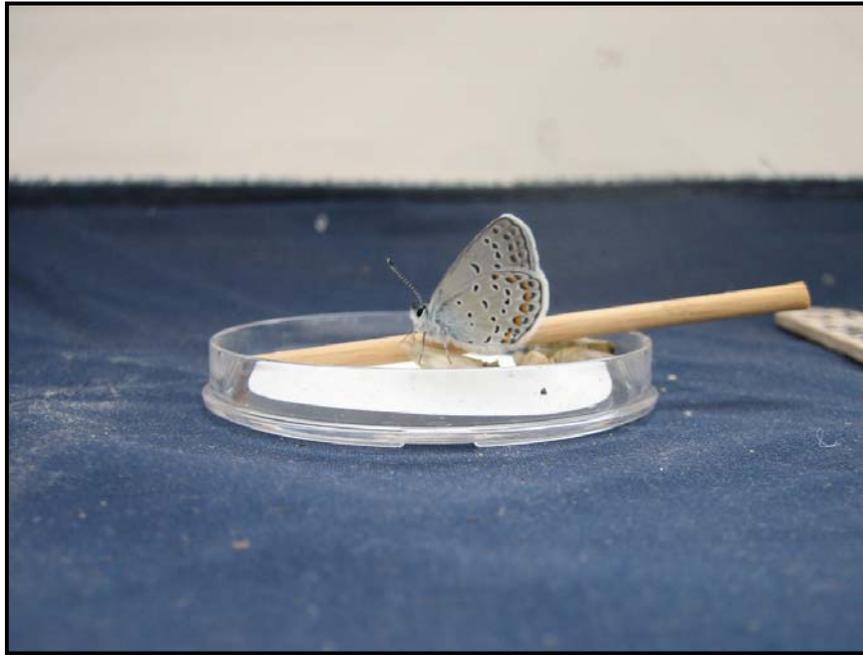


Propagation Handbook for the Karner Blue Butterfly,
Lycaeides melissa samuelis

First edition, November 2010



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Background and Summary

On November 7, 2007, the Karner Blue Butterfly Recovery Team, recognizing the importance of drawing upon lessons learned from successful ongoing captive rearing programs, established the Karner Blue Butterfly Captive Rearing Subteam to produce a comprehensive manual on captive rearing techniques for the butterfly. The Subteam was lead by Lindsay Webb of the New Hampshire Fish and Game Department who coordinated development of the manual and drafted the majority of the text.

This resultant manual provides a comprehensive guide on the various techniques that are being successfully used in captive rearing programs for the Karner blue butterfly (*Lycaeides melissa samuelis*) (KBB) by various organizations in Indiana, Michigan, New York, New Hampshire, and Ohio (refer to Table 1 for contact information). The manual includes information on, among other topics, egg, larvae, pupae, and adult care, release methods, vendors for captive rearing supplies, and suggestions on how to prevent disease. In addition, those starting new captive rearing programs with KBBs or other butterflies, will find this manual useful in determining which techniques will work best for them based on the location and size of the captive rearing facility, funds, and staff time. For more information pertaining to KBB translocations including reintroductions and population augmentations refer to the KBB Recovery Plan, especially Appendix I (USFWS, 2003).

Each captive rearing organization works closely with the U.S. Fish and Wildlife Service (USFWS) on their KBB reintroduction or population augmentation programs. An Endangered Species Act Section 10 (a) recovery permit is needed from the USFWS prior to engaging in a captive rearing program to authorize any take of the KBB that may result from the activity. In addition, a permit from the Animal & Plant Health Inspection Service (APHIS) is required if KBBs will be translocated across state lines. Check with the state's wildlife agency to determine if a state permit is required. This manual will be available to others interested in captive rearing of KBBs and serve to help streamline the permit process associated with captive rearing programs.

Table 1. Contacts for Captive Rearing Organizations

State	Contact Person(s)	Address	Email/Phone
Indiana	John Drake	The Nature Conservancy Southern Lake Michigan Rim Project 5690 Chase Street Merrillville, IN 46410	jhdrake@tnc.org (219) 981-9183
Michigan	Tom Schneider	The Detroit Zoo 8450 W. 10 Mile Road Royal Oak, MI 4806	tschneider@detroitzoo.org (248) 541-5717
New Hampshire	Lindsay Webb Steve Fuller	New Hampshire Fish and Game Nongame and Endangered Species Program 11 Hazen Drive Concord, NH 03301	Lindsay.Webb@wildlife.nh.gov Steven.Fuller@wildlife.nh.gov (603) 271-2461
New York	Neil Gifford	Albany Pine Bush Preserve Commission 195 New Karner Road Albany, NY 12205	ngifford@albanypinebush.org (518) 690-2768
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Introduction

Historically, the federally-listed endangered KBB has declined by 99% or greater throughout its range in the past 100 years. Historically, the KBB occurred in a geographic band between 41° and 46° North latitude extending from Minnesota to Maine (Dirig 1994) and was found in 12 states and at several sites in the Canadian province of Ontario. Currently, the butterfly is extant in only seven states (New Hampshire, New York, Ohio, Indiana, Michigan, Wisconsin and Minnesota) with the greatest number of occurrences in the western part of its range (Michigan and Wisconsin). It is considered extirpated from five states and the province of Ontario. The KBB is currently being reintroduced in three locations, Concord, New Hampshire, northwest Ohio, and southeastern Michigan. KBB population augmentation is on-going at the Albany Pine Bush in New York and at Indiana Dunes National Lake Shore and West Gary in Indiana. The KBB is a flagship species of the globally imperiled oak savanna and pine barrens ecosystems. Oak savannas are characterized by prairie plants dispersed among stands of widely spaced oak trees. Pine barrens are typified by structurally diverse vegetation dominated by scrub oaks and pitch or jack pines interspersed with grassy openings with wild lupine and nectar plants. These ecosystems have evolved to thrive with periodic fire disturbances. KBB reintroduction and/or augmentation programs should include concurrent habitat restoration and/or management activities to ensure suitable habitat is maintained for the butterfly.

Manual Description

Each section of the manual provides a brief description of an activity associated with captive rearing or information on the KBB life stage discussed. In addition, information on techniques used by each organization are presented in a table format in each section. Roger Williams Park Zoo in Providence, Rhode Island also participates in KBB captive rearing (larval rearing only) for New Hampshire. Their protocols follow New Hampshire's, unless otherwise noted throughout the manual. The techniques in this manual are those used up to the time of the completion of the manual edition (noted on cover page). Improvements to individual captive rearing techniques are made every year and sometimes mid-season. Subsequent editions of this manual will include any new improved captive rearing techniques.

Principles of Insect Rearing (Basic Environmental Conditions and Handling)

There are several basic requirements to insect care that should be applied to any captive rearing program. These include maintaining appropriate temperature and humidity levels, providing the appropriate light quality and duration in the captive facility, and using proper butterfly handling techniques (Singh and Ashby 1985). KBB care and rearing is done simultaneously with the *wild* KBB life cycle and therefore environmental conditions in the captive rearing facility should correspond with current outdoor conditions (this care is different from other butterfly laboratories where producing flying adults for commercial purposes requires facilities to mimic temperatures and humidity levels that do not normally occur at that geographic region or season. For more information on commercial breeding techniques see Venters and Rogers 2001.) To promote high survival rates and increased breeding success in the captive rearing facility there are some basic environmental conditions that should be maintained in order to provide ideal environmental conditions regardless of rainy or cloudy weather in the field. Keep in mind that less than ideal conditions outside and optimum conditions in a laboratory can result in uneven distribution between captive raised and wild KBB. If the goal of your program is to augment the wild population you will want to have both captive reared and wild KBB interacting with one another during the same time period in the field. Below is a brief review of the basic requirements for captive rearing programs. **More information on these requirements can be found throughout the manual.**

Temperature: Ideal temperatures for rearing KBBs range between 23°C (73.4°F) and 28°C (82.4°F) (Lane and Welch 1994). Extreme high or low temperatures can desiccate eggs, retard or speed up life cycles, and affect fecundity and fertility (Singh and Ashby 1985). The captive rearing programs have found that adult care especially during mating and egg laying can tolerate temperatures into the 90's °F.

