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Captive Rearing of Laguna Mountain Skipper (*Pyrgus ruralis lagunae*) Final Report

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Introduction

Laguna Mountains skipper (*Pyrgus ruralis lagunae*) was listed as an endangered species on January 16, 1997 (63 Federal Register 2313–2322). In the intervening years, extensive surveys to determine distribution and status have been conducted, and critical habitat was proposed (71 Federal Register 19157–19158) and designated (71 Federal Register 74592–74615). As recently as the early 1990s, the skipper occurred in multiple meadow habitats on Laguna and Palomar mountains. Wild populations continued to decline since listing, however, and the skipper appears to currently be restricted to a single mountain, Palomar Mountain, which is highly threatened by wildfire. One of the six remaining inhabited meadow systems was burned by the Poomacha fire in October 2007 and it is unknown if any skippers remain within the burned area. The current population status and the short lifespan (1–2 years), combined with the high risk of fire throughout the range of the skipper on Palomar mountain necessitates removing and captive rearing wild-bred individuals as insurance against extinction of the species should a catastrophic event, such as additional wildfire, occur in 2008.

The Carlsbad Fish and Wildlife Office (CFWO) contracted with The Urban Wildlands Group to evaluate the feasibility of and initiate, if necessary and appropriate, a controlled propagation program to prevent the extinction of this species. The Laguna Mountains skipper has not been reared in captivity previously and it is currently unknown how this subspecies will behave in captivity; however, methods have been developed with surrogate species that will be applied to Laguna Mountains skipper (Osborne 2007). The purpose of the current project was to (1) capture and captive rear wild-bred adult female Laguna Mountain skippers and/or larvae that can be released in the event that a catastrophic event causes the extirpation or extinction of remaining wild individuals and (2) develop captive rearing techniques specific to the Laguna Mountains skipper, ob-

serve the life cycle of the species in laboratory conditions, and determine the feasibility of utilizing controlled propagation to recovery this species. The housing and rearing of wild-bred individuals for later reintroduction or recovery related research without controlled propagation is exempt from the Service's Policy Regarding Controlled Propagation of Species Listed Under the Endangered Species Act (65 FR 56916).

The description of captive rearing methods below is derived predominantly from a handbook for propagation of the Lange's metalmark butterfly (Johnson et al. 2007b) and relies on techniques developed by Osborne (2007), Johnson (Johnson et al. 2005, 2007a, Johnson et al. 2008), Pratt (Pratt et al. 2000), and Mattoni (Mattoni 1988, Mattoni et al. 2003).

Methods

Permitting

We developed a rearing plan and solicited peer input from K. Osborne. The plan then underwent a series of revisions in consultation with CFWO staff to ensure exemption from the national "Policy Regarding Controlled Propagation of Species Listed Under the Endangered Species Act" (56916 FR 65: 183). The plan emphasized the importance of the research effort to perfect captive rearing techniques for this species. The effort had a secondary purpose as providing an "insurance" population in the event of a catastrophic event affecting the wild population. This insurance value was limited, however, because no permission for captive breeding was provided and all captive individuals would die without mating, putting an end date on the population of Summer 2009, no matter what success in rearing larvae was achieved. The plan was finalized in June 2008.

Ken Osborne was recruited to provide a secondary location for captive rearing when logistics allowed. It was established that all collection of wild material could take place under the authority of the CFWO Recovery Subpermit (hereafter "Subpermit"). Johnson was added to the Subpermit to conduct captive rearing activities and Osborne is authorized under the Subpermit to perform captive rearing and collection activities. Ten student workers were recruited and hired to assist with the care and maintenance of captive stock (feeding, climate control, care of food-plants, cleaning of oviposition and larval containers). Their qualifications were submitted to FWS and all individuals were approved by the CFWO Recovery Permits Program to conduct activities under the direct supervision of Jana Johnson and were approved by the CFWO to work in this manner. All of these students had previous experience with all aspects of captive rearing of the Palos Verdes blue butterfly (see Johnson et al. 2009b).

The Subpermit authorizing collection and rearing of LMS was provided to us on July 7, 2008. The Subpermit authorized collection of up to 20 adult females (five from four sties on Mount Palomar) and the rearing of all life stages in captivity. Larvae can be collected in substitution for adults. The relevant sections of the Subpermit are in the Appendix.

Collection of Adults

Adults were captured in the field with butterfly nets and fed a 25% honey/water solution. Locations for collection were recommended by CFWO staff and species experts D. Faulkner and K. Osborne. Adults were sexed in the field and maintained in climate-controlled conditions until transported to the laboratory at Moorpark College.

Adults in Captivity

Facilities and Operation

Adults were maintained in and around our greenhouse facilities at Moorpark College. One greenhouse is kept at an elevated humidity level with a swamp cooler. The temperature of the greenhouse is maintained with the swamp cooler, space heater, and “blackout” cloth, which blocks 100% of the light from the roof of the greenhouse. The second greenhouse is kept at low humidity and temperature is maintained with an air conditioning unit, space heater, and “blackout” cloth. Firm greenhouse rules are established. These rules include 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.

Oviposition Containers

Adult females were housed individually in containers clearly labeled with their studbook number. They were alternated between multiplant boxes and oviposition containers to determine which they prefer for oviposition (there is individual variation in preference to containment structure) (see figures in Johnson et al. 2008). 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.

Both adult containers served as containment devices within the greenhouse setting. Attenuated longevity of butterflies in past propagation efforts was attributed to temperature and humidity issues. We will insure that the temperature is controlled and the humidity is elevated. The difference in containers is the allotment of space for movement and variety of vegetation available to the adult. All containers were kept ant free to minimize predation.

The multiplant box is constructed of wood, knit cloth sleeves and organza (24” by 24” by 18”). 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 was a *Horkelia clevelandii*, one surrogate nectar plant, and an alternate host plant.

The oviposition container consists of a plastic container (1 quart) with screened ventilation access on two sides and the top. The lid to which the container attaches 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.

