Methods Protocol for the Mountain White-crowned Sparrow Project

Welcome to RMBL!!

The Rocky Mountain Biological Laboratory (RMBL) is a unique high-elevation, non-profit research station located at 9,500 ft. in Colorado's Elk Mountains. RMBL is surrounded by the Gunnison National Forest and several spectacular Wilderness areas. Local forests are composed mainly of aspen, spruce, and fir. Near the East River, Copper Creek, and smaller tributaries the habitats are composed of willow, alder, and birch shrublands and mesic meadows of various grasses and forbs. In the vicinity of Gothic there are about 50 bird species that are commonly seen and several dozen other rare occurrences. Black bears, moose, elk, mule deer, yellow-bellied marmots, weasels, coyotes, snowshoe hares, pikas, and beavers live in or close to town. RMBL is a fascinating place to work! The following protocols are designed to get you familiar with the mechanics of the project and should be used as references in the field.

The larger goal of this study is to examine how avian wildlife handle different selective pressures and what tradeoffs they make during the breeding season. We are particularly interested in how food resources, parasites, and human disturbance interact to influence reproductive success in White-crowned Sparrows (*Zonotrichia leucophrys oriantha*). We are also interested in determining how nest success is mediated by certain mechanisms, such as stress level, feeding rate, and immune system response.

Field Sites

The White-crowned Sparrow (MWCS) project has four established plots*. Trap sites are distributed throughout all four plots. Food supplementation will occur on a variety of trap that will differ from year to year. We will find as many MWCS nests as possible on all four sites, territories will be mapped, all adult birds and nestlings will be banded, and nests will be monitored throughout the season.

- In 2004 there was also a 5th plot down valley from the town of Gothic.

Training and Feedback

We will provide extensive training to all research assistants prior to running these protocols. Please give us immediate feedback about any concerns, questions, or problems that you are having. We want the research experience this summer to be productive and positive for everyone – so speak up!

Weather Protocol

Work will be done every day during the summer unless the following weather conditions are met:

- Rain above a light drizzle
- Snow or hail
- Temperatures below freezing
- Lightning or imminent threat of lightning

We will still, however, meet in the morning during adverse weather to decide what to do that day. If these conditions occur while in the field, all traps must be locked open and birds immediately released. If you are caught in a thunderstorm in the field, keep calm and seek shelter. Avoid large trees that may attract lightning. You should be familiar with the RMBL wilderness recommendations.

Days Off

Research assistants are expected to work on average 50 hours per week and are allowed one day off per week. Days off will be arranged to avoid overlap and to ensure that necessary work gets completed. A monthly vacation schedule will be available in the lab. Many students will save their vacation days for longer vacations in July when conditions are more favorable for outdoor activities.

Daily Meetings

All researchers will meet at a pre-determined spot from 5:30 – 6:00 am for breakfast and a daily briefing. Make sure you have all your equipment with you (see equipment list below) so that you can proceed immediately to the field.

What to Bring into the Field

Binoculars
Camera
Notebook/pencils
Raingear
Sunscreen/hat
Layers of clothing
Permit copies
Bird banding and bleeding records
Color band combination list
Bird territory map (optional)
Cooler with orange putty sealant
Cloth bird bags
Roasted millet seed (one large bag)
Swiss army knife (optional)
Snack and water
Kit

Field Kit

Kit includes:
- 60 g Pesola
- Caliper
- Mesh mass bag
- Color bands and spreader
- Metal bands and banding pliers
- Microcapillary tubes
- Cotton balls
- Sterile needles
- Wing chord ruler
- Tail ruler
- Drug and syringe
- Water and syringe
- Alcohol
- Ectoparasite kit*:
  - Insecticide
  - White paper
  - Forceps/Nunc tubes

***Ectoparasite kits will not be used until later in the season.**

You will be assigned a field kit for the summer. Put your name and address in it in case you loose it. You are going to be responsible for keeping it well stocked, clean, and in good working order over the course of the summer. In particular make sure that you remove used consumables (e.g. bloody cotton balls) as well as old sharps (broken microcap tubes, used needles, etc.) which you should frequently discard into the appropriate sharps containers in lab. Make sure you clean your water, drug, and alcohol containers and replace their contents regularly.

Notebook Etiquette

You are responsible for the project notebooks that are assigned to you. Write your name on the front cover along with a sequential number and the year (e.g. Sarah, # 1, 2004), as well as your contact information on the inside cover. Keep your notebook clean, legible, and avoid cryptic expressions and abbreviations (refer to the standardized example notebook sheet). Additionally, include any other information that may be relevant to the project: location of singing males, possible nest sites, any unusual behavior, etc.

You are responsible for entering your field data into the lab computer at the end of each day. Each researcher must cross-check weekly his or her field notebook with the computer entries. This is done easiest with another person. Mail every 10 days a back-up copy of the updated field data file (labeled by the last date of entry e.g. MWCS 6-15-08) to Johannes (jfoufop@umich.edu).

Daily Work on the Plots

1. Food Supplementation

Trap sites on food supplementation plots must be food-supplemented daily – one large handful of millet seed per trap site. Before putting out seed, check for the presence of husks at each site; disappearance of whole seeds will indicate "mammalizing." In that case, or if the seeds are not eaten at all, consider moving the site to a better spot close by. You must, however, clear any trap movement with the principle investigator.

Immediately after the morning trapping session, the person responsible must supplement his or her plot. A sign up sheet will be available in the lab to allocate supplementation duties.

