

**Protocol for Mosquito Sampling for Mosquito Best Management Practices on
State of California-Managed Wildlife Areas**

July 2005

Developed for the Integrated Pest Management Committee of the Mosquito and Vector Control Association of California, Sacramento, CA by:

William Walton, Ph.D.,
Department of Entomology, University of California, Riverside, CA 92521

Protocol for Mosquito Sampling

Assembly Bill 1982 provides funding to implement mosquito best management practices (BMPs) at wildlife areas managed by the California Department of Fish and Game. The first year of the program is 2005-2006. A protocol for sampling immature mosquitoes (larvae and pupae) is outlined in this document. The document is intended to focus on three wildlife areas, but is likely applicable to other wildlife areas to be included in the Mosquito BMP Program.

1. Proposed Mosquito Best Management Practices

The primary BMP modifications to existing wetlands in Wildlife Areas are (1) source reduction through vegetation removal/reduction; (2) improved water conveyance through several approaches including changes in the size and orientation of water conveyance channels, repair or replacement of water control structures, enhancement of pumping, and removal of structures/fea-

Table 1. Proposed Mosquito Best Management Practices at Three California Department of Fish and Game Wildlife Areas.

| Location | Mosquito BMP | Expected outcomes |
|---|---|---|
| Gray Lodge Wildlife Area | 1) improve water conveyance in upland pasture habitat by modifications to drains, intakes and swale improvements | (i) reduce mosquito production (ii) increase quality of habitat for wildlife |
| | 2) source reduction by mowing: improve water flow by reducing vegetation on the side slopes and bottom of ditches | (i) reduce mosquito production (ii) improve (increase) water flow (iii) reduce vegetation favorable for mosquito production |
| | 3) replace water control structures (10/yr) | (i) reduce mosquito production (ii) increase water flow |
| Yolo Bypass Wildlife Area (focus on BMP #1; 2-4 funded previously) | 1) source reduction of jointgrass (<i>Paspalum distichum</i>) in 1000 acres (~ 55% of the surface area of 12 fields): 2005 i) disc 800 acres ii) herbicide 200 acres iii) replace 6 water control structures | (i) reduce mosquito production (ii) reduce coverage by jointgrass |
| | 2) reconfigure irrigation system: swale addition | |
| | 3) replace or repair leaky water control structures | |
| | 4) removal of contour berms in an irrigated pasture on Tule Ranch (summer 2006) | |
| Grizzly Island Wildlife Area | 1) add mosquitofish holding areas and improve water conveyance by creating channels for fish movement and changing pump locations | (i) reduce mosquito production (ii) increase larvivorous fish abundance (iii) reduce chemical treatments against mosquitoes |
| | 2) discing problematic areas | Reduce mosquito production |
| | 3) improve pump efficiency | Improve water flow |

tures in wetland basins that inhibit movement of water; and (3) enhancement of populations of biological control agents (mosquitofish, *Gambusia affinis*) for immature mosquitoes (Table 1). In addition to decreasing the time required for flooding and for draining of wetlands, (4) altering the timing of flooding to reduce overlap with peak annual activity of mosquitoes is another mosquito BMP.

2. Study Design and Consideration of Factors Influencing Mosquito Populations

To evaluate fully the success of the BMPs, it is critical not only to document changes in mosquito abundance and/or production but also to assess the changes in the processes and features that potentially influence mosquito numbers in each Wildlife Area. For example, an understanding of how effective the BMPs are for changing the speed of flooding and draining wetlands or for changing the proportion of the wetlands' surface supporting vegetation and the types of vegetation will greatly enhance interpretation of changes in mosquito abundance or species composition. An accurate assessment of these factors is also needed to assist evaluation of any changes in wildlife usage or habitat quality. While the protocols for measuring these other factors are not addressed here, refuge and mosquito abatement personnel are encouraged to work together to collect as much of these data as is possible.

The approach used to evaluate of the efficacy of the mosquito BMPs will depend on how the BMPs are implemented at each Wildlife Area. The study design used to monitor mosquito abundance and then to test statistically the efficacy of the mosquito BMPs depend on how modifications of wetland features and operations are carried out. Two general types of comparisons can be made to evaluate the effects of mosquito BMPs on changes in mosquito numbers: ***(1) before-and-after comparisons*** or ***(2) wetlands with mosquito BMPs versus wetlands without mosquito BMPs***. The latter comparison involves the contemporaneous operation of the two wetland types.

2.1 Before-and-After Comparisons

If all wetlands within a refuge or only those wetlands designated as being problematic for mosquito production are modified in 2005, then the only mechanism to assess the efficacy of the BMPs for reducing mosquitoes will be before-and-after comparisons. Such comparisons necessitate that adequate historical data are available and that current methods of mosquito monitoring complement those of the historical data set. Surveillance data of host-seeking mosquito abundance and immature mosquito abundance in dipper counts are two possible sources of data to be used in before-and-after comparisons. However, only an overall population mean (i.e., numbers of larvae per dip) for immature mosquito numbers in dipper samples often is retained in records rather than the details of mosquito abundance in individual or composite dipper samples; the variation of mosquito numbers in dipper samples and the number of samples taken might not be available in the historical records. Historical data relevant to assessing the impact of mosquito BMPs in wildlife areas on mosquito abundance include (i) immature

mosquito surveillance; (ii) adult mosquito collections in surveillance programs using carbon dioxide-baited suction traps or light traps; (iii) service requests adjacent to the Wildlife Area; (iv) frequency of mosquito abatement measures; (v) type(s) of mosquito abatement required.

Robust historical data are also required for before-and-after comparisons of the environmental factors influencing mosquito abundance which are altered via best management practices. Questions that might be asked when considering this approach include, Do background data exist for flow rates, filling rate, etc. so that comparisons can be made before and after BMP implementation? What was the proportion of the water surface of a particular wetland covered by vegetation before mosquito BMPs? Did the coverage change significantly across seasons? What is the impact of the mosquito BMPs on other wetland organisms that are likely to interact with immature mosquitoes and may also be important components of the diets waterfowl and water birds? Do adequate before-BMP data exist for the organisms of interest?

