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
E	10/09/2014	ECO-02353	Migration to new protocol template

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1 OVERVIEW

1.1 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.

1.2 Scope

This document provides a change-controlled version of Observatory protocols and procedures. Documentation of content changes (i.e. changes in particular tasks or safety practices) will occur via this change-controlled document, not through field manuals or training materials.

1.2.1 NEON Science Requirements and Data Products

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.

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]).

1.3 Acknowledgments

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

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2 RELATED DOCUMENTS AND ACRONYMS

2.1 Applicable Documents

Applicable documents contain higher-level information that is implemented in the current document. Examples include designs, plans, or standards.

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.014051	Field Audit Plan
AD[09]	NEON.DOC.000824	Data and Data Product Quality Assurance and Control Plan

2.2 Reference Documents

Reference documents contain information that supports or complements the current document. Examples include related protocols, datasheets, or general-information references.

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.001271	NEON Protocol and Procedure: Manual Data Transcription
RD[05]	NEON.DOC.001581	Datasheets for TOS Protocol and Procedure: Mosquito Sampling
RD[06]	NEON.DOC.001125	TOS Protocol and Procedure: Plot Establishment
RD[07]	NEON.DOC.001401	NEON Raw Data Ingest Workbook for TOS Mosquito Abundance, Diversity, and Phenology
RD[08]	NEON.DOC.001100	TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing

2.3 Acronyms

Acronym	Definition
CDC	U.S. Centers for Disease Control and Prevention

2.4 Definitions

N/A

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3 METHOD

Mosquito sampling involves preparing to sample (SOP A), collection in the field (SOP B), minor laboratory processing (SOP C), shipping to external facilities (SOP F) and data handling (SOP D). 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.

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.

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.

Standard Operating Procedures (SOPs), in Section 7 of this document, provide detailed step-by-step directions, contingency plans, sampling tips, and best practices for implementing this sampling procedure. To properly collect and process samples, field technicians **must** follow the protocol and associated SOPs. Use NEON's problem reporting system to resolve any field issues associated with implementing this protocol.

The value of NEON data hinges on consistent implementation of this protocol across all NEON domains, for the life of the project. It is therefore essential that field personnel carry out this protocol as outlined in this document. In the event that local conditions create uncertainty about carrying out these steps, it is critical that technicians document the problem and enter it in NEON's problem tracking system.

The procedures described in this protocol will be audited according to the Field Audit Plan (AD[08]). Additional quality assurance will be performed on data collected via these procedures according to the NEON Data and Data Product Quality Assurance and Control Plan (AD[09]).

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4 SAMPLING SCHEDULE

4.1 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 (Figure 1, described below). Plots will be randomly located in each of the major vegetation types (≥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.

