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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).

GOALS 2000, long version
April 24, 2000
Still under construction

The research conducted at MLBS and in Bloomington is divided into three parts: the effect of testosterone (T) on male behavior, physiology, and fitness (the male project), the effect of T on the behavior and physiology of the individuals that associate with testosterone-treated males (T-males) and controls (C-males) (the extended phenotype project), and, in the context of possible constraints on the evolution of T in males, the effect of T on the behavior and physiology of females (the female project).

Each year we prepare the study area by creating T- and C-males, then monitor the relative reproductive success and survival of males of both types in order to assess annual variation in the impact of testosterone on population structure (age ratio, return rate) and components of fitness (EPF rates, predation rates, survival of adults, mass of nestlings at fledging, etc.). To do this, we implant birds, map territories, find nests, bleed/band/weigh nestlings, enter data into the computer, and help to create the daily list.

We also pursue sub-projects that relate to the objectives outlined above. Some of the sub-projects will lead to publishable papers, and others will lay the groundwork for future studies. In 2000, we will continue to quantify effects of testosterone on male behavior and physiology in relation to fitness. We will also measure aspects of the male's 'extended phenotype' in his neighbors, mate, and offspring. and we will begin to assess the effects of testosterone in females.

We also have a tremendous amount of work to do summarizing already collected data on return rates, mate fidelity, and genetic relatedness. And this is the summer when we need to write a proposal to the NSF describing future work.

Listed here are projects for the summer, not all of which we will be able to pursue. Much will depend on how many birds we implant, whether predators are abundant this year, and the interests of members of the crew, but projects marked with ** are highest priority.

1. Map study area

- Obtain an accurate reading of the all nest locations using GPS. Get readings for all trap and net locations and make a junco map of MLBS (Neudorf, McGlothlin).

We hope to capitalize on Diane Neudorf's familiarity with GIS/GPS in order to obtain a more accurate representation of the distances separating nests, territories, and net/trap sites. These will help with the interpretation of data already collected on extra-pair paternity and might help us determine whether territories of T-males differ in size or some other attribute from those of C-males.

2. T and the male phenotype: effect of T on immune function, allocation to parental and

mating effort, volume of the song control system, aggressiveness, and behavior towards neighboring females with fledglings.

- **To assess effects of T on humoral immune function, compare antibody titers of T- and C-males in response to immunization with PHA and sheep red blood cells (SRBC) (Casto, Parker-Renga).****

Question? Does T affect susceptibility to disease and male survivorship?

Because we expect animals that deviate from the norm to have lower average fitness, we anticipate that T-males might exhibit poorer health or lower survival. What follows are two protocols by Joe Casto for testing cell-mediated and humoral immunity to be applied to both free-living and captive male juncos.

a. Assessment of cell-mediated immunity in free-living juncos (written by J. Casto in 1998, modified in 99 and 00 by EK)

When attempting to determine how a manipulation such as long-term elevation of T influences fitness, we must assess potential costs as well as benefits. One potential cost is suppressed immunity, and substantial evidence suggests that T can act directly or indirectly to suppress immune function in both birds and mammals. In 1998 we began a comparison of the ability of T-males and C-males to mount an immune response in response to administration of a mitogen, using a paradigm known as the cutaneous delayed-type hypersensitivity test (DHT).

In the DHT paradigm, a bird is sensitized with an initial subcutaneous exposure to a novel compound such as a plant lectin or shellfish protein. In response to this initial sensitizing exposure, the bird mounts a classical, delayed (24- 48 hr) immune response characterized by T-cell reactivity, inflammation, and edema at the exposure site. It also forms an immune memory so that if exposed again to the same mitogen, the immune response is faster and more intense. The response to a second local administration of the compound (a challenge) allows a determination of a bird's ability to mount an immune response based on its immune memory and to compare the immune responses of T- and C-males.