Feeding

Females were fed twice a day with artificial nectar. Artificial nectar is 1 part honey to 3 parts water. It was presented on a Q-tip with a “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. Butterflies in oviposition containers were fed in the greenhouse in a modified oviposition container to prevent escape.

Egg Maintenance and Larval Rearing

Egg counts were attempted weekly, however this was 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 depended on the containment preference of each female. No more than a dozen visible eggs would be allowed per container. When the female founder was transferred to a new section of plant, the eggs are contained within a larval container were 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 were monitored twice a week.

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

Any ailing larva were 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 meticulously cleaned their hands and tools prior to continuing with any rearing work.

Our protocol was that no more than 12 larvae were maintained in a container and the food was kept fresh and the frass removed. At various points during the later instars, we relocated larvae to 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 be identical to the initial larval containers.

Late instar larvae were 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. Individual rearing containers were monitored daily for

removal of frass and maintenance of foodplant. Upon pupation, excess vegetation was removed and creamers are opened to allow the pupae to harden properly.

Once hardened, pupae were assigned their own individual identification number and weighed (to the nearest mg).

Results

Collection of Adults

In Fall 2007, Johnson made a field visit to Mount Palomar to see habitat and plan for the upcoming season. She visited the Observatory Campground and a property near the Girl Scout Camp. After all permits and permissions were secured, Johnson, assisted by student workers Adam Clause and Damien Renner, made a collecting trip to Palomar Mountain on July 19–20, 2008.

On July 19, 2008, Johnson and students met up with Dave Faulkner. He took them to the vicinity of the Girl Scout Camp. Faulkner observed a dozen LMS, he netted 8, and 2 were females. An identification number was assigned to every LMS netted (regardless of sex) for consistent recordkeeping. 08001 and 08005 were female as determined by Johnson. Sex determination is challenging for LMS because it consists of detecting the male “hair pencils” (see Figure 1). We developed a technique of placing the netted individual in a small clear plastic vial that when rocked cause the individual to adjust their position to maintain balance. This reaction to the gentle rocking motion allowed quick and easy detection of the hair pencils when viewing the individual from a posterior/ventral position. Faulkner noted that he determined sex by behavior in the field but behavioral techniques are time consuming and can be problematic with individuals still free to leave the site evading capture.

A single *Horkelia clevelandii* plant was collected from the field, because it was our understanding at the time that only a single foodplant could be collected. Faulkner disagreed with collecting any foodplant from the field. However, a single foodplant was transplanted and used for establishing the oviposition containers that the foundresses were housed in. The foodplant was carefully chosen for size, health, amount of vegetation to serve for oviposition, and presence of seeds. The seed were harvested by hand and served as the source of our germination projects (Figure 2).

Unfortunately 08001 was very old and died that evening at the hotel, despite every effort to care for her, including assisting her with the unfurling of her proboscis to help her feed. 08005 looked good and fed readily. All males were released at the site of their capture. The females were maintained in containers in the field (Figure 3) until temperatures cooled in the evening such that the temperature in the vehicle could be maintained at the ambient temperature. The females were transported back to the hotel where they were kept with the team overnight.



Figure 1. Photograph of male Laguna Mountain skipper showing “hair pencil” (arrow).



Figure 2. Collecting seed from *Horkelia clevelandii* in the field.



Figure 3. Handling adult Laguna Mountain skippers in the field. Butterflies are confined in plastic containers with sides and ends cut out and replaced with mesh to allow air to flow through the container. The containers are inside a box that allows access through fabric sleeves.

On July 20, Johnson and the students met with Ken Osborne. They proceeded back to the vicinity of the Girl Scout Camp and ended up in the same spot as the previous day. Out of 12 netted LMS individuals, 2 were females. Males were assigned numbers and released at their site of capture. Females 08018 and 08019 fed readily on artificial nectar. Female 08019 was from a mating pair that Adam found. The mating pair was captured directly into an oviposition container in order to not disturb the mating. The mating pair had to be moved several times during their mating to insure enough sun exposure to allow success, while avoiding overheating the pair. During this time the mating was observed to include the pumping motion necessary in the male's abdomen to transfer the spermatheca to the female. This suggested that the mating had been successful. With the exception of 08019, all individuals were slightly worn.

During the two days, the team collected 4 female skippers from Site I200 of Iron Springs / Girl Scout Camp (GPS 11S 0511192 UTM 3688161) 0.05 km from center. They obtained one foodplant (*Horkelia clevelandii*, Cleveland's horkelia) used to accommodate the females in oviposition containers. At the time they thought that no more foodplant was to be collected in order to minimize impacts on the wild skipper population, although this restriction was not specified by the Service. Even though the Subpermit exempts the captive rearing program from any restrictions on collection of host and nectar plants, it was not deemed advisable to take foodplant from an existing population (this was discouraged by experts Faulkner and Osborne), and other foodplant in presumably non-occupied sites was on Forest Service land and no permission had been obtained to collect from their property, given the short time available after the approval of the

collecting plan. A roadside site later suggested by the Service as a site for collected plant was fenced and posted no trespassing and therefore not useful. The plant that was obtained at the collection site had some mature seeds on it, so the seeds from the newly potted plant were collected for future use.



Figure 4. Left: setting up butterflies on *H. clevelandii* in the field. Right: female Laguna Mountains skipper in oviposition container with leaves.



Figure 5. Female Laguna Mountain skipper nectaring on a Q-tip decorated with orange paper, to mimic a flower and protect the skipper from becoming sticky with nectar, and soaked in honey water.

Oviposition

The butterflies were transported back from Palomar Mountain in the evening to minimize disturbing their usual temperature regime. All females nectared well in the laboratory (Figure 5). Back at Moorpark College, the females were set up in several different sizes of oviposition containers over plants to see which worked best to obtain eggs. These oviposition containers were of different sized clear plastic containers as described previously for other butterflies (Johnson et al. 2007b, Johnson et al. 2007a, Johnson et al. 2008).