2. Trap Line Protocol

Pre-seeding

On all plots, traps must be locked open and pre-seeded at least one day before trapping. On non-supplemented plots, sprinkle a small bit of millet seed over the trap (be stingy). Trap sites on food supplemented plots do not need to be baited because they receive a daily "handout" anyway. Whoever is running the trapping session is responsible for this duty.

Trapping

Traps must be opened and baited by 6:30 am and closed (locked open) by 12:30-1 pm. If you are food-supplementing on that day, you may close traps a bit early to be able to break for lunch by 1:30 pm.

Handle traps carefully. Trap setting is an art and it pays to do it carefully. To unlock the trap, push the release bar in and remove the "safety stick." Be sure to check that the trap door shuts completely and is not obstructed by grass, branches, etc. Place the trap on a flat surface so the birds can reach the seed. A substrate of soil is best; avoid placing the trap on rocks, dead grass, or other uneven or soggy ground where seed is not visible or accessible. Good locations are spots under willows or where the trap can be backed up against a bush. A very small amount of "welcome" seed should be placed in front of the door and a small sprinkling should be placed inside the trap. If the sun is shining, cover the roof or sides of the trap with leaves or grass – you may have to weigh them down with pebbles or sticks so they don't get blown away. Take into account the movement of the sun to make sure trapped birds remain in the shade until next time you check the trap. It is better to be overly conservative in this regard as heat stress can kill a bird very rapidly. At the end of your trap session make sure to lock the trap open. Block the trap door with the release bar and pry a "safety stick" between the trap door and the sides. Record and release all non-MWCS captures immediately. If time allows between trap sessions, note singing males, territories, band combinations, etc. Any information collected, please pass on to Johannes.

Not every trap site will be used in a trapping session. The unused trap sites allow us the flexibility to change trapping concentrations based on need or trapping success. Weekly trapping assignments will be made every Sunday and written on the lab white board. At this time, we will also mark a running tally of the cumulative trapping effort for each plot.

3. Banding Protocol

Each MWCS captured on the study sites needs to be banded with 1 USFWS metal band and a unique combination of 3 color bands. Males receive the metal band on their right leg and females on their left ("males always think they are right!").

At the beginning of the season, each researcher should check out a set of USFWS metal bands and sign up for unique color band combinations that you will be using throughout the entire season. You reserve unique color combinations for yourself by talking with Johannes who handles the appropriate Excel file. At the end of the season, tell Johannes which bands were unused and return the unused USFWS bands. Do NOT lose your USFWS metal bands in the field or in the lab or you will be tarred and feathered.

If a bird is banded as a juvenile from the previous year (a single metal band on the left leg), make sure the metal band is on the proper leg to reflect the sex of the bird. If it is not, change the USFWS metal band to the correct leg and add color bands. If an adult bird is incorrectly

banded, do not change the bands and flag it in the data spreadsheet. If a bird has pox on its leg (thick or swollen scaly legs), and is unbanded, do not place bands on the affected area. If bands are irritating a pox infection on a banded bird, remove the band(s).

Holding the Birds

If you are right-handed take the bird into your left hand and form a loose cage around its body. It's best if you are holding the bird snugly, but not tightly, in your hand. If you squeeze the bird's body too much you can impede breathing. The neck of the bird should be placed between your index and your middle finger facing outward. Your hold should be loose enough that you are not squeezing the neck of the bird but tight enough that the bird cannot withdraw its head from between your fingers. Controlling the head is the key in controlling the whole bird. If the bird struggles too much it is better to let it go rather than risk injuring the animal. Be especially careful with the legs of the bird. Do not force them into any unnatural positions to avoid breaking the legs or damaging the joints.

If an unbanded MWCS is trapped, follow the banding protocol below. You'll first need to sex the bird.

Sexing

Record the sex of the bird based on the size of the cloacal protuberance (Male > 4 mm, Female < 4 mm) or brood patch on females later in the season. Although both sexes look alike, females are in general a bit smaller and have a shorter tail. Confusion is most likely early in the season when these differences are not as pronounced.

Banding

<u>Females</u>

The lower band on the left leg is the USFWS metal band. One additional color band is placed on the left leg, and two color bands are placed on the right leg. Bands are read from top left to bottom right. For example:

G / **S** B / M = Left leg Green over USFWS metal band ("service")
Right leg Blue over Mauve

<u>Males</u>

The lower band on the right leg is the USFWS metal band.

K / Y W / **S**

<u>Juveniles</u>

Nestlings and fledglings, which cannot be sexed, are banded with a single USFWS metal band on the left leg.

Key to Colors

D = Dark Blue	O = Orange
G = Green	R = Red
K = Black	W = White
M = Mauve	Y = Yellow

If a banded bird is missing color bands, consult your banding records and replace the missing band(s). If you catch a bird that is banded incorrectly (e.g. an obvious male that was banded on the left as a sexless juvenile) you should correct the band placement and give the bird a new color combination.

USFWS Metal Bands

Place first the USFWS band on a bird. Use the two prongs at the side of your banding pliers to remove band from the wire in ascending numerical order. Open the band so that it can slip easily over the bird's tarsus. Place band with the numbers right side up. Close the pliers until the two edges of the band are in complete contact with each other. Make sure that you do not put any pressure on the bird's leg. After you are done the band should be able to slip freely along the tarsus. The top of the number should face towards the foot of the bird (so when the bird is standing upright the number is upside down). This ensures that when you are holding the bird in your hand, you can easily read the number. If you have to move the USFWS band from one leg to another, use the band removing tool to carefully open the band.

