i.e. changes in particular tasks or safety practices) occur via this change controlled document, not through field manuals or training materials.

This document is a detailed description of the field establishment process, relevant pre- and post-field tasks, and safety issues as they relate to this procedure and protocol.

1.2 Scope

This document relates the tasks for a specific field sampling and directly associated activities and safety practices. This document does not describe:

- General safety practices
- Site-specific safety practices
- General equipment maintenance

It does identify procedure-specific safety hazards and associated safety requirements such as safe handling of small mammals or safe use of required chemicals and reagents.

1.3 Acknowledgements

Dr. Cara Gibson, Kali Blevins, and Patrick Travers contributed significantly to early versions of this protocol.

2 RELATED DOCUMENTS AND ACRONYMS

2.1 Applicable Documents

Applicable documents contain information that shall be applied in the current document. Examples are higher level requirements documents, standards, rules and regulations.

AD [01]	NEON.DOC.004300	EHS Safety Policy and Program Manual
AD [02]	NEON.DOC.004316	Operations Field Safety and Security Plan
AD [03]	NEON.DOC.000724	Domain Chemical Hygiene Plan and Biosafety Manual
AD [04]	NEON.DOC.001155	NEON Training Plan
AD [05]	NEON.DOC.050005	Field Operations Job Instruction Training Plan
AD [06]	NEON.DOC.000910	NEON Science Design for Mosquito Abundance, Diversity, and Phenology
AD [07]	NEON.DOC.000911	NEON Science Design for Vectors and Pathogens
AD [08]	NEON.DOC.001100	TOS Protocol and Procedure: Ground Beetle and Mosquito Processing
AD [09]	NEON.DOC.005003	NEON Level 0 Data Products Catalog
AD [10]	NEON.DOC.001125	TOS Protocol and Procedure: Plot Establishment
AD [11]	NEON.DOC.001581	Datasheets for TOS Protocol and Procedure: Mosquito Sampling
AD [12]	NEON.DOC.001401	NEON Raw Data Ingest Workbook for TOS Mosquito Abundance, Diversity and Phenology
AD [13]	NEON.DOC.001271	NEON Protocol: Manual Data Transcription

2.2 Reference Documents

Reference documents contain information complementing, explaining, detailing, or otherwise supporting the information included in the current document.

RD [01]	NEON.DOC.000008	NEON Acronym List
RD [02]	NEON.DOC.000243	NEON Glossary of Terms
RD [03]	NEON.DOC.005003	NEON Scientific Data Products Catalog
RD [04]	NEON.DOC.014051	Field Audit Plan
RD [05]	NEON.DOC.000824	Data and Data Product Quality Assurance and Control Plan

2.3 Acronyms

CDC	U.S. Centers for Disease Control and Prevention

2.4 Definitions

A **protocol** is a formal summary description of a procedure and its related rationale, and includes information on knowledge and resources needed to implement the procedure. A **procedure** is a set of prescribed actions that must take place to achieve a certain result, and can also be called a method. It differs from a science design in that science designs provide a more complete description of the rationale for selecting specific protocols. It differs from a training manual in that training manuals provide materials in support of skills acquisition in the topic areas including information on how to best train staff rather than detailing only the steps of the procedure.

3 BACKGROUND AND OBJECTIVES

3.1 Background

Mosquitoes are a diverse and widespread family of insects that have been extensively studied because of their ecological and epidemiological significance. As a dominant taxon in aquatic food webs, mosquitoes comprise a sizable proportion of the invertebrate biomass and act as a key food source for aquatic and terrestrial predators (e.g., fish, amphibians, spiders, birds). Mosquitoes also act as vectors for numerous parasites and pathogens of humans, livestock, and wildlife, and their biology and ecology have been extensively studied to characterize and mitigate impacts of associated diseases. Because of their sensitivity to environmental gradients and perturbations mosquitoes represent an ideal sentinel taxon for evaluating the ecological effects of global change phenomena. Their short generation time and high fecundity allow mosquitoes to respond quickly to environmental change, but because of the group's high diversity and varied ecological niches the nature and magnitude of these changes can differ markedly among species. Changes in global climate are predicted to affect the distribution, demography, and seasonal phenology of many mosquitoes, and associated effects on pathogen transmission cycles have also been posited. Because of their frequent association with humans and ability to thrive in human-modified environments, mosquito ecology is also likely to be significantly affected by land use changes. Based on these reasons mosquitoes were selected as a sentinel (focal) taxon to be monitored within the National Ecological Observatory Network.

3.2 NEON Science Requirements

This protocol fulfills Observatory science requirements that reside in NEON's Dynamic Object-Oriented Requirements System (DOORS). Copies of approved science requirements have been exported from DOORS and are available in NEON's document repository, or upon request.

3.3 NEON Data Products

Execution of this protocol procures samples and/or generates raw data satisfying NEON Observatory scientific requirements. These data and samples are used to create NEON data products, and are documented in the NEON Scientific Data Products Catalog (RD [03]).

4 PROTOCOL

Mosquito sampling involves preparing to sample (SOP A: Preparing for Sampling), collection in the field (SOP B: Field Sampling), minor laboratory processing (SOP C: Laboratory Processing and Analyses), shipping to external facilities (SOP D: Sample Shipment) and data handling (SOP E: Post Identification Lab Processing). Field collection of live mosquitoes is conducted using CDC CO_2 light traps. A CO_2 light trap consists of (listed from the top to the bottom of the assembled trap): a cylindrical insulated cooler to hold dry ice, a plastic rain cover attached to a light/fan assembly (battery-powered), and a mesh collection cup. The light part of the light/fan assembly will be disabled. During deployment, dry ice in the insulated cooler releases CO_2 as it sublimates, and this gas attracts mosquitoes to the vicinity of the trap. The battery-powered fan sucks these mosquitoes into the mesh collection cup, where they remain alive until the trap is collected.

Following minimal in- house processing, samples will be sent to one or more external facilities where mosquitoes will be identified to species to characterize patterns of mosquito abundance, diversity, and phenology at NEON sites. A subset of identified mosquitoes will be tested for infection by pathogens to quantify the presence/absence and prevalence of various arboviruses. Some mosquitoes will be set aside for DNA barcode analysis as well as for long-term archiving.

5 QUALITY ASSURANCE AND CONTROL

The procedures associated with this protocol will be audited according to the Field Audit Plan (RD[04]). Additional quality assurance will be performed on data collected via these procedures according to the Data and Data Product Quality Assurance and Control Plan (RD[05]).

Because of the wide range and variance of mosquito abundance and pathogen prevalence, algorithms that check mosquito data for irregularities may catch some errors but will not be a dependable way to fully quality control mosquito data from the field. Following the protocols exactly at each domain will be required to ensure data quality and this can only be known by conducting “hot checks” where someone who knows the protocols well goes out into the field with technicians and observes their data collection. For work done by external laboratories, QA/QC plans will be developed based on pre-existing laboratory protocols modified as needed to meet NEON requirements.

When unexpected field conditions require deviations from the field protocols outlined in this document, contingent decisions outlined in Table 1 below should be followed in the interest of maintaining data quality.