Relative Humidity: Ideal relative humidity levels for rearing adult KBB range between 50% - 80% for oviposition and 40% - 60% for rearing immature stages (Lane and Welch 1994). Relative humidity can be critical for insects especially during the egg stage where mold and fungal growth can be detrimental (Singh and Ashby 1985). Humidity levels can be raised artificially by misting adult rearing cages, but care must be taken to avoid puddles of water which can be detrimental to butterflies if they crawl or fall into them.

Light: Ideal light quality and duration should mimic the natural photoperiod outdoors. Changing the quality of light (wavelength and intensity) and light duration can alter the insect's natural daily cycle. Full spectrum lights can be used to mimic sunlight if natural light is unavailable in the captive rearing facility or when it is overcast. If artificial lighting is used on a regular basis, the lights should be timed for a 16 hour day and an 8 hour night (Kamano and Sato 1985, Roberston 1985).

Handling: Different techniques are used to handle each KBB life stage. In general, handling should be kept to a minimum and done with care. Many studies have shown that the scales of Lepidopteran wings can cause allergic reaction in humans (Bellas 1989), and it is suggested that handlers wear protective clothing such as dust masks, gloves and outer clothing (Wirtz 1980, Wolf 1984).

Karner Blue Butterfly Habitat Needs and Release Site Description

It is essential that habitat management and/or restoration be conducted in conjunction with KBB captive rearing programs (USFWS 2003). All captive rearing organizations partner with other agencies, non-profits, contractors, or within other programs in their own agencies to conduct habitat management in areas where KBB's are to be released, currently inhabit, or may potentially occupy. Habitat management is needed to restore overgrown pine barrens and oak savannas to suitable habitat for the butterfly. These early successional habitats will always require management, especially since natural wild fires have been all but eliminated. Prescribed burns are necessary to reduce litter and the duff layers, to allow fire adapted plants to persist, and to create sandy openings for wild lupine establishment. In some cases, cutting or mowing may take the place of fire, however mechanical management does not result in reducing the litter and duff layer. Management of occupied KBB sites requires working with the USFWS on a permit to authorize take of KBBs that results from the management activity. Most burns conducted in occupied KBB habitat are done on a rotational basis so that not all the occupied habitat is disturbed at the same time; and sites burned one year are not adjacent to sites burned the following year. This strategy allows recolonization of the burned site prior to the next burn (refer to Appendix G of the KBB Recovery Plan (2003) for more guidance on KBB habitat management).

Prior to starting a KBB captive rearing program, potential release sites in managed habitat should be identified. Since specific habitat characteristics vary across the KBB geographic range (USFWS 2003), it may be difficult to identify ideal habitat release locations. In general there should be a combination of wild lupine (*Lupinus perennis*) and adult nectar plants, as well as a structurally diverse vegetation including grasses, forbs, shrubs, and trees. Unpublished studies in New Hampshire have revealed that shaded lupine (30%-60% canopy cover) is important to the fecundity of female KBBs, in addition to providing preferred oviposition sites (Grundel et al 1998b). Preliminary results of a New Hampshire and New York study on the timing of management and associated impacts to wild lupine and female fecundity demonstrated that an early April burn increased female fecundity during the first few days of oviposition. There is still much to learn about managing oak savannas and pine barrens habitats, particularly in understanding how technique and timing influences the KBB, what the correct combinations of management techniques are, and what exact acreage is needed to support a viable ecosystem. Many states are also challenged with restoring KBB populations in areas that are fragmented by development. Table 2 provides descriptions of areas that are part of the on-going KBB reintroductions/ augmentation programs.



A field of seeded wild lupine in a powerline corridor in New York (left). (Photo credit: L. Webb)



A prescribed burn in the Concord Pine Barrens, New Hampshire (right). (Photo credit: S. Fuller)

Table 2. Karner Blue Butterfly Site Description

State	Habitat Site Name	Total Acres at Site	Habitat Manager	Habitat Management
IN	The Nature Conservancy (TNC)-Southern Lake Michigan Rim Project, Indiana Toleston Macrosite-West Gary Recovery Area, Indiana Dunes National Lakeshore (IDNL) - Howe's Prairie Recovery Site	TNC-600 acres IDNL-200 acres	The Nature Conservancy National Park Service	Understory clearing, canopy opening and supplementing existing host plant populations. Eventual implementation of controlled burn plan.
	MI	Petersburg State Game Area	500-600 acres Joe Robinson, Michigan Department of Natural Resources and Environment	
NH	Concord Pine Barrens	300 acres	New Hampshire Fish and Game Department	Mowing, brush cutting, prescribed burning, planting native plants.
NY	Albany Pine Bush	3,100 acres	Albany Pine Bush Preserve Commission (APBP)	Prescribed fire, mechanical treatments (mowing, whole-tree harvest, grubbing), chemical treatments (herbicide), planting of native plants.
OH	Oak Openings Metro Park and Kitty Todd Preserve	1,000 acres	Toledo Area Metro Park, The Nature Conservancy	Mowing, brush cutting, prescribed burning, planting native plants.



A Karner blue release site in the Albany Pine Bush. (Photo credit: N. Gifford/APBPC)

Captive Rearing Facility Description

The size and location of captive rearing facilities varies (Table 3). Important considerations when choosing a captive rearing facility are the proximity of the facility to a food source (wild lupine), and to the release site(s); ideally, the facility should be close to both for convenience and to help minimize travel time. Other things to consider are the ability to control temperature and humidity, an area to sanitize equipment, and the use of natural lighting.



Greenhouse and butterfly containment area in Ohio. (Photo credit: P. Tolson)



The polyhouse roof in New Hampshire allows natural sunlight in to the rows of mating tents below (left). Office space (with regular roof) doubles as lab area for larvae and egg storage during the heat of the summer (right). (Photo credit: L. Webb)

Table 3. Captive Rearing Facility Description

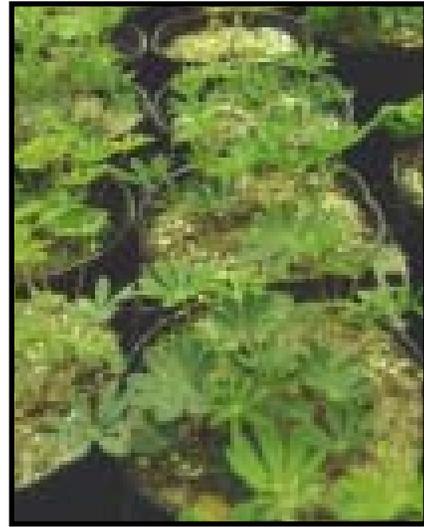
State	Captive Rearing Building Location	Distance to Wild Lupine Source	Distance to Release Site	Captive Rearing Descriptions
IN	Great Lakes Research Education Center and USGS Lake Michigan Research Station, Indiana Dunes National Lakeshore (IDNL), Porter, IN	1.5 miles	TNC-25 miles IDNL 1.5 miles	Greenhouse
MI	Detroit Zoo, Detroit, MI	40 miles	40 miles	Polyhouse facility
NH	New Hampshire Army National Guard Base, Concord, NH and Roger Williams Park Zoo, Providence, RI	50 feet in NH, potted wild lupine just outside door in RI	1 mile	Building in NH has part polyhouse roof and part regular roof. Building has heat control. Doors between outside and inside have small control space to prevent other insects from coming in and KBB's from exiting. Windows are covered with antivirus insect screening. RI captive rearing occurs in a small room with sky light and door to the outside both covered with anti-virus insect screening.
NY	Farnsworth Middle School, Guilderland	Potted wild lupine	5 miles	Greenhouse structure (2' x 4' x 6') in a typical middle school science classroom. The greenhouse is covered in clear plastic and located in front of a large window so that indirect sunlight reaches the rearing chambers.
OH	Toledo Zoo, Toledo, OH	18.3 miles	18.3 miles	Polyhouse facility

Propagation of Host Plant-*Lupinus perennis*

Propagation of wild lupine (Table 4) requires a local seed source picked in July prior to dehiscence (pods bursting). In addition to plant propagation, many states also directly sow seed in the fall. If wild lupine is needed for first brood larval rearing, propagation should begin in late winter in a heated greenhouse. Most states have found that shading lupine plants with a shade cloth prevents direct sunlight from wilting or drying out the plants, thus promoting better nutritional forage quality for the larvae. In New Hampshire, a study on fecundity found that females who fed on shade grown lupine (30%-60% shade) oviposited a greater number of eggs earlier than females who fed on lupine grown in direct sunlight. Shaded lupine has also been known to affect larval growth rates and larval duration (Grundel et al. 1998a).



