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Propagation Handbook for Lange's Metalmark Butterfly, *Apodemia mormo langei*

First edition, August 2007

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1 Introduction

Lange's Metalmark butterfly ("LMB"), *Apodemia mormo langei*, is experiencing a dramatic population decline of unknown cause. Captive propagation will serve as an insurance policy against extinction in the wild while the conservation efforts at the Antioch Dunes become more active over the next few years. The propagation effort will also afford the opportunity to study the species and increase its population size, with the goal of reintroducing captive bred individuals to the wild.

This handbook is meant to serve as a flexible guideline for propagation efforts. There will be two rearing sites to guard against a stochastic event wiping out the entirety of the captive stock. Should either site experience difficulties, the other facility will contribute material to ensure two separate healthy captive populations. Use of multiple sites offers variation in rearing methods as well. This handbook is written to allow for multiple approaches to rearing, as well as modification to rearing methods as captive propagation proceeds. Each rearing season is a learning experience and methods should be adjusted as needed. The techniques presented here are the result of a collective effort by the U.S. Fish and Wildlife Service (USFWS) and the LMB working group.

Found only on the Antioch Dunes National Wildlife Refuge, LMB has a very restricted range. Located about 40 miles to the northeast of San Francisco, this 67-acre refuge has been seriously degraded by past human activities. Following the great fire of 1906, the city of San Francisco was largely rebuilt with bricks made of sand mined from the Antioch Dunes. This sand mining, coupled with industrial development, and invasion by exotic plant species has destroyed much of the butterfly's habitat. In 1976, the Lange's metalmark was listed as an endangered species under federal law.

Lange's metalmark was recognized as a subspecies by Comstock (Comstock 1938). It is univoltine, breeding once per year in synchronization with the flowering of their only host plant, naked-stemmed buckwheat, *Eriogonum nudum auriculatum.* Adult butterflies emerge from their

pupae in late summer (Arnold 1981, 1983). During their roughly one week lifespan in the wild, the butterflies mate and the females oviposit their eggs in small clusters directly onto the buckwheat. The eggs are thick-walled and it is difficult to differentiate between fertile and infertile eggs. The larvae develop within the egg (it is not a true egg diapause) and hatch at the onset of fall rains. The first instars will feed on the new, tender plant growth. During the winter, the larvae enter a period of dormancy. Through the spring and summer, the larvae resume feeding and mature before metamorphosing into pupae. About two weeks later, the adult butterflies emerge and begin the cycle anew (Arnold 1981, 1983).

The precise cause of the recent sharp decline of Lange's metalmark remains uncertain. Suspected factors include anthropogenic fire and invasion of the habitat by non-native plants, both of which impact the buckwheat on which the butterfly depends (Smith and Hurt 2005). Other possibilities include microclimactic changes due to the loss of sand, loss of natural disturbances, low perch site availability, and low alternative nectar source availability. Restoration of the habitat is an ongoing and vitally important step toward species recovery, including providing suitable sites for reintroduction of the captive stock.

2 Propagation of host plant – Naked-stemmed Buckwheat, *Eriogonum nudum auriculatum*

USFWS cultivated seed and distributed seedlings to The Native Seed Bank with the San Diego Zoo for propagation.

2.1 Taxonomy

Eriogonum nudum Benth. is an herbaceous perennial to 2 meters and is common to dry open areas in many plant communities throughout California. The species is a known food plant for several butterfly species, including members of the genera *Apodemia*, *Euphilotes*, and *Gaeides*. The species *E. nudum* is highly variable and contains 13 described varieties. *E. nudum* var. *auriculatum* (Benth.) Jepson is an herbaceous perennial to 1.5 meters, common to many plant communities of central western California. It is known from an elevational range of 0–1200 meters, with its approximate center of range being Alameda County. This taxon has been described as the host for the federally endangered Lange's metalmark butterfly (*Apodemia mormo langei*), and while

we accept this naming convention herein, the reader is cautioned that the taxon known from the Antioch sand dunes of Contra Costa County may indeed be a yet undescribed variety within the complex and intergrading *E. nudum* group.