Plastic Color Bands

Remove the color band first from the aluminum storage stick by slipping it onto the little shoehorn spreader. Force it back enough that the band is now open. Lay the shoehorn with the color band onto the tarsus (the shoehorn should be in snug contact along the full length of the tarsus). Using your fingernail push the band onto the leg and pull the shoehorn off. You'll do yourself a favor if you place first the bottom band (the one closest to foot) onto a tarsus – that way the first band is not in the way during the placing of the second band. In other words, band in this order: service, color band above service, color band closer to foot, color band farther from foot.

4. Bird Measurement and Bleeding Protocol

Measuring

Researchers should take the following measurements every time a bird is trapped, unless the bird is trapped twice in one day.

Sex: Record the sex of the bird based on the size of the cloacal protuberance (Male > 4 mm, Female < 4 mm) or brood patch on females later in the season. Sexing the bird first will help you determine on which leg to put the bands on an unbanded bird.

Band: Record the USFWS band number. If this is a newly banded bird, make sure to put a box around the band number in your notebook.

Color: Record the color and service band combination.

Mass: You should tare the Pesola balance to the mesh bag every couple of days. Place bird in mesh bag and secure the opening. Record the bird's weight (to the nearest 0.5 g) with the Pesola. Because birds can escape easily, researchers should do this measure last.

Drug: The antiprotozoal drug cocktail is administered to odd-numbered birds, and a water placebo is administered to even-numbered birds. Birds less than 25 grams should be given 0.1 cc, birds between 25 and 30 grams are given 0.13 cc, and birds over 30 grams are given 0.15cc. Administer the drug one drop at a time by placing the liquid in the corner of the beak. Drug should be stored in the fridge overnight. New master batches need to mixed every 6 weeks or so.

Fat: Gently blow the feathers away from the region above the cloacal protuberance (lower abdomen) and from the region below the keel (furcula). Fat is visible through the transparent skin as a white, opaque stratum layered over dark red muscle tissue. Record the fat level as 0 through 5 with the lower abdomen value on the left and the furcula value on the right (LA / F).

	Abdomen	*Furcula*
0 =	No fat	No fat
1 =	Trace, streaks on the belly	Trace
2 =	Thin cover, less than half covered	Small middle pad
3 =	Thick cover	Partially filled
4 =	Bald streak	Flush with edges of furcula
5 =	Bulging (fat motherfucker)	Spills onto keel

You will never see a fat level of 4 or 5 during the breeding season.

Cloacal Protuberance (CP): Gently blow apart feathers in the posterior ventral region of the bird. Measure the cloacal protuberance (to the nearest 0.1 mm) from the anterior base of the CP to the end of it. Because the tip of the CP is obscured by feathers, for practical purposes we measure from the base of the CP to the beginning of the light-colored feathers.

Brood Patch: A brood patch is developed by incubating birds as a means of transferring as much heat as possible to eggs in a nest. Females pluck feathers in their abdomen about 3-5 days before the first eggs are laid. Gently blow apart the feathers above the keel. Record the stage of the brood patch as 0, 1, 2, or 3.

0 = No brood patch

1 = Slight defeathering
2 = Bare
3 = Bare and scaly with grayish skin (swollen or blistery appearance)

Wing chord: Insert the ruler under the wing with the bend of the wing pressed snugly against the stop. Do not apply additional pressure to the wing with the ruler. Make sure that the line between the carpal joint and the tip of the longest primary lies parallel with the edge of the ruler. Gently place the wing tip on top of the ruler so that it touches the ruler and read the wing chord length (to the nearest 0.5 mm). For females, wing chord length is generally between 63 – 80 mm. For males, wing chord length is generally between 67 – 84 mm.

Tail: Hold the ruler parallel to the tail, and insert it between the tail and the under tail coverts. Record the tail length (to the nearest 0.5 mm). Tail length in females is generally between 62 – 74 mm and in males generally between 64 – 78 mm.

Tarsus: The tarsus is the length between the top of the intertarsal joint and the distal end of the last leg scale before the toes emerge (the "heel"). Hold the bird's left tarsus between your thumb and index finger so that the heel is bent at 90° and the tarsus is parallel to the long axis of your caliper. The front prong of the caliper should be pressed against the bent back toes of the bird, while the rear prong should be pressed against the heel. Make sure that the tarsus is now snug between the two prongs and cannot be moved backwards or forwards. Record tarsus length (to the nearest 0.1 mm) to the first decimal.

Crown Width: Place the ends of the caliper along the outer black edges of the crown (at the height of the eyes), and record the width (to the nearest 0.1 mm).

Stripe Width: Measure the width of the white stripe (to the nearest 0.1 mm) in line with the eyes. If the line is very irregular, you might have to approximately estimate where a straight line would be running.

Photograph of Crown Stripe [*Optional*]: For each MWCS we need two digital photographs of the head patterns: one from above and one profile. For scale, place the ruler with the USFWS band number written on it next to the bird's head and take the photographs. Make sure to fill the frame while including the entire head and that the number and scale are legible. Also make sure that the pictures are well lit, free of shadows, and not at an angle.

Bleeding

Preparation of the area

Take several microcapillary tubes out of their container; prepare a clean dry cotton ball and place everything at a clean place where you can access it quickly.