The three females produced 355 eggs after a range of manipulations designed to induce oviposition described below (Table 1). The females laid eggs at different rates (3.8, 6.6, and 9.7 eggs per day) and with variable total egg production (72, 99, and 184) (Table 1; Figure 6).

The team utilized many techniques to encourage oviposition, including Osborne's *ruralis* container plus additional ventilation windows, companion butterflies (Lycaenid males), a ten-minute temperature and light rotation cycle from 8 A.M. to 5 P.M. daily between cool/shady and warm/sunny, and wind from a fan.

Table 1. Total daily egg production from Laguna Mountain Skipper females at Moorpark College rearing facility. These are only eggs that could be observed without removing oviposition containers and therefore represent an undercount of the total number of eggs laid. Numbers given for each day the female was alive.

	08001	08005	08018	8019	Total
19-Jul	DOA	0	0	0	0
20-Jul		0	0	0	0
21-Jul		0	9	13	22
22-Jul		8	0	21	29
23-Jul		5	3	18	26
24-Jul		8	4	13	25
25-Jul		5	23	29	57
26-Jul		2	14	22	38
27-Jul		1	2	8	9
28-Jul		0	4	6	10
29-Jul		7	11	15	33
30-Jul		6	19	9	34
31-Jul		3	10	10	23
1-Aug		6	0	5	11
2-Aug		7	0	4	11
3-Aug		3		4	7
4-Aug		4		3	7
5-Aug		6		3	9
6-Aug		1		1	2
Total		72	99	184	355

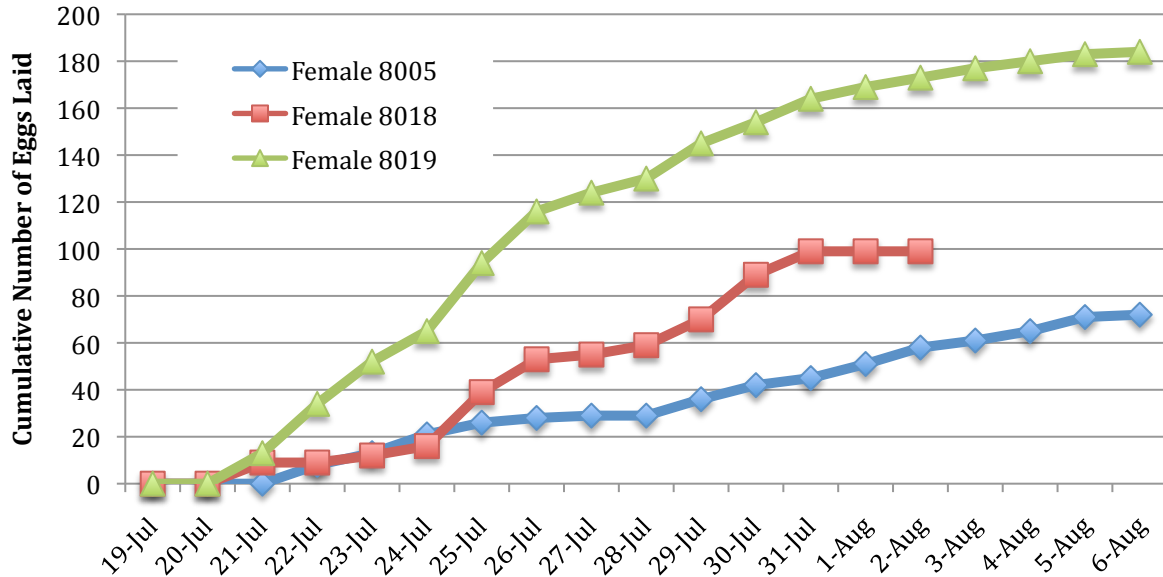


Figure 6. Cumulative egg production by captive Laguna Mountain skippers. More eggs were located after oviposition containers were removed, but the data of laying of those eggs is unknown. These curves show individual variation in oviposition rate and total production. Overheating episode for female 8018 is at July 22.

“Osborne Container”

The females did not oviposit in either of the clear oviposition containers we have used previously so we switched over to the styrofoam oviposition container that Osborne developed when working on *Pyrgus ruralis ruralis* (Osborne 2007). The “Osborne container” is a Styrofoam food container with a ventilated lid and holes in the bottom that allow for the petioles of leaves to extend through the holes into a reservoir in a second “nested” Styrofoam cup. The difficulty with the Styrofoam cup was temperature. Therefore, we modified it after some experimentation (see below) to include “windows” cut out of the Styrofoam with mesh hotglued to cover the opening and allow ventilation and heat dissipation (Figure 7). Two large windows are added per Styrofoam container. The plastic lid is also equipped with a large cutout window that is secured with mesh hotglued into place. There are holes punched in the base of the container that allow cut foodplant leaves to be inserted into the holes with the petioles extending below the oviposition container. If this entire “cage” is inserted into a second Styrofoam container with matching “windows” then the ventilation is maintained and water can be placed in the second container with the petioles obtaining water and maintaining the cut foodplant for a longer period of time. These containers were key for oviposition. We did not try them for larval rearing, but it may be warranted. The physiognomy of the *Horkelia* does not match well with our present larval rearing containers, resulting in a rather quick demise of the *Horkelia*.



Figure 7. Styrofoam oviposition containers modified from designs by Osborne. Foodplant in the inner container obtains water from the second, outer container.

Light and Temperature

We experimented with several containers and lighting/temperature regimes to induce oviposition by the females. Skippers were maintained both in the greenhouse and “outside” (where they were still housed in two layers of containment). There is no substitute for direct sunlight so skippers are moved during the day to change their exposure to sunlight, simulating the changes they would experience moving through a natural habitat far larger than a rearing container. They were exposed to “dappled light,” morning light, afternoon light, and other variations by changing where on the rearing grounds they are housed for the given period of time. Frequently we used “rotations” of 20–30 min. to change their exposure throughout the day.