Wild lupine seed comes in various colors.
(Photo credit: L. Webb)



Wild lupine propagated in Ohio.
(Photo credit: P. Tolson)



Wild lupine just emerging in New Hampshire. (Photo credit: L. Webb)

Table 4. Propagation of Host Plant-*Lupinus perennis*

State	Seed Storage	Scarification Methods	Pots	Soil and Amendments	Water	Other
IN	Dry	Manual with light sandpaper.	2.5" x 2.5" x 6", plastic.	60% sand/40% peat. <i>Rhizobium</i> inoculate, type H (Prairie Moon Nursery, Winona, MN) used.	As needed.	Plants are grown only for restoration. Host plants for captive rearing are wild collected.
NH	Freezer	Seeds soaked in hot water over night. Sand paper and coffee grinder prior to soaking used in past, but this step is not needed if hot water soak is performed.	2 7/8" x 2 7/8" x 9" pots to accommodate the long tap root.	One part sand per two parts metro-mix. <i>Rhizobium</i> inoculate, type H (Prairie Moon Nursery, Winona, MN) used. Soil pH checked regularly. To lower pH a liquid acidifier, bought at local garden center, works the quickest.	Soil kept damp until leaves emerge. Watered weekly or more if soil is dry.	Pine needles (white pine or pitch pine) placed in bottom of pots to help keep acidity at the proper level. Seeds more than one year old have low viability.
NY	Freezer	Seeds soaked in hot water over night		Seeds mixed with <i>Rhizobium</i> inoculate.		
OH & MI	Dry, outside	Seeds are scarified with medium grade sandpaper and are placed in hot water for one hour.	2-gallon pot.	Faford 52 soil mix. Seeds mixed with <i>Rhizobium</i> inoculate, type H (Prairie Moon Nursery, Winona, MN), and placed in a clear plastic box for 24 hours in sunlight. Soil fertilized once a week with 50% dilution of fish emulsion.	Seedlings are watered thoroughly every day in summer, once a week in the winter months.	Soil mix needs to be well drained to prevent root rot. In the event of spider mites or white fly infestation, foliage is cleansed with diluted dish soap (1/4 tsp/cup water) applied with spray bottle. The treated plants should be set aside for several days and then thoroughly rinsed with tap water before allowing them to be eaten by larvae.

Karner Blue Collection

Collecting KBB females in the wild should occur a few days to one week after the first sighting of wild KBBs. Females usually mate within the first three days of flight, but not all females may be mated. Slightly tattered wings on females and a heavy looking abdomen are useful indicators that females are older, but there is a risk that she may have already laid the majority of her eggs. Females can lay eggs everyday, however an absence of egg laying behavior on a particular day does not indicate that she is done laying eggs. Most organizations collect females over the course of several days and at several locations to maximize the potential that they were successfully mated, and to ensure genetic diversity among the females collected. Peter Tolson (Toledo Zoo, pers. comm. 2008) reported that females collected the third week of the first flight had the highest fecundity. In many cases, those doing the captive rearing are not the same individuals that work in the field monitoring for wild KBBs, and therefore it is imperative that groups communicate with field staff to ensure that collection is taking place during the appropriate time period.

The number of females to be removed from the wild that will not impact the wild population must be determined prior to collection, as well as the staff effort needed to raise the next brood in captivity. The average number of adult KBB females removed from the wild by captive rearing groups is 20 per brood. The number removed should be discussed with the field/monitoring staff to ensure that it will not be detrimental to the overall population numbers at the site. All of the captive rearing groups collect females in the field with a butterfly net with soft netting, except in Michigan, where they wait until the butterfly lands, and then place the transport boxes over them. This reduces the risk of injury that may be caused by netting. Containers used to transport the butterflies vary (Table 5). All groups transport the containers in a cooler (except in New Hampshire where the transport time is only five minutes); the cool, dark environment keeps the KBB's relatively inactive and calm during transport (via car). In addition to collecting females in the wild for captive rearing, New York and New Hampshire have collected wild eggs and larvae from New York and transported them to New Hampshire for rearing. Eggs were kept on original substrate and put in jars or plastic boxes. Larvae were placed in a paper towel-lined plastic box, and the top of the box was kept slightly open and out of direct sunlight. Collecting adults seems to be the most reliable and more efficient technique, as it is much harder to search the plants for larvae or eggs.



Searching for female KBB's in a field of wild blue lupine in New York (above). (Photo credit: L. Webb)

Collecting adult female KBB's in Ohio (below). (Photo credit: P. Tolson)



Table 5. Karner Blue Collection

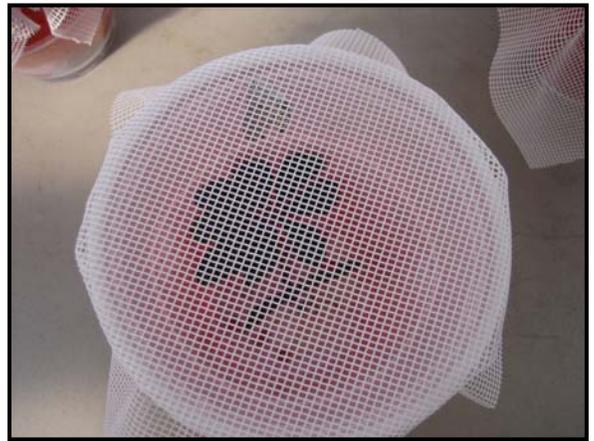
State	Female Container	Transporting Container
IN	6 oz. plastic food containers with a vented lid supplied with a lupine cutting, a native nectar source cutting (<i>Coreopsis</i> or <i>Phlox</i>) and an artificial nectar source consisting of cotton soaked with a 10-20% honey-water solution held in a 0.5 inch section of clear plastic tubing.	A solid lunchbox type cooler and transported by car from the field to the rearing facility. KBB's in containers for up to 2 hours.
NH	Wild NH females are put in separate 12"x12"x12" tents (Bioquip Products).	Female container is the transporting container. Wild NH females are transported by air conditioned car for five minute drive from the pine barrens to the rearing facility.
NY	Same as IN.	A cooler with towel wrapped ice packs and transported by car. KBB's in containers for 3 hours to NH or 15 minutes to Farnsworth Middle School.
OH & MI	10 cm x 10 cm x 18 cm transparent, plastic container (AMAC Plastics Products Corp., Sausalito, CA) with artificial nectar source fastened inside of each plastic container consisting of a rubber-capped florist tube with a cotton wick protruding through the cap filled with a 10% clover honey/water solution.	Thick-walled 32 quart Styrofoam cooler that contain a single ice pack separated from the butterfly containers by bubble-pack insulation. KBB's in containers for 3 hours from Michigan to Ohio.

Holding and Care of Wild Caught Females in Captive Rearing Facility

Several facilities collect wild female KBBs and hold them for oviposition, subsequently care for the larvae through the four instars and release the captive reared butterflies as adults. Premature death of a wild female may result in the loss of 100+ eggs, therefore it is very important to reduce or eliminate premature death of wild females. This may be accomplished by both limiting accidental mortality and increasing longevity by a variety of methods, including careful attention to holding enclosures, adequate feeding strategies, and providing appropriate supplemental light, heat, air flow, and humidity. Successful methods for holding and caring for wild caught females are summarized in Table 6 below.



