Ketterson / Nolan Research Group Collection

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.2008
Instructions for egg steroids
Revised on June 6, 2009

(Earlier drafts were May 10, 1998, April 25, 2000, May15, 2002**,** May 5, 2003, May 11, 2004, May 11, 2005, May 18, 2006, May 15, 2008)

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 2009.

A. Eggs

History

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. Sample sizes were small.

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). One of the questions addressed included: 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? We found based on a very small sample that they did (Jawor et al. 2007). This has important implications for whether yolk T affects behavior or vice versa.

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 for an experiment that Joel was conducting. 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 acting 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, we would favored collecting them, but we not see how it would. We did, however, visit the nests daily during laying to add to our information about delayed laying in T-females, and to see whether T-females are more likely to skip days between eggs or differ from C-females in the length of the incubation period.

By 2007 we had become quite interested in comparing yolk T across populations and to adult female behavior and morphology – VA, SoDa, and CA, so we again collected eggs. This continued in 2008 and 2009.

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 know whether the female needs banding, implanting, bleeding, etc., depending on the year.

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 Kristal, D, or A. You might mark the eggs without removing them from the nest (one spot for egg 1, 2 spots for egg 2, a circle for egg 3.

In the past we marked like this: 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 spots. If there are more than 2 eggs or incubation is underway (eggs warm) leave them unmarked. Be sure to record all this for later entry onto nest logs.

4. *Return the egg to the nest*. To return an egg to the nest, nestle the 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!

5. *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.)*. Be sure to fill out data sheet in lab each day. This will allow whoever is doing the daily list 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 K, D or A, 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, collect the third egg. If the laying order is unknown, determine which egg is largest by eye and collect 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, carefully carefully return the collected egg to the lab and notify Kristal of its existence. We have egg carriers that have cotton on all sides to keep the egg from cracking in transit (e.g. film canisters or empty containers that once held microhematocrit tubes).

Insert from Eric Snajdr 6/8/09, About the egg collecting, I took a quick look at the Egg collection data sheet paper files in the lab. In 2003 we were collecting the day after the last egg was laid. As I recall this is how we started things off with the egg collecting... I seem to recall a conversation with you and Joe, deciding to wait until the the day after the last egg, for those cases where there might be a 5th egg. However, in later years (e.g. 2007 we collected the egg on the day of the 4th egg. As I recall, we started doing this because of fear of losing too many egg collections in years of high predation.

7. Measuring, weighing and storing eggs. Kirstal, or someone trained who feels confident, will use calipers to measure the long axis and wide axis to the nearest 0.1 mm. She will 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. Weigh the egg to the nearest 0.01 g.

Wrap the egg 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. D*o 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.

B. Sex ratio

Question: do females that respond more strongly to GnRH or have more masculine digit ratios also have higher yolk T or produce more sons? 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 we did in 2005 and 2006. Starting in 2007 and this year too we have decided to collect one tube on day 3 just to be sure we get as many samples as we can before the broods are lost to predators. **Note Nicki says that the DNA is dilute in these samples so do your best to get a whole tube but don't injure young.**

1. On hatching day weigh the young and marked them by clipping feathers on the head, the back, both, or neither. After marking young, weigh them and measure tarsus.

~~Previously we did this 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).~~

~~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 K, D, or A how to do this).~~ [As of 2009 decided to no longer do this.]

3. On day 3 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 or 3 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 1000 ul of Longmire's and needles. Note, it is essential that the Longmire's and the Eppendorf tubes not be contaminated. 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 head clip, back clip, both clip, neither clip, etc.*** If a bird disappears during the nestling phase, we have to know which one if we are to compute secondary sex ratios.

4. On day 6 weigh and measure again when you band and bleed taking both and A and a B tube (again coordinate with K, D, A, see the NEST protocol). Be sure to associate the correct feather clip 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 (These instructions are for future generations)

Question: does treatment of the male or the female with T or response to GnRH predict that 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 and juncos are one of the few passerines in which people have attempted this, and 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?