One of the females (08018) experienced an overheating episode, but recovered well. The overheating occurred when we were working at stimulating ovipositing. Light and heat are necessary for oviposition, but can lead to overheating. The signs of overheating — increased agitation and flopping onto the side (off of her legs) — occurred during these adjustments of light and heat, which are closely observed at all times. We had also inserted a thermometer into the cup to monitor the temperature. The increased activity was not out of the ordinary until right before she fell over. Johnson immediately removed her back to cooler conditions and revived her by uncurling her proboscis and providing nectar. After this experience we modified the containers with extra ventilation windows, which then allowed us to expose them to sun/heat without overheating them and stopped exposing the females to full afternoon sun. This series of events was the

key to getting successful oviposition. Without having gone through a series of protocol modifications we would not have obtained eggs from the collected females.

Foodplant

Eggs were obtained from all 3 of the surviving females after manipulations with light and temperature and presence of *Horkelia clevelandii*. All eggs were mapped (See figure 6). Interestingly enough, the presence of *H. clevelandii* was helpful in stimulating oviposition by 08005 & 08018, but 08019 was actively ovipositing without the presence of *H. clevelandii*. Despite the increased probing when *H. clevelandii* was present, 08005 & 08018 would oviposit on the *H. truncata*. These numbers will not equal the total number of eggs as some were oviposited in unknown locations and found rolling around free in the container. These are of limited aid in indicating preference of substrate, as the majority of substrate available was *H. truncata*. That being said, 08018's majority on *H. clevelandii*, when that was the limited substrate present strengthens our assertions from observations that she needed *H. clevelandii* for stimulation. Female 08019 was less selective in oviposition and was switched onto strictly the surrogate *Horkelia truncata* to reserve *H. clevelandii* leaves for 08005 and 08018 which would not oviposit without *H. clevelandii* leaves present.

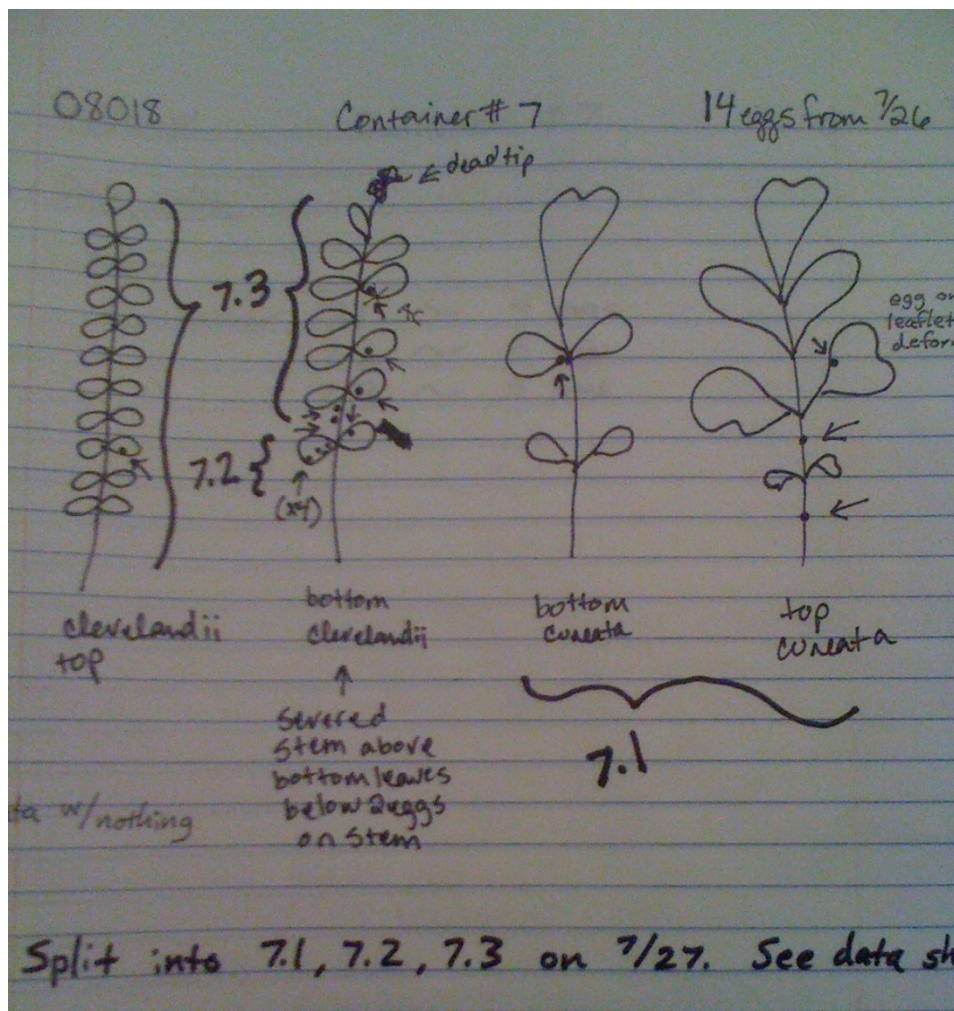


Figure 8. Lab notes showing oviposition location on plants.

Once light and temperature were suitable, the females oviposited on *Horkelia truncata*, but two females also required presence of *H. clevelandii*. By the end of their extended lives, the females were ovipositing on the containers and the eggs were unattached and found rolling freely around the container, these eggs were difficult to maintain due to their not being attached to any substrate. The older the female upon collection, the earlier on in captivity this occurred. This is a more pronounced occurrence that also has been observed to a lesser extent in the other species we have reared. We transferred eggs that were not attached to plants with a moistened brush (Figure 9).



Figure 9. Transfer technique using moistened brush for eggs not attached to plants.

Table 2. Number of ova produced by each collected female by foodplant.

Female #	# of eggs oviposited on <i>H. truncata</i>	# of eggs oviposited on <i>H. clevelandii</i>
08005	33 (70%)	14 (30%)
08018	39 (43%)	52 (57%)
08019	174	6*

* very little *H. clevelandii* was used with 08019 due to its limited supply and 08019's willingness to oviposit without *H. clevelandii* present.