Mesh tents covering a potted lupine plant in Ohio. (Photo credit: P. Tolson)



Female ovipositing container in New Hampshire. (Photo credit: L. Webb)



A 10% clover honey/water solution is provided in a rubber-capped florist tube with a cotton wick protruding through the cap in Ohio. Notice the wear of the wings, indicating an older female, and the heavy abdomen, indicating an egg load. (Photo credit: P. Tolson)



A female KBB. (Photo credit: D. Leopold)

Table 6. Holding and Care of Wild Caught Females in Captive Rearing Facility

State	Female Enclosure	Nectar Source	Environmental Conditions
IN	<p>One female is placed on a marked host plant. Host plants are potted lupine plants started from the previous year that have reached about 6 inches in diameter and height. The potted lupine is covered with a green mesh fabric (tent) and secured with string. The mesh tents are held fairly close over the host plant to limit movement of females away from the host plant and to stimulate ovipositing.</p>	<p>A native nectar plant cutting placed in a florist sipper tube and an artificial nectar source consisting of a 0.5 inch cube sponge soaked in honey-water solution (10-20%) are included within the mesh tent covering the lupine.</p>	<p>Netted host plants with females are kept in a natural light setting within USGS Lake Michigan Research Station greenhouse. Host plants are misted regularly. Temperature is kept as close to 80-90° F as possible.</p>
NH	<p>Females are placed in either their own ovipositing container which is a 10 oz SOLO cup or together with other females in a mesh tent. Putting each female in her own container allows an accurate count of eggs per female.</p>	<p>A honey soaked and water soaked cotton ball is placed at the bottom of the cup around a small hole where one or two stems of lupine leaves and a stem of a nectar flower reach down to a smaller cup containing water. A mesh cloth is held over the top of the cup with a rubber band. Lupine, nectar flowers, and cotton balls are refreshed daily or as needed.</p>	<p>Cups or tents are placed on a counter where they are exposed to natural light or under a UV full spectrum light during cloudy or rainy days. To keep temperature between 80° F and 90° F, halogen heat lamps or air conditioners are used. To keep humidity above 50%, cups are misted with water and the floor is soaked with water. A fan is turned on or windows are opened (anti-viral screening used in windows) to allow for air flow.</p>

State	Female Enclosure	Nectar Source	Environmental Conditions
NY	<p>Each female is placed in a rearing chamber with a lupine plant growing in a half gallon milk carton. A rectangular mesh covers the plant leaving a few inches of space above the plant. The bottom of the mesh is held in place with a rubber band.</p>	<p>Native plant cuttings and honey water solution in sipper tube.</p>	<p>A heat lamp connected to a thermostat maintains a temperature of 72-80°F. Open trays of water next to the plants insure a high humidity. Two Reptisun® UVB bulbs placed 6 inches above the plant are placed on a 12 hour cycle.</p>
OH & MI	<p>Butterflies are placed in a tented enclosure consisting of a mesh-covered potted lupine plant in a 2-gallon plastic pot. This covering is a cylinder of white poly mesh netting #65-50 (Jason Mills, Westwood, NJ) sewn together with the seams arranged on the outside of the enclosure to assure that none of the butterflies can become trapped in the seam and harm themselves. The nets fit snugly over the pot rim, and are secured with a #107 (7" x 5/8" x 1/16") rubber band to prevent escape of butterflies and to deny entrance to predators.</p>	<p>A nectar source is provided from a rubber-capped florist tube with a cotton wick protruding through the cap filled with a 10% clover honey/water solution (see picture). This is placed in the soil of the potted plant. The honey/water solution is refilled every day and replaced and disinfected in diluted common household bleach every other day. Disinfected tubes and wicks are rinsed 10x in tap water and 5x in distilled water before being reused. We also hand feed adults daily to maximize longevity. In this process, adults are encouraged to climb on the honey-moistened wicks of the feeding tubes. They are then gently placed with the tubes in one of the 10 cm x 10 cm x 18 cm transparent plastic containers used for transport. After the proboscis is withdrawn, the butterflies are placed back into their netted enclosures. A small 3" pot of <i>Lantana camara</i> or <i>Pentas</i> sp (commercially grown) is also placed in the tent as a nectar source.</p>	<p>To prevent dehydration the mesh-covered enclosures are misted by hand or, during periods of heat and low humidity, by an Ecologic Technologies® Rainmaker misting system (Ecologic Technologies, Pasadena, MD). The Rainmaker is set up to mist for a 2-minute duration every 15 min. from 11:00 am - 6:00 pm. In extremely hot weather (>30°C) a garden soaker hose is placed on the cement pad near the enclosures to provide added humidity through evaporation. During low light levels and cooler temperatures, quartz lighting and propane heaters are used.</p>

Egg Collection

Females will oviposit whether they have been fertilized or not, and eggs are usually laid singly on lupine leaflets, stems, or on the surrounding enclosure. After egg laying (or if the female does not lay eggs), the female is removed from the enclosure. Some organizations release females back into the wild if she does not lay eggs during the first few days, while other states release females after a predetermined number of eggs are laid or the number of days of laying eggs is met. In other states, females are kept until they expire. Oviposition ranges from zero to more than one hundred eggs per day. Refer to Table 7 for egg collection methods.



Egg on wild lupine stem in Ohio.
(*Photo credit: P. Tolson*)



Looking through a microscope you can see the larva through the egg. (*Photo credit: L. Webb*)



In New Hampshire ten eggs per Petri dish, labeled with colored tape, are stored in plastic crisper boxes by lineage. (*Photo credit: L. Webb*)

Table 7. Egg Collection

State	Egg Collection Notes	Egg Collection Tools	Egg Collection Container
IN & NY	<p>After the females are returned to the field, the mesh tent is carefully removed from the host plant. When dissecting the host plant for eggs it is important to leave some leaves intact as not every egg is discovered and those larvae that hatch out onto the host plant will need a food source. When inspection is completed the mesh tent is again placed around the host plant (and the pots returned to a frame rack). The host plants are inspected daily for larvae that hatched out of undiscovered eggs.</p>	<p>Eggs on the mesh enclosure are cut around using cuticle scissors. If eggs can be brushed off safely they are brushed off with 0 gauge model paint brushes.</p>	<p>Eggs on mesh are placed in larval rearing containers (Table 8). If eggs are brushed off with paint brushes, no more than five eggs are brushed together into larval rearing containers.</p>
NH	<p>Nectar flowers, wild lupine, and containers are checked for eggs everyday, or the plant material is set aside with the date printed on a cup to be searched later. All plant material is kept for a second (and sometimes third) check of eggs. Plant material is discarded near the release site in case it contains undiscovered eggs.</p>	<p>The eggs on the plant material are brushed off with a damp #2 camel hair paintbrush or the plant material is cut into small sections with the eggs still attached. Scissors used to cut plant material and paintbrush are sterilized between individual or lineage groups with a 1:4 bleach:water solution and double rinsed in water.</p>	<p>Ten eggs are placed in one small Petri dish lined with filter paper. The dish is marked with the lineage and the number of eggs in the dish.</p>
OH & MI	<p>Wild caught females are monitored every day for egg laying. An official egg count is performed every 2nd day by visually inspecting each leaflet and stem of the lupine plant. The undersides of the leaflets are viewed with a dentist mirror. When 10 eggs have been laid the female is moved to a new host plant. Nectar plants that are replaced when the flower heads are spent are also checked for eggs. In the event that a host plant becomes infected with spider mites, the foliage supporting the eggs is gently washed, clipped from the infected plant, and transferred to a healthy host plant.</p>	<p>To remove eggs, the area of foliage containing the egg/s is clipped off and placed in the host plant pot. Loose eggs, such as those oviposited on the substrate, are gently transferred with a damp #2 camel hair artist brush.</p>	<p>When 10 eggs have been laid the female is moved to a new host plant. The ideal carrying capacity of each healthy host plant is estimated to be between 10-12 larvae/lupine. Some females may lay over 50 eggs in a single night.</p>

Inventory and Care of Larvae

Eggs oviposited by first brood females begin to hatch immediately up to and through seven days after being laid. Some eggs may not hatch (unfertile) and some eggs may become moldy or dried out. Larvae go through four instars and reach pupation in about three weeks. Second brood eggs that overwinter (refer to Second Flight Egg Overwintering Protocol) begin to hatch in early to mid-April, and usually take an extra week (about four weeks) to reach pupation. Handling larvae during the first two instars should be done very carefully: as larvae move into third and fourth instars they tend to be a little more “tough.” Tools used in larvae rearing and handling include magnifying glasses to locate larvae, paintbrushes to gently brush early instars off or onto to something, and soft forceps to pick up late instars (see Table 8 below). As larvae move into their third and fourth instars, they start eating much more lupine and care must be taken to provide enough lupine for feeding, especially if larvae are not reared directly on a lupine plant. Care should be taken to not overcrowd larvae and larvae of different sizes should not be left in the same container as larger larvae may cannibalize their smaller conspecifics. Larvae can also be cooled to slow the growth rate, but this is not recommended as some mortality is common when this technique is applied.