Even if long-term records are available, before-and-after comparisons are less preferred than are contemporaneous comparisons of wetlands with and without mosquito BMPs. Besides theoretical statistical considerations of temporal differences of sampling effort, randomization of sampling sites, sampling bias of different investigators and other considerations, interannual variation in climatic conditions and temporal changes in wetlands (i.e., succession) that might differ between the two groups of wetlands are among the other concerns when making before-and-after comparisons. The effects of annual wetland management on mosquito and aquatic insect populations are likely to be comparatively strong relative to other factors affecting the ecologies of wetland organisms so before-and-after comparisons of highly and frequently managed wetlands might be less problematic than for comparisons of natural vs. restored wetlands or wetlands that have not been managed for several years vs. recently managed wetlands.

Before-and-after interannual comparisons are appropriate for assessing the efficacy of mosquito BMPs with comparatively short-term effects (< 1 year) on mosquitoes. Each year wetlands can be classified as being managed with or without the particular BMP(s). For example, half the wetlands would have the BMP implemented in one year and then not have the BMP implemented in the following year. A second group of wetlands would not have the BMP implemented in the first year and then have the BMP implemented in the second year. This study design would help to control for interannual variability in environmental factors but nevertheless requires simultaneous operation of wetlands with and without the BMP(s) of interest.

2.2 Comparisons of Contemporaneously Operated Wetlands

Comparisons between wetlands with mosquito BMPs and wetlands without mosquito BMPs (control wetlands) are preferred to before-and-after comparisons because the effects of interannual environmental factors and possible sampling bias from different investigators are eliminated or lessened, respectively. In order to compare mosquito abundance in wetlands that have undergone modifications and are operated to reduce mosquito production versus wetlands without mosquito BMPs, the wetlands in the two treatment groups should be as similar as possible. Factors such as wetland area, vegetation before modifications, position on the refuge,

orientation to prevailing winds, etc. should be as similar as is practical for the wetlands in each of the two treatment groups. It is permissible to use these factors to group (block) wetlands so that similar wetlands receive one or the other treatment; within a particular block, a wetland in one treatment must be paired with a wetland in the other treatment. For example, if two equivalent sized wetlands were found at one end of a refuge, then one wetland could be assigned for BMP implementation and the other wetland could be assigned as a control¹.

If BMPs are to be implemented in a subset of the wetlands in a Wildlife Area in subsequent years, then contemporaneous comparison of the two wetland types is possible. For 2005, mosquito BMPs could be implemented in a subset of the wetlands and those wetlands scheduled for implementation of mosquito BMPs in subsequent years could serve as non-BMP (control) wetlands during 2005. The number of wetlands of each type should be equivalent. Therefore, even though monitoring of mosquito abundance in all wetlands is recommended for mosquito control purposes, mosquito populations in that subset of wetlands assigned to BMP and non-BMP treatments need only to be monitored for the assessment of the efficacy of the BMPs. Wetlands should be assigned to a respective treatment group at the start of the study.

Contemporaneous comparison of wetlands with and without mosquito BMPs is not without other considerations/drawbacks. For example, mosquito production differs among wetlands of different age and successional state, and is highly associated with emergent vegetation. Whereas the relevant comparison for this study is between wetlands with a mosquito BMP and wetlands without a mosquito BMP, the latter wetland group should nevertheless be managed typically. It would not be appropriate to forego an annual procedure that is routinely carried out in the non-BMP wetlands. The time period since vegetation management was carried out in the control (non-BMP) wetlands will be an important consideration. The dynamics of mosquito populations in wetlands that have undergone significant manipulations within the last year usually differ appreciably from wetlands that have been in continuous operation (or left fallow in the case of intermittent/seasonal wetlands) for 2 or more years. Moreover, sampling strategies require careful consideration when assessing the effects of source reduction because wetlands that have undergone particular types of vegetation management will have less surface area containing emergent vegetation than will wetlands that have not had vegetation managed. These considerations are discussed briefly below.

Last, sampling methods that might be appropriate for situations where all wetlands are modified are not appropriate in situations where wetlands with mosquito BMPs are run adjacently to wetlands without mosquito BMPs. For example, given the large area of some wetlands, it might be possible to supplement the assessment of the efficacy of the mosquito BMPs by counts of immature mosquitoes with adult collections using carbon dioxide-baited suction traps. This approach makes sense when all of the wetlands are modified or the area undergoing modification is large enough to limit immigration of significant numbers of host-seeking mosquitoes from outside the modified wetlands. Collection of host-seeking adult female mosquitoes to assess the efficacy of mosquito BMPs is not appropriate when wetlands with and without mosquito BMPs are run adjacent to each other.

¹ Each treatment is replicated only once in each block. An assumption of no statistical interaction between the blocking factor and the treatment factor is implicit in experimental designs using randomized blocks. One should be familiar with the assumptions and requirements of the experimental design and analyses beforehand.

2.3 Assessment of the Efficacy of Multiple BMPs

For many of the Wildlife Areas included in the Mosquito BMP program, multiple BMPs are being applied to particular wetlands on a refuge. In most instances there is insufficient replication to be able to assess the efficacy of each BMP for reducing mosquito populations independently, moreover to be able to assess interactions among BMPs in a cross-classified manner. For example, adding mosquitofish and reducing emergent vegetation cannot be expected to have the same effect on mosquito populations as does either reducing emergent vegetation or adding *Gambusia* sp. alone; it would be inappropriate to utilize wetlands with each of these BMPs as replicate “mosquito BMP” wetlands. ***It is therefore important to replicate the same BMP(s) in as many sites as possible.*** However, given cost considerations (e.g., labor, equipment) and wildlife management needs, it may not be practical to do so. Therefore, another approach that is consistent with practical wetland management considerations will be needed.