During April-May 1998, we captured and implanted approximately 90 male dark-eyed juncos with empty or T-filled Silastic implants and, while the birds were anesthetized for implant, we administered a sensitizing dose of phytohemaglutinin (PHA), a T-cell dependent mitogen (Sigma L-8754, 0.25 mg of PHA in 50 μl of complete Freund's adjuvant via a subcutaneous injection in the loose skin of the neck). PHA is a harmless plant lectin derived from red kidney beans and has been used successfully in a variety of avian species including chickens, bobwhite quail, jungle fowl, and barn swallows. Swelling caused by exposure to PHA is very localized and the cutaneous hypersensitivity is short-lived and does not negatively influence a bird's ability to survive in the wild.

Over the course of the summer we gave injections of a challenge dose of PHA to males captured at their nests on the 6th or 7th day after their young hatched. At capture, we measured the thickness of the "web" of each wing (the skin between the wing and the neck) with a spring-

loaded thickness gauge. Birds were injected in one wing with 5 mg of PHA (??) in phosphate buffered saline (PBS) and in the other wing with an equal volume of PBS.

Between 24 and 48 hrs after the challenge injection males were recaptured at the nest and the thickness of each wing web was measured again and a blood sample taken. The magnitude of the immune response to the local challenge was calculated by subtracting the change in thickness of the PBS-injected wing web from the change in thickness of the mitogen-injected wing web.

Although the time between sensitization and challenge differed for individual males, it was necessary to perform the challenge at a time when we could insure capture of the male twice within a 48-hour period. Both T- and C-males are most easily captured when they are caring for young at the nest.

During the last two weeks of July and the first two weeks of August we recapture implanted males and remove their implants. It would be possible at that time to challenge any males not already challenged and to keep them in holding cages for 24 hrs in order to measure their immune responses, but to date we have not done this, but we may in 2000.

Results to date: Under free-living conditions, T-males exhibited elevated T titers, and suppressed cell-mediated immunity (70% less wing web swelling than C-males, $p < .05$)(Casto et al. under review).

b. Assessment of cell-mediated and humoral immunity in captive male juncos (also by J. Casto, written in 1998, modified in 1999 and 2000)

In 1998, we used two immune tests to compare the influence of artificially elevated levels of circulating testosterone on cell-mediated immunity and humoral (antibody-mediated) immunity in the same individuals. The tests were conducted on captives in order to insure a high degree of control over the timing of immunizations and assessment of immune responses. The results formed the basis for the experiments that were conducted in 1999.

Thirty male dark-eyed juncos were captured in the areas surrounding MLBS, housed alone in outdoor aviaries at MLBS, and given *ad libitum* access to food and water. Fifteen males were implanted subcutaneously with testosterone-filled silastic tubes and the remaining 15 males received empty silastic tubes.

Ten days after implantation, we initiated a DHT test to compare cell-mediated immunity. Each male was injected with a subcutaneous sensitizing dose of PHA; 10 days later each received a subcutaneous challenge injection of PHA in the web of one wing and a subcutaneous control injection of phosphate buffered saline (PBS) into the web of the other wing. At 24 and 48 hours post-challenge, swelling of each wing web was measured and the difference between wings served as an index of cell-mediated immune responsiveness (see above). At 48 hours post-challenge, we also collected a blood sample to determine the concentration of circulating testosterone and to determine a baseline antibody concentration for the humoral immunity assay.

On the day following termination of the DHT procedure, all males were immunized with 100 μl of a solution of 2% sheep red blood cells (SRBC,ICN biochemicals) in PBS via intraperitoneal injection. At 6 days post-immunization a blood sample was collected and plasma was used to determine antibody concentration using a hemagglutination assay.

Briefly, findings to date have shown that captive T-males exhibited elevated T titers, elevated CORT titers, suppressed humoral immunity (36% lower antibody titers than C-males, $p < .05$), and suppressed cell-mediated immunity (52% less wing web swelling than C-males at 48 hrs post- immunization, $p = .05$). Three weeks after hormone implants were removed, T-males and C-males did not differ in their cell-mediated immune responses (Casto et al. under review).

The elevated CORT in T-treated males is something that we find consistently (Ketterson et al. 1991, Klukowski et al. 1997, Schoech et al. 1998), and is intriguing because CORT itself has been shown to be immunosuppressive and future experiments will focus on this aspect of the findings to date.