When KBB larvae feed, they eat the leaf's mesophyll, and leave the epidermis creating a “window pane” pattern on leaves of wild blue lupine. *(Photo credit: L. Webb)*



KBB larvae on lupine in Ohio. *(Photo credit: P. Tolson)*



Larval rearing chamber in Indiana under ultraviolet lights. *(Photo credit: National Park Service)*



KBB larvae starting to morph into pupae. *(Photo credit: P. Tolson)*



Looking through a microscope, note hairs on this freshly hatched larva. *(Photo credit: L. Webb)*

Table 8. Inventory and Care of Larvae

State	Larvae Containers	Number of Larvae per Container	Handling Larvae and Sanitation	Keeping Track of Larvae	Environmental Conditions	Feeding Larvae
IN & NY	Two oz. clear food portion containers with lids. Containers are stacked and placed inside clear plastic Rubbermaid storage boxes covered with clear plastic film. As larvae began to hatch they are transferred to new containers.	When the larvae are very small, 2-3 may be held in each container but as soon as possible all larvae are transferred to individual containers which are placed in storage boxes. Containers with larvae from the same female are placed in the same storage box.	Every effort is made not to touch or transfer frass between containers and all equipment and tools used are cleaned with alcohol if contact with frass occurs. Caretakers wash hands with anti-bacterial soap if they come in contact with frass and between handling separate lineages. Each storage box is supplied with an individual set of transfer tools to be used only with those larvae. To reduce the chance of infection, the larvae should be transferred to new containers each day.	Each egg container lid is marked with an E and the number of eggs. As larvae began to hatch they are transferred to new containers with host leaves and marked with an L and the number of larvae. Each container is opened, larva accounted for, growth and health noted, and host leaves discarded and replaced with fresh host leaves. The total number of larvae is recorded each day and all mortality events are accounted for.	Storage boxes are placed within a chamber lit with a UV light along with the collected host plant cuttings (refer to picture).	Each container is supplied with lupine cuttings to serve as a food source for developing larvae. Upon initial transfer to these containers eggs laid on host plant leaves are placed in the containers without adding more leaves. New fresh host plant leaves are collected on a 1-2 day basis from local lupine plant sources but may also be collected from greenhouse or nursery stock if available. Fresh leaves are added as needed. Small leaves tend to dry out quicker, so larger leaves or several small leaves are used.

State	Larvae Containers	Number of Larvae per Container	Handling Larvae and Sanitation	Keeping Track of Larvae	Environmental Conditions	Feeding Larvae
NH	Larvae emerge from eggs stored in small Petri dishes (50x11mm, Fisher-Scientific) lined with filter paper. Dishes are stacked four high in large crisper boxes (12x9 x3.5”) and placed on top of a multi-dimensional plastic liner (looks like a plastic egg container) and lined with a moist paper towel. The multi-dimensional plastic liner allows air flow under the Petri dishes. After the second or third instar, larvae are put in either small (7x5x3.5”) or large crisper boxes lined with paper towels.	Up to 10 larvae per small Petri dish. After second or third instar, approx. 20 larvae are put in a small crisper box, or approx. 40 in large crisper box. If larvae are placed in large containers too early they will crawl under the paper towel, possibly seeking warmth and food, and may die there. Put lupine under towel if this is suspected.	Handling early instar larvae is done very carefully with a camel hair paintbrush. If larvae are very still and are thought to be going through an instar, they are not touched as interruption of this process results in death. Late instar larvae can be handled with butterfly forceps. Instruments used in handling larvae, frass, or lupine in each dish is rinsed in a 4:1 water/bleach solution and double rinsed in water. If a group of larvae turn black they are immediately removed. If a disease is suspected, a sample of the population is sent to Mississippi State University for an insect pathology report (See Insect Pathology Resource Section).	Once larvae start to hatch the dish is numbered and recorded on a data sheet. Larvae are counted everyday or every other day as time permits. Any missing larvae are noted on the sheet and searched for the next day. Half eaten lupine leaves are kept in a container labeled with the lineage name and “refuse”. The refuse boxes are checked daily for “lost” larvae. Larvae found in refuse boxes are placed in new containers, labeled and kept isolated because of higher risk of virus or bacteria due to the moldy vegetation.	The current lab has two rooms: one with a polycarbonate roof that allows sunlight in and a smaller room with a regular roof and no heat. Between these two rooms is a small enclosed walkway with an air conditioner with humidity control. Among these three rooms, the larvae can be cooled down or warmed up to mimic conditions in the wild.	One lupine leaflet is placed in each petri dish for newly hatched larvae. Full lupine leaves are put in larger containers. Fresh leaves are added daily and half eaten lupine leaves are kept in refuse container. All lupine plants brought in from the wild are rinsed in a 10% bleach solution, double rinsed in water, and patted dry to eliminate any viruses or bacteria on the leaves. Left over lupine is stored dry in a refrigerator. Nutrients begin to leach out of lupine leaves if stored in water. Ideally, lupine is picked fresh daily.

State	Larvae Containers	Number of larvae per Container	Handling Larvae and Sanitation	Keeping Track of Larvae	Environmental Conditions	Feeding Larvae
OH & MI	Larvae are placed on potted lupine plants that are netted and placed in a Rubbermaid plastic box with a substrate of paper toweling. Larvae are transferred to new host plants as leaves are denuded. It is important not to overcrowd the larvae or to keep larvae of differing sizes on the same host plant to reduce the chance of cannibalism. When pupation is imminent, several large pine bark chips are added to the pots.	10	Larvae are not handled unless it is absolutely necessary. Larvae are moved with a damp #2 camel hair artist's brush.	The larvae and pupae in each pot are counted and recorded three times a week on the data sheet and a running total is kept for each instar. Condition of larvae and pupae are noted. A running total is kept for all eggs, larvae, pupae, and adults produced by a given female in a studbook maintained in an informal Microsoft Excel [®] spreadsheet.	Each lupine plant must be thoroughly checked (i.e. examination of each leaflet) for predators before placing a KBB of any life stage upon it. Polyester netting covering the plants excludes predators, but each plant is checked every 2-3 days to ensure that no predators have entered the pots nor have hatched from undetected eggs. Any detected predators are simply crushed with fingers.	Larvae are normally kept on netted, potted lupine plants. Larvae are fed fresh lupine leaves; stems are placed in a rubber-capped florist tube.

Pupae

After about 2-3 weeks (3+ weeks first brood, 2+ weeks second brood) most larvae have reached fourth instar and have begun the process of pupation. Larvae will slow down and often become fixed at a point on a host leaf or the base of the container. Larvae may also burrow 1-2 cm into the soil at the base of the host plant. Larvae will stop feeding at this time, contract, and the exoskeleton will begin to harden and take the shape of the pupa. When pupae first form they are still very soft and fragile. It is best not to disturb them until 24 hours after the pupae is formed, at which point they are harder and can withstand handling. Pupae can be picked up with blunt forceps or they can remain on the substrate they pupated on (lupine leaf, pine bark chip, paper towel) and be moved (if necessary) to their eclosure tent. Table 9 summarizes the methods used for holding pupae.



Pupae are first green (right) then turn brown and yellow, then blue, eventually their wings, antennae, and abdomen can be seen through their transparent chrysalid (left). (*Photo credit: L. Webb*)

Table 9. Pupae

State	Pupae Container
IN & NY	Pupae are transferred to larger 4 oz. food portion containers with lids. Up to 5 pupae of the same age groups are put in each container. Pupae are monitored daily for the onset of eclosion (transformation into adult). As the green pigment begins to turn to light brown and the darkened eye spots begin to form the pupae are ready to be placed in transfer containers which are 10-12 oz. plastic food containers with some grass stems criss crossing through the container to serve as supports and climbing structures in case of accidental premature eclosion. Once all pupae have been inspected and those ready to eclose have been determined, the transfer containers are taken to the field to be placed in the release nets. NY keeps pupae separated by originating female.
NH	Pupae are moved to filter lined Petri dishes without the tops and placed in a 12"x12"x12" mesh tent. A small wooden dowel is placed in the dish to provide a place for newly eclosed adults to crawl out onto and dry their wings. If pupae turn black and do not eclose after 15 days they can be prepared for slide smears or sent for an insect pathology report to determine cause of death (refer to Insect Pathology Resources section). A daily count of pupae is recorded.
OH & MI	Pupae are left to pupate on the host plant, under a pine bark chip, or in the soil. Pupae that have been removed from the host plant and placed in containers are then placed on damp sand in a Petri dish.