Consideration of the direct and indirect effects of mosquito BMPs on other BMPs and on non-BMP wetlands will be needed. For example, the effects of changing hydroperiod will extend beyond the direct effect of the time of year (or duration) when water is present. Changing the hydroperiod will affect plant species composition. The palatability of plant species differs among herbivores. Differences of plant growth form and density are related to the potential to provide harborage for immature mosquitoes. The effects of these factors are therefore inter-related. Even though the goals of this program are not to study the subtle interactions among ecological factors affecting mosquito abundance in wetlands, the potential for significant effects on mosquito abundance from linked effects of BMP implementation should be appreciated.

Measures may need to be implemented to prevent effects of mosquito BMPs on non-BMP wetlands. If non-BMP (control) wetlands are not routinely stocked with larvivorous fishes or wetlands are interconnected by water flow, then measures need to be implemented to prevent fish added to BMP wetlands from entering the non-BMP wetlands. Such considerations also are relevant if the enhancement of mosquitofish populations is a BMP under consideration and the enhanced fish populations can potentially enter control (non-BMP) wetlands. Such considerations may also be relevant to issues of water conveyance and flooding schedules.

3. Sampling to Assess Immature Mosquito Abundance

3.1 Hand-dipper samples

Larval mosquito counts collected using a hand dipper are the preferred type of abundance data for evaluating the efficacy of mosquito BMPs because dipper samples (a) are comparatively easier to collect than are other types of collections for immature mosquitoes and other mosquito life stages (e.g., substrate samples for eggs of some mosquito species, emergence trap samples for adult mosquitoes), (b) can be processed quickly compared to other sampling methods, and (c) are more indicative of mosquito abundance in a particular place than are adult host-seeking mosquito collections which could include individuals produced elsewhere than in the immediate

vicinity of the trap. Sampling methods for all stages in the mosquito life cycle are summarized by Service (1993).

A hand dipper is essentially a ladle, usually a white plastic cup with a volume of 350-500 mL, attached to a handle (Figure 1). The number of mosquito larvae can be counted and staged for each dipper sample in the field without the need to preserve the sample; although this approach requires considerable experience identifying genus and stages of larvae. It is preferable to preserve samples and examine the contents in the laboratory. The use of a dissecting microscope (at magnifications between 25-50X) to process samples in the laboratory will permit accurate staging of the larvae and species identification of the late larval instars using Meyer and Durso (1998)² or another key.

In the field, samples are concentrated through a small mesh ($\leq 100 \mu\text{m}$ mesh aperture) screen using a concentrator cup (Figure 1). See the Appendix for instructions to fabricate a concentrator cup. The sample is swirled to reduce the volume of water by forcing excess water through the screen windows in the concentrator cup. Gentle tapping of the screen will help to facilitate the draining of samples that are filtering slowly. The sample is then preserved with 95% ethanol to a final concentration of approximately 50% ethanol. Plastic snap-cap vials with a volumetric capacity of $\sim 55 \text{ mL}$ work well for preserving field samples that are a composite of 3-5 dipper samples. Snap-cap vials should be labeled using pencil or indelible ink; the minimum information on the label should include the marsh, sample site, and date.



Figure 1. Picture of a dipper, a concentrator cup, and a snap-cap vial. Photos by M. R. Sanford.

It is important that sampling is standardized to the greatest extent possible. While dipping is a fairly simple, straight-forward method for sampling the aquatic stages of the mosquito life cycle, dipping techniques differ among individuals and among habitat types. Differences in dipping

² The recent split of the genus *Aedes* into *Aedes* and *Ochlerotatus* occurred after this key was published.

technique among individuals can result in significant differences in the numbers of immature mosquitoes collected. When > 1 person is taking dipper samples, standardization of sampling among individuals is strongly recommended. (1) When possible, the same individual should sample the same group of habitats on each sampling date. (2) A particular individual should not sample only wetlands that are in one particular treatment group. If this were done, differences in dipping techniques among individuals might contribute significantly to the variation in mosquito counts between BMP treatments. (3) In a particular mosquito habitat, a single method of dipping should be used. If more than one habitat is being sampled and each habitat requires a different dipping technique, then consistency of sampling for the respective habitats is recommended. The same dipping technique (see below) should be used by persons taking samples in particular mosquito microhabitats; although the dipping technique might differ among microhabitats. A worst case scenario might include one sampling method used in habitats that have undergone BMP modifications and a second method used in control (non-BMP) habitats.

Standardization of dipping technique should include, at a minimum, a discussion of dipping techniques among the individuals taking the samples. To appreciate the differences in dipping technique, it might be instructive to have individuals sample the same habitat repeatedly (e.g., a wading pool with vegetation and mosquito larvae) and record the number of larvae in each replicate of 5-10 samples per person. If this is done, be sure to allow an adequate time after replacing mosquito larvae (> 1 minute) to permit larvae to return to the water surface between samples.

3.2 Dipping techniques

There are several techniques used to sample immature mosquitoes with a dipper. The differences in techniques are primarily a function of the aquatic microhabitat being sampled and mosquito genera found in a particular microhabitat. Water depth, vegetation type (i.e., emergent, floating, vs. submerged) and density are important factors influencing dipping among microhabitats where immature mosquitoes are typically found.

Collins and Resh (1989) describe two general styles of dipping: quick and slow dips. The two styles of dipping are appropriate for different mosquito species and microhabitats. The quick dipping technique is most appropriate for most mosquito species, but especially *Culex* larvae, and is applicable in most types of vegetation. The slow dipping technique is most appropriate for sampling *Anopheles* larvae (which spend more time at the water surface than do larvae of other genera in California wetlands) and is not suitable when sampling in robust emergent plants because the emergent plants interfere with the dipper as it is skimmed along the water surface. In the slow dipping technique, the dipper is slightly submerged and drawn along a distance that is comparatively longer than that sampled by the quick flick of the wrist used in the quick dipping technique. Up to ten-times more microhabitat is sampled by the slow dip technique than by the quick dip technique. Consequently, more mosquito larvae tend to be collected by the slow dip technique.