The propagation of *E. nudum* var. *auriculatum* for use in Lange's metalmark butterfly *ex situ* rearing efforts will consist of four project elements: seed collection, seed processing and storage, germination, and plant grow-out.

2.2 Seed collection

The typical flowering period for *E. nudum* var. *auriculatum* ranges from May through October, with an expected peak seed set in September. General plant phenology, onset of flowering, and seed maturation will be monitored in the field by U.S. Fish and Wildlife Service Refuge personnel. Seed maturity will be informed by a "seed cut test." For this test, a small representative subset of the seed available on any given day will be cut longitudinally and inspected by use of a hand magnifier to determine ripeness. Seed will be collected from mature inflorescences when the cut test reveals hard seed with a high fill percentage. All seed for this project will be collected at the Antioch Dunes National Wildlife Refuge. Seed will be collected from a minimum of fifty individuals. No more than five percent of the seed available during any collection event will be taken. Seed will be stored temporally in open containers, or in closed containers containing silica desiccant, and protected from temperature extremes (i.e. direct sun, closed vehicles, etc.) until it can be transported to the CRES Native Seed Gene Bank (NSGB) for cleaning and additional processing. Seed may be collected throughout the maturation period to form a single aggregate collection.

2.3 Seed processing

Upon receipt at NSGB, collected materials will be processed per standard cleaning protocols. Chaff and other debris will be separated from the seed and discarded. A subset of the collection will be tested for filled-seed percentage by the cut test method. The collection will be stored in a desiccation chamber and dried to 10–15% total moisture. Following desiccation, an approximate seed count will be made using a 500 seed count weight. The dried seed collection will be stored at –20˚ C, until use.

2.4 Germination

Germination testing will be conducted on the post-processed seed collection at the CRES Beckman Center. A standard germination test will be conducted consisting of a twenty-four hour room temperature imbibition period followed by agar planting and an alternating temperature/light regime of 28˚ C /light and 10˚ C /dark. Tests will be conducted on four replicates of twenty-five seeds (4X25) and score weekly. Alternative methods will be determined (e.g., stratification, scarification, etc.) if standard test results are unsatisfactory.

Greenhouse germination will be conducted at the CRES Botanical Conservation Center. Seeds will receive a twenty-four hour imbibition, followed by sowing on a suitable germination media (e.g., SunGro Sunshine Mix #3). Seeding trays will be kept uniformly moist in a shaded ambient outdoor temperature greenhouse. Seedlings will be transplanted to 2 1/4 X 2 1/4 X 4 inch "rose" pots. Potting media will be well-draining and made up of a 1:1 mixture of SunGro Sunshine Mix #3 and coarse sand (or a equivalent mixture).

2.5 Plant grow-out

Rose pot seedlings will be monitored for root development and transplanted to one-gallon commercial nursery pots when warranted. Transplants will be held under fifty percent shade for two weeks before removing to full sun for hardening off and grow-out. No pesticides are to be used during any stage of nursery propagation. Any outbreak of undesirable insects may be controlled by washing and/or hand picking. Outbreaks of undesirable soil organisms may be controlled by transplantation and solar sterilization of infected soils.

Plants should be groomed for removal of invertebrates prior to use with captive LMB stock. This grooming should be vigilantly maintained throughout the rearing season. Plants exposed to captive LMB stock will subsequently be watered with a subterranean delivery system to limit the threat of drowning larvae.

2.6 Surrogate Host Plant

There is the possibility to use surrogate foodplants with LMB captive stock (Arnold 1981). Pratt and Ballmer used *Eriogonum fasciculatum foliosum* successfully in captive rearing *Apodemia*

mormo subspecies (Pratt and Ballmer 1991). However, *Eriogonum fasciculatum fasciculatum* has been implicated in mass larval death of *Apodemia mormo mormo* (Pratt, pers. comm.). Dunn (pers. comm.) notes that each variety is found in sympatric distribution with its neighboring varieties and hybridization is common. Add to this that these variants are often difficult to distinguish morphologically (Flora of North America Editorial Committee 1993+) and caution should be exercised when employing surrogate foodplant.

