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This document is part of a collection that serves two purposes. First it is a public archive for data and documents resulting from evolutionary, ecological, and behavioral research conducted by the Ketterson-Nolan research group. The focus of the research is an abundant North American songbird, the dark-eyed junco, *Junco hyemalis*, and the primary sources of support have been the National Science Foundation and Indiana University. The research was conducted in collaboration with numerous colleagues and students, and the objective of this site is to preserve not only the published products of the research, but also to document the organization and people that led to the published findings. Second it is a repository for the works of Val Nolan Jr., who studied songbirds in addition to the junco: in particular the prairie warbler, *Dendroica discolor*. This site was originally compiled and organized by Eric Snajdr, Nicole Gerlach, and Ellen Ketterson.

Context Statement
This document was generated as part of a long-term biological research project on a songbird, the dark-eyed junco, conducted by the Ketterson/Nolan research group at Indiana University. For more information, please see IUScholarWorks (https://scholarworks.iu.edu/dspace/handle/2022/7911).

EGGS, SEX & INDEPENDENCE.2005
Instructions for egg steroids
Revised on May 18, 2006

(Earlier drafts were May 10, 1998, April 25, 2000, May15, 2002**,** May 5, 2003, May 11, 2004, May 11, 2005)

Our methods for sampling eggs, sex ratio, and survival to independence are described here. They have varied from year to year, depending on the question, so this document is both a history and a description of what to do in 2006.

A. Eggs

In1997 and 1998 we were interested in extended phenotypic effects of male testosterone treatment on the eggs laid by females. We marked eggs as they were laid and weighed and measured them the day after the clutch was complete (1997) or on the day the eggs were laid (1998). We also collected eggs to determine steroid concentrations.

In 1999 and 2000, the last years in which we implanted males, we attempted to relate laying order to hatching order and obtain precise measurements of the incubation period to determine whether status of the mate affected female incubation behavior and hatching asynchrony.

We began to implant females with T in 2001-2002. In 2001, we measured eggs mostly in complete clutches and did not attempt to determine whether egg size varied with laying order. In 2002, we marked eggs as they were laid, and all the eggs were measured by one person (Eric) on the day the clutch was complete. We also collected one egg (egg 3 or the largest egg) for later determination of steroid concentrations. Daily visits during laying helped us to detect delayed laying in T-females. We also found that yolk T was higher in the eggs of T-females than in the eggs of C-females, but the sample size was small (Clotfelter et al. 2004).

In 2003 and 2004 we were interested in variation in endogenous T, and we marked eggs as they were laid, measured them when the clutch was complete, and collected the 3rd egg if laying order was known (or the largest egg if it was not). Questions still to be answered because the assays still need to be run include: do females that respond more strongly to GnRH (or that have whiter tails or that are mated to males with higher tail white, etc.) also produce higher yolk levels of T?

In 2005, we decided not to collect eggs because to do so might make it less likely that we would detect differences in tail white development. It would have been good to confirm that T-implants caused females to increase yolk T, because our sample to date is small, but we decided against egg collection.

In 2006 we again decided not to collect eggs, this time because we will be measuring any effect of T on immune development of offspring and delivery of food to offspring, and any treatment-related differences are likely to be greater if family size is larger.

Any effect of female T on immune development and offspring begging could be a maternal effect of directly on the offspring during early development, or an indirect effect mediated

through effects of T on adult parental care, or both. Without cross fostering, which is not realistic in our system, we won't be able to distinguish between these two kinds of effects. If collecting eggs could help us distinguish between these two explanations, I would favor collecting them, but I don't see how it will.

Despite not collecting eggs, we will need to visit the nests during laying to add to our information about delayed laying in T-females, and also whether T-females are more likely to skip days between eggs or differ from C-females in the length of the incubation period.

Please proceed as follows -

1. *When possible find nest during building*, ideally before egg 1 has been laid.

2. *Identify the parents*. During the laying stage, males often stay with the female while she is foraging. Go to territory where nest is and listen for call notes. The male will sing occasionally and accompany the female nearly all the time. When you return each day to mark eggs (see below), use the opportunity to determine adults. If you find out, be sure to report IDs right away (!!!!) so others don't spend time trying to do something you have already done. We need to whether the female is banded/implanted.