O'Malley (1995) categorized dipping techniques into seven types that differ according to the mosquito genera characteristic of the site, water depth, and microhabitat (Table 2). The partial

submersion and complete submersion (modified for sampling *Culex* and *Culiseta*) techniques work well in most habitats supporting emergent vegetation.

Proceed slowly and carefully to avoid disturbing the water surface as much as possible. Waves or vibrations from footsteps or rapid changes of the light regime caused by casting a shadow or by increasing light levels when opening vegetation will stimulate immature mosquitoes to dive from the water surface. The sensitivity of larvae to disturbance differs appreciably among wetland mosquito species (Workman and Walton 2003). Try to approach sampling sites while facing the sun, using quiet slow steps, moving vegetation only as necessary and from downstream if water flow rates are high enough to merit doing so (but this factor is unlikely to be important in the large wetlands on most Wildlife Areas).

Immature stages (larvae and pupae) of mosquitoes commonly found in wetlands are usually found **at the water surface** and **associated with vegetation or debris on the water surface**. Larvae of species associated with organic enrichment, such as *Culex stigmatosoma*, *Cx. quinquefasciatus*, and *Cx. restuans*, can be occasionally found in large numbers in the open water adjacent to emergent vegetation; however, even these larvae tend to be found at the margins of habitats and not in deep, open water. Immature mosquitoes can be concentrated in vegetated regions of habitats by prevailing winds. Dipping should be focused near objects providing physical structure at the water surface such as floating and emergent vegetation and debris floating at the water surface. Ideal microhabitats for immature mosquitoes provide protection from predation and from disturbance (e.g., wave action). These habitats can include downed, floating vegetation and root masses (e.g., *Typha* spp.) that become exposed as water levels decrease.

3.2 What to count and identify to species

The numbers of young larvae, old larvae and pupae should be recorded. While it is preferable to separate the mosquito larvae in samples into the four stages, it is also time consuming to do so. Separating the larvae into two subpopulations, young larvae (1st and 2nd instars) and old larvae (3rd and 4th instars) can be done comparatively quickly and usually aids interpretation of population trends in subsequent analyses. Because larval head capsules are sclerotized and change size abruptly at each molt, larvae can be readily separated into stages using the relative size of the head capsule. (An absolute measure the head capsule width can be obtained using a calibrated ocular micrometer!) The width of the head capsule, across the eyes and perpendicular to longitudinal body axis, is the most reliable way to distinguish among the larval stages. Body size is not always reliable for separating larvae among stages, especially for newly molted 3rd and 4th instars when body size is small relative to head capsule width. Genus-level categorization is recommended for young instars and pupae.

In the unlikely instance that a sample contains several hundred larvae, it is permissible to count a subsample of the larvae. A known proportion of the sample should be counted. The use of a counting chamber or a dish with a grid marked off to $\frac{1}{4}$, $\frac{1}{2}$, and $\frac{3}{4}$ of the sample is recommended. If a subsample is counted, then it is imperative that a random subsample is enumerated. Larvae should be equitably distributed across the bottom of the dish or be in representative abundance within the subsample withdrawn from the sample. Elliott (1977) and

Table 2. Seven techniques of hand-dipping modified from those described by O'Malley (1995).

| Dipping Method | Mosquito genera targeted | Method | Notes |
|---------------------|--|--|--|
| Shallow skim | <i>Anopheles</i> | The leading edge of the dipper is submerged at approximately 45° and about 1 inch (2.5 cm) below the water surface. The dipper is drawn along the water surface and filled at the end of the stroke. | This method is analogous to the slow dipping technique of Collins and Resh (1989). It works better for sampling <i>Anopheles</i> larvae because they remain at the water surface comparatively longer than do <i>Culex</i> , <i>Aedes</i> and <i>Ochlerotatus</i> larvae after the dipper enters the water. A good technique for sampling submerged macrophytes that have leaves just below the water surface. |
| Complete submersion | <i>Aedes</i> , <i>Ochlerotatus</i> , <i>Psorophora</i> (<i>Culex</i> , <i>Culiseta</i>) | The dipper is submerged quickly in open water, usually in floodwater habitats. The dipper is brought up to water surface through the submerging larvae that have reacted to the disturbance created by submerging the dipper. | This method is used primarily to sample mosquitoes whose larvae respond rapidly to the dipper entering the water, but are visible. (This technique also is appropriate for sampling larvae adjacent to vegetation. The dipper is brought to the water surface while contacting the emergent vegetation.) |
| Partial submersion | <i>Culex</i> , <i>Culiseta</i> | The dipper is submerged at approximately 45° along the emergent vegetation. Water flows rapidly into the dipper. The dipper is not moved horizontally. The dipper can be moved vertically to scrape along the edge of emergent vegetation. | This method works well when sampling in robust emergent vegetation such as cattail and bulrush. The suction created by water flow into the dipper and scraping also collects small insect predators and herbivores associated with mosquito larvae on or near the vegetation. |
| Flow-in | <i>Aedes</i> , <i>Ochlerotatus</i> (<i>Culex</i>) | This technique is used in shallow water that has a depth < the height of the ladle on the dipper. The bottom of the dipper is pushed into the substrate and the water with associated larvae and debris are allowed to flow into the dipper. | This method works well in shallow habitats, root masses and other habitats that are shallower than the dipper's profile. |
| Scraping | <i>Coquillettidia</i> , <i>Mansonia</i> | The dipper is scraped against the underside of floating vegetation to dislodge attached larvae. The scraping action is usually a vigorous back-and-forth motion. | Used to sample larvae that reside under, and usually attached to, the underside of floating vegetation or the roots of floating plants. Because a vigorous back-and-forth motion is used with the dipper completely submerged, this technique works best with dippers having a screened bottom. |
| Simple scoop | <i>Culex</i> | A quick flip of the wrist is used to submerge completely the dipper just below the water surface. The technique is similar to taking water to drink. | Not a preferred method, especially if the sample is not taken adjacent to a mosquito microhabitat. Might be closest to the quick dip technique of Collins and Resh (1989). This technique would be adequate in hypereutrophic situations where the abundance of larvae often approaches 1000/dip. |
| Background | <i>Aedes</i> , <i>Ochlerotatus</i> | The dipper is used to provide a light background against which darker colored immature mosquitoes are more easily seen. After mosquitoes are found, they are collected by quickly pulling the dipper through the water surface. | A technique used primarily to identify mosquitoes inhabiting woodland ponds and pools. |