First make sure the bird appears to be in good condition (birds that are shivering strongly, are injured, wet, or are closing their eyes should not be bled). Start by holding the bird in the standard banders' grip (see description above). Stretch its left wing out so as to expose the

underside of the wing. You can carefully trim the undercover feathers that cover the elbow, but if doing so be extremely careful not to cut the bird. Alternatively you can just wet the area of the joint with an alcohol-soaked cotton ball. This will mat down the feathers and expose the elbow joint. You can further expose the area by carefully plucking the little down feathers growing around the elbow. This is probably painful to the bird and should be done with utmost care to avoid haematomas under the skin. Clearing the area around the sticking point from feathers is important; blood coagulates the moment it touches feathers. You should now be able to see the brachial vein running as a dark purple thread across the elbow joint.

Collecting blood

Keeping the needle as perpendicular as possible to the plane of the wing (this reduces the incidence of subcutaneous haematomas [bloodblisters]), puncture the portion of brachial vein located between the radius and the ulna. Only a small poke should be necessary – you may have to withdraw the needle before you see the blood beading up. Once blood flow is initiated, place a microcapillary tube under the puncture – capillary action will suck the blood into the tube. Keep the tube slightly slanted downwards so that gravity will assist the flow of the blood into the tube. If flow slows down, move the tip of the capillary tube to find a better spot facilitating flow. If that fails, you can gently 'pump' the wing by alternately bending and extending it. If that fails also you may have to carefully stick the bird again.

Fill each tube until it is ca. 95% full (you will need the remaining 5% of the tube length for pushing the tube into the sealant). If possible collect at least two full tubes of blood (60 microliters each). At the end of bleeding, place a piece of sterile cotton on the wound, and fold the wing over cotton. Hold the bird quietly and snuggly in your hand. Wait for approximately 1 minute and then check to see whether bleeding has stopped. Do not pull cottonball if it is stuck to the wound. If bleeding has not stopped, hold the bird longer and increase the pressure on the particular spot. Make sure, however, that you don't squeeze the bird! To release the bird walk it over to the base of a bush, set the bird on the ground, and let go gently. Do not toss the bird in the air.

Roll blood tubes horizontally [not vertically as this will cause the blood to run out!] between your fingers to break up any blood clots. Then push the capillary tubes into the orange sealant (again keep tube of blood horizontal through this process). The orange or white sealant should enter the bottom of the tube by at least 1 mm. Positions are marked from 1-4 along the periphery of each orange thingy. Multiple tubes from the same individual taken at the same time should be placed right next to each other at the same position. Make sure to record the amount of blood (e.g. 3.5 tubes = 3.5 Tb) as well as the position of each sample in your notebook. Place the orange thingy into your snow or ice-pack filled cooler. The orange thingy should be placed snuggly and vertically into a hole in the snow. If melt water has accumulated inside the cooler make sure you pour it out regularly. This is important because such water has the tendency to work itself into the blood tubes and destroy the samples. Place the cooler in the shade. (When you return to the lab at day's end, remember to put your orange thingy with blood in the shade or fridge, but not in the freezer!)

Important: Birds, like humans, can donate only so much blood before suffering ill effects. We never take more than 6 full tubes (360 microliters) every 2 weeks. You will need to carry with you bleeding records for your site in order to determine whether a bird can donated any more blood.

After bleeding, continue to walk the trap line.

Other Work on Plots

1. Nest Searching and Monitoring Protocol

Territory Mapping

We will be mapping territories of singing males throughout the season. This allows us to estimate the number of territorially active males on each plot, understand their distribution and evaluate whether we have been successful in finding all the nests on a plot. To map territories you will need to walk slowly through each plot early in the morning when birds are singing. Make sure it is light enough to identify color bands. The locations of their singing perches and other non-singing movements need to be then recorded on a map and eventually transferred into the appropriate *Photoshop* file. Unbanded birds should be recorded in the field for reference (especially for focusing trapping effort), but only color-banded birds (full and partial) should be transferred into the *Photoshop* file.

Nest Searching

Male territories will be mapped throughout the season. Territory mapping can help in locating nests by showing the researcher an approximate area in which the nest may be located. Nest finding can be a frustrating task; patience is an important asset. It is a good idea to set the goal of finding on average one nest daily. We will search for MWCS nests on four previously established plots, and one additional plot. Searchers should alternate plots every 2 – 3 days to eliminate temporal bias. Nests should be located during construction to provide the best estimates of nest success. This is also usually the easiest time to find nests due to the high level of activity and lack of concealing vegetation. Females build nests, and males exhibit mate-guarding behavior. Thus, the most effective way of finding most nests is by locating and following females, although males may provide some cues. Follow a bird with nesting material from a distance to avoid disturbance. Disturbance at this early stage can cause abandonment.

Egg-laying is the most difficult stage for finding nests because the female may visit the nest only when she lays an egg. During incubation some nests can be found by observing females who call just before leaving, or just after leaving, the nest – a characteristic “seep-seep-seep” call. It is best to sit quietly and somewhat concealed 20-30m from a possible nest site (choosing an elevated site makes observation much easier) and wait for the female to return. This will generally occur once every 30-60min. Often the female will fly into the general nest

area, drop to the ground and then walk the last meters to the nest. You may want to look then very carefully for moving branches or vegetation. After a female has returned to the nest, wait 3-5 min for it to settle down and then walk quietly to the suspected nest site. By flushing the female at closer range, you can generally find the nest easily. Females usually will only flush from the nest if the researcher walks very close to the nest, so caution should be taken in this situation to avoid stepping on the nest. Nests also may be found by flushing the female by walking nearby a nest during traplining.