Use of Companion Butterfly

08005 produced 8 eggs after adding a Pygmy blue (captured at Moorpark College) to her container to provide a bit more activity. Osborne and Johnson have observed greater oviposition success with surrogate butterfly activity in the oviposition container. A healthy male of a completely unrelated species (Pygmy blue) is used to eliminate any issues of mating/non-target eggs. Interestingly, the days when there were meetings with lots of activity were the peak oviposition days, so perhaps simply having any activity around the butterflies stimulates further activity.



Figure 10. Pygmy blue butterfly was added to containers to interact with females to stimulate oviposition.



Figure 11. Location of eggs on underside of *Horkelia clevelandii* leaves.

Egg Hatch

All eggs were packaged in larval containers on *Horkelia truncata* in groups of 7 eggs or fewer per container. All foodplant (in use, or awaiting use) was housed in the greenhouse with the eggs and females. The *Horkelia clevelandii* we obtained from the field underwent shock and every effort was taken to nurse it through in preparation for eclosion. We did this by purchasing special “water release” soil. We split the side of the pot, opened one side, and then duct taped it to a second large pot to allow root expansion without shocking it with a complete transplant a second time. It was hand groomed for any non-target insects that were found on its leaves. It was moved throughout each and every day to keep it in dappled light as the sun moved. During this time all larvae were reared on the surrogate *H. truncata*. The leaves for the *H. clevelandii* were used to induce oviposition from the foundresses, unfortunately the foundresses would not oviposit on the living plant, so the leaves had to be harvested, further shocking the *H. clevelandii*.

Table 3. Disposition of eggs from each of the three female skippers.

Female	Eggs hatched	Collapsed/unhatched eggs	Missing eggs	Total eggs
08005	10 (13.9%)	41 (56.9%)	21 (29.2%)	72
08018	25 (25.3%)	58 (58.6%)	16 (16.1%)	99
08019	132 (71.7%)	28 (15.2%)	24 (13.0%)	184*

* 3 eggs from 08019 given to Ken Osborne on 8/6/2008. Osborne reared these three larvae, photographed them, and stored them in alcohol for use as reference material or in future DNA analysis.

On July 29, eggs from all three females had visible larval development (Figure 12). Eight-day-old eggs from females 08018 and 08019 hatched. The first instars were inflicting feeding damage on the *Horkelia truncata*. The hatch rate for eggs of individual females was 14% (08005), 24% (08018) and 71% (08019) for an overall hatch rate of 46%. Hatch was around 10 days from oviposition (Figure 14).



Figure 12. Development of Laguna Mountain skipper egg on *H. clevelandii*. The first photograph, taken July 22, shows a fresh egg. In the second photograph, taken on July 29, the dark head of the developing larvae is visible through the chorion.



Figure 13. A misshapen Laguna Mountains skipper egg on *Horkelia truncata*. This egg did not hatch.



Figure 14. Hatched egg on drying *Horkelia* leaf.

Larval Development

Larvae and foodplant were monitored daily. The first instar larvae built shelters and began to inflict feeding damage on the surrogate foodplant, *Horkelia truncata* (Figure 15). However, beginning the first week in September, some of the larvae refused to eat and began wandering around the containers. There seemed to be some cannibalism, but some other losses due to an unknown cause. Hypotheses at that point included too high humidity, too little direct light, or use of the surrogate foodplant. Larvae always die during the early instars, so the wandering behavior was the indicator that something was wrong. There was no evidence of disease, which we have dealt with before at other facilities (Mattoni et al. 2003). The behavior suggested to us a problem with the environmental conditions in the containers rather than disease. Because the first two instars had consumed *H. truncata* without issue, foodplant was the last factor that was investigated. Therefore, we immediately began to experiment with various lighting, temperatures, and humidity. These efforts included:

- Placing containers outside in locations with more light or less light;
- Placing containers inside the greenhouse in locations with more and less light;
- Lowering greenhouse humidity by running the air conditioner; and
- Raising greenhouse humidity by running the swamp cooler.
- Soil added to the base of the larval container in case they required access to substrate

In each instance, larvae were observed to see if they would return to the plant and feed or they would continue to walk about. We also varied the container and the contents of the container, but the second instar larvae repeatedly left their plants and wandered the container (regardless of the health of the plant). This is frequently referred to as “going walkabout” in the breeding community and is worrisome as it indicates that the larvae are looking for some factor that is not provided in the container.



Figure 15. First instar skipper larva and feeding damage on *Horkelia truncata* leaf (to left of larva).

We experienced a spike in larval mortality associated with the behavior exhibited by the larvae. This continued over the course of ten days as we experimented with temperature, humidity, and lighting (days 60–70 after eclosion; Figure 16). As a result our number of larvae declined rapidly, with a loss of over 20 on day 60 after eclosion and consistent losses following. During this period the larvae were all on surrogate foodplants in early October, the *H. clevelandii* was recov-

ered enough to support larvae and 5 surviving larvae were placed on it (though one of those 5 was already failing to thrive). By mid-October, we had 4 healthy larvae (4 of the 5 that were moved to the *H. clevelandii*) that were stable for 10 days since being relocated to *H. clevelandii*. All larvae on *H. truncata*, regardless of all of the environmental manipulations described, stopped eating, went “walkabout,” and died.