Downing and Rigler (1984) discuss subsampling and the statistical evaluation of counts to assure a random sample has been taken. Generally, processing subsamples until between 50 and 100 larvae have been counted provides a sufficient measure of total larval mosquito abundance. Samples should be then scanned for rare species.

Identify the 4th instars to species; often the 3rd instars can be assigned to species. It will be assumed that the relative abundance of species among the older instars provides a reasonable approximation of the species composition for the younger instars. If desired or required for identification, a representative sample of live pupae can be returned to laboratory to collect adults upon emergence.

To minimize errors in data transcription and reproduction, standard data sheets and forms of notation should be used for all data records. The person(s) collecting particular samples should be recorded.

3.3 Sampling frequency

When monitoring mosquito abundance for mosquito control activities, the minimum number of times sampling should occur is once during the time interval between the egg and adult stages for a particular cohort of mosquitoes. More frequent samples are often needed. The development rate of immature mosquitoes is strongly dependent on water temperature; the warmer the temperature, the faster immature mosquitoes develop to adulthood. However, water temperatures above 34°C are usually detrimental to mosquitoes common in California wetlands. Low food levels, often associated with high densities of 3rd and 4th instars, can significantly slow immature development. Development time (the inverse of development rate) for the period from when an egg is laid until emergence as an adult for California wetland mosquitoes is approximately 10-12 days at summer water temperatures (25-30°C). Semiweekly samples (or even more frequently) would be required to study the mortality schedules of the two larval subpopulations. Samples taken once per week will be sufficient to document mosquito population trends. Whereas weekly samples are preferred to less frequent sampling, **biweekly samples are probably sufficient to assess differences between wetland management strategies**.

3.4 Stratification according to ecological factors

Stratified random sampling is preferable to simple random sampling (where every location in a wetland has an equal chance of selection as a sampling site) because not all habitats are equally conducive to mosquito production. Moreover, wetlands usually cover a large area and it is impossible to sample adequately in the majority of sites where mosquitoes might be found. Sampling efficiency is increased by dividing the mosquito population in a wetland into several sub-populations or *strata*. A goal of stratification is to minimize the variability of field data by taking into account the effects of different strata on mosquito numbers (Collins and Resh 1989). Because mosquito production differs among strata in a wetland, mosquito abundance in the strata

should be more homogeneous than in the whole wetland. Strata should be well defined areas of known size. If the strata are unequal in area, the sampling sites are divided unequally among strata and the number of sites allocated to each stratum is proportional to the area of the stratum³. Even though an investigator is utilizing knowledge of mosquito ecology to define strata, the sites sampled (= sampling units as per Elliott 1977) must be representative of the whole mosquito population and therefore must be selected without bias.

Stratification of sampling sites should be carried out according to interactions among historic, physiographic, hydrologic and biological factors that strongly influence the distribution and abundance of mosquitoes (Collins and Resh 1989). Each important environmental factor strongly influencing mosquito populations should be stratified with regard to one or more other factors and quantified within each resulting stratum (Table 3). For mosquito density (the factor being quantified in this protocol; category A in Table 3), hydroperiod and vegetation type are characteristics that are likely to be the focus of most of the BMP modifications proposed. Hydroperiod differences are expected to define the treatments (one type of BMP vs. non-BMP wetlands) and, because hydroperiod effects are accounted for by the treatments, stratification of sampling sites by differences in hydroperiod of wetlands within a treatment is unlikely to be important except during flood-up and drawdown phases. If hydroperiod differs among mosquito BMPs (e.g., source reduction was carried out in one group of wetlands with no change in hydroperiod and water conveyance improvements were carried out in a second group of wetlands), then these differences need to be taken into account in the subsequent analyses of mosquito BMP efficacy. Any differences in flooding schedules among wetlands should be noted. The distribution of water relative to sampling sites should also be noted. That is, for sampling during flood-up and drawdown, is a particular site dry?

Stratification of sampling sites by vegetation type is likely to be needed because mosquito production often differs appreciably among vegetation types. The predominant species of aquatic vegetation of a wetland (or field) should be identified and the relative abundance of each species (% cover) estimated. Sampling sites should be distributed among the different vegetation species roughly in proportion to the proportion of the vegetated surface area covered by that species. The vegetation associated with each sampling site should be recorded.

Sampling needs to be replicated within each stratum and should be representative of that stratum. For example, if sampling is stratified by vegetation species then representative samples should be taken from sites within a stand of that plant species and not taken on the edge of the stratum where the effects of open water might influence the number of mosquitoes in the sample.

Other factors (e.g., larval fish density, herbivore density, flow pattern) will be important at some of the BMP sites; sampling and subsequent analysis of mosquito BMP efficacy need to

³ Elliott (1977) stated that the theoretical optimum allocation of units in the sample is one that minimizes the standard error of the estimated mean for a given cost of taking the sample, not the proportional allocation which is used most frequently. The optimum allocation of sampling units is achieved when the sampling fraction for each stratum is proportional to the standard deviation for the stratum. This method is rarely used because standard deviations are hardly ever known before sampling. Moreover, standard deviations will change as the mosquito population abundance changes temporally.