3. Place a small bit of leaf in the nest and note whether it is there the next day. This will usually tell us whether the female visited the nest, even if she did not lay an egg.

~~*3. Mark the eggs each day/add a leaf*. Knowing each egg's order in the laying sequence is important. Until further notice, please mark with a sharpie or as advised by Eric. Carefully remove egg from nest, holding it gently in your left hand. Use sharpie to mark egg with a I, II, or III. Don't poke the shell! Note change in methods from earlier years: leave 4th or 5th eggs unmarked to reduce handling of eggs. Never mark with a pencil.~~
~~What to do under other circumstances: If there are two eggs in the nest at the time you find it and they are fresh, mark them both with a I. If there are more than 2 eggs or incubation is underway (eggs warm) leave them unmarked.~~

~~4. *Return the egg to the nest*. Nestle egg in your fingers and let your fingers lead the way into the nest to avoid puncture of the egg by vegetation or nest material. Gently release egg into cup of nest. Again don't poke the egg!~~

[deleted instructions, decided not to mark eggs in 2006]

4. *Back at the lab record what you did on the nest log* ~~***and*** *the egg data sheet (marked egg, (saw/did not see leaf when you arrived, left new leaf, etc.)*~~. Let's use these data sheets, even when we are not collecting eggs. Be sure to fill out data sheet in lab each day. This will allow Eric to be certain that each egg has been marked. If we miss one, the sequence is lost to us, so please make and keep your commitments to mark eggs on the day they are laid~~.

Beware: sorting things out later *depends on* our writing the correct dates in the nest logs. Check the calendar each time you record the date regarding the presence of 2 eggs, 3 eggs, etc. If you

notice anything odd, please report it to Eric so we can figure out what's up right away. E.g., did the female skip a day? Did more than 2 days pass between nest completion and the arrival of egg 1?

~~*6. When the clutch is complete, measure the third egg. If the laying order is unknown, determine which egg is largest and measure it.* Clutches are typically 4 eggs in the early season, 3 eggs later on. On the day *after* the 4th egg (or 3rd, if there is no 4th) is laid, measure only egg 3 or determine which egg is the largest by sight and measure only it. Using calipers, measure the long axis and wide axis to the nearest 0.1 mm. You want the calipers to hug the egg, but not too tightly. Take care not to break the eggs, and if you do have an accident, please keep complete notes. *Before we settle this for sure, we might designate a small number of people to measure eggs, e.g. Eric and Joel are quite experienced. Broken eggs will be a serious loss, so I think we should have a few egg specialists.*~~

~~7. The next step would be to *collect the 3rd egg or, if laying order is not known, the largest egg*, but we are still deciding what to do. These instructions will apply if we later decide to collect eggs. Place the egg in a carrying container (e.g., film cassette lined with cotton), and return it to the lab. Weigh it. Wrap it tightly in Parafilm (which will catch any albumin that leaks when the egg cracks during freezing) and place it in a glass storage vial with a plastic top, labeled completely, freeze. Fill out the egg data log, noting the criterion used to select egg (3rd, 4th, or largest) and the egg's dimensions. [Note in 2002 and 2003 we collected the largest egg or the 3rd egg. In 2003 we reconsidered why we did not collect the 4th egg, but recalled that would mean an extra trip back to the nest and also remembered no effect of laying order on egg size, so decided to stick with 3rd or largest.]~~

~~8. Even if we later decide to collect eggs, *do not collect more than one egg from a given female. Subsequent clutches should be left complete*. Goal here is to obtain eggs from as many females as possible but also to anticipate that many first clutches will fail, allowing observations of behavior to be made on replacement clutches that will be of full size.~~

5. *Return on the expected day of hatching and note eggs have hatched and that have not.*

B. Sex ratio

Question: do T-females produce more sons than C-females? To answer precisely, we would need to determine sex ratio of broods *as close to hatching as possible before any mortality occurs* and then follow later survival of individuals young. This is a big job that requires multiple trips to the nest, marking individual young, and possibly increasing the risk of predation. **Alternatively we can simply determine the sex ratio at day 6 when we collect blood for paternity. This is less intrusive and good enough for our purposes, so this what I recommended for 2005, let's do the same in 2006.**

Therefore these instructions go beyond what we plan to do in 2005 or 2006, but I leave them here for future years.