During 2000, Casto will again test free-living juncos for effects of T on cell-mediated immunity using PHA and effects of T on humoral immune function using SRBC. Although we get about 33% non-responders, which makes the SRBC assay less than perfect, it is still a good one for us because the time required to mount an immune response after a challenge corresponds with the length of the juncos' nestling period. Thus we can perform one challenge early in the nestling stage and a second challenge near the end of the nestling stage, knowing where the males will be throughout. We may also compare for humoral response to KLH using Hasselquist's ELISA since he thinks that the same time period required for SRBC will also work for KLH ??

In the future, Casto is planning to elevate T in one group, CORT in a second group, and have a third group as a control in order to determine if the immuno-suppression caused by T can be accounted for by changes in CORT. He will use the same assays as last year and also include a KLH ELISA in collaboration with Dennis Hasselquist of Sweden. In addition to the initial assays, he will re-immunize birds after implant removal to assess further the effects of T and CORT on immune memory formation and recovery from immuno-suppression.

- **To assess effects of T on aspects of parental behavior, compare T- and C-males for load size and actual feeding behavior at the nest (video) (Clotfelter, Schubert). ****

Reduced parental effort by male dark-eyed juncos with experimentally elevated testosterone: the importance of motivation to feed and responsiveness to competing stimuli **(excerpted from proposal by E. Clotfelter, 1999, modified 2000**).

When given subcutaneous implants of testosterone (T), male dark-eyed juncos (*Junco hyemalis*) increase their mating effort and decrease their parental effort relative to control (C) males. Females mated to implanted males (T-males) increase their own rates of nestling feeding but nevertheless nestling survival is reduced in nests of T-males. The causal relationship between elevated T and reduced male parental effort is poorly understood. In this study, I will

use a combination of laboratory and field experiments to elucidate the mechanism(s) involved. My specific objectives are:

a. To measure the quantity of food delivered to nestlings by T-males and C-males under field conditions.

b. **To quantify the stimuli presented by nestlings of T-males and C-males by measuring gaping behavior and mouth coloration of junco nestlings taken from the field to determine if T-males reduce their parental effort as a result of differences in salient stimuli rather than differences in motivation to feed.**

Significance: Testosterone affects a suite of behavioral, morphological, and life history parameters in vertebrates, including vocal behavior, secondary sexual characteristics, home range size, food consumption, and circadian rhythms. Elevated circulating levels of testosterone are associated with increased agonistic and sexual behavior and also with decreased body mass, immune system function, and survival. By experimentally manipulating testosterone levels in free-living animals we can evaluate the various costs and benefits associated with this trait. In birds, one of the most widespread tradeoffs of elevated testosterone is a reduction in male parental effort. Despite numerous studies that have produced similar results and shown that reduced male parental effort reduces reproductive success, we know little of the mechanism by which testosterone influences parental behavior. One potential explanation is that testosterone affects male motivation to respond to external stimuli, including food, begging nestlings, and receptive females. Another explanation is that the stimuli received by males with elevated testosterone are different from stimuli received by normal males, perhaps as a result of changes in nestling condition or female behavior. If this is the case, then behavior of the female or the nestlings may feed back to further suppress male parental effort. Distinguishing between these potential explanations will help us understand how natural selection acts on male behavior, and perhaps on female or offspring behavior as well, to regulate testosterone levels in males.

Background. Despite the abundant evidence that testosterone reduces male parental effort, we know very little about how this effect is produced. In some species testosterone reduces foraging rate or feeding periodicity (Andrew and Clayton 1979, Das 1991, Guyomarc'h and Guyomarc'h 1994); in others, including juncos, it increases food consumption (Deviche 1995, Lynn et al. in press, Jones et al. in prep.). And numerous studies report that testosterone increases muscle mass and decreases lipid content (Stetson and Erickson 1972, Schwabl and Farner 1989, Ketterson et al. 1991). The inhibitory effect of testosterone on foraging rates reported in some of these studies suggests that reduced male parental effort could be a consequence of reduced motivation to feed. Alterntively, an increase in appetite could cause males to consume more of the food they encounter and leave them less to deliver to their young.