Second Flight Adult Care and Breeding

KBB fertility and fecundity may be affected by several factors: health, nutritional status, longevity, size of the female, quality of the sperm packet delivered by the male, and the environmental conditions of the facility where the butterflies are held. It appears that serial mating is not common in KBBs, so the nutritional status of KBBs at the time of mating may be very important and a honey solution should be given to adults. Some organizations have tried various flavors of Gatorade® and found that KBBs drink it readily, but staining to the abdomen from the colors may occur. Gatorade® is used as a supplement in captive rearing programs for the Miami blue butterfly. In the table below you will find the specific environmental variables and nutritional resources that each facility makes use of when caring for adults and encouraging mating in the lab. In general, KBB mating occurs when temperatures are over 80° F, humidity is over 75%, and the sun is shining brightly, usually between 11:00 am and 3:00 pm.

Captive rearing programs should address genetic concerns such as inbreeding and genetic drift. Due to genetic concerns, most organizations only captive rear KBBs in a limited capacity, bringing in “fresh genetics” (wild females) each year. Detailed records on each individual are kept in order to prevent inbreeding. Hand pairing butterflies is a common breeding technique used by commercial butterfly breeding companies (Venters and Rogers 2001). However, KBB captive rearing organizations do not use this type of strategy, but instead rely on providing the ideal environmental and nutritional factors that encourage KBB’s to mate when they feel ready to do so on their own. Captive rearing of the endangered Palos Verde blue butterfly also does not use the hand pairing because it is tedious, not conducive for mass rearing, and adults chosen for pairing may be subject to artificial (and unwanted) selection (Mattoni et al 2003). The strategies used by the various organizations for care and breeding of 2nd flight KBB adults are presented below in Table 10.



Looking through a mating tent in New Hampshire. (Photo credit: L. Webb)

The mating scheme and number of males and females in each tent drawn up on a white board keeps New Hampshire organized. (Photo credit: L. Webb)

Table 10. Second Flight Adult Care and Breeding

State	Second Flight Adult Care and Breeding
IN & NY	All adults are released into the wild. No breeding in captivity occurs.
NH	<p>After adults eclose out of their pupa case they are left alone to dry their wings for about a half hour. Depending on each adults' genetic lineage and sex, they are either marked and released into the wild or placed into a mating tent. Within the mating tent (24"x24"x24", Bioquip), a bouquet of lupine stems, lupine flowers, and other nectar flowers are placed in a vase which has been capped with a parafilm cover. A small dish of sand soaked with protein solution is placed on the bottom. The protein solution is made up of: 0.10 g NaCl, 7.2 g KCl, 0.24 g CaCl₂, 5 g casein, and 1 liter of water. This sand dish is replenished with plain water on a daily basis or as needed. A honey solution soaked cotton ball is also placed on the bottom of the tent. The honey solution is made up of: 4 g ascorbic acid, 1 g sorbic acid, 150 ml honey, and 1000 ml water. This as well as another cotton ball with just plain water is refreshed on a daily basis or as needed. Ideally, one mating tent contains 10-15 males and 5-10 females. After three to four days, the bouquet of flowers and lupine leaves is replaced. Mating is usually observed when the sun is shining, temperatures are high (94-96° F) and humidity is high (75-80%). In order to maintain these optimal conditions, UV full spectrum lights are used to mimic sunlight, a portable heater and halogen lights are turned on to raise the temperature, air conditioners to lower the temperature, the floor is wet down for evaporative cooling, and tents misted to raise the humidity. When mating is observed, length of time paired, temperature, and humidity are recorded. If an individual female's eggs need to be tracked, she is pulled out and put in her own oviposition container (See Holding and Care of Wild Caught Females Section). As adults perish within the mating tents they are removed and newly eclosed individuals are added. After two weeks or sufficient number of eggs have been collected, any remaining adults are marked and released into the wild.</p>
OH & MI	<p>Second brood adults are placed into breeding enclosures that are essentially individual camping tents with "no see-um" mesh (Tropic Screen II®, Bioquip Products, Inc.). These mesh tents have a nylon floor and are placed approximately 4" off of the cement pad of the polyhouse by plastic shipping pallets to keep them dry and allow ventilation. This prevents the formation of dangerous pools of water (that will catch and drown small butterflies) or harmful mildew growth. The parabolic shape of the tent prevents the adults from getting caught in corners or on the peak as they would with a conventional tent. The breeding enclosure (mesh tent) is equipped with six large, healthy lupine plants, six large <i>Lantana camara</i>, <i>Pentas sp.</i> or <i>Asclepias tuberosa</i> plants, and two large artificial nectar sources placed on pedestals. These are fashioned from disinfected 4" diameter white scrub pads cut to fit into 4" diameter Petri dishes and then saturated with the 10% clover honey/water solution. Elevating the artificial nectar source on a pedestal prevents the attraction of harmful pests like ants to the breeding enclosure and makes them more visible to the butterflies. Males and females from the same locality (10 males, 10 females) are placed within the breeding enclosures for mating and oviposition. Under this system</p>

females have the opportunity to serially mate. Serial mating may be important in this species, as we have seen egg fertility decline in subsequent clutches of wild-mated females. The adults are provided with a timed overhead misting system. Dead adults are removed from the floor of the tent on a daily basis and replaced by new butterflies. It is difficult to count eggs oviposited in the tents because of the potential harm to the breeding adults. Oviposition is estimated by viewing the host plants through the mesh and determining whether or not adequate numbers of eggs have been laid. After the target numbers of eggs are reached for each locality (usually 50 eggs), the remaining 2nd brood adults are released at the reintroduction site. Deaths and daily releases of 2nd brood adults are recorded on a daily basis and tracked on a separate chart and a tag on each tent. Butterflies are encouraged, by gentle nudging, to walk on the saturated wick of one of the artificial nectar tubes. They usually immediately commence feeding. Using this technique, adult survival has increased as much as four weeks in the breeding facility. For breeding males, a slurry of animal dung is provided to help provide essential nutrients for the sperm packet. Nectar plants must be adequately watered to ensure that they are producing nectar. The condition of the host plant and nectar plant is also monitored every other day.



A breeding tent in Ohio. (*Photo credit: P. Tolson*)



KBBs mating in Ohio. (*Photo credit: P. Tolson*)

Release

KBBs can be released into the wild at all life stages: eggs, larva, or adult. While other life stages have been tried, the captive rearing organizations release adults, either as pupae or flying adult butterflies. The advantages of releasing pupae are: ease of handling, after eclosion adults can move freely and immediately to their desired location, and there is little chance of imprinting. The advantages of releasing adults are: captive reared adults can be marked and easily identified in the field, and individuals can be chosen to be kept for captive rearing mating. Raising pupae through the adult stage also virtually eliminates predation during the pupal stage. Prior to release of pupae or adults it is very important to check the upcoming weather conditions. Pupae and adults should not be released during inclement weather. Thunderstorms can cause pupae to become too damp in release tents. Extreme heat conditions (over 100°F) multiple days in a row may also be detrimental to pupae, since they have no way of cooling themselves during this stage. If extreme heat conditions are forecast, raise pupae to adults in the captive rearing facility and release. It is also very important to locate and evaluate the release sites. Release sites should have ample nectar and wild lupine, include some shade, and be close enough to travel to frequently. Table 12 reviews butterfly release methods.