1. On hatching day weigh the young and marked them with polish as red-R, red-L, red-both, and nothing. Nail polish works fine, but it can rub off, so it needs to be applied very carefully (a

piece of grass dipped in the bottle works better than the brush to mark just the toes and not the foot - it takes a little while to dry, so think of this as an important task that needs to be done carefully). After marking young, weigh them and measure tarsus.

2. After hatching is complete (the day after the last young has hatched), bring any un-hatched eggs back to the lab. Be sure to note on nest logs and on data sheet regarding any un-hatched eggs. Make a determination as to whether the egg developed by external examination. If the egg developed, we may be able to determine its sex later from embryonic tissue, so we should freeze it in an egg vial (carefully marked, ask Eric how to do this).

3. On day 2 please collect one microhematocrit tube of blood from the wing. Ideally we would do this as close to hatching as possible, but experience has shown that bleeding before day 2 can be too hard on the nestlings. Later we will isolate the DNA from this blood, amplify the product using PCR, and identify sex-specific sequences on the female chromosome. We may also use it for parentage analyses.

To prepare for taking the blood samples, take the proper number of Eppendorf tubes with 500 ul of Longmire's and needles. Note, it is essential that the Longmire's and the Eppendorf tubes NOT BE CONTAMINATED with human sequences. Use gloves when aliquoting and don't let foreign matter get into the Eppendorf tubes. It is also essential that the DNA be associated with the right individual!! So be sure not to switch the DNA of the nestlings; keep track of whether the blood came from red-R, red-L etc. If a bird disappears during the nestling phase, we have to know which one!

3. On day 3 to ID, measure and weigh the nestlings. Renew the polish before it wears off to keep track of individuals.

4. On day 6 weigh and measure again when you band and bleed (again coordinate with Joel and Dawn's teams, see the NEST protocol). Be sure to associate the correct polish combination with the band number. Once again, this is essential for the sex ratio question.

5. Check the nest on day 9 and count young (if the situation will permit) but don't touch (!! Young will fledge prematurely if touched).

6. On fledging day, weigh and measure again, note which young survived in order to know both the sex ratio at hatching (really day 2) and the sex ratio at nest-leaving.

C. Survival to independence (again these instructions are not for 2006 or 2006 but for future generations)

Question: does treatment of the female affect number of young surviving to independence? Do male and female offspring survive equally well (sex to be determined later from blood samples)?

This is a tough assignment = very challenging but very satisfying too. Perhaps one of you 2006ers will decide whether to make this a priority for yourself because it relates to offspring care. Again it's hard, but juncos are one of the few passerines in which people have attempted

this and we did do this to compare T- and C-males Juncos are birds of the understory, so while this is very hard, it is not impossible.

1. Our procedure is to attempt to determine the number of young that survive to 1 day after fledging, 2 days after fledging, 3 days after fledging, 6 days after fledging, 9 days after fledging, and 14 days after fledging. However this is nearly impossible to do. The families move around a lot, and you may find them one day and not find them the next. Still we do a census on each of these days.

When looking for young use all your senses (including ESP). *The key to success*: is to go prepared. Have the information about band colors written down, so you know what leg and colors to look for. You will get only a few seconds, so preparation will make all the difference.

2. The day-14 measure is the most important, but you have to visit the territories all along in order to know the likely places to look on day 14. See data sheets for recording fledgling sightings. Be sure to record what you saw ASAP, so others will not duplicate your efforts.

3. If on day 14 you find all the fledglings that you think are alive, then you can quit looking. If you have not seen one or more of them, keep going to the territories even after day 14 (says days 15 and 16 and 17, until you are CONVINCED that certain young are not alive. On day 21 you may quit in any case.

4. Return to the lab and record your findings on the fledgling survival sheets. Note where you looked, how much time you spent, how sure you are of your observations, etc.

5. Be on the look out for signs of re-nesting. Is the female still around the fledglings? Did you see her feed young or just the male? I think that the earliest turn around is a case in which a female laid her first egg 8 days after the first brood fledged. But 12 to 15 days is more common.

6. Select a few nests for observations of post-fledging behavior. Do parents divide the brood in the same way if females are treated with T?