Table 1. Contingent decisions for field season sampling

Sampling Delay	Required Action	Adverse Outcome for Data Quality?	Outcome for Data Products
<3 hours	Resume/continue with normal sampling at conclusion of delay. (Note the duration and cause of the delay)	None, but note that excessively delayed retrieval of mosquitoes from traps increases the likelihood of mosquito mortality, especially under hot/dry and wet conditions. Dead mosquitoes are more difficult to identify and test for pathogens.	Quality of samples reduced, creating potential for complications with processing (identification, pathogen testing).
3 hours to 1 day	Scenario 1: If the delay occurs prior to trap deployment and prior to the start of the sampling bout then push the start date for the bout back one day. Scenario 2: If the delay occurs after the initial deployment of traps during a bout but prior to the collection/resetting of traps, then repeat any missed trapping on the subsequent day. In both cases, a) do not adjust (push back) dates for subsequent sampling bouts, and b) note the duration and cause of the delay	Consistent temporal interval of time series data interrupted.	Compromise statistical analysis of temporal trends in the data
1-7 days (core), 1-14 days (relocatable)	Scenario 1: If the delay occurs prior to trap deployment and prior to the start of the sampling bout then push the start date for the bout back for the duration of the delay. Scenario 2: If the delay occurs after the initial deployment of traps during a bout but prior to the collection/resetting of traps, then repeat the entire sampling bout at the conclusion of the delay. In both cases, a) do not adjust (push back) dates for subsequent sampling bouts, and b) note the duration and cause of the delay	Consistent temporal interval of time series data interrupted (moderately).	Compromise statistical analysis of temporal trends in the data
>7 days (core), >14 days (relocatable)	Cancel the impacted sampling bout and stop sampling until next scheduled sampling bout. Contact associated TOS staff scientists. Note duration and cause of the delay.	Consistent temporal interval of time series data interrupted (maximally)	Reduction in sample size as sampling bouts are missed

6 SAFETY

Personnel working at a NEON site must be compliant with safe field work practices as outlined in the Operations Field Safety and Security Plan (AD[02]) and EHS Safety Policy and Program Manual (AD[01]). Additional safety issues associated with this field procedure are outlined below. The Field Operations Manager and the Lead Field Technician have primary authority to stop work activities based on unsafe field conditions; however, all employees have the responsibility and right to stop their work in unsafe conditions.

Follow guidelines provided in (AD[02]) to prevent mosquito bites. If used, insect repellent must be applied at least 30 minutes prior to arriving in the field. If using insect repellent in spray form DO NOT apply in the vicinity of sampling equipment. After applying insect repellent, clean the palms of hands (e.g., with soap/water or alcohol swabs) before handling any sampling equipment.

Field personnel are collecting biting insects but there is no increased risk of infection by zoonotic pathogens during implementation of this protocol than in general field work.

7 PERSONNEL REQUIREMENTS

Prior experience collecting mosquitoes or working with related insects (i.e., entomological fieldwork) is desirable but not required. Personnel should have good fine manual coordination for handling individual specimens.

8 TRAINING REQUIREMENTS

All technicians must complete required safety training as defined in the NEON Training Plan (RD[04]). Additionally technicians complete protocol specific training for safety and implementation of protocol as required in Field Operations Job Instruction Training Plan (RD[05]).

9 SAMPLE FREQUENCY AND TIMING

9.1 Sampling Frequency and Timing

There are two distinct types of sampling associated with mosquito trapping. Mosquito sampling will consist of a combination of field season and off season sampling

1. **Field season sampling:**

Field season sampling bouts will involve ~40 continuous hours of sampling using one CDC CO_2 light trap at each plot (Fig. 1, described below). Plots will be randomly located in each of the major vegetation types ($\geq$5% of total cover), with the number of plots per vegetation type proportional to the percent cover of that type at the site. Plots will be located within 30m of a road to facilitate expedient sampling. The specific timing of these activities depends on local patterns of seasonal phenology.

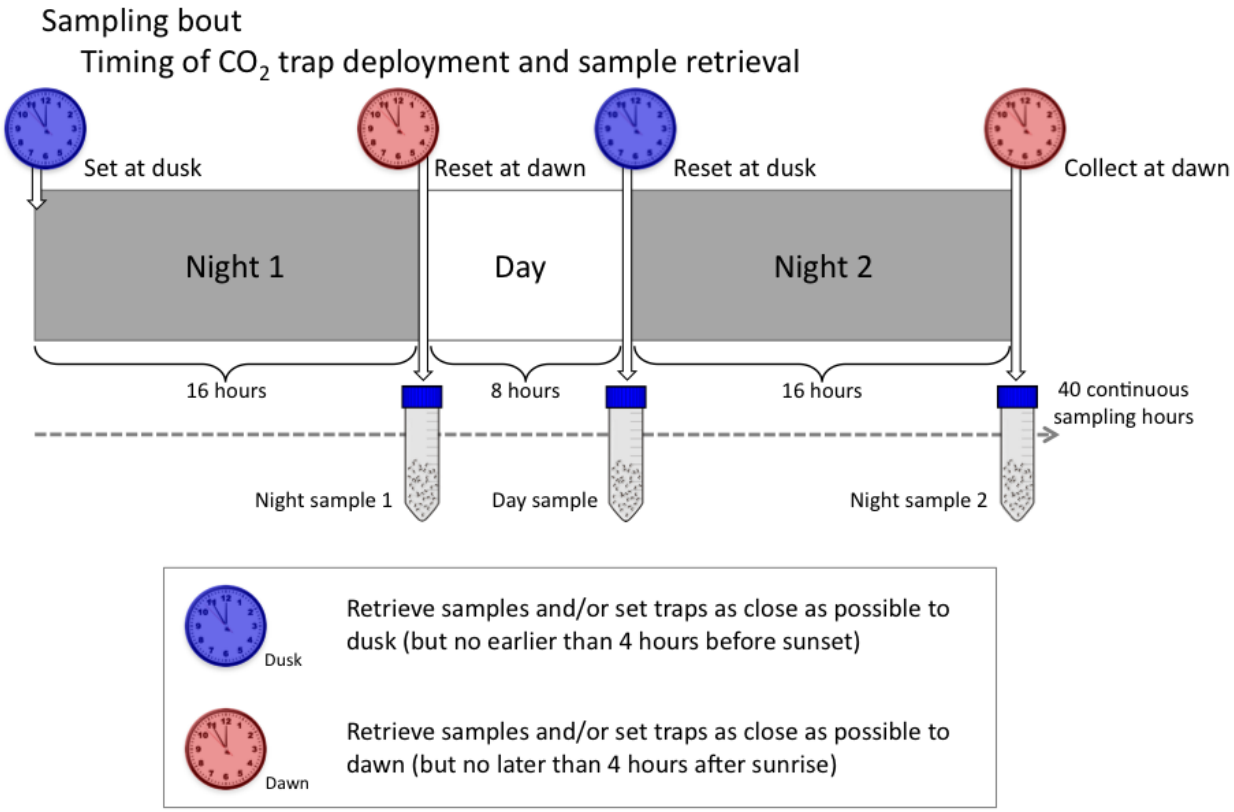


Figure 1. Timing of a mosquito sampling bout that generates three samples (designated by vials), two trap-nights and the intervening day. (Daytime and night-time durations shown are for illustrative purposes only.)

During a bout of field season sampling, traps will be deployed and initially set as close as possible to dusk (but no earlier than 4 hours before sunset) on the first day of the bout (Fig. 1). Traps will be checked (full catch cups retrieved and replaced with new/empty catch cups) and reset (coolers refilled with dry ice) as close as possible to dawn (but no later than 4 hours after sunrise) on the second day of the bout. Traps will be checked and reset again as close as possible to dusk (but no earlier than 4 hours before sunset) on the second day of the bout. Traps will be checked and retrieved as close as possible to dawn (but no later than 4 hours after sunrise) on the third day of the bout.