Finding nests during the nestling period is relatively easy because both males and females bring food and remove fecal sacks. Nests can be found from a distance using binoculars or from closer using a blind. (For a detailed description of nest searching and monitoring, see Martin and Geupel. 1993. Nest-monitoring plots: Methods for locating nests and monitoring success. Journal of Field Ornithology. 64(4):507-19.)

Nests are primarily found at the base of willows or in clumps of forbs and grass. Nests are usually found between 0 – 0.2 m from the ground (occasionally found at 0.5-1.5 m). Mark each nest with 2 pieces of orange string tied to surrounding vegetation such that the nest is in line with and halfway between the strings. It is extremely important to record color bands of nearby chipping parents. Transcribe nest data daily into the database.

Nest Monitoring

GPS coordinates, distance to road, height nest is above ground, and substrate vegetation are recorded for each nest. Name (don't number) each nest and check it every two to three days to determine its status. Record the number of eggs and nestlings for each visit (see sample field notebook for other details).

When the nest is finished, record either number of nestlings fledged or how the nest failed. Do NOT attempt to rescue abandoned nestlings – this is prohibited by law.

Processing Nestlings

Nestlings should be processed 6 to 7 days after hatching (nestlings fledge approximately 9 days after hatching). If a nest is found during the nestling stage, the age of the nestlings should be approximated by morphological characteristics. Ask Johannes for pictures.

Removing Nestlings

Take care when approaching the nest; the parents will be alarmed and may dive-bomb the researcher. Keep calm in this event: the safety of the nestlings is a priority.

Cover nest with hand and gently remove one nestling at a time. Try to keep nest covered to prevent the nestlings from jumping out of the nest (nestlings processed after 7 days are more likely to attempt to escape). Nestlings may be placed together in one bag, and transferred to another after processing.

Processing

Avoid placing nestlings in direct sunlight or processing them in strong winds. Avoid processing a nest if it is cold or raining. It is better to wait a day to process a nest than place the nestlings' survival at risk.

Mass and tarsus length are the only measurements made on the nestlings. 0.5 – 1 tubes of blood should be taken from the tarsal vein (follow procedures listed in the Lab Protocol section for blood processing; DNA dots take priority over the other measurements). These measurements should be taken before a USFWS band is placed on the nestling (left leg only).

Replacing Nestlings

Replace nestlings in the same manner as removing them. Keep the nest covered so the nestlings do not escape. Survival may be reduced if the nestlings fledge early. After all the nestlings are replaced, keep the nest covered for 0.5 – 1 minute, or until the nestlings are calm. This will reduce the likelihood of nestlings attempting to escape.

2. Stress Series Field Protocol

Walking the Trap Line

When collecting stress series you'll need to work in pairs and you will open only a reduced number of traps so that you can get to each trap relatively quickly. You will also need to use the absolute minimum amount of seed possible since the presence of food can affect corticosterone levels in a trapped bird. Check traps from a distance using binoculars-this will be more effective if you shift the traps a bit into a more visible spot. If no bird is present, do not approach the trap. If the habitat does not permit this (often the case later in the season with increased vegetation), approach the trap slowly until the yellow stripe on the trap door is visible.

If no birds are caught during a round, wait approximately 20 minutes before you begin the next round.

If a bird is present, start your stopwatch immediately. Walk, do not run, to the trap. Carefully open trap do and remove bird with a bander's grip. Only perform the stress series protocol ONCE per bird – consult your records before you have the bird in hand! If bird is successfully removed from trap, then your partner will continue checking the trap line.

Bleeding

Birds need to be bled within three minutes from the first sighting. Extract at least two full tubes of blood (60 microliters each) for the baseline corticosterone sample. Use the same bleeding technique as described above. Because you will need to take the first sample rapidly you may have to skip many of the preparation steps outlined previously (especially

plucking or cutting feathers around the prick). Place the bird into a cotton opaque bag for 30 minutes, making sure to tie the drawstring securely. Place the bag in complete shade. Ideally you will hang it from an elevated position so it cannot be squashed or stepped on. Continue walking the trap line.

Return to the trap site with bird at exactly 30 minutes. Extract the bird from the bag carefully. Draw two more tubes of blood (60 microliters each) for the elevated corticosterone sample and for blood smears/DNA dots. Place the blood into the cooler.

If one bird is bled during the round, wait approximately 5-10 minutes before starting the next round. If two birds are bled, release all birds caught in remaining traps and begin the next round immediately.

When you come in from the field for lunch, place your cooler in a fridge (not freezer).

3. Ectoparasite Collection Protocol

Take bird in hand and dust bird with the flea/tick powder over a folded piece of white paper. Rub the flea/tick powder into the bird's feathers, especially around the head and neck, making sure not to get any of the powder into the bird's eyes. Any ectoparasites that fall into the crease of the sheet of paper should be poured into an alcohol filled Nunc tube. The Nunc tube should be labeled with a black sharpie as follows: MWCS, USFWS number, Color bands, Date (mm/dd/yy), and ECTO for ectoparasite sample.