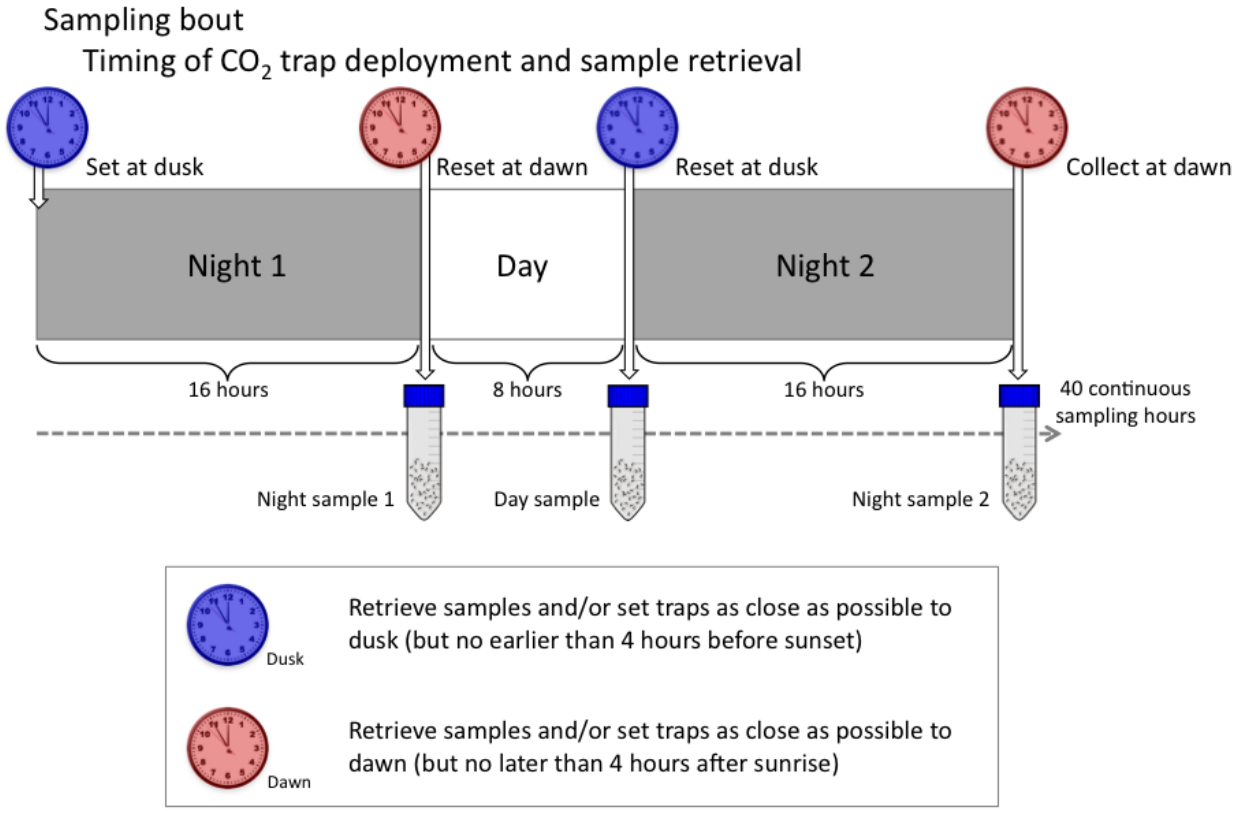

Figure 1. Timing of a mosquito sampling bout that generates three samples (designated by vials), two trap-nights and the intervening day. (Daytime and night-time durations shown are for illustrative purposes only.)

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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 (Figure 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.