Table 3. Matrix of interactions among ecological factors in non-tidal, palustrine wetlands in relation to ecological mosquito control developed by Collins and Resh (1989: Table 15).

| A. Quantify by these factors | | | | | | | | | |
|-------------------------------------|------------------|-------------------|-----------------|-----------------|-----------------|----------------|-------------|--------------|-------------|
| Water Chemistry | | | | | | X | X | X | X |
| Water Turbidity | | | | | | X | X | X | X |
| Vegetation Type | | | | X | X | X | X | X | X |
| Herbivore Density | | | X | X | X | X | X | X | X |
| Predator Density | | X | X | X | X | | X | X | X |
| Mosquito Density | X | X | X | X | X | | | X | X |
| | Predator density | Herbivore Density | Vegetation Type | Water Chemistry | Water Turbidity | Substrate Type | Water Depth | Flow Pattern | Hydroperiod |
| B. Stratify by these factors | | | | | | | | | |

account for differences in the distribution and abundance of these factors. Flow pattern includes factors such as presence or absence of eddies, position relative to site(s) of inflow, flow rates, etc. Sampling sites should be chosen to minimize these effects (e.g., sampling sites should not be adjacent to water control structures). Strata that are defined by the spatial variability of a factor will also have a temporal component that is influenced by seasonal and other changes in site conditions. Data from strata where mosquito abundance does not differ significantly can be combined during analysis to increase the power of statistical analyses.

3. 5 The number of samples

The number of samples that should be taken depends on the dispersion of the mosquito larvae, the mean number of mosquito larvae per dip, and the size of the acceptable error in the estimate of the population mean. The dispersion of mosquito larvae in developmental sites is usually either clumped or approximately random (Service 1993). Two statistical distributions used to describe the frequency distributions of counts for these dispersion patterns are, respectively, the negative binomial or the Poisson. Acceptable error is the degree of precision for the estimate of the population mean and can be thought of as being related to how small a reduction in mosquito abundance by mosquito BMPs can be detected statistically. While the statistical underpinnings for the calculations of sample size and the additional parameters needed to estimate optimum sample sizes are beyond the scope of this protocol (examples are provided in the Appendix), given that mean larval mosquito abundance in dip samples from wetlands is often < 1 larva per dip and that the variance for larval mosquito abundance is often equal to or greater than the mean, large sample sizes (> 50 in most scenarios) are needed to be able to estimate mean mosquito abundance with a moderate degree of certainty (Table 4).

Table 4. Optimum sample size (n) required for random (Poisson) and clumped (negative binomial) dispersion patterns to estimate the mean abundance of mosquito larvae at several error rates. (ξ) is the mean for number of mosquito larvae per dip. k is the exponent of the negative binomial distribution; clumping increases as k approaches 0.

| mean no./dip (ξ) | | 0.1 | 0.25 | 0.5 | 0.75 | 1 | 2 | 5 | 10 | 20 | 25 |
|------------------------|-----|---------------------|------|-----|------|-----|----|----|----|----|----|
| Poisson | | Sample size (n) | | | | | | | | | |
| 10% error | | 1000 | 400 | 200 | 133 | 100 | 50 | 20 | 10 | 5 | 4 |
| 20% error | | 250 | 100 | 50 | 33 | 25 | 13 | 5 | 3 | 1 | 1 |
| 40% error | | 63 | 25 | 13 | 8 | 6 | 3 | 1 | 1 | 1 | 1 |
| Negative binomial | k | | | | | | | | | | |
| 20% error | 0.5 | 300 | 100 | 83 | 75 | 63 | 55 | 53 | 52 | 51 | 51 |
| 20% error | 1 | 275 | 75 | 58 | 50 | 38 | 30 | 28 | 27 | 26 | 26 |
| 20% error | 2 | 263 | 63 | 46 | 38 | 25 | 18 | 15 | 14 | 14 | 14 |
| 20% error | 3 | 258 | 58 | 42 | 33 | 21 | 13 | 11 | 10 | 10 | 9 |
| 40% error | 2 | 66 | 16 | 11 | 9 | 6 | 4 | 4 | 4 | 3 | 3 |

One needs to balance the tradeoff between statistical accuracy and the time spent collecting and processing samples. Elliott (1977) recommends taking 5 sampling units (where sampling units are replicate samples within a random sample; dipper samples here) at random and calculating the arithmetic mean. Then take 5 more sampling units at random and calculate the mean for 10 unit samples. Repeat for intervals of 5. When the mean value ceases to fluctuate, then an adequate number of sampling units have been taken for a particular sampling site. This approach is somewhat impractical for surveys of large areas. Elliott (1977) and Sokal and Rohlf (2002) provide examples of estimating sample size for a given level of precision.

If historical data of mosquito abundance are available, or if baseline data are collected, one can use these dipper count data to estimate the optimum number of samples per wetland using the mean for number of larvae per dip and the 20% error rate for the Poisson or negative binomial with $k = 2$ in Table 4. However, if historical data are to be used in before-and-after comparisons, then the sampling protocol should follow that used previously. If the staff who collected the previous (“before”) samples are available, then they should collect the samples for the “after” component of the study. Even though larval mosquito abundance is probably based on total numbers of larvae in the “before” samples, separating the larvae into the two subpopulations in the “after” samples is still worthwhile; numbers can always be summed to estimate total larval mosquito abundance. If the number of samples is too large for personnel to collect and process given time and/or budgetary constraints, then an alternate approach is needed.

An alternative approach is to establish 50 sampling stations per wetland. At each station, 3-5 dipper samples are taken at random within a 2-5 m area circumscribing each sampling station and combined into one sample for the sampling station. This is a form of stratified, systematic sampling that has been used successfully in large wetlands to monitor mosquito population trends by repeatedly sampling particular vegetation types at particular stations (Keiper et al. 1999, 2003); this sampling was supplemented with less frequent basin-wide surveys of mosquito abundance to confirm that focal sampling on comparatively few stations provided a reasonable

measure of mosquito abundance and that hot-spots of mosquito production did not exist elsewhere in the large (~ 500 acres) wetlands. Focusing sampling at predetermined sampling locations is technically is not truly random sampling because pre-established sampling stations are being used (i.e., not assigned randomly) and replicate samples are taken haphazardly (replicate samples at each sampling station are not determined using a set randomization procedure) at each sampling site. More than 50 sampling stations can be established if needed.