4. Immunology Field Protocol

PHA (Phytohemagglutinin) Test

The PHA test is designed to measure the nonspecific (cellular) response of a host's immune system to a standardized foreign protein (PHA). The protein is injected into the skin of a bird, and the response, in the form of the thickness of a swelling, is measured after 24 hours.

Researchers should spend three days on each of the four plots:

Day 1 – Initial Thickness Measurement

Set up a trap line of approximately 20 – 25 traps, which will be walked continuously. When a MWCS is caught, hold the bird in the bander's grip. Turn the body of the bird upside down. Have your partner stretch the wing out so that the wing-membrane (the thin membrane that is at the front edge of the wing and connects the hand-joint of the wing to the body) is stretched out and taught with no wrinkles. Sterilize the area first with ethanol-soaked swab and then wipe any small plumes out of the way. Measure the initial web thickness by using the spessimeter, or thickness gauge. Let the gauge on the spessimeter touch the skin only briefly, to prevent squeezing the intracellular liquid out of the tissue and resulting in artificially small measurements. Take three thickness measurements, and record the average for each bird.

Day 1 – Injection

Inject 100 microliters into the center of the underside of the wing web. While inserting the needle, turn the needle so that you can see the beveled edge and bore. Slowly work the needle into the wing membrane between the two paper-thin skin layers. A good injection results in a bubble forming between the two skin layers with no liquid spilling out. Record the amount of solution injected (this should be included as a covariate in the analysis). If a minimal amount of liquid spills out from the injection area, the injection is fine. If approximately more than 50% of the liquid spills out then move to the other wing and try again. Record the amount of PHA solution injected and the side of the bird the injection was given. Release the bird.

Day 2 – Thickness Measurements and Injection

Walk the same trap line of 20 - 25 traps and attempt to recapture treated birds after 24 hours. If a bird is recaptured, measure the injected wing web for a final thickness measurement. The measurement area will have turned yellow and is swollen after 24 hours. Before releasing the bird, follow the bird measurement protocol as described above. If new birds are captured, measure the initial wing web thickness and administer the injection (see above methods).

Day 3 – Final Thickness Measurements

Walk the same trap line and attempt to recapture treated birds from Day 2. Follow the Day 2 procedure outlined above. If new birds are caught, do **not** inject them with PHA, but do follow the bird measurement protocol as described above.

<u>KLH (Keyhole Limpet Hemocyanin) Test</u>

This test measures the response of the humoral (antibody) component of a host's immune system to a standardized foreign protein (KLH). The antibodies in a baseline blood sample are compared to those of a sample collected approximately 14 days after injection with KLH.

Each of the four plots will be focused on for a two day period, with a 14 day break, and another two day period for a total of four days.

First 2 Days – Initial Capture and Injection

Walk a trap line of about 20 – 25 traps. After capture of a MWCS, take a baseline blood sample of two tubes from the bird. Inject 80 microliters into the bird in either the breast muscle (Hasselquist 1999) or into the triangle of fat tissue on the side of breast muscle (Lindstrom-House sparrows and Darwin finches). The injection should occur between the intramuscular and subcutaneous layers of the skin. After the injection, measure the bird using the above bird measurement protocol. After all measurements are taken, release the bird.

Second 2 Days – Recapture (12 – 14 days later)

Walk the same trap line of about 20 – 25 traps. Attempt to recapture the birds caught within the first two day period. Take another two tubes of blood to measure how antibodies have changed in response to the KLH injection (immune response evaluation).

All blood samples need to be spun down and frozen (although not in a –70C freezer).

Lab Protocols (102 Willey Lab)

1. General Lab Protocol

Introduction

Immediately after lunch, process your blood samples. Do **not** process your samples in the evening because by that time the blood will have started to degrade. When removing a sample from the orange sealant, make sure that the tube is pulled out horizontally. Additionally, make sure that the orange / white sealant plug remains in the edge of the tube. After removing the tubes from the orange sealant, stick your tubes into the small holes in the side of the Critoseal holder and write ona separate sheet of paper the tube holding position and the corresponding USFWS band number (optional). You will need blood for the following samples:

a. 1 parasite smear (15% of one microcapillary tube)
b. 3 blood dots (amount highly variable: 25-50% of one tube total)
c. 2 immune smears (15% of one tube each =30% total)
d. Hematocrit
e. Plasma for immunoglobulin and steroid assays (at least one full tube)

If you have a limited amount of blood (often the case), and assuming you have no previous samples from an animal, you should prioritize blood use in the above order. If an animal has been caught previously, prioritize blood use based on what information is lacking for the bird. If the amount of blood is sufficient, and at least two weeks have passed since the previous capture, resample each of these five measures. Blood quality may influence your decision in what samples to prepare; you can use coagulated blood only for blood dots, hematocrit, and plasma extraction.

Preparing Smears

Take your sample from the slug and break off the end that contains the putty. This will allow the blood to flow out. Tap out a small droplet of blood next to the white side of a clean microscope slide. Handle the slide only from its edges. Use the smaller edge of another clean slide, slanted at 45^{o} to make contact with the blood. Once the blood has run along the edge of

the second slide, drag the second slide across the first slide to make your smear (one red blood cell layer thick) in a deliberate, smooth motion. You can control the thickness of the smear by changing the angle of the top slide: higher angles (70 ^{o}) will result in thicker smears, while lower angles (30^{o}) result in thinner smears. Dry the slides on a paper towel and store in a plastic bag in the freezer.