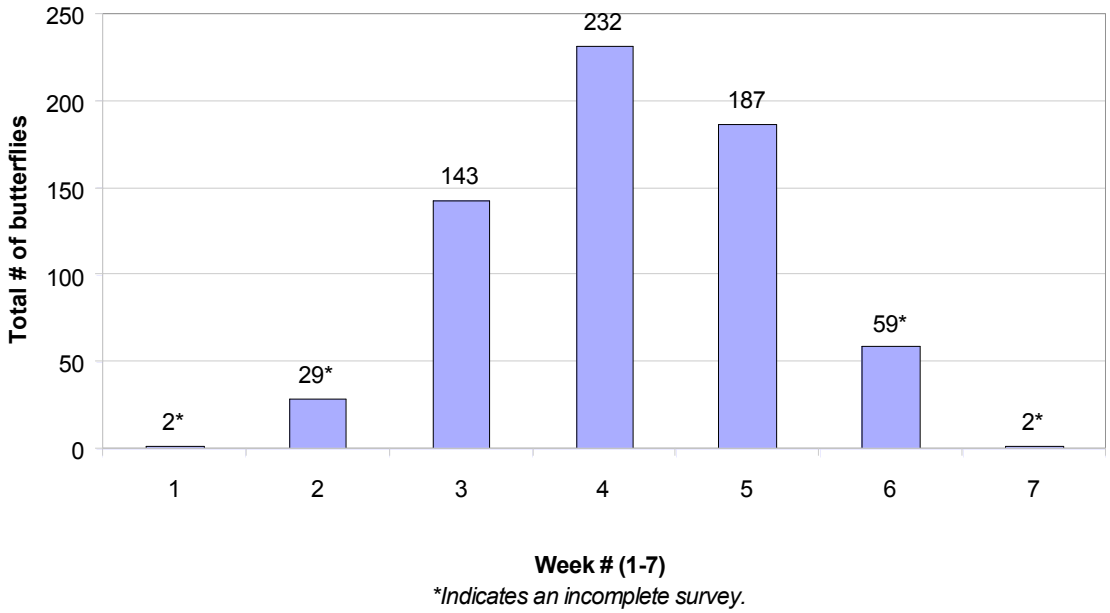

Figure 1. Weekly counts of Lange's metalmark at Antioch Dunes NWR (Smith and Hurt 2005). Week 1 started August 11.

3 Collection of Adults

Collection should occur immediately after peak flight. This will allow determination of the number to be collected (based on a percentage of the maximum number observed in a day) and limit the effect on the wild population. USFWS begins the surveys prior to the flight period and surveys weekly thereafter. USFWS should notify the rearing facilities of their weekly survey results. Collection will occur immediately following peak flight (usually the first week of September based on past data, although this varies with the weather; Figure 1) (Smith and Hurt 2005).

Each captive rearing facility will collect their own stock.

Six females or 10% of peak flight, whichever is greater, will be collected and split between the rearing facilities. 10% of peak flight should be less than 3% of the total wild population based on a comparison of results between peak flight count and mark and recapture studies. We tested this relationship also with survey data for the Palos Verdes blue butterfly (*Glaucopsyche lygdamus palosverdesensis*) (Longcore 2007) and found that 10% of peak flight was 1.34±0.58% of total brood size for the years 1994–2006, when total brood size was estimated from transect counts (Mattoni et al. 2001).

Additional collection of 1–2 males will occur for mating (see below).

To maximize genetic diversity, collection should occur throughout the remaining occupied habitat. Therefore, no more than half of the females in a given location should be collected. This will also serve to minimize the effects of collection on the wild population.

Females collected to serve as founders should be slightly older to insure they are gravid. They should not have extensive wing wear as they may be too old or stressed to serve effectively as founders for the propagation efforts. If a fresh female is observed, she should be collected only if a successful mating is observed.