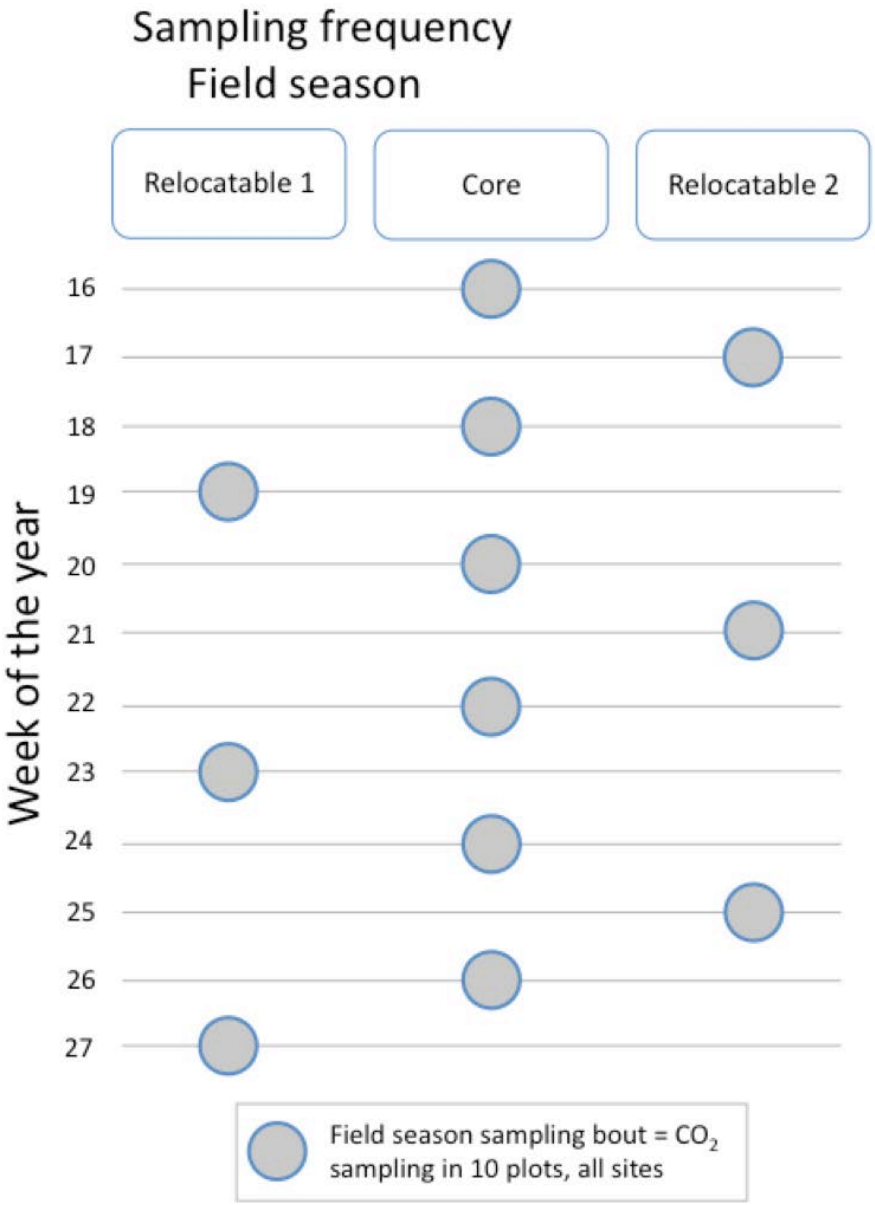

Figure 2. Frequency of sampling bouts at a domain; weekly sampling alternating between core and relocatable sites.

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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.

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 (Figure 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.

4.2 Criteria for Determining Onset and Cessation of Sampling

Sampling will be conducted until specified collection thresholds that designate the end of field season sampling and beginning of off season sampling are met (Figure 3, Figure 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 (Figure 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 (Figure 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 (Figure 3, Figure 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.