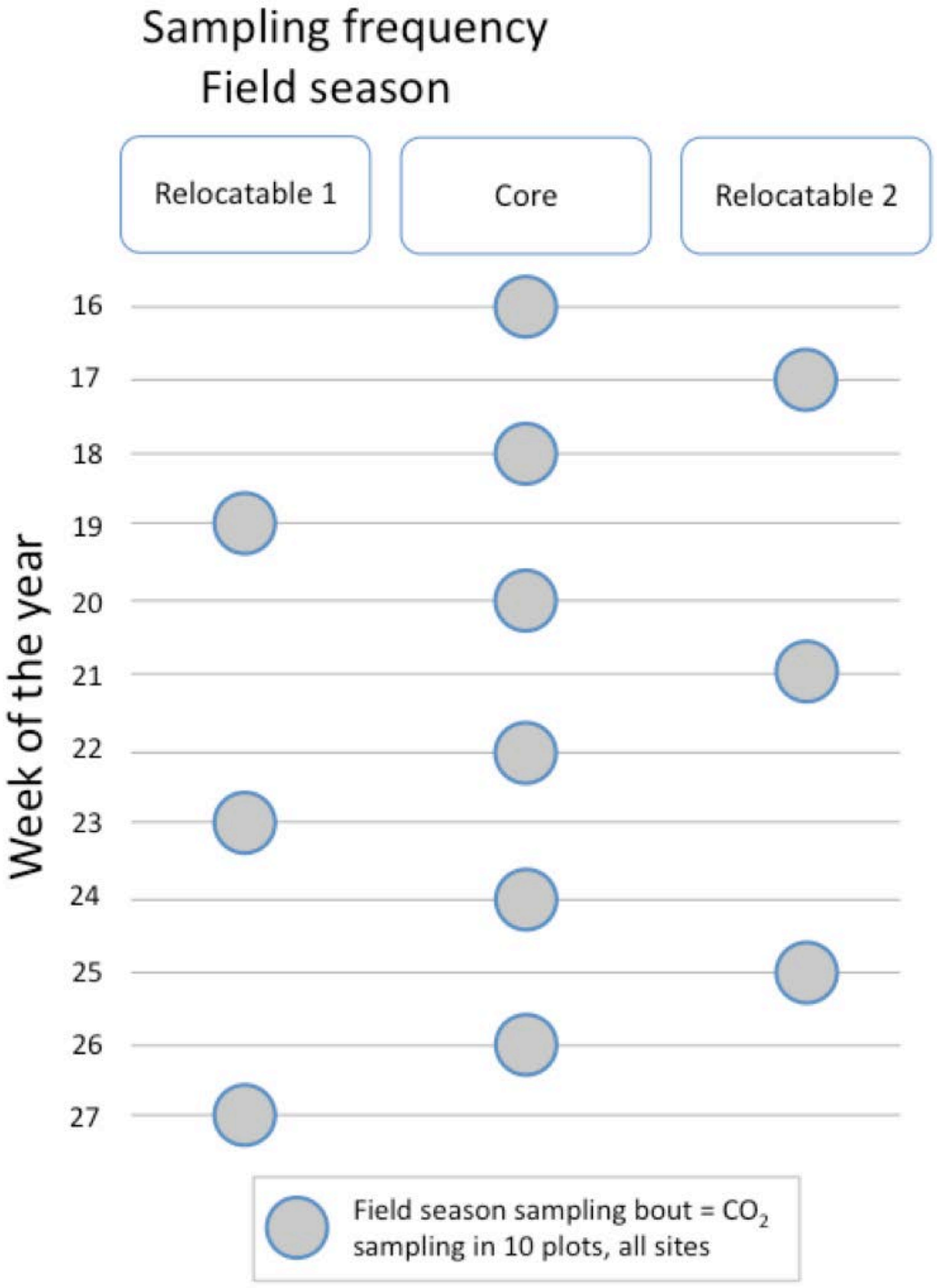


Figure 2: Frequency of sampling bouts at a domain; weekly sampling alternating between core and relocatable sites.

2. **Off season sampling:** Off season sampling is conducted exclusively at core sites for the purpose of determining the start of field season sampling each year. Information from this sampling will contribute to the monitoring of changes from year to year in mosquito phenology (the timing of life cycle events).

   During a bout of off season sampling, traps will be deployed as close as possible to dusk (but no earlier than 4 hours before sunset) on the first day of the bout and retrieved as close as possible to dawn (but no later than 4 hours after sunrise) on the following day. In contrast to field season sampling, a bout of off season sampling involves only a single night of trapping.

9.2 Sampling Frequency

Within a domain, bouts of field season sampling will occur every two weeks at the core site and every four weeks at each relocatable site, with sampling alternating between relocatable sites such that sampling is conducted at one site every week at the domain scale (Fig. 2). Off season sampling will be conducted weekly but a given bout will only occur if the mean daily high temperature for the previous 5 days was above 4°C.

9.3 Sampling Timing Parameters

Sampling will be conducted until specified collection thresholds that designate the end of field season sampling and beginning of off season sampling are met (Fig. 3, 4). A bout of off season sampling is conducted in the week following the first zero-catch bout of field season sampling at the core site (Fig. 3). The following week a bout of field season sampling is conducted as scheduled. If all three of these are zero-catch bouts then field season sampling stops and off season sampling continues (only at the core site). In contrast, the collection of mosquitoes results in the continuation of field season sampling until three consecutive zero-catch bouts have occurred (Fig. 3).

Off season sampling will only occur if the mean daily high temperature at the core site (or nearest location for reliable temperature data) for the previous 5 days was above 4°C. Off season sampling will continue until the first mosquito is collected during an off season bout. This will mark the resumption of field season sampling at all sites in the associated domain (Figs. 3 and 4). At that time, if resources are not sufficient to initiate formal sampling at all sites within the domain (e.g., spring arrived very early and seasonal technicians are not yet available) sampling will be prioritized at the core site.

Table 2. Estimated Dates of Historical Temperature Thresholds

	Average 5-day Temperature above 10°C		**Average 5-day Temperature above 4°C**	
Site	**Start**	**End**	**Start**	**End**
Harvard Forest	March 28	November 20	February 22	December 17
Bartlett Experimental Forest	April 14	November 2	March 18	December 4
Smithsonian Conservation Biology Institute	February 23	December 5	Year Round	
Ordway-Swisher Biological Station	Year Round			
Disney Wilderness Preserve	Year Round			
Jones Environmental Research Center	Year Round			
UNDERC	April 14	October 26	March 19	November 13
Oak Ridge National Lab	February 12	December 21	Year Round	
Talladega National Forest	Year Round			
Woodworth Station	April 9	October 30	March 21	November 12
Central Plains Experimental Range	February 27	November 22	January 9	December 19
Sterling, CO	February 27	November 22	January 9	December 20
Onaqui	March 8	November 15	January 21	December 18