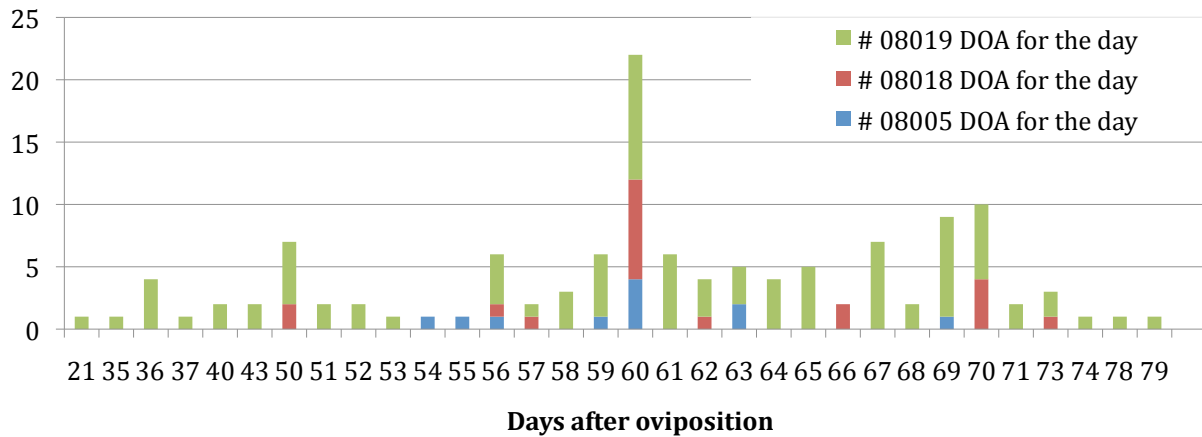


Figure 16. Mortality of larvae by day after oviposition.

We continued to check larvae daily in the fall (October–November), and they continued to eat intermittently on the single *H. clevelandii* plant. On November 11, one of the larvae pupated. The second pupated November 16. The final two larvae did not pupate.

At the end of December the two pupae were kept on a base of crushed walnuts in Styrofoam eclosion cups. These cups have ridges scratched on the inside surfaces to allow the imago to climb up and expand its wings upon eclosion. The cups have mesh lids and are kept in boxes with mesh sides inside the greenhouse and visually checked each day.

We located and collected 121 dead larvae to measure head casings and preserved them in ethanol for future genetic analysis (Figure 17). Other dead material was collected and archived at Moorpark College. Dead larvae have been archived in ethanol in small plastic containers in the freezer. Dead adults are stored at ambient temperatures and one is used in an educational display at the rearing facility. In our collaboration with UCLA on the genetics of Palos Verdes blue butterfly we have found that DNA can be extracted from imagoes that have been in dry storage (Johnson et al. 2009a). With permission of CFWO, we provided dead material to Texas State University to test for evidence of infection by *Wolbachia* (Nice et al. 2009) and to the USGS to develop a microsatellite library for future population genetic studies of the species.



Figure 17. Staff measured head capsule of deceased larvae (left) and shared material for development of a microsatellite library and testing for the bacterium *Wolbachia* (center and right).

Our measurements of the head capsules did show peaks that could provisionally be construed as being associated with different instars (Figure 18). Using these measurements alone, we ran a clustering algorithm (Ward's method of agglomerative clustering) to create five clusters (Figure 18). Assuming that each cluster represents an instar, the data suggest that larval head capsules increase 0.35–0.45 mm each instar (Table 4). This analysis is extremely conjectural because we were unable to record the instar of each larva at time of death, given the difficulty of observing larvae on the plants. Future research could take these data as a starting point. The distribution head capsule sizes does reflect our observation that mortality occurred when a certain size was reached (3rd instar), after which we had difficulties keeping the larvae from trying to leave the surrogate host plant.

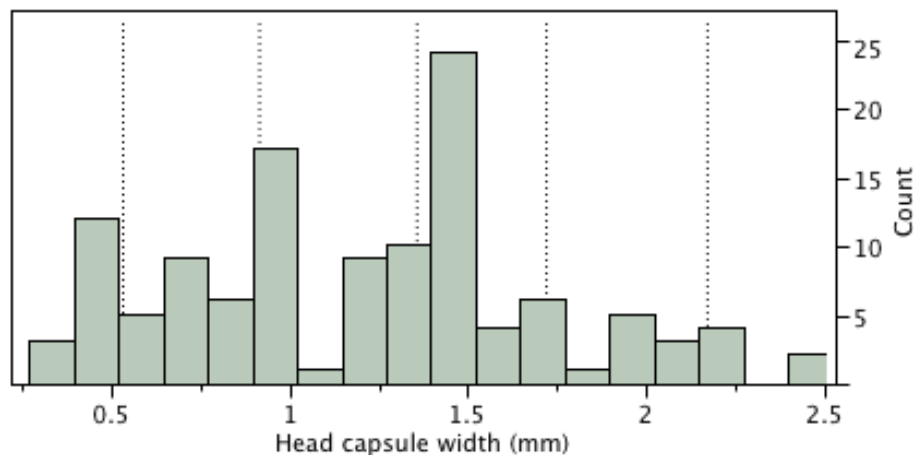


Figure 18. Distribution of head capsule widths for dead larvae (n=121). Vertical lines indicate midpoints of five clusters derived from Ward's agglomerative clustering method on width values.

Table 4. Clustering of head capsule sizes as a preliminary, unverified indication of growth by instar.

Cluster (instar?)	Number	Mean (mm)	Growth from Previous
1	29	0.53	
2	24	0.92	0.39
3	43	1.36	0.44
4	14	1.72	0.36
5	11	2.17	0.45

Pupal Storage

The pupae were stored at room temperature and checked visually each day.

Pupae were stored at ambient temperatures. They did not eclose during the spring flight period and were checked thereafter. The pupae had desiccated and were not viable. One weighed 7 mg and the other 10 mg and broke open when weighing. A partially developed imago was inside.

Plant Propagation and Storage for Genetic Analysis

We have attempted to grow the seeds our *H. clevelandii* plant produced. We solicited advice from multiple native plant growers and botanists and began the germination process. We have two protocols for germination and growth of the foodplant. One protocol was developed by the Moorpark College botanist Katherine Courtney, and the other is from a grower of *Horkelia cuneata*. There were 373 seeds of *Horkelia clevelandii* from the original collection. We split the seeds between the two protocols to determine the best method for germination. In the first small trial 10% germination was obtained on agar. A second trial used a potting mix and yielded some seedlings.