Stations should be stratified among vegetation types and located where the mosquitoes tend to be abundant such that larval counts are indicative of the maximum mosquito density for each collection date. It is standard practice not to use open water as a stratum in sampling programs for immature mosquitoes. Because some of the mosquito BMPs will eliminate emergent vegetation, it will be informative to estimate total mosquito production for the site; to do so requires an estimate of the relative coverage of each stratum (different vegetation types/species and open water) as well as an estimate of mosquito production from each ecological stratum. Therefore, including a minimum of 3 sampling sites in open water (in addition to the minimum 50 in vegetated habitats) will provide a record of mosquito abundance in the open water ecological stratum. Such are samples are relevant (even if the samples do not contain mosquitoes) because increasing the amount of surface area as open water is expected decrease overall mosquito production.

To estimate the mean mosquito abundance and the variation around that mean, calculations need to be corrected for the number of replicate sampling sites in each stratum. Comparisons among sites should standardized to a per dip measurement, so numbers will need to be corrected for the number of replicate dips taken at each sampling site. Therefore, it is important to use a consistent number of replicate dipper samples. If there are s strata, with proportional stratified sampling, the weighted arithmetic mean used to estimate the population mean is calculated as

$$\xi = \frac{\sum x}{n} = \frac{n_1\xi_1 + n_2\xi_2 + n_3\xi_3 + \dots + n_s\xi_s}{n}$$

where ξ_s is the arithmetic mean of mosquito abundance in each stratum, n_s is number of sampling sites in a stratum and n is the total number of sample sites. The overall mean is corrected for number of dips.

To calculate the variation around the mean, the following calculation is used:

$$\text{standard error of mean} = (1/n) \sqrt{n_1s^2_1 + n_2s^2_2 + \dots + n_s s^2_s}$$

where s^2_s is the variance for each stratum.

4. Sampling to Assess Adult Mosquito Abundance

Surveillance of adult host-seeking populations might be useful if the effects of mosquito BMP implementation operate on a scale larger than the flight range of the mosquitoes. Standard surveillance techniques used by the vector control districts should be followed.

5. Summary

- The primary mosquito BMP modifications to existing wetlands in California Department of Fish and Game Wildlife Areas are (1) source reduction through vegetation removal/reduction, (2) improved water conveyance through several approaches, (3) enhancement of populations of biological control agents (mosquitofish, *Gambusia affinis*) for immature mosquitoes and (4) altering the timing of flooding to reduce overlap with peak annual activity of mosquitoes.
- Two general types of comparisons can be made to evaluate the effects of mosquito BMPs on changes in mosquito numbers: (1) before-and-after comparisons or (2) wetlands with mosquito BMPs versus wetlands without mosquito BMPs. The latter comparison involves the contemporaneous operation of the two wetland types. Before-and-after comparisons necessitate that adequate historical data are available and that current methods of mosquito monitoring complement those of the historical data set.
- Multiple BMPs are often being applied to particular wetlands on a refuge and, in most instances, there is insufficient replication to be able to assess the efficacy of each BMP for reducing mosquito populations independently. It is therefore important to replicate the same BMP(s) in as many sites as possible.
- Larval mosquito counts collected using a hand dipper are the preferred type of abundance data for evaluating the efficacy of mosquito BMPs. While dipping is a fairly simple, straight-forward method for sampling the aquatic stages of the mosquito life cycle, dipping techniques differ among individuals and among habitat types. It is important that sampling is standardized to the greatest extent possible.
 - The numbers of young larvae, old larvae and pupae should be recorded.
 - Identify the 4th instars to species; identify young larvae and pupae to genus.
 - Biweekly samples are probably sufficient to assess differences between wetland management strategies.
 - The optimum number of samples per wetland can be estimated using the mean for number of larvae per dip and the 20% error rate for either the Poisson distribution or negative binomial distribution with $k = 2$ in Table 4.
 - An alternative approach is to establish a minimum of 50 sampling stations per wetland. At each station, 3-5 dipper samples are taken at random within a 2-5 m area circumscribing each sampling station and combined into one sample for the sampling station.
 - Stratify sampling sites by vegetation type.
- To evaluate fully the success of the BMPs, it is critical not only to document changes in mosquito abundance and/or production but also to assess the changes in the processes and features that potentially influence mosquito numbers in each Wildlife Area. An accurate assessment of these factors (e.g., vegetation distribution, hydroperiod, vagaries of flooding regime) is also needed to assist evaluation of any changes in wildlife usage or habitat quality. While the protocols for measuring these environmental factors are not addressed here, refuge

and mosquito abatement personnel are encouraged to work together to collect as much of these data as is possible.