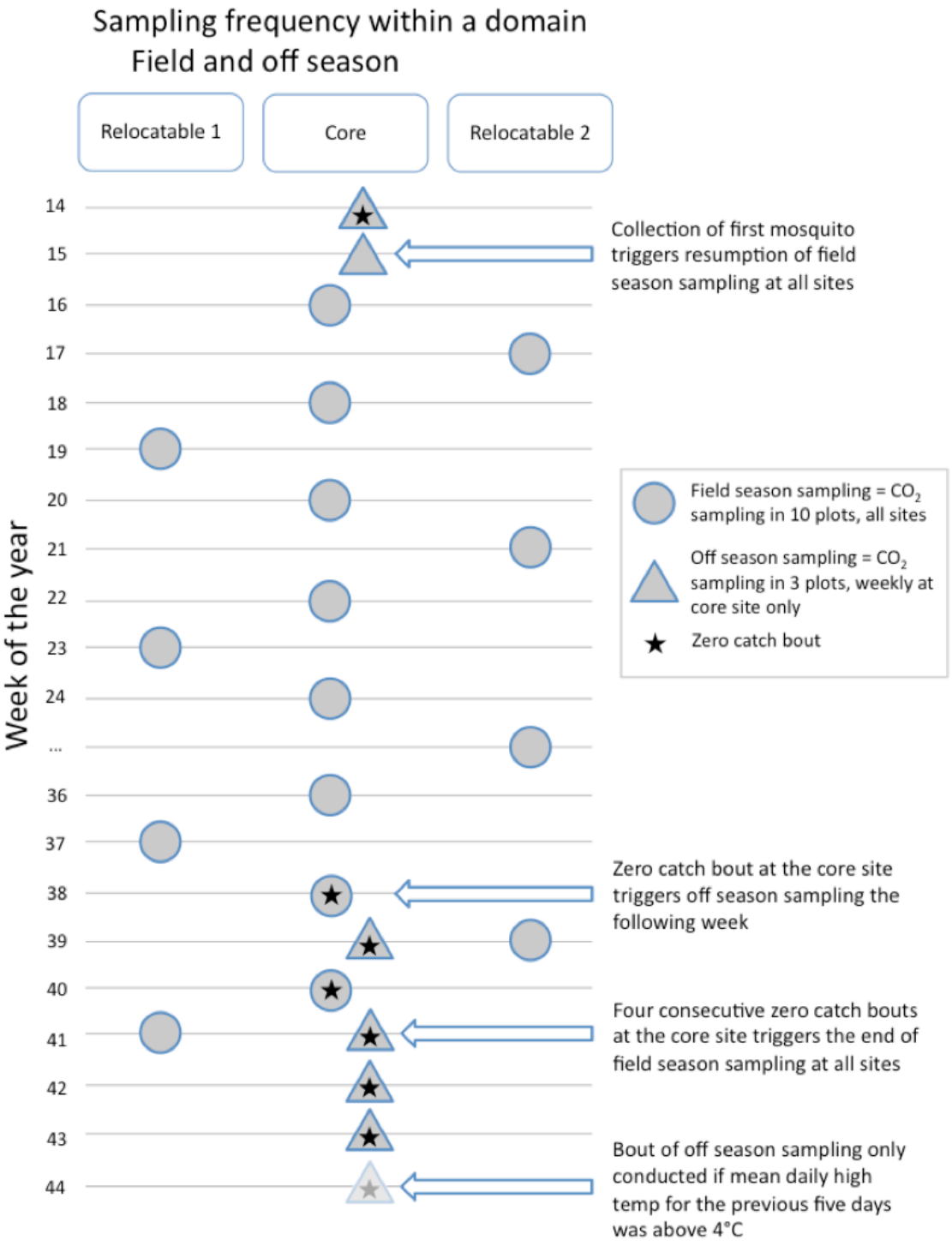


Figure 3. An example domain sampling schedule highlighting the transitions between field season and off season mosquito sampling.

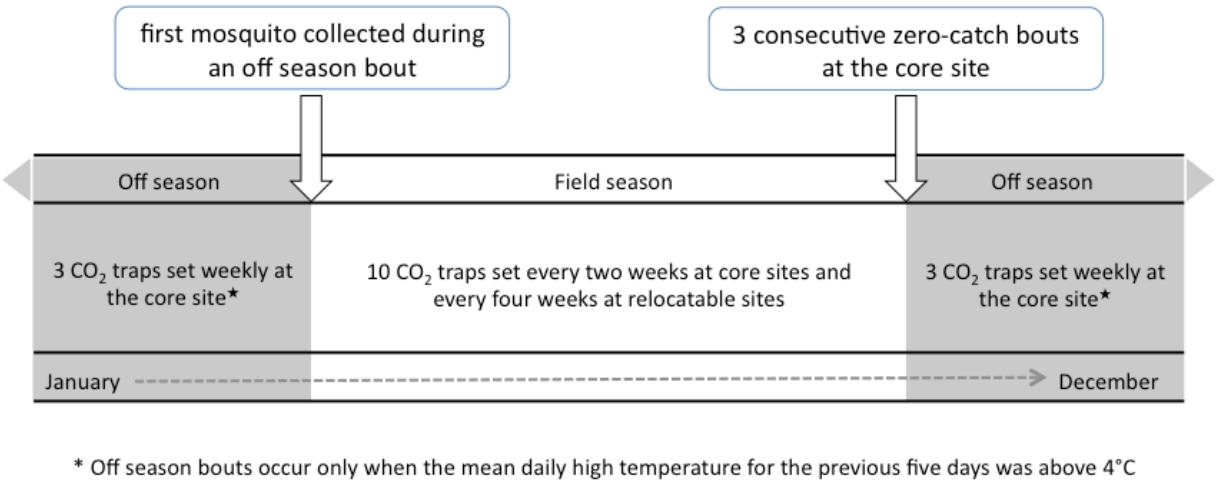


Figure 4. Annual summary of mosquito sampling intensity including transitions from off season to field season and back to off season sampling.

10 STANDARD OPERATING PROCEDURES

SOP A: Preparing for Sampling

Prior to a sampling bout:

1. For each CO_2 light trap, remove the rain cover and make sure that in the red circuit assembly, the first switch is in the closed position and the second and third switches are in the open position (Fig. 5). On this setting the trap fan remains on at all times.

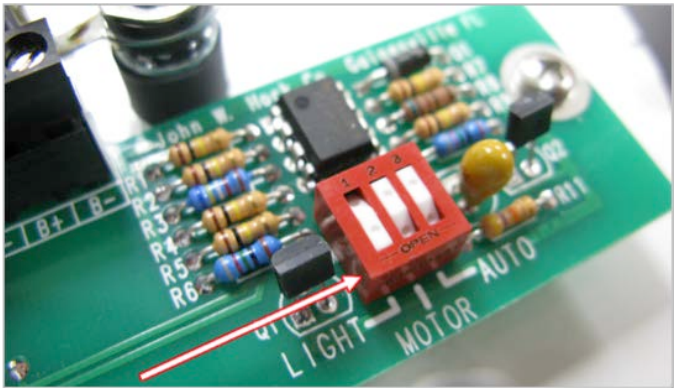


Figure 5. Switches inside circuit assembly of a CDC mosquito trap.

2. Remove the light bulb from each trap before the first use. Cover the hole with tape (masking tape recommended) and recycle the light bulb. Traps are deployed without lights.
3. Test all trap components for proper functionality. This includes making sure that electronics are working (e.g., fan turns on when connected to a battery and spin in the proper direction) and mesh of collection cup sleeves is not torn. If the metal clip used to connect the rain cover to the insulated cooler is difficult to open, consider adding a small carabineer.
4. Make sure batteries are charged or charging (see SOP B: Field Sampling for battery charging instructions).
5. If they will be used, make sure that reusable ice packs are frozen or being frozen.
6. Identify the locations of plots used for mosquito sampling (use GPS and/or maps).
7. Print field datasheets and sample locality labels. Label preparation is described in the Lab Protocol for Beetles and Mosquitoes (AD[08]).

Just prior to heading to the field for sampling:

1. Obtain enough dry ice to be able to fill the cylindrical insulated cooler of each trap (e.g., ~1.5kg of ice in pellet form) and transport any samples from the field to the lab in larger insulated coolers. To ensure that enough dry ice will be available in spite of sublimation that will occur between ice pickup and trap deployment consider the duration of this period and the manner (e.g., temperature) in which dry ice is stored and transported to the field. If trap coolers are filled individually in the lab

and transported to the field with dry ice already in them, cover the vent hole on the bottom of each cooler with tape.

2. If used, insect repellant must be applied at least 30 minutes prior to arriving in the field. If using insect repellant in spray form do not apply in the vicinity of sampling equipment. After applying insect repellant clean the palms of hands (e.g., with soap/water or alcohol swabs) before handling any sampling equipment.

Field Equipment and Materials

Table 3. Equipment list, Preparing for sampling

Maximo Item No.	Item Description	Purpose	Quantity	Habitat-Specific	Special Handling
optional	GPS receiver, recreational accuracy	Locating plot	1	No	No
MX103541	CO_2 light trap, battery-powered fan assembly	Mosquito trapping	10	No	No
MX100695	CO_2 light trap, cylindrical insulated cooler	Mosquito trapping	10	No	No
MX100694	CO_2 light trap, mesh sleeve and collection cup	Mosquito trapping	12	No	No
Required	CO_2 light trap, rain cover	Mosquito trapping	10	No	No
Required	CO_2 light trap, red rain cover screws	Mosquito trapping	30	No	No
Required	Dry ice (pellet form recommended)	Mosquito bait	1.5kg per trap for 12-18 hours of sampling	No	Yes
Required	Cryogenic gloves (to handle dry ice)	Safety		No	No
Required	Tape (masking recommended)	Cover trap holes		No	No
Required if necessary	Shepherd's hook	Hanging traps	Up to 10	Yes	No
Optional	Ice packs, -20 C	Keep samples cool		No	No
Required	Rope (need less if using shepherd's hooks)	Hanging traps		No	No
Required	Scissors	Cutting		No	No
Required	Aluminum foil (optional)	Cattle chewing deterrent		No	No
Required	6V Battery (for CO_2 light trap)	Powering mosquito traps		No	Yes
Required	Paper towels	Absorb excess moisture		No	No