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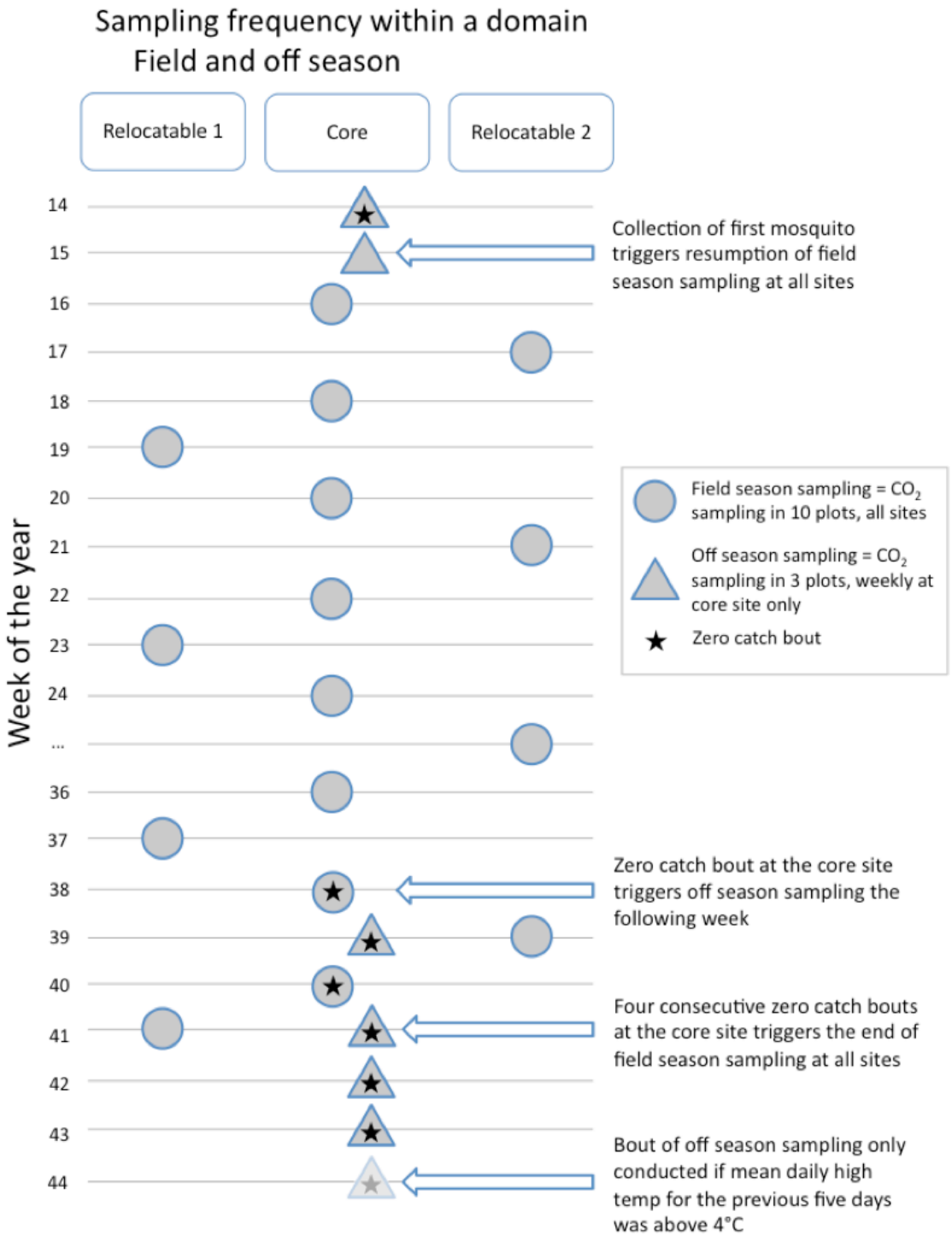

Figure 3. An example domain sampling schedule highlighting the transitions between field season and off season mosquito sampling.

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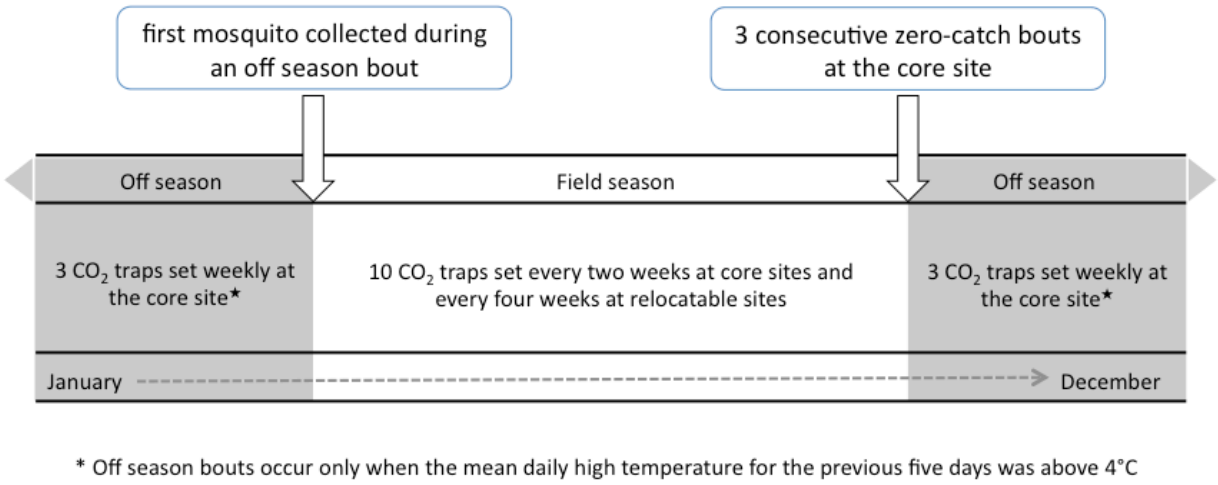

Figure 4. Annual summary of mosquito sampling intensity including transitions from off-season to field season and back to off-season sampling.