The adults will be fed with artificial nectar as soon as possible after capture. Artificial nectar consists of a buckwheat honey/water solution.

The adults will be assigned stud numbers immediately to insure each maternal gene line is isolated and tracked. "F1.1" is an example of this notation with The Butterfly Project. "F1" indicates female 1. The number after the decimal indicates the sequential numbering of the oviposition container that the female is housed in. In this case female 1 was in her 1st oviposition container. If there are at least one dozen eggs observed in an oviposition container, the laying female is transferred to a new oviposition container to prevent overcrowding the larvae when they hatch Information regarding their capture date, capture location, condition upon capture, and behavior regarding feeding in the field will be recorded and maintained in the studbook.

Each rearing site will keep detailed records to maintain gene line data. One possibility for this is the software program PopLinks although a spreadsheet is sufficient. As the season progresses, data regarding longevity and fecundity should be maintained in the studbook.

Female butterflies will be packaged in their first oviposition container on cut wild foodplant with the stems inserted into flower vials to prevent wilting. Females should be carefully monitored to prevent overheating or prolonged chilling.

3.1 Transport

The adults will be transported back to the rearing facilities by automobile at night to limit stress (this will avoid altering photoperiod and limit and potential temperature shock).

4 Adults in Captivity

Upon arriving at the rearing facilities, the adults will be reared according to each rearing facility's methods.

4.1 Methods for The Butterfly Project at Moorpark College

4.1.1 Qualifications

Prior to handling LMB captive stock, research associates are required to prepare a Curriculum Vitae, which is submitted to US Fish and Wildlife Service, Sacramento office for the purpose of subpermiting the research associates. Research associates are also required to study the Propagation Protocol and pass a test for each section regarding the care and maintenance of LMB).

4.1.2 Facilities and Operations

The rearing will be performed in and around the greenhouse on America's Teaching Zoo grounds. This is a secure facility with 24-hour surveillance. The lab is kept at a constant humidity level with a swamp cooler. The temperature of the greenhouse is maintained with an air conditioning unit, space heater, and "blackout" cloth that blocks 100% of the light from the roof of the greenhouse.

Firm greenhouse rules are established. These rules included addressing security of the butterflies (keeping the door shut, posting warning signs, entering associates waiting for clearance by associates already working inside), exclusion of predatory threats (no food/drink, immediate extermination/removal of any other species including but not limited to spiders, earwigs, ants and

aphids) and shoes are excluded from the greenhouse to decrease the amount of foreign material introduced.

4.1.3 Oviposition Containers

The LMB founder females will be housed individually and clearly labeled with their studbook number. They will be alternated between multiplant boxes and oviposition containers to determine which they prefer for oviposition (there can be variation in preference to containment structure). The oviposition containers afford greater control to the keepers, but females frequently prefer the multiplant boxes which afford a range of nectar sources and room for flight. The goal is to maximize egg production. Eggs may be harvested out of the multiplant boxes into larval containers to afford greater control over offspring.

Figure 2. Multiplant oviposition box. Note plants are inserted into cutouts, this particular multiplant box will be modified to organza on three sides (no plexiglass roof) and soapy water will be added to the containers surrounding each leg to prevent intrusion by ants.

The multiplant box will be an outdoor oviposition container. It is constructed of wood, knit cloth sleeves and organza (Figure 2). Two sides and the top are covered in organza (cloth with small enough mesh to serve as an exclusion boundary to the smallest parasitoid). The remaining two sides consist of plywood with circular cutouts associated with sleeves to allow instant access to

the captive stock with no chance of escape. The floor is plywood with cutouts for the potted plants to be inserted into (the lip of the pot will secure it in the hole and suspend the plant). The entire multiplant box has sturdy legs that insure that the pots are suspended above the substrate. The legs are in containers of soapy water to prevent access. Inside the multiplant box, there will be two pots of naked-stemmed buckwheat, one surrogate nectar plant (*Encelia virginiensis, Senecio flaccidus* var. *douglasii* or *Bidens laevis*) and one *Lotus scoparius* (a preferred roosting spot for LMB) (Arnold and Powell 1983).