Required	Plastic bag, 1 gal, resealable	Storage		No	No
Required	Utility knife	Cutting labels		No	No
Required	Safety pins	Attaching labels		No	No
Required	Cooler(s)	Holding chilled/frozen samples		No	No
Required	Cardboard cards or cloth	Insulating samples		No	No
Required	Copy paper, Rite in the Rain(for datasheets and labels)	Printing datasheets		No	No
Required	Pencils	Recording data		No	No
Required	Label tape (optional, must be able to handle -80 C)	Labeling sample vials		No	No
Required	Permanent marker, archival, black	labeling		No	No
Required	Battery chargers	Charging 6V batteries	10 (or 5 with dual capacity)	No	No
Required	Plastic bins	battery secondary containment		No	No
Required	Laundry detergent, fragrance free	General cleanup		No	No

SOP B: Field Sampling

Setting traps

1. Travel to the sampling plot using maps and/or a handheld GPS as necessary.
2. Hang a single trap at each mosquito trapping plot.
3. Hang the trap from a natural structure (e.g., a tree branch) or installed post (e.g., a Shepherd's hook) such that the height of the hole in the bottom of the insulated cooler is between 1.2 and 1.8 meters (4-6 feet) above the ground. On more exposed sites, if possible deployment locations should be adjacent to and on the west side of elevated vegetation to allow shading from the morning sun and on the leeward side to afford protection from prevailing winds as these locations provide protection from the elements. Hang the trap within 10 meters of the mosquito sampling point assigned during plot establishment (AD[10]). At each sampled plot, hang the trap in the same location throughout all bouts of a season. If resampling the same plot over multiple years, continue to use the same location. In many cases the number of suitable locations for hanging traps will be limited but if necessary, make note of the location for use in subsequent seasons.
4. Hang the trap's insulated dry ice container from the elevated external structure and use the clip on the underside of the cooler (or optional carabineer) to attach the rain guard/fan assembly. Secure the mesh collection cup to the lower end of the fan assembly using the elastic band sewn into the mesh.
5. Connect the fan to the power source by color-matching the wire leads and the battery terminals (red to '+' terminal, black to '-' terminal). The fan should immediately come on.
6. If possible, tie any loose battery cord around the natural feature or Shepherd's hook from which the trap is suspended. Place the battery in a re-sealable plastic bag to keep it dry. At sites with cattle present, wrap the battery cord with aluminum foil to provide protection from chewing livestock.
7. Remove the tape covering the hole in the underside of the insulated cooler containing dry ice. **Easy to forget but critical step!
8. Attach three locality labels to the catch cup, using safety pins to secure the labels to the nylon cuff of the mesh sleeve and not through the mesh itself. Write a "1", "2" or "3" on the back of the locality labels to identify the sample as being from the 1st collection (first night), 2nd collection (day) or 3rd collection (second night). This will ensure that each catch cup can be assigned to a single sample line on the datasheet.
9. Record appropriate information about the visit to the sampling plot on the field datasheet, including the sampleID.

Retrieving samples from traps

1. Travel to the sampling plot using maps and/or a handheld GPS as necessary.
2. Note that ice may have formed on or around the vent hole of the trap's insulated cooler. During deployment, condensation drips down the sides of the cooler and may freeze around the vent hole depending on ambient conditions. This is normal and should only be considered a problem if the insulated cooler is still more than half full of dry ice at the conclusion of a deployment.
3. With the fan still running, gently tap flying mosquitoes down towards the bottom of the sleeve and into the cup. Tie the laces on the catch cup mesh sleeve to seal the opening. Be careful not to crush any mosquitoes while tapping them down and tying the laces. Keeping the fan running during this process ensures that the mosquitoes cannot escape from the collection cup during this process. This step may be best completed as a two-person operation.
4. Remove the collection cup by sliding the mesh sleeve off of the fan assembly. Keeping the fan running.
5. If possible gently stuff/tuck sleeve material into the hole in the top of the catch cup but only to the extent that this does not crush mosquitoes.
6. Ensure a triplicate locality label is still attached to the collection cup. Write the collection date on the locality label in the space left for this purpose using archival ethanol-safe ink.
7. Redeploy trap as necessary. Remember to attach new locality labels.
8. Transport catch cup containing sample back to field vehicle
9. Prior to placing the catch cup into an insulated cooler for transport back to the lab, use paper towels (recommended) to remove any water that has accumulated in the catch cup. Do this by swabbing the mesh-covered hole in the bottom of the catch cup. Do not untie the laces.
10. Place catch cup into insulated cooler for transport back to the domain lab. The cooler should ideally contain dry ice but may contain frozen reusable ice packs if logistics (e.g., duration of field visit, local availability of dry ice) preclude the use of dry ice.
11. Place a cardboard card or cloth between the catch cups and the ice so that they do not come into direct contact. Moisture on the outside of the catch cup or mesh bottom can freeze to the ice and cause cups to stick, potentially damaging equipment or samples. Once frozen, samples must remain frozen at all times.
12. Record appropriate sampling information on the field datasheet including the label ID of each catch cup.
13. If using a Shepard's hook, after initial deployment leave the hook at the plot for the duration of the sampling season.

Sample preservation

1. Upon returning to the lab, immediately transfer samples into an ultra-low freezer. Keep samples from different collection events separate (e.g., 1st collection samples should be in a different labeled bag than 2nd collection samples to aid in differentiating them during the transfer to vials in the lab).
2. Once frozen, samples must remain frozen at all times.

Refreshing the sampling kit

1. Test traps to verify that they are still fully functional.
2. If used, refreeze reusable ice packs.
3. Obtain fresh consumable items.
4. Print new datasheets as needed.

Equipment maintenance, cleaning, and storage

1. If mesh sleeves/collection cups are wet or dirty following trapping, gently wash them by hand using fragrance-free laundry detergent and hang/stack to dry. Make sure all trap components are clean and free of insect parts.
2. If the mesh sleeve is damaged or torn it should be replaced as captured mosquitoes may be able to escape through holes in the mesh.
3. Clean any other equipment as necessary using fragrance-free laundry detergent.
4. Make sure all equipment is dry before placing it in storage.
5. Charge mosquito trap batteries
    a. The batteries used to power the CDC CO_2 light traps are a 6V sealed gel electrolyte type. They pose little risk, but proper handling procedures should be followed. Use plastic covers or tape to cover terminals when not in use. Charging should be performed in a designated area with batteries placed in plastic bins for secondary containment. Particular care should be exercised when charging depleted batteries as chargers can become hot and potentially cause burns. The green light indicates charging is complete and batteries on chargers should not be touched until after the green light comes on. Warning signs should be placed around the batteries while charging.
    b. To charge a battery, first connect the color-coded leads to the battery. Next, plug the charger into the AC outlet. If the battery is mistakenly connected backwards (negative to positive) or the charger leads are shorted together, a red light on the charger will be illuminated to indicate a possible mistake. When the charger is first plugged in it sends intermittent current pulses into the battery and monitors the battery's response to determine the charge state. A red light may come on briefly if the battery is excessively discharged, but it should go off within the first few minutes of charging.
    c. Once the charger determines that the battery can safely take a charge, it goes into the bulk charging mode. During bulk charging, indicated by a yellow light on the charger, the full capacity of the charger is applied until the battery reaches 80% of its capacity.
    d. Once the battery has reached 80% capacity charging enters absorption mode: the yellow light on the charger remains on but the charge output is reduced from 100% to 33% of capacity until the battery reaches full charge.
    e. Once full charge has been reached a green light on the charger becomes illuminated to indicate that the battery is now in its ideal charge state. As long as the batteries remain connected, the charger will maintain them in this state. This means that batteries may be left connected to the charger overnight, over the weekend, or indefinitely, and when they are picked up, they are in the ideal state.