Adding pupae to a release net in Indiana.
(Photo credit: *Post-Tribune of Northwest Indiana*)



Releasing adults in New Hampshire. (Photo credit: *B. Kimball/ NH Natural Heritage*)



Releasing adults in Ohio.
(Photo credit: *P. Tolson*)



Pupae release net in New York.
(Photo credit: *N. Gifford/APBPC*)



Inside pupae release net in New York.
(Photo credit: *N. Gifford/APBPC*)

Table 11. Release

State	Release Technique
IN & NY	Pupae near eclosure are placed in release nets which are inverted mosquito hats with the neck opening facing upward and suspended by fishing line from tree limbs about 3-4 feet above the ground. Release nets can also be hung from metal plant hangers, aka Sheppard’s Crooks, instead of tree branches to reduce the potential for wind damage. A plastic lid is placed over the top opening to keep rain from soaking the interior of the release net. Enough space is allowed for adults to climb up to the opening and fly away freely. Dried grass stems are placed inside the nets for supports and climbing as the adults emerge from the pupal case. NY sterilizes the grass stems collected in the field by placing them in a conventional or micro-wave oven. Release nets are also secured with fishing line from the base to the ground and usually attached to a large heavy branch to keep the nets from blowing around in wind or severe weather. In NY the fishing line is coated with Vaseline to deter ants. Pupae are simply placed in the bottom of the release nets and then left to eclose unassisted. Adults usually emerge in the morning, climb up the sides of the nets or the grass, unfold and allow their wings to dry and harden and then fly out of the top opening. Any pupae that do not complete metamorphosis are discovered when the nets are taken down about 5-10 days following the last placement of pupae. The final number of adults released is then recorded.
NH	After eclosure in the captive rearing lab, the sex of the adult is recorded and a unique number is marked on the wings of the butterfly with an ultra fine point Sharpie®. Adults are placed in a small tent and driven in an air conditioned car five minutes to the release location. The release site has a healthy population of wild lupine, New Jersey tea and other nectar sources. Adults are released any time of the day as long as it is not raining. If it is raining, adults are placed in a larger tent in the captive rearing lab with nectar flowers and released at the next available time.
OH & MI	Second brood adults that have previously been in breeding enclosures or are freshly eclosed are transported to the reintroduction site in a mesh-covered pot containing lupine and a nectar plant. They are released in early afternoon in fair to good weather conditions in an area where there are adequate nectar and host plants.
NH & IN	<u>Note Regarding Release of Eggs:</u> NH and IN occasionally release eggs into the wild when anticipating a high hatch rate and a lack of ability to raise all larvae adequately in the captive rearing lab. Releasing eggs does not allow you to keep track of hatch success, larval survivorship, or adult emergence and therefore should be done only when it is predicted that lab resources would be overwhelmed. To release eggs, approximately five eggs should be placed directly on lupine whorls. Lupine should be checked regularly for hatch and evidence of larval feeding.

Monitoring

Monitoring KBBs should be an integral component of all captive breeding, translocation, reintroduction and accelerated colonization programs. Monitoring can be done using a number of methods and target one or more life stages. However, since recovered population thresholds are based on achieving/maintaining a minimum number of adult butterflies, monitoring this life stage appears to be preferred (USFWS 2003). The KBB Recovery Plan (USFWS 2003) recommends that viable populations of the KBBs have at least 3000 butterflies and large viable populations have at least 6000 butterflies. Maintaining good records on the number of adult butterflies observed within release sites over time will also assist in establishing guidelines on the minimum release size (how many animals) and timing (how many years) needed to establish a self-sustaining subpopulation or meta-population, since such guidelines are currently lacking.

There are a number of monitoring methods that can be used to determine adult KBB population numbers. The degree to which these methods can be used to estimate population size varies. Methods currently used to monitor adult KBBs include: exhaustive searches, Pollard-Yates and modified Pollard-Yates transects (PY), Distance Sampling (D), and Mark-Release-Recapture (MRR). For smaller populations, collecting at least annual trend data using one of the methods to determine whether the reintroduced population is stable, increasing, or decreasing is recommended; for larger populations nearer the recovery criteria, estimate the population density using Distance Sampling methodology (Cathy Carnes, USFWS, pers. comm., 2010). Readers interested in learning more about monitoring methods should consult the appropriate literature including Appendix H, Monitoring Requirements and Guidelines, of the KBB Recovery Plan (USFWS 2003) and “A guide to the use of distance sampling to estimate abundance of Karner blue butterflies” (Grundel 2008).

Second Flight Egg Overwintering Protocol

Second brood eggs laid in July enter diapause, overwinter, and hatch in April the following spring. In order to release first brood KBB adults into the wild, a few organizations have developed ways to keep the eggs protected from predators and desiccation in an environment that allows for the natural temperature, humidity, and photoperiod to occur. Data collected from the field at the Allegan State Game Area, Michigan, indicates that overwintering eggs in the duff are subjected to very high humidity in the winter, usually 98-100% Relative Humidity (RH) with infrequent spikes to lows of 80% RH in a near-condensing atmosphere. Temperatures under snow cover are remarkably stable between 0° C to - 5° C. In New Hampshire, dataloggers placed in with overwintering eggs recorded temperatures between 0° C to - 5° C with an occasional spike up to 5° C and down to -10° C and RH levels between 90-100%. Table 11 summarizes methods used for overwintering eggs.

At least two of the captive rearing organizations have experienced hatch of 2nd brood eggs, calling them (possibly inaccurately) a 3rd brood. It is uncertain what environmental factor(s) are causing these eggs in the captive rearing facility to not enter diapause. Eggs should be checked regularly for this pre-mature hatch; if a 3rd hatch does occur and larvae survive they can be reared, but most biologists caution against releasing these insects into the wild.



In New Hampshire, eggs in Petri dishes are placed in mason jars and put inside cardboard boxes. Boxes are placed outside and wrapped in polypropylene row cover. Row cover is a material used as a protective covering to shield plants from the undesirable effects of cold and wind, and also from insect damage. (*Photo credit: L. Webb*)

In Ohio, eggs in modified mason jars are buried partially in the snow. (*Photo credit: P. Tolson*)



Table 12. Second Flight Egg Overwintering Protocol

State	Overwintering Procedure
IN	N/A
NH	Second brood eggs are stored in the captive rearing lab until the fall when the Petri dishes (10 eggs per dish) are put in mason jars. These jars are placed in cardboard boxes during the first week in December and secured with row cover. A HOBO datalogger is placed in one of the boxes to record temperature and humidity through the winter. Eggs are recovered in early April.
NY	N/A
OH & MI	Eggs are placed in Mason jars containing a 2” diameter insert constructed from Plexiglas tubing. The tubing has a support of chiffon fabric 1” below the lip of the tubing. To construct this support, a 9” section of tubing is sawn into two pieces-one of 1” length, and one of 8” length. A circular patch of fabric is glued between these two pieces of tube using Silastic aquarium cement. Replacing the dome lid with a chiffon fabric insert glued into the band with silicone aquarium cement further alters the Mason jar and protects the eggs from marauding ants. Ten to 12 eggs are placed in each jar and the jar is labeled with the number of eggs and the collection locality of the parents. Eggs are whitish green when deposited but change color to a dirty gray as they over-winter. This color change is normal. In the extremely hot and dry months of July and August, ca. 200 ml of distilled water is added to the bottom of each jar to keep humidity levels high for the eggs. Care must be taken to ensure that no water condenses around the eggs as the chiffon fabric insert in the band may inhibit evaporation, particularly under rainy conditions when ambient RH is high. Lids can be removed from jars with condensation to allow condensed water to evaporate. Jars should never be left without tops overnight, as the eggs may be predated or parasitized. Jars can be placed in a shaded location protected from rain. We prefer to bury the jars, protected by covering them with a sheet of polyfilm, beneath snow cover when possible.

Pathogens

Pathogens can be detrimental in a captive rearing facility, very quickly decimating stock. The confined space within a laboratory can promote the growth of viruses and bacteria. Prevention is critical in eliminating the potential risk of pathogens. Most importantly, the captive rearing facility must be kept sanitary with a pre-season cleaning and maintenance of a clean environment throughout the captive rearing season. Wild lupine leaves should be sterilized to prevent naturally occurring plant viruses from entering the laboratory. Lab employees should be cognizant of butterfly behavior and physical characteristics for early warning signs of infections. A quarantine area in the lab should be identified for potential outbreaks and specimens should be sent for analysis if a pathogen is thought to be infecting the captive population. Three insect pathology labs are listed in the Insect Pathology Resource Section of this handbook that may be able to assist in pathogen identification.