Once smears are stained, they can be stored in slide holders. Label slide holders with a black Sharpie as follows: Type of smear, MWCS, RMBL, Summer 200X, Box #. Line the bottom of each new slide holder with paper towel. Place the slide holder away from the sun on the shelf in the lab.

Parasite Smear

Parasite smears are used to identify blood-parasite infections. Prepare and store one smear as described above. Label the smear as follows: MWCS, USFWS number, color band, date (mm/dd/yy) collected, and PS (parasite smear).

Staining Parasite Smears

Stain the parasite smears within 2-3 days after they were made or the quality of the smears will significantly degrade. Place smears in the staining rack. Place the containers holding the 4 baths onto a kitchen paper towel (ideally one of those backed with a layer of plastic). Dip the rack into four consecutive baths:

a. Methanol – Dip rack 2-3 times for one second each. Careful, do not touch or inhale this toxic substance. Drain the rack for a few seconds.
b. Red stain – Dip rack 6 times for one second each. Drain the rack again.
c. Blue stain – Dip rack 4 times and drain.
d. Deionized water – Dip rack 4 times and drain.

After slides are dry, store them in their appropriate slide holder.

Please be careful with the stain because it is impossible to remove from surfaces and clothing. After a while you will need to replace the baths. Stop using the red and blue stain when an iridescent scum forms. Discard the red and blue stains in the same, well-sealed container. Stop using the deionized water when it becomes dark. You can pour the used deionized water down the drain with the faucet running. Methanol will evaporate with time, so replace it until it becomes discolored. Used, discolored methanol should go in a separate, well-sealed container. Label all waste containers appropriately.

Immune Smears

These smears are used to evaluate immune cell populations in the blood. Prepare two smears using the above methods. Immune smears are equally thin, but cover a larger area of the

slide. Label each smear as follows: MWCS, USFWS number, Color bands, Date of collection (mm/dd/yy), and ISA or ISB for immune smear A or B. Store smears in a plastic bag in the freezer. Immune smears are **not** stained. Use a new zip lock bag every week, and add slides as quickly as possible to limit the amount of time the slides are exposed to air and humidity. If you do not do this, overtime condensation will form ice crystals on the slides destroying your samples. Label the plastic bags with a sharpie as follows: Initials of PI, MWCS, RMBL, and Date (mm/dd/yy).

Blood Dots

Blood dots are blotches of dried blood on absorptive paper (round filterpaper pieces are used in general) they are used to assess paternity and to identify blood parasites through DNA analysis. Tap the edge of the microcapillary tube against the round filter paper until a blood dot forms (4-6mm in diameter). Place a radial row of three heavy dots of the remaining blood onto the paper. Label blood dots at the rim with MWCS, USFWS band number, color band combination, and date (mm/dd/yy). Let the dots dry before covering them with a clean sheet of paper. Place the sheet of paper in a plastic bag, and place the bag in the freezer. Label the plastic bag with a black sharpie as follows: PI initials, MWCS, and Date (mm/dd/yy). During this process do not touch the blood dots to avoid contamination with foreign DNA.

Centrifuging the Blood

To centrifuge blood use the large desk-top IEC MicroMB Microcentrifuge. Before placing the tubes into the centrifuge, reseal the sealed end with at least 3-4 mm of Critoseal putty. Place each tube into one of the grooves of the centrifuge rotor with the sealed edge towards the outside snugly touching the outer rim of the rotor. The sealed end should always be the intact end, and **not** the broken end to ensure a tight fit of the tube against the rotor. Counterbalance each tube of blood on the opposite groove with another tube of blood or an empty mircocapillary tube. Make a list of which tube corresponds to which groove using the USFWS band number. Once the tubes are in the centrifuge, tightly screw on the rotor lid (if this is not done, an instantaneous loss of all the samples can occur, as well as a huge mess to clean up). Close the heavy lid of the centrifuge and set the timer to 5 minutes. Do not open the lid to the centrifuge until it comes to a complete stop. Remove the samples and return them into their appropriate slug or orange “thingy” positions using the list you just prepared. The tubes of blood now have separated into packed red blood cells (dark reddish brown color) and clear plasma (yellowish color). Past experience has shown that despite their heavy construction, IEC centrifuges are quite delicate and break easily – please treat them very carefully, clean them from any glass or blood remnants using an alcohol wetted piece of cotton. Report any malfunciton / concerns immediately to Johannes.

Hematocrit

To measure the hematocrit (the amount of red blood cells) in a given volume of blood, place a spun-down microcapillary tube on a hematocrit card reader. Place the centrifuged microcapillary tube vertically on the chart with the bottom edge of the packed red blood cells in line with the “0” percent line. Slide the tube along the chart until the meniscus of the

plasma intersects the “100” percent line. The height of the packed red cell column is then read directly as percent cell volume. This value (usually ranging between 35-55%) is the percentage of the blood volume made up of cells and is called hematocrit. Repeat this procedure for all the spun tubes belonging to the same bird and average them out for the final hematocrit value.