Field Equipment and Materials

Table 4. Equipment list, Field sampling

Maximo Item No.	Item Description	Purpose	Quantity	Habitat-Specific	Special Handling
Optional	Handheld GPS	Locating plots		No	No
MX103541	CO_2 light trap, battery-powered fan assembly	Mosquito trapping	10	No	No
MX100695	CO_2 light trap, cylindrical insulated cooler	Mosquito trapping	10	No	No
MX100694	CO_2 light trap, mesh sleeve and collection cup	Mosquito trapping	12	No	No
Required	CO_2 light trap, rain cover	Mosquito trapping	10	No	No
Required	CO_2 light trap, red rain cover screws	Mosquito trapping	30	No	No
Required	Dry ice (pellet form recommended)	Mosquito bait	1.5kg per trap for 12-18 hours of sampling	No	Yes
Required	Cooler (i.e. Yeti)	dry ice storage and transport		No	No
Required	Cryogenic gloves (to handle dry ice)	Safety		No	No
Required	Masking tape	Cover trap holes		No	No
Required	Shepherd's hook	Hanging traps	Up to 10	Yes	No
Optional	Ice packs, -20 C	Keep samples cool		No	No
Required	Rope	Hanging traps		No	No
Required	Scissors	Cutting		No	No
Required	Aluminum foil	Cattle chewing deterrent		No	No
Required	6V Battery (for CO_2 light trap)	Powering mosquito traps		No	No
Required	Paper towels	Absorb excess moisture		No	No
Required	Resealable plastic bags (gallon size recommended)	Storage		No	No
Required	Razor-blade knife	Cutting labels		No	No
Required	Safety pins	Attaching labels		No	No

Maximo Item No.	Item Description	Purpose	Quantity	Habitat-Specific	Special Handling
Required	Cooler(s), 16 aq	Holding chilled/frozen samples		No	No
Required	Cardboard cards or cloth	Insulating samples		No	No
Required	Printing paper (for datasheets and labels)	Printing datasheets		No	No
Required	Pencils	Recording data		No	No
Optional	Adhesive-backed, paper	for labeling of sample vials		No	No
Required	Pens with archival, ethanol-safe ink	Writing on labels		No	No
Required	Battery charger	Charging 6V batteries		No	No
Required	Plastic bins	battery secondary containment		No	No
Required	Fragrance-free laundry detergent	General cleanup		No	No

SOP C: Laboratory Processing and Analyses

Sample processing timing

1. Process samples as soon as possible after returning from the field, ideally within one week of sample collection.

Transferring mosquitoes from catch cups to sample vials

1. Prior to processing samples clear and clean off bench space.
2. Obtain enough sample vials to hold samples from each catch cup. In the case of large volume samples, you may need multiple vials for each catch cup. Approximate the number of vials based on typical catch cup volumes at your site. Preparing extra vials is recommended in the event that cup contents are higher than expected.
3. Prepare enough locality labels to be able to insert one into each sample vial. As with estimating the number of vials, we recommend printing extra labels.
4. Externally label each sample vial. It is important to do this before chilling the vial (described below) as chilling will cause condensation on the external surfaces of the vial that will make it difficult to apply a label. External labels can be pre-printed on adhesive-backed paper or written directly onto sample vials using a permanent, ethanol-safe marker. Label the body rather than the lid of the vial.
5. The external label format (vialID) consists of the sampleID and a vial number (the number of the vial relative to the total number of vials containing mosquitoes from a single catch cup, 1 or higher, depending on the number of vials associated with each catch cup) (Fig. 6). The sampleID includes a plotID, the collectTime (YYYYMMDDHHMM, a combination of the collection date and time into a single numeric string) and the letter “D” or “N” (indicating whether the sample represents day or night sampling). As an example, “OSBS_002.201308021608.D2” would indicate that the labeled vial is the second vial containing mosquitoes caught during daytime sampling in plot 002 at Ordway Swisher Biological Station and collected on August 2, 2013 at 4:08PM.

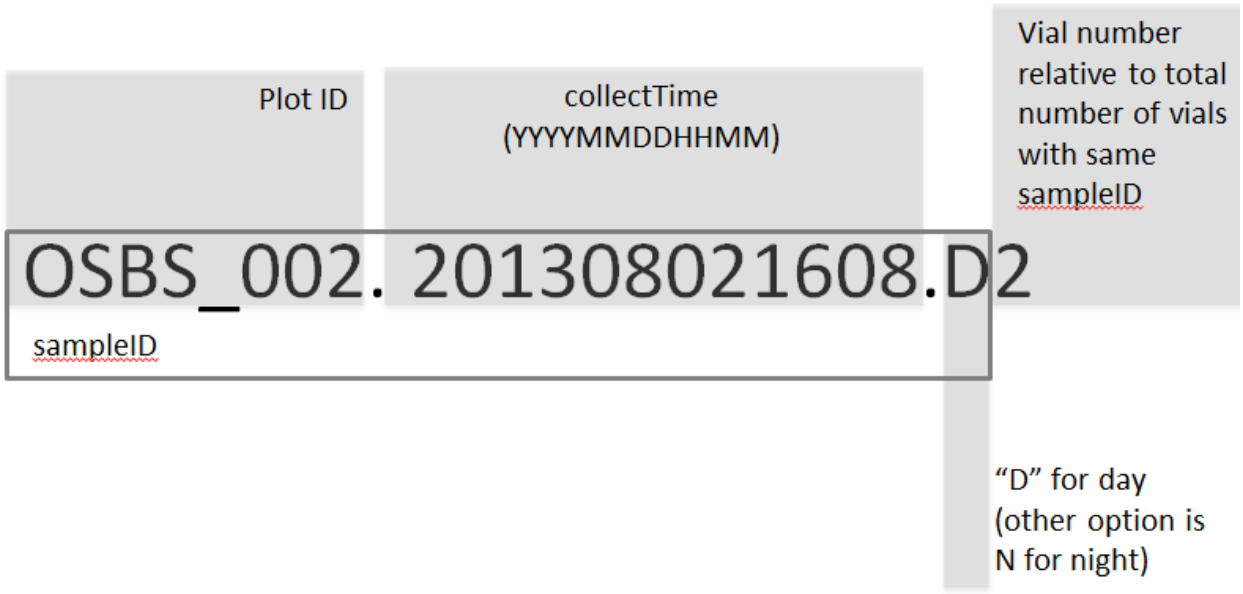


Figure 6. Annotated vialID example

6. Place the empty, labeled sample vial(s) into a freezer for 5 minutes so the walls of the vial(s) become frozen. This will prevent thawing frozen mosquitoes by transferring heat from warm vials.
7. Gather and/or prepare any equipment necessary for transferring mosquitoes from catch cups into sample vials (Table 4).
8. Set up a chilling station to keep sample vials cold following removal from freezer and during transfer. A simple version of such a station involves a small cardboard box that has holes cut into the top into which chilled sample vials can be inserted. During sample transfer, the box is filled with ice or dry ice to keep the sample vials cold.
9. Remove a catch cup and the corresponding labeled empty sample vials from the freezer. Place empty vials into chilling station.
10. Quickly transfer mosquitoes from the catch cup into the empty sample vial(s) to ensure that samples remain frozen.
    a. Insert a funnel into a frozen sample vial
    b. Unscrew the lid of the catch cup and remove mesh. Be sure that no mosquitoes are trapped in the mesh.
    c. Flip catch cup over and tap sharply onto a piece of paper on the lab bench. This should dislodge most mosquitoes.
    d. Use the paper to guide mosquitoes down into the funnel.
    e. Remove any obvious bycatch during transfer (e.g., moths that are difficult to pass through the funnel) but prioritize keeping mosquitoes frozen. If bycatch is frozen to mosquitoes, do not attempt to disentangle.

f. Use forceps to transfer any mosquitoes that may remain in the catch cup or associated mesh into the sample vial.
g. Do not overfill the sample vial. Leave space to accommodate a small piece of tissue paper and a locality label. Use additional vials as necessary until all mosquitoes from a catch cup have been transferred into sample vials.

11. Once all of the mosquitoes from the collection cup have been transferred into the frozen sample vial(s), place part or all of a piece of tissue paper into the top and bottom of each vial. This will prevent samples from shifting and being damaged during subsequent handling and shipping.
12. Place a locality label into each vial. Be sure to fill out any blank fields on the label with an ethanol-safe pen, and check that the label information matches sampling details for the processed catch cup (e.g., date and location of collection). If using small vials, consider slipping locality label along the edge of the vial to avoid crushing mosquitoes and if necessary, insert locality label before mosquitoes.
13. Seal each vial and immediately place it into an ultralow freezer. When storing samples, take steps to keep samples of similar origin together. For example, multiple vials from a single site/bout/trap combination may be bound together with a rubber band or small bag, and all vials from a sampling site/bout combination can be stored together in a re-sealable plastic bag or vial rack. Vials from different sampling bouts within a site, and from different sites, should not be mixed. This organization will reduce the probability of samples thawing when they are inventoried and sorted at external processing facilities.
14. For each sample vial, enter relevant information into a computer using the mosquito trapping spreadsheet in the NEON Raw Data Ingest Workbook for TOS Mosquito Abundance, Diversity and Phenology (AD [12]).