4.3 Timing for Laboratory Processing and Analysis

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

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4.4 Sampling Timing Contingencies

When field conditions require deviations from the protocol, follow the contingent decisions outlined in Table 1 below to maintain data quality.

Table 1. Contingent decisions

Delay/ Situation	Action	Outcome for Data Products
< 3 hours	Resume/continue with normal sampling at conclusion of delay. (Note the duration and cause of the delay)	Quality of samples reduced, creating potential for complications with processing (identification, pathogen testing). Note also 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.
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	Brief interruption in consistent interval of time series data compromises 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	Moderate interruption in consistent interval of time series data compromises 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.	Maximal interruption in consistent interval of time series data compromises statistical analysis of temporal trends in the data. Reduction in sample size as sampling bouts are missed.

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5 SAFETY

This document identifies procedure-specific safety hazards and associated safety requirements. It does not describe general safety practices or site-specific safety practices.

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.

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6 PERSONNEL AND EQUIPMENT

6.1 Equipment

The following equipment is needed to implement the procedures in this document. Equipment lists are organized by task. They do not include standard field and laboratory supplies such as charging stations, first aid kits, drying ovens, ultra-low refrigerators, etc.

Table 2. Equipment list – Trap deployment and sample retrieval during field season sampling

Item No.	R/S	Description	Purpose	Quantity	Special Handling
Durable items					
MX103541	R	CO_2 light trap, battery-powered fan assembly	Mosquito trapping	10	N
MX100695	R	CO_2 light trap, cylindrical insulated cooler	Mosquito trapping	10	N
MX100694	R	CO_2 light trap, mesh sleeve and collection cup	Mosquito trapping	12	N
	R	CO_2 light trap, rain cover	Mosquito trapping	10	N
	R	CO_2 light trap, red rain cover screws	Mosquito trapping	30	N
	R	Cryogenic gloves (to handle dry ice)	Safety		N
	R	Shepherd's hook	Hanging traps (if woody vegetation or other natural structures not available)	Up to 10	N
	R	Scissors	Cutting	1	N

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Item No.	R/S	Description	Purpose	Quantity	Special Handling
	R	6V Battery (for CO_2 light trap)	Powering mosquito traps	10-20	Y
	R	Utility knife	Cutting labels	1	N
	R	Cooler(s)	Transporting chilled/frozen samples back to domain lab	Depends on size/volume	N
	R	Pens with archival, ethanol-safe ink	Labeling	1	N
	R	Battery chargers	Charging 6V batteries	10 (or 5 with dual capacity)	N
	R	Plastic bins	Battery secondary containment	Depends on size relative to batteries	N
	S	GPS receiver, recreational accuracy	Locating plot	1	N
	S	Ice packs, -20 C	Keep samples cool	Depends on number and size of coolers	N
Consumable items					
	R	Dry ice (pellet form recommended)	Mosquito bait	1.5kg per trap for 12-18 hours of sampling	Y
	R	Masking tape	Cover vent holes in trap coolers		N

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Item No.	R/S	Description	Purpose	Quantity	Special Handling
	R	Rope (need less if using shepherd's hooks)	Hanging traps	Depends on height of vegetation from which traps are hung	N
	R	Paper towels	Absorb excess moisture		N
	R	Plastic bag, 1 gal, resealable	Protect trap batteries from rain/moisture	10	N
	R	Safety pins	Attaching labels		N
	R	Cardboard cards or cloth	Insulating samples		N
	R	Copy paper, Rite in the Rain	Printing datasheets, labels		N
	R	Pencils	Recording data		N
	R	Label tape (must be able to handle -80 C)	Labeling sample vials		N
	R	Laundry detergent, fragrance free	General cleanup		N
	S	Aluminum foil	Cattle chewing deterrent		N