The second round of germination experiments yielded six *H. clevelandii* seedlings that were tended during the quarter. We have had difficulties with fungus, water balance, soil, and pest invertebrates so growth has been slow. The plant that we collected in the field has been growing and reproducing vegetatively.

The appearance of differences at the genetic level is quite likely considering that both species of *Horkelia* plants have various macroscopic dissimilarities. While *H. clevelandii* has palmately shaped, numerous, slightly resinous leaves; *H. truncata* has pinnately shaped, few, pungent, and profusely resinous leaves.

The first step of genetic analysis consists of collecting and storing material from both *Horkelia* species for use in genetic testing. The materials used to collect plant tissues consist of silica gel, small sealed plastic bags, a permanent ink pen, the use of a freezer, and two pairs of scissors.

On recommendation of Dr. Paul Kores we used silica gel as a drying agent to preserve the plant tissues. This was done as follows: place $\frac{3}{4}$ to an inch of silica gel into the sealable plastic bag, using a clean pair of scissors cut samples of *H. clevelandii* by cutting close to the node (select

material clean of any foreign plant seeds or debris), make sure to collect from many different plants of the same species, cut up plant material into small pieces and place into prepared bag, label the bag and store in a dry environment. I repeated this process three more times for *H. clevelandii* before proceeding onto the next species. Using a different pair of scissors and materials clean of any foreign plant DNA, I prepared four samples of the *H. truncata* and placed them in silica gel (as is described above).

Per recommendation of Professor Katherine Courtney we froze plant tissue from both species; by storing plant material two ways the margin of error decreases. Freezing plant material was done as follows: using clean scissors that have only touched *H. clevelandii*, cut samples that are free from foreign plant DNA by cutting close to the node (collect from many plants of the same species), these samples do not need to be cut into pieces and were placed directly into re-sealable plastic bags. This process was repeated three more times for *H. clevelandii*. The above process was repeated four times for *H. truncata* using a clean pair of scissors that had only touched *H. truncata*. All samples meant for the freezer were labeled and stored below zero degrees Celsius.

In total, four silica-dried samples and four freezer samples for each *Horkelia* plant were prepared and stored, giving a total of sixteen samples collected.

Discussion

Most of the larvae from the eggs obtained from the collected females died and this has raised many questions from CFWO. This experimental effort was, however, successful in many respects.

- We developed a quick, effective method for accurately identifying sex in the field.
- We showed for the first time that female Laguna Mountains skippers can be induced to oviposition in captivity with manipulation of conditions that include the type of containment, light, heat, activity in the cage, and presence of foodplant.
- We showed variation between individual females in preference for oviposition site and that females could be induced to oviposit on congeners of their *Horkelia* foodplant.
- We showed that first and second instar larvae *will* consume a surrogate hostplant and have strong indications that later instars will *not* accept a surrogate and require a native foodplant.
- We documented the early instar larvae for future reference in field identification and preserved material for genetic analysis.
- We provided tissue to Texas State University for testing for evidence of the bacterium *Wolbachia* (Nice et al. 2009).
- We provided tissue to the USGS Forest and Rangeland Ecosystem Science Center in Corvallis, Oregon for development of a microsatellite library. The laboratory was able to obtain high quality DNA from the samples.
- We obtained head casing measurements, which provide suggestive data for development of a classification scheme.
- We have gathered information from other captive rearing programs that could impact success of future efforts.

These are significant findings and the ultimate result of no production of adults has the same biological effect as if all larvae survived, since no permission was granted to mate imagoes or to release individuals.

Surrogate Foodplant

These plants had been provided to us by Osborne who had purchased them from a nursery as *Horkelia cuneata*, but were easily keyed out to *Horkelia truncata* (Hickman 1993). Our early reports therefore indicate *H. cuneata*, when in fact *H. truncata* was used. We were cautious from the beginning about bringing in *P. p. lagunae* without the specific foodplant and voiced these reservations early and frequently. However, since being assured that there was no *H. clevelandii* available and because these plants were the same plants that Osborne had used to rear *ruralis* we were cautiously optimistic. We now know that *lagunae* does not exhibit the same hostplant flexibility as *ruralis* at the later instars, but did not know this at the time. The impact of the misidentification of the *truncata* as *cuneata* is unknown. *H. cuneata* physiognomy is much more similar to *H. clevelandii* than *H. truncata*, but without overages of captive reared stock, the determination of edible surrogate foodplant for *P. r. lagunae* is ill advised. Another approach would be to examine the molecular similarity of the *Horkelia* species. We have preserved clippings of the *H. clevelandii* and *H. truncata* if funding for such a study becomes available. These clippings are preserved in a drying silicate and a second set is preserved in a freezer, per two Moorpark College botanists, Katherine Courtney and Paul Kores.

By way of explanation, the rationale for the use of *truncata* with the larvae was many-fold. First, Osborne had reared *ruralis* on this species (although he thought it was *cuneata*). The preliminary work for rearing any endangered species is to work with a non-endangered sister taxon. Often this yields information about the endangered taxon. In this instance it did not, but it was a reasonable starting point for learning in this exploratory effort. Second, we tried to obtain *H. clevelandii* from commercial nurseries but were unable to locate any. It was discovered after the fact that there was a large supply of *H. clevelandii* being reared for restoration efforts, but this was unknown at the time of the rearing. Third, the logistics of obtaining *H. clevelandii* in the field were not good. Despite the permission to take foodplant under the Subpermit, this action was opposed by experts Faulkner and Osborne when in the field. We were unwilling to take foodplant from private or federal property without permission and those permissions and legal access were not easily obtained in the height of the season within our time constraints with butterflies in the lab. Finally, we were encouraged by the initial acceptance of *H. truncata* for oviposition and by early instar larvae. This initial acceptance led us to believe that subsequent difficulties were with other aspects of the environment and not from the foodplant. However, Toledo Zoo found that Michell's Satyr larvae imprint on the foodplant that they start to eat and will not switch regardless of whether or not it is the correct foodplant, dying from either malnutrition or secondary compounds when they began to eat the incorrect foodplant, despite being offered the correct foodplant to later instars (P. Tolson, personal communication).