Symptoms vary among the different pathogens, however some things to watch for include a dark coloring in larvae or pupae, inability of larvae to pupate, pupae turning black and not eclosing, small-sized adults and changes in colors, or deformities. A few pathogens known to infect captive rearing labs include cytoplasmic polyhedrosis virus (CPV), nuclear polyhedrosis virus (NPV) (two naturally occurring plant viruses), Oe (virus found in Monarch and Queen butterflies), *Wolbachia* (a virus that has been known to infect KBBs) (Nice et al 2009), and bacteria such as *Serratia marcescens*. This is not a complete list of potential infections, and every effort should be made to keep up to date with potential pathogens. The following is a list of actions that can be taken to prevent pathogen outbreaks. If an outbreak does occur, individuals should be quarantined (never released into the wild), and specimens should be sent to an insect pathology lab for analysis.

Prevention:

- Designate the captive rearing facility for only KBB rearing to prevent cross-contamination with other plant and animal pathogens.
- Use a double door entry system in heavy traffic entrances to prevent outside insects and air-borne pathogens from entering the rearing area.
- Replace window screens with a smaller mesh advertised as anti-viral (refer Table 13: Supplies and Vendors).
- Replace light bulbs with ultraviolet, full spectrum lights. UV light helps break down some viruses and bacteria (Ali and Sikorowski 1986).
- Wash walls, floors, and other surfaces with a bleach solution during the off season.
- Use bacterial fighting sprays or wipes to clean up work stations at the end of each day.
- Wash containers and tools according to the Dish Washing Section and as described in the other sections (refer to NH in Inventory and Care of Larvae section). Sterilize wild lupine leaves by rinsing in a 10% bleach solution, rinse twice, and pat dry with paper towels (refer to NH in Inventory and Care of Larvae section).
- Keep KBB's in sterilized containers in small groups to keep potential outbreaks isolated (refer to Inventory and Care of Larvae section).
- Larvae must have clean containers and as little contact with frass as possible.

Dish Washing Protocol

All captive rearing containers, tents, and cages need to be washed to remove unwanted bacteria and help promote a sterile rearing environment. Below are two dish washing protocols.

Lane and Welch (1994) utilized a dishwashing protocol used by the University of Minnesota – St. Paul, Insect Ecology Laboratory with the following stepwise elements:

Step 1. Remove any debris and pre-soak in a Zephiran chloride (Benzalkonium chloride) solution (36 ml of 17% Zephiran chloride solution/ gallon distilled water).

Step 2. Wash dishes in a sink full of water (approximately 10 gallons), 8 oz. of bleach, and 4 oz. of liquid soap.

Step 3. Drain sink or washing container and rinse dishes well with distilled water (many laboratories rinse 10 x with tap water and 5 x with distilled water). All soap residue must be removed before transfer to the final soak, as it may inactivate the Zephiran chloride.

Step 4. Soak dishes once again in a Zephiran chloride solution (36 ml of 17% Zephiran chloride solution/ gallon distilled water), then soak in a solution of ¼ oz. Roccal II/ gallon distilled water.

Venters and Rogers (2001) suggest a simpler method described as follows:

Step 1. Soak dishes in a sink or tub in 20% cold bleach water.

Step 2. Rinse thoroughly with water. Some containers (Highland Plastic) can be run through an automatic dishwasher after soaking in bleach water.

Step 3. Allow dishes to air dry. Stack dry containers well away from livestock production to reduce the risk of contamination. You may also wipe the container dry with a clean paper towel.

Step 4. Just before the container is used, spray it with a 70% grain-alcohol solution, which evaporates quickly.

Table 13. Supplies and Vendors

Supplies	Vendors
Alcohol	Local Pharmacy
Anti-bacterial hand soap	Local Pharmacy or Grocery Store
Antivirus insect screen	Bioquip Products: www.bioquip.com
Bleach	Local Grocery Store
Cotton balls	Local Pharmacy
Cuticle scissors	Bioquip Products: www.bioquip.com
Dental wick	Quick Medical: www.quickmedical.com
Distilled water	Local Grocery Store
Ecologic Technologies® Rainmaker misting system	Ecologic Technologies: www.cloudtops.com
Eye dropper	Local Pharmacy
Filter paper	Fisher Scientific: www.fishersci.com
Fishing line	Local Sporting Goods Store
Florist sipper tubes	Florist Supplier
Food containers, 2-12 oz	Local Grocery Store
Forceps	Bioquip Products: www.bioquip.com
Full Spectrum Lights and Fixtures	Local Home Improvement Store
HOBO data logger	Forestry Suppliers: www.forestry-suppliers.com
Honey	Local Grocery Store
Magnifying hand lens	Bioquip Products: www.bioquip.com
Mating and Transporting Tents	Bioquip Products: www.bioquip.com Tropic Screen: www.campmor.com
Mosquito hats	Local Sporting Goods Store
Paintbrush, 0-2 gauge, camel hair	Local Arts and Crafts Store
Parafilm	Fisher Scientific: www.fishersci.com
Petri dishes	Fisher Scientific: www.fishersci.com
Plastic boxes	Pioneer Plastics, Inc: www.pioneerplastics.com AMAC Plastic Products: www.amabox.com Highland Plastics: www.highlandplasticsinc.com
Plastic cups with lids	Bio-Serve: www.insectrearing.com
Polyester mesh fabric	Jason Mills www.jasonmills.com
Sponges	Local Grocery Store
Tall pots	Anderson Die & Manufacturing: www.andersonpots.com
Ultra fine point Sharpie®	Local Office Supplier Store
Vaseline	Local Pharmacy

Survival Rates

Each KBB captive rearing organization does things slightly different from one another, adjusting to the needs of the butterfly, the environmental conditions in the captive rearing facility, and the resources available for rearing. This manual is intended to provide a variety of options to new captive rearing organizations and help to prevent the sometimes cumbersome trial and error process associated with established captive rearing programs. Rearing insects in a confined setting must be controlled by maintaining high sanitation standards, be extremely organized and cognizant of subtle stress indicators. Summary data from the 5 on-going captive rearing programs is provided in Table 14 below. The KBB captive rearing organizations have calculated survival rates for the various life stages of the butterfly in their captive rearing facilities which are provided to the readers for informational purposes only. The numbers in some cases may vary widely due to the length of the captive rearing program and bacterial or viral outbreaks. In addition, organizations that started captive rearing many years ago, went through a trial and error process before using improved protocols that result in higher survival rates.

Table 14. Survival Rates

	Egg to Larvae (over-winter)	Larvae to Pupae (1st brood)	Pupae to Adult (1st brood)	Egg to Adult (1st brood)	Egg to Larvae (2nd brood)	Larvae to Pupae (2nd brood)	Pupae to Adult (2nd brood)	Egg to Adult (2nd brood)
IN ¹	-----	-----	-----	-----	68.00 %	93.50 %	98.50 %	63.00 %
MI ²	-----	-----	-----	-----	66.30 %	74.52 %	47.40 %	23.42 %
NH ³	83.23 %	70.00 %	96.93 %	28.33 %	71.60 %	72.39 %	92.57 %	39.51 %
NY ⁴	-----	-----	-----	-----	-----	-----	65.31 %	-----
OH ⁵	28.76 %	-----	-----	-----	50.47 %	75.44 %	80.70 %	30.72 %

¹ Indiana does not over-winter eggs. Second brood average from 2007 – 2008.

² Michigan does not over-winter eggs. Second brood average is from 2008. Low survival rates attributed to poor lupine plants and unusually high temperatures during rearing season.

³ New Hampshire averages from 2001-2008, with the following exceptions: Egg to Larvae (over-winter) is 2008 only, Egg to Larvae (2nd brood) and Egg to Adult (2nd brood) is 2007 – 2008. There was a change in the protocol during second brood 2007 to only collect eggs from documented mated females or wild caught females, which dramatically changed hatch rates. A viral outbreak in 2006 lowered survival rates drastically during second brood and first brood 2007.

⁴ New York does not over-winter eggs. Average is from 2008 where they believe inclement weather contributed to low survival.

⁵ Ohio averages from 1998 – 2008, excluding 2004 where no collections were made. Egg to larvae (over-winter) average is from 2002 – 2004, where they were released as larvae. In 2007 a bacterial outbreak contributed to a low survival rate.

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A male KBB on butterfly milkweed in Ohio. (*Photo credit: P. Tolson*)

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