R/S=Required/Suggested

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Table 3. Equipment list – Laboratory processing and analysis

Item No.	R/S	Description	Purpose	Quantity	Special Handling
Durable items					
	R	Permanent marker, archival, black	Writing labels	2	N
	R	Centrifuge tubes (15 or 50 mL volume recommended)	Storing mosquitoes	25	N
	R	Cryovial freezer storage box	Holding tubes in freezer		Y
	R	Jewelers forceps, straight	Manipulating specimens	3	N
	R	Petri dish	Temporary storage; separating bycatch from mosquitoes		
	S	Centrifuge tube rack	Holding tubes	1	N
	S	Plastic funnel (use paper funnel if preferred)	Transferring mosquitoes to tubes	1	N
Consumable items					
	R	Copy paper, Rite in the Rain	Datasheets and labels	2	N
	R	Tissues paper	Protecting mosquitoes in tubes	25	N
	R	Laundry detergent, fragrance free	General cleanup	1	N

R/S=Required/Suggested

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Table 4. Equipment list – Sample shipment

Item No.	R/S	Description	Purpose	Quantity	Special Handling
Durable items					
(None)					
Consumable items					
	R	Shipping materials (TBD: to be specified by CLA)	Protecting specimens during shipping		N
	R	Hard-copy shipping manifest	Inventory of specimens being shipped	1	N

R/S=Required/Suggested

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6.2 Training Requirements

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

6.3 Specialized Skills

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.

6.4 Estimated Time

The time required to implement a protocol will vary depending on a number of factors, such as skill level, system diversity, environmental conditions, and distance between sample plots. The timeframe provided below is an estimate based on completion of a task by a skilled two-person team (i.e., not the time it takes at the beginning of the field season). Use this estimate as framework for assessing progress. If a task is taking significantly longer than the estimated time, a problem ticket should be submitted.

An experienced two-person team will require approximately 15-25 minutes to complete deployment or retrieval of samples from at a single plot. This entails travel time between the plot and the road and time required for either a) hanging a trap for an initial deployment, b) transferring a catch cup containing samples into a cooler and replacing the catch cup (and potentially battery) for a redeployment, or c) transferring a catch cup containing samples into a cooler and taking down trap equipment at the conclusion of sampling bout.

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7 STANDARD OPERATING PROCEDURES

SOP A Preparing for Sampling

A.1 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 (Figure 5). On this setting the trap fan remains on at all times.

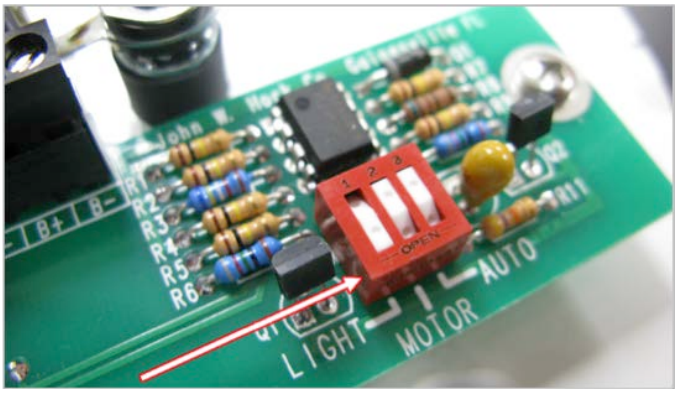

Figure 5. Switches inside circuit assembly of a CDC mosquito trap.

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 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 (RD[05])and sample locality labels. Label preparation is described in the TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing (RD[08]).

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A.2 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 pick-up 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.

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SOP B Field Sampling

B.1 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 (RD[06]). 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 (RD[05]), including the sampleID.

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B.2 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 (RD[05]) 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.