Future suggestions

Based on our experiences with this effort, we offer the following suggestions for future captive propagation of Laguna Mountain skipper:

A member of the CFWO or a designated representative should be present for the collection process to make the decisions in the field that arise unexpectedly. There is no cell phone service from the collection site in the field, virtual representation will be to the disadvantage of the success of the endeavor.

We should collect during the first flight period, just after its peak. This is the standard for univoltine species and although LMS has been shown to be bivoltine, the second flight is considerably smaller than the first. Impacts on the flight will be less if we collect at the tail end of the primary flight period, rather than from the drastically smaller second flight, thereby taking a smaller percentage of the flight. By collecting on the first flight the rearing cycle would be done in a shorter window and not face overwintering issues. Overwintering issues are a source of loss for multiple captive programs (e.g., Oregon Zoo, Toledo Zoo, University of Florida). The goal should be to collect on the first flight and either release on the second flight or breed the adults when they eclose. This will reduce expenses, labor, and impact on wild population while increasing the likelihood of success. It will be easier to collect females: it took us two full days in the field to find 4 females (and we netted every butterfly observed).

The collection should be of younger females, if possible, from whom eggs are collected in situ, and then rereleased. The higher productivity, increased hatch rate and the eggs remaining attached to the plant for the younger female, and the decreased impact by taking eggs instead of females from the wild are the basis for this suggestion.

Any program must start with an adequate supply of *H. clevelandii*. Although rearing in the small oviposition containers on *H. clevelandii* was successful, there was an increased impact on the plant from the interaction between the container and the feeding on the plant. This method may be employed, if there is a large supply of *H. clevelandii* available. Alternative designs could be to use cut leaves from *H. clevelandii* inserted into the modified "Osborne" container or using the Toledo Zoo's Mitchell's Satyr set up in the cement tub. In any arrangement, increased exposure to direct sun is vital to larval health and survival. If this is accomplished outside of the greenhouse, either multiplant boxes or popup rearing containers from LiveMonarch can provide a secondary level of containment.

Increased monitoring will allow a more detailed and accurate picture of hatch window, timing between instars and behavioral aspects of rearing. However, with increased monitoring, there is increased manipulation of the stock and increased losses of eggs and larvae from this disturbance alone.

Additional captive rearing should not be undertaken unless that stock can be released, mated or both. Captive propagation, within reason without the intention of producing excessive overages, would make sense in terms of being able to figure out a protocol for mating and any progeny from successful breeding would serve as an experimental population to iron out rearing issues, use for LD50 testing to aid in developing restoration dosage limits for herbicides, additional DNA work, and further *Wolbachia* testing without further impacts on the wild population.

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Appendix: Permit Conditions

21. Taking of the Laguna Mountains skipper (skipper):

The subpermittee is authorized to survey, capture, handle, and collect adult individuals; survey for larvae and eggs; collect and hatch eggs; release hatched larvae at the collection site; and remove from the wild adult females and larvae and rear in captivity all life stages for refugia and research within the geographic boundaries specified above, and the time limitation specified in the subpermit, provided that:

...

e. The subpermittee is authorized to take up to twenty adult female skippers from the wild and rear all life stages in captivity for the purpose of research and as refugia against catastrophic events, provided that:

i. No more than five adult females shall be collected from four sites on Mount Palomar. Larvae may be collected in substitution for, but not in addition to, adult females.

ii. Adult females shall be collected late in the flight season to ensure minimal effect on the wild population. Slightly older females shall be collected to insure they are gravid. Fresh females shall be collected only if a successful mating is observed.

iii. Individuals from each of the four sites shall be kept segregated to avoid intercrossing, unless otherwise approved by the CFWO.

iv. Captive propagation is not authorized at this time. Measures shall be taken, as necessary, to prevent mating. Captive propagation activities shall not commence prior to Region-8 approval of a captive propagation plan and compliance with the Service's Policy Regarding Controlled Propagation of Species Listed Under the Endangered Species Act (65 FR 56916).

v. Skippers of any life stage (adults, larvae, eggs) shall not be released from captivity into the wild, unless approved by the Region-8 office. For post catastrophic events, captive individuals shall be re-introduced only if skippers are extirpated from the area, habitat conditions will support butterflies, and all regulatory and policy requirements have been met.

vi. Captive individuals shall be reared and maintained at two facilities: the Moorpark College Teaching Zoo, Ventura County and the residence of Kenneth Osborne, Riverside County, California. Additional facilities require written consent of the CFWO.

vii. The number of individuals that shall be maintained in captivity is dependent upon the reproductive success of the females brought into captivity.

viii. All collection and rearing activities shall be conducted consistent with the proposal, Capture and Rearing Plan for Laguna Mountains Skipper, dated June 19, 2008, on file at the CFWO. Modifications to the methods shall be submitted in writing and require written approval from CFWO.

ix. Captive reared skippers shall not be released or used as founders for captive propagation until the permittee demonstrates that adequate disease prevention methods have been implemented in the rearing of captive stock.

x. The transfer of captive stock to another facility requires written approval of the CFWO.

xi. In the event of an emergency that would require immediate transfer of captive stock to prevent possible harm or injury, captive stock shall be secured in a safe and secure location, and report this activity to the CFWO within 24 hours by telephone and 2 days in writing. Prior approval is not required in emergency situations. If the location where the captive stock is being housed needs to be changed because of a

situation that a reasonable person would consider beyond the authorized individual's control, and if the timing of the change is such that the CFWO can not be notified ahead of time, then such a situation may be considered an emergency.

xii. Deliberate termination of the captive rearing program requires approval of the CFWO. If termination is deemed appropriate, captive individuals shall live out their lives without mating, and shall be appropriately preserved and made available for research.