References

- Collins, J. N. and V. H. Resh. 1989. Guidelines for the Ecological Control of Mosquitoes in Non-tidal Wetlands of the San Francisco Bay Area. CMVCA, Inc. and U.C. Mosq. Research Program. Berkeley.
- Downing, J. A. and F. H. Rigler (eds.) 1984. A Manual on Methods for the Assessment of Secondary Productivity in Fresh Waters. 2nd ed. IBP Handbook. Blackwell Scientific Publications. Oxford.
- Elliott, J. M. 1977. Some methods for the statistical analysis of samples of benthic invertebrates. 2nd ed. Freshwater Biological Association Scientific Publication. No. 25. Cumbria, U.K.
- Keiper, J. B., J. Jiannino, J. Beehler, and W. E. Walton. 1999. Distribution and abundance of Culicidae and Chironomidae (Diptera) following storm damage in a southern California constructed wetlands. Proc. Mosq. Vector Control Assoc. Calif. 67: 47-54.
- Keiper, J. B., J. A. Jiannino, M. R. Sanford, and W. E. Walton. 2003. Effect of vegetation management on the abundance of mosquitoes at a constructed treatment wetland in southern California. Proc. Papers Mosq. Vector Control Assoc. Calif. 70: 35-43.
- Meyer, R. P. and S. L. Durso. 1998. Identification of the Mosquitoes of California. Mosquito and Vector Control Association of California, Elk Grove, CA.
- O'Malley, C. 1995. Seven ways to a successful dipping career. Wing Beats 6(4): 23-24.
- Service, M. W. 1993. Mosquito Ecology: Sampling Methods. 2nd ed. Chapman and Hall. London.
- Sokal, R. R. and F. J. Rohlf. 2002. Biometry: The Principles and Practice of Statistics in Biological Research. 3rd ed. Freeman. N.Y.
- Workman, P. D. and W. E. Walton. 2003. Larval behavior of four *Culex* (Diptera: Culicidae) from treatment wetlands in southwestern United States. Journal of Vector Ecology 28: 213-228.

Appendix

A.1 Concentrator cup fabrication

A concentrator cup can be easily fabricated using a 1-cup (~250 mL) plastic kitchen measuring cup. A modified soldering iron can be used to cut 2 windows in the measuring cup. Do not extend the windows to the bottom of the cup because, when sampling in the field, a small amount of water needs to be retained to facilitate transfer of the sample to the vial. Small-mesh metal screen can be affixed to the inside of the cup to the plastic by melting the plastic along the edge of the window or by gluing the screen to the cup.

A.2 Optimum number of samples

For a particular standard deviation (s), the standard error is a function of the number of sampling units (n) within a random sample. The ratio of the standard error ($\sqrt{s^2/n}$) to the arithmetic mean (ξ) is an index of precision (D). If a standard error of 20% can be tolerated, then n for a random sample is given by the formula:

$$n = \frac{s^2}{D^2 \xi^2} = \frac{s^2}{0.2^2 \xi^2} = \frac{25 s^2}{\xi^2} \text{ for a 20\% error}$$

For a Poisson series:

$$D = 1/\sqrt{(n\xi)}$$

The precision of the estimated population mean therefore depends on the number of animals in the sample! A tolerable error of 20% requires the product of $n\xi$ to equal 25 ($= 1/0.2^2$). The number of sampling units is $25/\xi$ for a 20% error.

Approximate optimum sample sizes (n) for a 20% error and for the mean number of individuals per dipper sample (ξ) for the Poisson distribution are:

| | | | | | | | | | |
|-------|-----|------|-----|------|----|---|----|----|----|
| ξ | 0.1 | 0.25 | 0.5 | 0.75 | 1 | 5 | 10 | 20 | 25 |
| n | 250 | 100 | 50 | 33 | 25 | 5 | 3 | 2 | 1 |

For a negative binomial distribution, the optimum sample size (n) is estimated as:

$$n = (1/D^2) [(1/\xi) + (1/k)]$$

$$\text{for a 20\% error: } n = 25 [(1/\xi) + (1/k)]$$

For $k = 2$ and a 20% error, the optimum sample sizes for the mean number of individuals per dipper sample (ξ) are:

| | | | | | | | | | |
|-------|-----|------|-----|------|----|----|----|----|-----------|
| ξ | 0.1 | 0.25 | 0.5 | 0.75 | 1 | 5 | 10 | 20 | ≥ 50 |
| n | 263 | 113 | 63 | 46 | 38 | 18 | 15 | 14 | 13 |

This sampling effort would be equivalent to tolerating 95% confidence limits of $\pm 40\%$ of the true value because the formula for n has to be multiplied by t^2 where t is the Student's t distribution (~ 2) and $D = 0.4$. If the tolerable 95% CLs are reduced by half, to $\pm 20\%$ (which is equivalent to a standard error of $\sim 10\%$ of the mean), four times the sampling units are required!

A.3 Sampling vegetation

Mosquito production is intimately linked to wetland vegetation that provides immature mosquitoes refuge from predation and disturbance. Emergent macrophyte density and species composition can be quantified using 0.25 m² or 1 m² quadrats. Quadrats should be selected randomly from the regions supporting emergent vegetation. A map of the vegetation in the habitat is required. The position of each quadrat is determined using a random numbers table or using the random numbers functions found in spreadsheet software. For the latter approach, all possible quadrats are assigned an individual number and quadrats are then selected using a random number function that allows the specification of the range of numbers across which selection takes place. If quadrats are selected more than once, then the selection process is repeated for those quadrats until quadrats occur only once in the group to be included in the census. The numbers of live standing, dead standing, and dead floating stems of emergent macrophytes can be assessed within the area of each quadrat. Vegetation types (i.e., emergent, submerged, floating) and species should be noted and can be used to assign ecological strata within the wetland.

A comparatively more complex approach to quantifying vegetation is provided by Collins and Resh (1989). The three-dimensional architecture of vegetation and the intersection line value will determine the quality and quantity of mosquito habitat. Intersection line value is equivalent to the density of menisci (potential hiding places for mosquito larvae) created by plants on or penetrating through the water surface. Vegetation data should be collected at permanent stations within the wetland and should be the same stations that are used to collect mosquito larvae. Seasonal changes in the vegetation, such as growth and senescence, will influence its potential as mosquito habitat. The impact of these comparatively long-term changes in the vegetation on mosquito abundance will be influenced by comparatively short-term factors such as changes in water quality or herbivory.

Vegetation maps should indicate the areas of the wetland covered by different plant species and may also indicate vegetative conditions. Architecture of vegetation can be described by the vertical patterns of change in plant growth habit (i.e., the angle of stem growth relative to the water surface) and standing crop (oven-dry or fresh weight of plant tissue per volume or surface area of habitat). Collins and Resh (1989) provide a discussion of the calculation of plant architecture index, intersection line density, and an index of the overall condition of mosquito habitat within a wetland for comparisons among sites.