Rather than affecting males' motivation to feed themselves or their offspring, elevated testosterone may affect the way in which males respond to competing stimuli. Most studies have shown that males with experimentally elevated testosterone increase their vocal behavior, home range size, and time spent with females (Wingfield 1984, Logan and Carlin 1991, Chandler et al. 1994, Saino and Møller 1995), suggesting an increase in sexual motivation. Sexual and parental behavior are often assumed to be mutually exclusive (Dittami et al. 1991, Whittingham 1994),

but to my knowledge no study has tested directly male preferences between parental and mating behavior.

These explanations assume that stimuli received by males are equivalent and that the effect of testosterone on male parental effort is the result of differences in motivational state. However, variation in male parental effort may also result from variation in external stimuli. Nestling gapes, mouth coloration, mouth markings, and vocalizations are all selected to maximize feeding rates by parents (Mondloch 1995, Price et al. 1996, Kilner 1997). If female behavior (e.g., increased nestling feeding rate) or nestling condition (e.g. hunger) causes deviations in these stimuli, males may respond by increasing or decreasing their feeding rates. Therefore, decreased male parental effort may be unrelated to differences in motivation.

The proposed research will test the following hypotheses using dark-eyed juncos (*Junco hyemalis*) as a model organism: (1) Reduced parental effort in males with elevated testosterone follows from a reduced motivation to feed; (2) reduced parental effort in males with elevated testosterone results from differences in salient stimuli and not motivational differences.

Materials and Methods.

(1) To measure the quantity of food delivered to nestlings by T-males and C-males under field conditions. These measurements will be made at MLBS during the field seasons of 1999 and 2000. Previous work has shown that T-males make fewer feeding trips than do control males (Ketterson et al. 1992). In addition to continuing observations of male feeding rates, I will measure the quantity of food brought to nestlings. This will be accomplished using two techniques: nestling neck ligatures and serial mass measurements of nestlings. Neck ligatures prevent nestlings from swallowing so that prey loads can be measured (Mellott and Woods 1993). Both techniques will be used in 1999 to evaluate their relative effectiveness for continued use in 2000. Females will be temporarily removed before prey-load size data are collected to reduce confounding effects of compensatory feeding.

(2) To quantify the stimuli presented by nestlings of T-males and C-males. I will examine the following characteristics of nestlings of T-males and C-males at MLBS in 1999 and 2000: nestling begging vocalizations, gaping behavior, and mouth coloration. Begging vocalizations and gaping behavior will be measured by removing nestlings from nests, food-depriving them for approximately 1.5 hours, and stimulating gaping by tapping their bills (E.D. Ketterson, pers. comm.). Mouth coloration, an honest signal of hunger in nestling birds, will be recorded with Hi8 video cameras and transferred to Adobe Photoshop software for analysis (Kilner 1997, Kilner and Davies 1998). Considerable preliminary data exist on nestling begging vocalizations, but not for gaping behavior or mouth coloration. Measurements of nestling stimuli will be conducted with the assistance of an NSF REU research assistant at MLBS.

The proposed changes/additions outlined below are part of our larger research program (99-048) on parental behavior in male dark-eyed juncos (*Junco hyemalis*). In particular,

these experiments will focus on the evolution of a visual signal in nestling juncos and the nature of parent-offspring communication.

Proposed changes/additions:
1. We will examine the effect of food type on changes in mouth color in nestling juncos.

2. We will measure nestling mouth color at different stages of development to document age-related changes in mouth color.

3. We will temporarily manipulate nestling mouth color in the field and monitor parental responses.

Methods:
This study (99-048) will non-destructively utilize up to 30 broods of dark-eyed juncos at the Mountain Lake Biological Station in southwestern Virginia (see BIACUC study number 98-107). Using methodology that we employed successfully in 1999, we will remove two nestlings from each nest and bring them in an insulated container to the MLBS laboratory, where their mouths will be photographed in a darkroom. Food will be withheld for one hour, a treatment utilized in many behavioral studies. In 1999 there was no mortality or other ill effects as a result of this manipulation.