Plasma Samples

To collect plasma samples, first obtain a Hamilton syringe, a cup of deionized water, and NUNC tubes. Suck up clean water into the barrel of the Hamilton needle and eject it. Repeat twice to ensure that the barrel is clean. Insert the tip of the needle into the spun-down capillary tube and extract the plasma. Be careful not to insert the tip of the needle too deep into the plasma to avoid plasma spilling out. Remove all plasma until you reach the packed-cell layer. Be careful not to contaminate the plasma sample with any red blood cells. Eject extracted plasma into a Nunc tube (one Nunc tube per bird). Repeat this process with the remaining tubes of blood collected for that bird. Label the Nunc tube with a black Sharpie as follows: MWCS, USFWS number, Color bands, Date (mm/dd/yy), and number of tubes of blood used. Place a piece of clear tape over the label, and carry Nunc tubes to the freezer and place them in the Nunc tube holder. Do not take tube boxes out of the freezer to add new samples. New Nunc tube holders should be labeled with a black Sharpie as follows: Plasma samples, PI initials, MWCS, RMBL, Summer 200X, and Box #. Place the new box in the freezer. Before proceeding to the next bird, make sure the Hamilton is rinsed appropriately with deionized water.

2. Stress Series Lab Protocol

Remove microcapillary tubes slowly and horizontally from the jar, ensuring that the orange putty remains in the sealed end. Reseal the putty end with hematocrit sealant. Your two initial tubes of blood from the stress series will be used to measure baseline corticosterone (stress hormone). One tube from the final sample will be used to measure elevated corticosterone, while the other tube will be used for parasite/immune blood smears and DNA dots.

Record the bird band number and designate which tube will be used for initial corticosterone levels (ICORT) and final corticosterone levels (FCORT). Record which numbered centrifuge slot each tube is placed using the provided data sheet.

To centrifuge, follow the above methods. After centrifuging the blood, measure the hematocrit and extract the plasma for each tube as described above. Label Nunc tubes as follows: MWCS, USFWS number, Color bands, Date (mm/dd/yy), ICORT or FCORT, and number of microcapillary tubes of blood used to extract plasma for that Nunc tube. Place Nunc tubes in Nunc tube holder, and place the holder in the freezer. Label each new Nunc tube holder as follows: Stress series, PI initials, MWCS, RMBL, Summer 200X, and Box #.

Make parasite/immune smears and DNA dots with the remaining tube of blood. Use the above methods.

3. Immunology Lab Protocol

PHA (Phytohemagglutinin) Test

Materials:

PHA	- Phytohemagglutinin from *P. vulgaris* (10mg) (Sigma Code: H 9017)
PBS	- Phosphate buffered saline Powder in foil pouches, 10 packets (Sigma Product Code: P 3813).
Water	- Sterile (Sigma Product Code: W 3500, 1 liter bottle)
Needles	- 1cc, 28gauge insulin syringes from BD with permanently affixed needle (Fisher Cat. Nr. 14-829-1B)
Test tubes	-10cc, sterile with screw-on top.
Thickness gauge	- Mutymoto digital pocket gauge, "1/2," obtain through Penn Tools on the web [79$]).
Ethanol	- 70%
Cotton Swabs	

Preparation of solution:

Dilute PHA with sterile PBS (Phosphate buffered saline) to a 1 mg/ml solution (REFS: Martin 2003 Proc R. Soc. B). You will only get 10 ml in total, so divide it into little vials (of for example 2ml) and take one each day into the field so you do not ruin the entire solution (Mix at least 2x of what you'll need daily). You should try to keep it cold once it's mixed (that is, fridge cold (2-8^{o}C), do not freeze) and use within a week.
When taking into the field keep cool in Ziplock bag containing 10ml sterile tube with PHA solution and a blue ice-pack).

KLH (Keyhole Limpet Hemocyanin) Test

Materials:

KLH - Hemocyanin preparation from Keyhole Limpet [*Megathura crenulata*](KLH) - Lyophilized powder (20mg / 39.50$)(Sigma Product Code: H 7017)

PBS - Phosphate buffered saline - Powder in foil pouches, 10 packets (Sigma Product Code: P 3813).

Water - Sterile (Sigma Product Code: W 3500, 1 lt bottle)

Freund's Incomplete Adjuvant - (Sigma Product Code: F 5506) (buy ca. 2 x 10ml)

Luer Lock mixing Syringes - (Sigma?) (Becton-Dickinson Safety Lock 10 ml Reorder Nr: 305559) – need 2 at a time

Luer lock couplers - (Small but important) (Sigma C-4681)

Other syringes (5-10ml) with removable needles to inject birds - (one syringe per day, one needle per bird). (Sigma)

Preparation of KLH:

Dissolve PBS powder packet in 1 lt of sterile water. Then use sterile PBS to bring KLH to an 1mg/ml solution (hence the 20mg container will produce good 20ml of solution). I think (check with Karin) that solution should be good for up to ca. 15 days.

Then emulsify KLH/Saline with the adjuvant. Mixing ratio is 1:1. Prepare this mixture daily. Put 2ml of adjuvant in one syringe and 2ml of KLH solution in second syringe. Connect with Luer lock and start pushing back and forth. Mix for 15 minutes. Solution will eventually turn thick and creamy white, making it increasingly difficult to push it back and forth.

Once done, replace Luer lock with needle and slowly pour the thick mixture into the barel of a syringe. Make sure that no bubbles get into the syringe since no air must be injected into the bird. Store syringes vertically with tip down so that any bubbles formed inside the injectable can rise out. After finished mixing, keep cold and use the same day.You will need about 2-5ml a day.

Laboratory cleaning

Every day, one person will be responsible for sweeping the lab at the end of the afternoon and wiping any dirty surfaces down. Once a week, the floor needs to be moped, all surfaces thoroughly cleaned, windows washed and trash taken out.