Sample preservation

1. After each sample is processed, transfer the storage vial into an ultralow freezer.

Equipment Maintenance, Cleaning and Storage

1. Clean off the surface of the lab bench where processing activities were performed.
2. Clean any non-disposable equipment used during processing (e.g., funnel) with ethanol.
3. Put away all supplies in their designated storage locations.
4. All materials should be put away in clearly marked receptacles or cabinets after each bout of laboratory work.

Laboratory Equipment and Materials

Table 5. Equipment list, Laboratory processing and analyses

Maximo Item No.	Item Description	Purpose	Quantity	Habitat-Specific	Special Handling
Required	Copy paper, Rite in the Rain	for datasheets and labels	2	No	No
Required	Permanent marker, archival, black	Writing labels	2	No	No
Required	Centrifuge tubes (15 or 50 mL volume recommended)	Storing mosquitoes	25	No	No
optional	Centrifuge tube rack (recommended)	Holding tubes	1	No	No
Required	Cryovial freezer storage box	Holding tubes in freezer		No	Yes
Required	Jewelers forceps, straight	Manipulating specimens	3	No	No
Required	Petri dish (for separating bycatch from mosquitoes)	Temporary storage			
Required	Tissues paper	Protecting mosquitoes in tubes	25	No	No
optional	Plastic funnel (use paper funnel if preferred)	Transferring mosquitoes to tubes	1	No	No
Required	Laundry detergent, fragrance free	General cleanup	1	No	No

SOP D: Sample Shipment

1. Samples will be shipped overnight on dry ice.
2. Other shipping details (e.g., acceptable days of the week for shipping, ship to address, manner of shipping etc.) will be specified by the taxonomic ID facility and communicated to FOPS by CLA.
3. Whenever a batch of samples is shipped, the batch must be accompanied by a hard-copy shipping manifest enclosed within the shipping container AND a corresponding electronic version of the manifest (Excel file) emailed to the testing facility.
4. The hard-copy shipping manifest lists every sample vial in the shipped batch. Include the following fields from the mosquito_trapping spreadsheet in the NEON Raw Data Ingest Workbook for TOS Mosquito Abundance, Diversity and Phenology (AD [12]): vialID, boutNumber, senderID, dateSent and receiverID. An example of a populated hard-copy manifest is provided in Figure 7.

vialID	boutNumber	senderID	dateSent	receiverID
OSBS_001.201307121608.D1	1	R. Nelson, D03	20131101	D. Weissmann, VDCI
OSBS_001.201307120814.N1	1	R. Nelson, D03	20131101	D. Weissmann, VDCI
OSBS_001.201307120814.N2	1	R. Nelson, D03	20131101	D. Weissmann, VDCI
OSBS_001.201307130932.N1	1	R. Nelson, D03	20131101	D. Weissmann, VDCI
OSBS_002.201307121012.N1	1	R. Nelson, D03	20131101	D. Weissmann, VDCI
OSBS_002.201307130822.N1	1	R. Nelson, D03	20131101	D. Weissmann, VDCI
OSBS_003.201307120858.N1	1	R. Nelson, D03	20131101	D. Weissmann, VDCI
OSBS_003.201307120858.N2	1	R. Nelson, D03	20131101	D. Weissmann, VDCI
OSBS_003.201307121733.D1	1	R. Nelson, D03	20131101	D. Weissmann, VDCI
OSBS_003.201307130808.N1	1	R. Nelson, D03	20131101	D. Weissmann, VDCI
OSBS_003.201307130808.N2	1	R. Nelson, D03	20131101	D. Weissmann, VDCI
OSBS_003.201307120712.N1	1	R. Nelson, D03	20131101	D. Weissmann, VDCI
OSBS_004.201307130923.N1	1	R. Nelson, D03	20131101	D. Weissmann, VDCI
DSNY_004.201307220947.N1	1	R. Nelson, D03	20131101	D. Weissmann, VDCI
DSNY_010.201307221655.D1	1	R. Nelson, D03	20131101	D. Weissmann, VDCI
DSNY_010.201307230805.N1	1	R. Nelson, D03	20131101	D. Weissmann, VDCI
DSNY_010.201307220904.N1	1	R. Nelson, D03	20131101	D. Weissmann, VDCI
DSNY_011.201307220904.N2	1	R. Nelson, D03	20131101	D. Weissmann, VDCI
DSNY_011.201307231732.D1	1	R. Nelson, D03	20131101	D. Weissmann, VDCI
DSNY_012.201307230949.N1	1	R. Nelson, D03	20131101	D. Weissmann, VDCI

Figure 7. Example of a hard-copy shipping manifest

5. The electronic manifest is an excel file that should be emailed to the taxonomic ID facility as soon as possible after a batch of samples has been shipped. It is an electronic version of the corresponding hard-copy manifest that additionally contains all of the remaining data columns in the full mosquito trapping spreadsheet in the NEON Raw Data Ingest Workbook for TOS Mosquito Abundance, Diversity and Phenology (AD [12]). These remaining data columns, which are blank, will be filled in with taxonomy data and metadata by the testing facility. The order of samples in the electronic manifest should be the same as the order in the corresponding hard-copy shipping manifest.

Laboratory Equipment and Materials

Table 6. Equipment list, Sample shipment

Maximo Item No.	Item Description	Purpose	Quantity	Habitat-Specific	Special Handling
Required	Shipping materials (TBD: to be specified by CLA)	Protecting specimens during shipping		No	No?
Required	Hard-copy shipping manifest	Keeping an inventory of specimens being shipped	1	No	No

SOP E: Post-Identification Lab Processing

After identification at external facilities, some mosquitoes will be shipped back to the domain lab for additional processing, including pointing, photographing and tissue removal for DNA barcoding.

DNA barcoding

1. After mosquitoes are identified to species by taxonomists at one or more external facilities, they will be returned to the lab in labeled vials separated by species. Prepare and submit a subset of these mosquitoes for DNA barcoding.
2. The process of DNA barcoding involves 3 steps: (1) pointing mosquitoes, (2) removing a leg to submit as a tissue sample, and (3) photographing mosquitoes (not necessarily in that order). These steps are essentially the same for both ground beetles and mosquitoes. Consider doing ground beetle and mosquito barcoding at the same time and refer to the detailed instructions in the Common Insect Lab Protocols (AD [08]).

Pointing

1. Because a physical specimen is required for every individual submitted for barcoding, the same individual mosquitoes selected for barcoding need to be pointed. Pointing involves mounting the mosquito on a small paper triangle on a pin. Detailed pointing instructions are provided in the Lab Protocol for Beetles and Mosquitoes (AD [08]) and will not be repeated here. All mosquitoes that need to be mounted on pins will be pointed (as opposed to direct pinning).

Photographing

1. Similar to pointing, all mosquitoes that are submitted for DNA barcoding must be photographed. Detailed instructions for photographing pinned/pointed insects are provided in the Lab Protocol for Beetles and Mosquitoes (AD[08]) and will not be repeated here.

SOP F: Data Entry and Verification

1. Enter data from field datasheets and laboratory processing of sampling into the "mosquito_trapping" spreadsheet in the NEON Raw Data Ingest Workbook for TOS Mosquito Abundance, Diversity and Phenology (AD [12]) within 14 days of returning to the lab after a bout of data collection, according to instructions in the NEON Protocol: manual data transcription (AD [13]).
2. Scan datasheets and save in PDF file format.
3. Save paper copy of datasheets.

11 REFERENCES

N/A

APPENDIX A QUICK REFERENCES FOR MOSQUITO SAMPLING

Quick Reference: Processing Mosquito Specimens

STEP 1 – Quickly transfer contents of catch cup into frozen sample vials. Remove any obvious bycatch during transfer but prioritize keeping mosquitoes frozen.

STEP 2 – Place tissue paper and a locality label in each sample vial.