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B.3 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.

B.4 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 (RD[05]).

B.5 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.

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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.

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SOP C Laboratory Processing and Analysis

C.1 Transferring mosquitoes from catch cups to sample vials

1. Clear and clean off bench space prior to processing samples.
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.

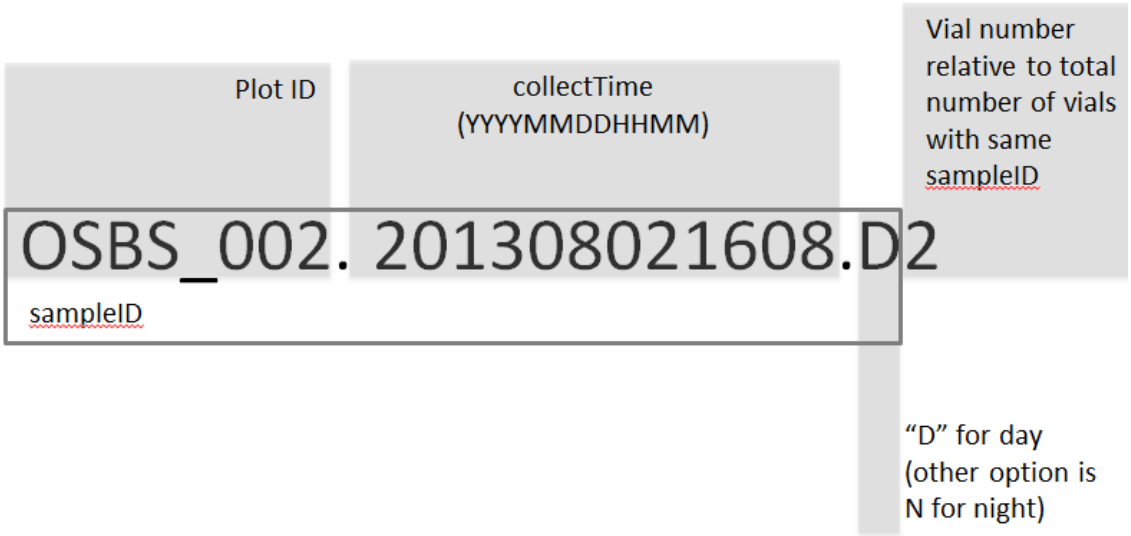

Figure 6. Annotated vialID example

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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 3).
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

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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 (RD[07]).

C.2 Sample preservation

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

C.3 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.

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SOP D Data Entry and Verification

As a best practice, field data collected on paper datasheets should be digitally transcribed within 7 days of collection or the end of a sampling bout (where applicable). However, given logistical constraints, the maximum timeline for entering data is within 14 days of collection or the end of a sampling bout (where applicable). See RD[04] for complete instructions regarding manual data transcription.

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 (RD[07]), according to instructions in the NEON Protocol and Procedure: Manual Data Transcription (RD[04]).
2. Scan datasheets and save in PDF file format.
3. Save paper copy of datasheets.

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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.

E.1 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 TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing (RD[08]).

E.2 Pointing

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 (RD [08]) and will not be repeated here. All mosquitoes that need to be mounted on pins will be pointed (as opposed to direct pinning).

E.3 Photographing

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 (RD[08]) and will not be repeated here.

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SOP F Sample Shipment

Information included in this SOP conveys science-based packaging, shipping, and handling requirements, not lab-specific or logistical demands. For that information, reference the CLA shipping document on CLA's NEON intranet site.

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.

Samples should be shipped frozen on dry ice via overnight delivery service to a taxonomic ID facility. **Samples must remain frozen at all times.**

F.1 Handling Hazardous Material

Dry should be handled according to the guidelines in the EHS Safety Policy and Program Manual (AD[01]) and the Domain Chemical Hygiene Plan and Biosafety Manual (AD[03]).

F.2 Timelines

From a technical/scientific perspective, mosquito samples stored at -80C will retain their integrity for many months. Samples will be shipped overnight on dry ice.