Figure 3. Oviposition and larval containers.

The oviposition container consists of a plastic container with screened ventilation access on two sides and the top (Figure 3). The lid that the container attaches to has a hole cut out of the center. This allows the lid to be wrapped around the stem of a foodplant and secured with duct tape. The lid is supported by a metal support taped to the lid and inserted into the soil of the potted foodplant to prevent mechanical stress on the stem of the foodplant. The portion of the plant extending through that hole and into the oviposition container is monitored for eggs. No more than a dozen eggs are allowed per oviposition container. The oviposition container is easily removed

from its lid to allow access to the female for care and feeding, the eggs for egg counts, and eases the relocation of the female to prevent overcrowding of eggs.

Egg counts will be attempted, however this will be easier in the oviposition containers rather than the multiplant boxes, which do not restrict laying sites for the female. The success of the egg count will depend on the containment preference of each individual LMB female. Egg counts will be documented in the stud book.

4.1.4 Feeding

Adults will be fed twice a day with artificial nectar. Artificial nectar is 1 part buckwheat honey to 3 parts water. It will be presented on a Q-tip® with a yellow "target" around it to aid in training the butterflies to feed and also to minimize the probability of the adults getting nectar residue on them. Multiplant boxes allow feeding within the unit itself. Females in oviposition containers will be fed in the greenhouse in an oviposition container modified specially for feeding. John Emmel has cleaned his butterflies when they have nectar residue on them and this is an option if precautions to avoid this fail (Pratt, pers. comm.).

Hand feeding with a Q-tip® has worked well for *Apodemia mormo mormo* in preliminary trials (Osborne, pers. obs.). If there are difficulties in stimulating a feeding response, a second option is to chill the adults and then unfurling the proboscis with a pin to train them to feed on the artificial nectar source (Pratt, pers. obs.).

4.1.5 Mating in Captivity

Metalmarks are difficult to mate in captivity. One pair has been cage paired in captivity (Pratt, pers. comm.). Successful cage pairing will be a hurdle to overcome for a successful captive propagation program. Wild females will be used initially with the assumption that they are gravid collected. Although there is the possibility of collecting infertile females after the flight season peak – due to temporal disjuncture in gender eclosion (Calabrese and Fagan 2004) and due to mere chance (Osborne, pers. obs.). Chilling of gravid females could result in sperm death and inability to fertilize eggs on oviposition. Thus, we will limit or avoid chilling of the founders.

We will collect one of two wild males in order to use them as captive mates for any founder females (such as a female reluctant to oviposit) we come to suspect as infertile. Observed captive mating will be documented and reported. The addition of a male into the oviposition chamber may additionally stimulate oviposition simply by the increased activity in the container (observed in Palos Verdes blue butterfly, J. Johnson, pers. obs.). Collection of one or two males from the wild will have a vastly smaller impact on this species than collection and maintenance of an infertile female that might otherwise have mated in the wild.

4.1.6 Egg Maintenance and Larval Rearing

No more than a dozen eggs should be allowed per container. When the female founder is transferred to a new section of plant, the eggs will be contained within a larval container labeled with gene line stud book number for their dame (similar to an oviposition container, only with organza instead of mesh to insure inclusion of first instars and exclusion of predators/parasitoids). Eggs will be monitored twice a week.

A log for each rearing container will be established and maintained. Detailed notes will be kept on each rearing container (including but not limited to the larval stage, plant health, and removal of aphids). Prior to opening a container, keepers will check the log.

Any ailing larva will be removed, noted in the log, and established in its own rearing container (creamer cup with foodplant and vent holes) in the "hospital." After handling an unhealthy larva, keepers will meticulously clean their hands and tools prior to continuing with any rearing work.