STEP 3 – Store vials in -80 °C freezer.

STEP 4 – Record date and time of specimen processing.

STEP 5 – Repeat procedure with specimens from next catch cup.

Keep samples frozen. The genetic material detected during pathogen testing degrades when a sample thaws.

Quick Reference: Getting Ready for Sampling

STEP 1 – Gather all needed supplies (and extras).

STEP 2 – Test functionality of mosquito trap components.

STEP 3 – Upload sample locations and obtain maps and datasheets.

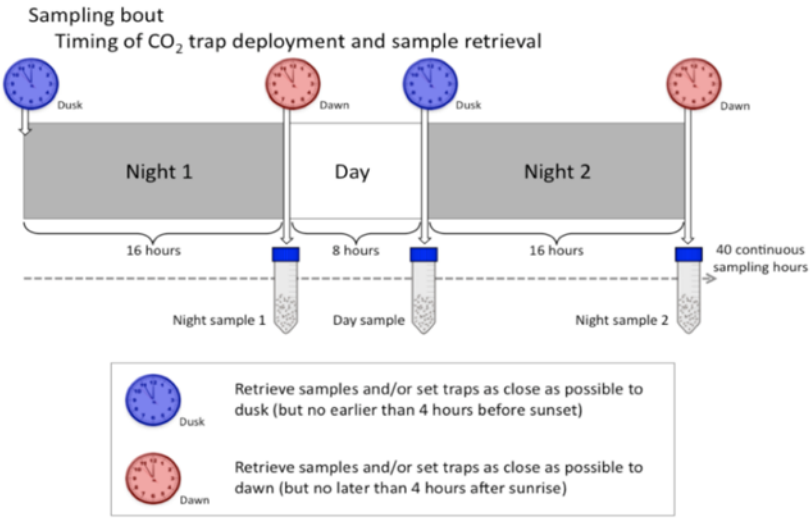
STEP 4 – Generate and print locality labels and cut into strips.

On the field day:

STEP 5 – Obtain enough dry ice to set (or re-set) traps. Add additional dry ice or frozen reusable ice packs to keep samples cold or frozen during transport back to lab.

STEP 6 – In coolers used to transport samples from field to lab, cover ice with cardboard or cloth.

Mosquito trap servicing during a bout occurs during a ~40 hour window, including two nights and the intervening day. This involves four trips to each sampling plot.



Keep samples frozen. The genetic material (that will be analyzed for pathogens) degrades when a sample thaws.

Quick Reference: Setting Trap and Collecting Mosquitoes

STEP 1 – Write the date on three locality labels and attach to mesh sleeve.

STEP 2 – Fill cylinder cooler with dry ice pellets.

STEP 3 – Assemble trap components. Attach fan assembly to rain cover using screws. Attach catch cup (with mesh sleeve). Connect to battery. Remove tape from cylinder vent hole.

STEP 4 – Return to trap after elapsed time.

STEP 5 – Keep fan running. Tie off mesh sleeve and remove from fan assembly.

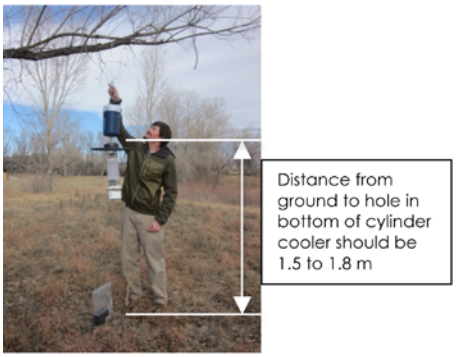
STEP 6 – Gently tuck mesh sleeve into catch cup.

STEP 7 – Reset trap, if required.

STEP 8 – Carefully transport catch cup back to vehicle.

STEP 9 – Place catch cup into cooler containing dry ice or frozen reusable ice packs.

STEP 10 – Record all metadata and any irregularities on data sheet.



Trap Setup:

- Suspend with hole in bottom of cylinder cooler approximately 1.5 to 1.8 m above the ground.
- Hang from tree or sturdy shrub.
- Shield from heavy wind, sunlight, and rain:
  - Hang on leeward side, away from wind.
  - Hang on west facing side, away from direct morning sunlight.
- Where tree or shrub is not available, secure trap to a shepherd's hook.
- Seal battery in resealable freezer bag and place on ground.

APPENDIX B CHECKLISTS FOR MOSQUITO SAMPLING

Checklist: Processing Specimens in the Lab

Specimen quality: Be sure to…

- ☑ Store specimens in ultra-cold freezer (-80° C).
- ☑ Work with specimens from one catch cup at a time.
- ☑ Look carefully for mosquitoes caught in folds of mesh sleeve.
- ☑ Work quickly, sorting mosquitoes from obvious bycatch so that mosquitoes remain frozen.
- ☑ Provide ample room in vials so as not to damage specimens.
- ☑ Put locality label (with date) in every sample vial.

Data entry: Did you…

- ☑ Record date and time of specimen processing?
- ☑ Describe irregularities or deviations from protocol?

Work quickly when you separate mosquitoes from obvious bycatch. Frozen mosquitoes must remain frozen.

Checklist: Getting Ready for Field Sampling

(At least one to two days before sampling bout)

Avoid looking like this guy.

Make sure you have everything you need for field sampling (including extras of just about everything).

Gather supplies and test equipment at least one day before a sampling bout.

Locality labels: Be sure to…

- ☑ Print labels with correct month and locations.
- ☑ Cut labels into strips.
- ☑ Bring safety pins for attaching locality labels.

Equipment: Be sure to...

- ☑ Inspect catch cup and mesh sleeve for tears.
- ☑ Check circuit switches on traps (1st switch closed, 2nd and 3rd switches opened).
- ☑ Test fan by connecting battery.
- ☑ Charge batteries.
- ☑ Print datasheets.
- ☑ Upload sample coordinates to GPS and obtain maps.
- ☑ Check that sufficient amounts of dry ice and/or reusable ice packs are available.
- ☑ Cover ice in transport cooler with cardboard or cloth.
- ☑ Assemble mosquito traps.

Checklist: Collecting Quality Mosquito Specimens

Before heading into the field: Make sure you have…

- ☑ All supplies (and extras).
- ☑ Datasheet and locality labels.
- ☑ Taped the hole in cylinder cooler (if it was filled with dry ice in lab).
- ☑ If used, applied insect repellent away from sampling equipment. Do this ½ hour before heading into the field. Wash hands before handling sampling equipment.

Sample collection: Be sure to…

- ☑ Plan your day so that you service traps within the required window of time.
- ☑ Double check that your actual location matches the one on the locality label.
- ☑ Write the date on three locality labels.
- ☑ Check mesh sleeve for tears or holes.
- ☑ Freeze and store specimens on dry ice or frozen reusable ice if dry ice is not available.
- ☑ Record all required data and any irregularities on Mosquito Field Datasheet.

Before leaving trap: Check that…

- ☑ Cylinder cooler has been refilled with dry ice if trap reset is required.
- ☑ Tape is removed from cylinder cooler and CO_2 is subliming.
- ☑ Locality labels (with date) are attached to mesh sleeve.
- ☑ Battery is connected to fan housing and fan is running.

Transporting samples: Make sure…

- ☑ Mosquitoes remain frozen or as cold as possible
- ☑ Mosquitoes are not in direct contact with ice in transport cooler.
- ☑ Cooler is secure in vehicle and will not tip over during transport.
- ☑ Catch cups are transferred to -80C freezer in domain lab as soon as possible.

APPENDIX C CHANGE NOTES

The following changes have been made between Rev C_DRAFT and Rev D of this protocol:

- The light bulb will be removed from mosquito traps prior to deployment
- Spatial sampling in year-1 has been removed. Sampling in 10 plots will be the same in year 1 and all subsequent years.
- The format of sample vial labels has been updated (use vialID)
- The number 1, 2 or 3 is now written on the back of locality labels attached to mosquito catch cups
- The protocol involving transfer of mosquitoes from catch cups into sample vials has been formalized
- Shipping instructions for samples (e.g., to external taxonomic facilities) have been detailed
- Datasheets have been updated and aligned with newly approved ATBDs