For each of the following experiments, we propose to use approximately 10 broods. In experiment #1 we will randomly assign a different food type to each nestling (canned cat food, mealworms *Tenebrio molitor*, and crickets *Gryllus* spp.). These food types are all standard for bird husbandry. Following food deprivation nestlings will be fed to satiation with one type of food and photographed again. Twenty minutes after feeding, we will return them to the nest.

For experiment #2, we will only use nests in close proximity to the MLBS laboratory to reduce disturbance to nestlings. We will remove nestlings at days 3, 5, and 7 after hatching, and follow the same procedure outlined above. All nestlings will be fed a standard food type at the end of the trial and returned to the nest.

For experiment #3, we will manipulate the mouth color of nestling juncos following established protocols (Gotmark and Ahlstrom 1997, Saino et al. 2000). Redness will be enhanced with several drops of food coloring, which produces a temporary change in mouth color. Feeding rates by males and females will be monitored for one hour before and after this manipulation to determine the effect of mouth color on parental behavior.

Rationale for changes/additions:
Our research on nestling mouth color in 1999 yielded interesting results. When deprived of food for one hour, junco mouths increased in redness. This is consistent with the hypothesis that mouth color is a signal of nestling hunger (Kilner 1997). After feeding nestlings to satiation with canned cat food, however, their mouths continued to redden. Experiment #1 will determine if our choice of food type was inappropriate or if the increase after feeding is a robust phenomenon. We will compare mouth color changes for nestlings fed cat food with those fed natural food types such as crickets and mealworms.

Saino et al. (2000) proposed that nestling mouth color was a signal of nestling quality (body size, immunocompetence, etc.) rather than of nestling hunger. Our 1999 data do not support this hypothesis, but to further examine it we will measure mouth color at different stages of development (experiment #2). Our belief is that mouth color may reflect hunger at early stages (day 3), but may indicate nestling quality once offspring pass the critical period when starvation is likely to occur (day 7).

Finally, an assumption implicit in our research is that junco parents respond to the signals produced by their nestlings. Previous studies in other species (Gotmark and Ahlstrom 1997, Saino et al. 2000) report that parents increase their feeding rates when nestling mouth color is experimentally enhanced, but this has yet to be tested in juncos (experiment #3).

Effects of these changes/additions:
Our experiments from 1999 showed that these manipulations have no lasting effects on nestlings or their parents. We fed nestlings before taking them back to their nests, and parents immediately fed them once they were returned. Experimental enhancement of mouth color with food dye will result in only transient changes in mouth color (Gotmark and Ahlstrom 1997, Saino et al. 2000).

Literature cited:

Gotmark, F. & Ahlstrom, M. 1997. Parental preference for red mouth of chicks in a songbird. Proc. Roy. Soc. Lond. B 264: 959-962.

Kilner, R.M. 1997. Mouth colour is a reliable signal of need in begging canary nestlings. Proc. Roy. Soc. Lond. B 264: 963-968.

Saino, N., Ninni, P., Calza, S., Martinelli, R., De Bernardi, F., & Moller, A.P. 2000. Better dead than red: carotenoid-based mouth coloration reveals infection in barn swallow nestlings. Proc. Roy. Soc. Lond. B 267: 57-61.

- **To assess neuro-anatomical effects of experimental elevation of T, compare song control system in T- and C-males in (Casto, Smulders; involves analysis of already collected data).****

In 1997 Tom Smulders collected brain tissue from a set of T- and C-males studied north of MLBS. He has published his results on the relative size of the hippocampal formation (HF)

and found no effect of treatment on HF (Smulders et al. in press). However, other areas of the brain, particularly the song control nuclei are expected to be sensitve to T, so this summer Casto will examine the same slides used to study HF in order to see whether he finds an effect of T.