First instar larvae are very fragile and a source of high losses to any rearing program. With proper care and maintenance of the plants it will not be necessary to move these first instars from their initial rearing containers. If issues arise with the plants, then larval relocation to a fresh foodplant will be performed by the lead biologist.

There should never be more than 12 larvae in a container, the food must be kept fresh, and the excess frass that accumulates at the lowest point in the container must be removed. At various points during the later instars, the rearing container will become strained as foodplant is con-

sumed. At this point the larvae will have to be relocated into a new container with fresh food. When establishing a new rearing container, the larvae are transferred without direct contact whenever possible. The larvae can be transferred as they cling to a section of plant. If it is a thick stem, it needs to be severed slowly and steadily in order not to catapult the larva. The plant section and larva are then transferred into the new container. These new containers will identical to the initial rearing containers.

Late instar larvae will be removed from the larval rearing containers and established in individual rearing containers. Individual rearing containers are creamer cups with ventilation holes in the lids and cut foodplant provided ad lib (Figure 4). Individual rearing containers will be monitored daily for removal of frass and maintenance of foodplant. Upon pupation, excess vegetation is removed and creamers are opened to allow the pupae to harden properly.

Figure 4. Individual rearing containers.

Once hard, pupae will be assigned their own individual identification number and weighed (to the nearest mg).

4.1.7 Pupal Care

Pupae may be used for reintroduction to the Antioch Dunes. If the pupae remain in the captive rearing program, then they will then be stored in an eclosion container. Pupae of known lineage will be placed in an eclosure chamber *only* with siblings. In other words, there will be an eclosure chamber strictly for F1 offspring, a separate eclosure chamber strictly for F2 offspring, and so on. This guarantees that we know the lineage of the butterflies that eclose in any given chamber.

a)  b) 

Figure 5. a. Eclosion dish with eight pupae in assigned "seats." This allows tracking to the individual level throughout eclosion. b. Eclosion dish inside eclosion chamber to afford another level of containment.

The eclosure chamber consists of the pupae on gravel or dirt substrate in plastic dishes (Mattoni et al. 2003). The substrate will be heated via solar sterilization prior to use to reduce risk of pathogens. Within the plastic dish, there is a "seating arrangement" for the eight pupae in that dish. This allows us to track each individual.

Inside the dish, there is a metal semicircle of mesh for the adults to ascend while their wings expand and harden. These are placed inside of mesh boxes to provide containment for adults in the lab. Access to the eclosure chambers is possible by lifting the eclosion mesh chamber directly up. The pupae will be artificially warmed via timer-operated heat lamps from 9 A.M. – 6 P.M. The timed heat lamps allow eclosures to be "scheduled" for 9 A.M. when personnel will be prepared to immediately manipulate the adults as they eclose.

Adults will not be disturbed during the eclosion process. If they fail to eclose properly, there is no way to "help" them without destroying their wings, and then they will not be able to mate. They must make it out of their pupae on their own. If they are unable to complete this process successfully, they are immediately frozen in a labeled container and will serve as part of future genetic study. Any anomalies of this sort will be recorded.

Once the butterflies emerge from the pupae, they will climb up the mesh that was provided while their wings expand and harden. Once again, it is essential that they are allowed to complete this process prior to any manipulation.

Adults will then be sorted and mated according the methods noted above for captive mating.

4.2 Methods for Murrieta Rearing Site

The female Lange's metalmarks collected at the Antioch Dunes will be brought back to the Murrieta Facility. Each female will be given a number for following female lines. The females will be fed each evening on a piece of paper toweling soaked with 1/3 buckwheat honey to 2/3 filtered water (Figure 6). Each female will be set up in an oviposition/rearing container provided with cuttings of California buckwheat (Figure 7). The containers will be filled with buckwheat branches, so they will frequently encounter the food plant as they move about inside the containers. The reasons for this method are high quality branches can be chosen and the more frequently the female encounters the buckwheat the more eggs will be oviposited.

Figure 6. Female *Apodemia virgulti* feeding on buckwheat honey/water soaked paper toweling.