F.3 Conditions

Samples should be stored dry in vials at -80C until shipped to an external facility. Samples should be shipped in insulated shipping containers containing dry ice.

F.4 Grouping/Splitting Samples

All samples collected during any given site/bout combination should be shipped together. Sample vials containing samples collected as part of the same site/bout combination could be taped or rubber-banded together, or placed in a separate bag, to allow them to be easily inventoried/sorted at the external facility.

F.5 Shipping Inventory

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.

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 (RD[07]): vialID, boutNumber, senderID, dateSent and receiverID. An example of a populated hard-copy manifest is provided in Figure 7.

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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

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 (RD[07]). 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.

F.6 Laboratory Contact Information and Shipping/Receipt Days

See the CLA shipping document on CLA's NEON intranet site.

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8 REFERENCES

N/A

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APPENDIX A DATASHEETS

The following datasheets are associated with this protocol:

Table 5. Datasheets associated with this protocol

NEON Doc. #	Title
NEON.DOC.001581	Datasheets for TOS Protocol and Procedure: Mosquito Sampling

These datasheets can be found in Agile or the NEON Document Warehouse.

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APPENDIX B QUICK REFERENCES

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.

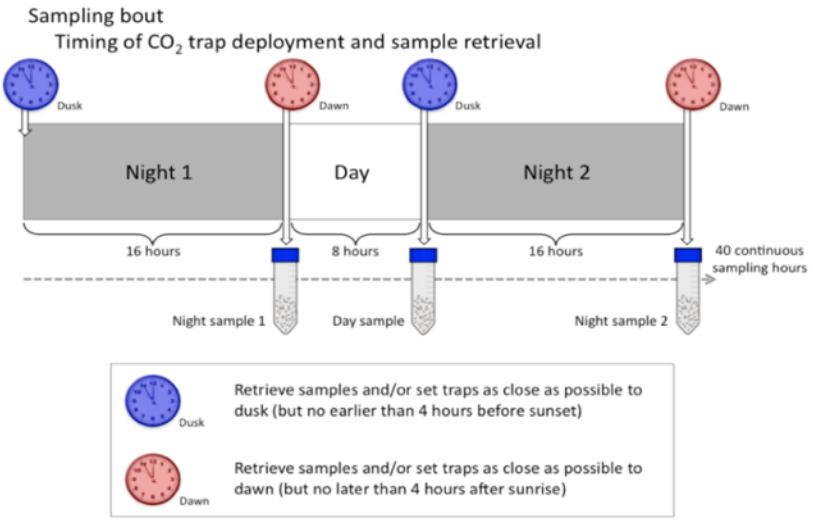
STEP 4 – Generate and print locality labels and cut into strips.

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.



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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.

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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.

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APPENDIX C REMINDERS

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.

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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.

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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.

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APPENDIX D ESTIMATED DATES FOR ONSET AND CESSATION OF SAMPLING

The dates in the table below are based on historic records and are estimates for the start and stop dates of sampling. It is essential that domain staff monitor real-time conditions to determine when to start (and stop) sampling, as described in Section 4 of this protocol.

This table will be completed in a future revision of this document, as data become available.

Table 6. Estimated dates of historical temperature thresholds

	Average 5-day temp above 10°C		Average 5-day temp above 4°C	
Site	**Approx. Start Date**	**Approx. End Date**	**Approx. Start Date**	**Approx. End Date**
HARV	March 28	November 20	February 22	December 17
BART	April 14	November 2	March 18	December 4
OSBS	February 23	December 5	Year Round	
OSBS	Year Round			
DSNY	Year Round			
JERC	Year Round			
UNDE	April 14	October 26	March 19	November 13
ORNL	February 12	December 21	Year Round	
TALL	Year Round			
WOOD	April 9	October 30	March 21	November 12
CPER	February 27	November 22	January 9	December 19
STER	February 27	November 22	January 9	December 20
ONAQ	March 8	November 15	January 21	December 18

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