- **To assess effect of T on aggressiveness, compare T- and C-males for their behavioral response to a simulated territorial intrusion (STI) (Team). ****

- **To learn why EPFs are higher in years of low nest predation, particularly for C-males, perform fledgling watches when females are fertile to quantify rates of intrusion by T- and C-males (Team). ****

A. Endogenous T in males: effect of relatedness, repeatabilities and individual variation in plasma and fecal T.

- Begin assessment of heritability of T by collecting plasma T under similar conditions from *any* related males or females found on the study area (father-son, brothers-sisters, etc.). [Can also collect samples from unrelated individuals, so long as a DNA sample has been collected from both to assess relatedness.] (Team). **

- To document repeatability and individual variation, collect plasma and fecal samples repeatedly from same males during the same stage of reproduction (Team? May do in Indiana). **

B. Heritability, repeatability on captives

- Rear young in Bloomington to produce experimental system and opportunity to compare siblings for hormone levels and behavior (Ketterson, Lipar, Wolf, Sumner)**

- Transport newly caught juncos to Bloomington in late summer to help maintain the colony of juncos there. Possibly practice hand-rearing related (sibling) young for later measurement of T and T-mediated traits. Whether we do this will depend on success of breeding efforts in Bloomington.

C. Demography and data analysis

- Continue to compare treatments with respect to reproductive success, analyze new paternity data from Parker lab (Ketterson, Snajdr)**

- Demographic data, 94-present, return rates of adults and young, mate fidelity, fledgling mass and numbers, annual variation in rates of predation, opportunities for EPFs, etc. (Nolan, Ketterson, Snajdr). **

- Keep up with USFWS banding schedules, fitness correlates sheets, and other summary sheets as we go.

D. Projects likely to be postponed.

2. Testosterone and flexibility in behavior (to be postponed)

In an earlier proposal to the NSF, we proposed to investigate treatment-related differences in allocation of effort to mating and parenting and the *coordinated* physiological and behavioral changes involved. We also promised to determine whether fixed hormone profiles (i.e., T-implants) limit male flexibility in a maladaptive way.

The idea would be to create environmental opportunities that would be expected to tilt the fitness equation towards mating effort or parental effort and then to compare the responses of T- and C-males. One prediction might be that as the potential gains from parental effort increase, male physiology should reflect greater readiness to behave parentally. Alternatively, the greater the potential gains from mating effort, the more male physiology should represent readiness to mate.

The manipulations would be alteration in brood size and induced fertility in mates of neighboring females. Dependent variables would be song and feeding rates (including time structuring), T and Prl, and possibly sperm or responsiveness to predators. We would also look at self-maintenance, i.e., self-protection, grooming, and self-feeding (foraging) vs. any form of reproductive effort.

We might predict that if brood size is enhanced, males would sing less, feed young more, preen more, possibly eat more themselves, be more likely to detect a predator, pay less attention to an intruder or a female in a pre-copulatory display, have more sperm (because they are not copulating?). Simultaneously we would predict that they would turn down their T and possibly have higher Prl. If brood size were reduced, all predictions would be reversed. We would also predict that T-males would be less flexible than C-males.

If a neighboring female becomes fertile, we might predict that all males would feed less and raise T, but the decline in feeding would be greater in T-males than in C-males.

This project has been on the books for several years but predation rates have been too high to pursue it. This might be a year to get a start on the effect of brood enhancement using control males on 714.

3. Sperm counts and copulatory behavior (to be postponed)

Are T-males more likely than C-males to mount a stuffed female in the field? Do sperm reserves refill more rapidly in captive T and C-juncos?

We would like to know whether T affects mounting behavior in the field. If T-males copulate more frequently in the field, we might expect them to copulate more readily with a stuffed female mounted in a precopulatory display and placed on their territories.

If we found an effect of testosterone, I would be interested in the future in trying to take this apart by designing an experiment to distinguish the effects of straight T, an estrogen implant, or an aromatase blocker.

In order to determine whether males are likely to be sperm-limited, we need to know their 'refilling rate' in captivity and in the wild. This could be accomplished by milking a set of captives at 24-hour intervals and by capturing males at their nests on successive days. We tried this in 96 on captives with limited success, and I am not really sure why except to say that the captives did not respond well to repeated efforts to sample sperm. This work may have to be done in the field. It may not be worth pursuing unless we can obtain behavioral evidence that T-males copulate more readily.