Figure 7. Oviposition/rearing containers with cuttings of California buckwheat.

The buckwheat branches for eggs and larvae will be checked thoroughly before being placed into the oviposition/rearing containers for both ovipositing females and rearing larvae of the Lange's metalmark. This careful checking will reduce contamination of *Apodemia virgulti* eggs and larvae and various predators and potential parasites to the eggs and larvae. Laboratory grown buckwheat plants can be more attractive than buckwheat in the field to ovipositing Behr's Metalmarks because they are well fertilized and watered.

The major contaminating predator is expected to be lacewing larvae. Other predators can be various bugs, earwigs, and ladybugs. These lacewing larvae are very good at hiding on buckwheat plants. These larval predators will in a very rapid period feed upon and kill all metalmark larvae and eggs that are found in the same container. Large numbers of larvae can be lost to this insect predator. Parasitic wasps can also take a fair number of eggs and larvae as well.

The containers will be placed in a window exposed to morning sunlight (when temperatures are coolest), so the females of each container will be stimulated to oviposit. Each day the eggs will be collected from each female and placed into a sterilized yogurt container. The containers with

these eggs will be labeled as to the female line. The eggs will be collected by cutting the leaves and plant parts with eggs and sometimes peeling eggs off of the branches. The eggs of members of the mormo complex have very thick chorions (egg shells) so they are much easier to handle than most other butterflies. The leaves and plant parts will be dried to prevent fungal growth.

Figure 8. ***Apodemia virgulti*** **eggs on** ***Eriogonum fasciculatum*** **branches.**

The eggs (Figure 8) will be brought back to Pratt's house in Riverside to examine daily in the early morning. Most eggs of the Behr's metalmark hatch shortly after sunrise. Once the eggs begin hatching, which should be sometime in late November or December, they will be transferred back to the Murrieta facility where they will be monitored twice daily. The newly eclosed larvae will be immediately transferred to buckwheat branches within oviposition/rearing containers (Figure 7). Larvae that hatch that are not immediately fed will die within a few hours. Hatching of eggs can run over a month to a month and a half period (Pratt and Ballmer 1991). Each larva will be carefully placed into an oviposition/rearing container with branches of California buckwheat and labeled as to female number. Up to 20 first instars will be transferred to an oviposition/rearing container per female line and a new one will be started after the 21st larva of

position/rearing container per female line and a new one will be started after the 21st larva of each line.

A separate container with *Eriogonum nudum* will be set up for each female line. This will compare larval development on California buckwheat to that of development on nude buckwheat. Twenty first instars from each female line will be reared on a potted plant of *Eriogonum nudum* grown from seed collected at the Antioch Dunes within an oviposition/rearing container. The larvae will be reared inside these containers until the plant material has been completely fed upon or the larvae have pupated. Once the plant material has been fed upon the larvae will be transferred to a new container placed over another potted plant of *Eriogonum nudum*.

Figure 9. Freshly eclosed Behr's Metalmark.

The pupae will be weighed to the nearest milligram and then pasted onto paper toweling with Elmer's glue. Female pupae are consistently larger than male pupae. The pupae will have a drop of glue placed on the abdominal tip just prior to placing on the paper toweling. This is to help prevent the wings of the freshly emerged butterflies getting stuck to the eclosed chrysalis. The

wings and other body parts can get stuck to the pupal shell when it is not fastened to something. To weigh each pupa, it will have been removed from the silken shelter that would have secured it to a surface. After 2 to 3 weeks the adults will eclose from the pupae (Figure 9).

Two types of releases can be performed at the Antioch Dunes. One will involve release of late last instar larvae and the other will involve either older pupae or freshly emerged adults. The larvae will be placed on buckwheat plants at the Antioch Dunes. If pupae are used they should be placed in a container that will not be found and fed upon by natural predators. The advantage of release of larvae is that they can move around and find a secure location to pupate. If adults are to be used they should be released immediately after they have completely expanded and dried their wings.

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