

## 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>).

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## PROJECT SUMMARY

Males and females typically share some attributes and differ in others. Differences arise when one sex is subject to greater reproductive competition than the other (sexual selection) and when the two sexes differ in their ecology (natural selection). Because the sexes share a common genome, they are also subject to correlated selection, which occurs when attributes directly favored in one sex are also expressed in the other. Correlated selection can hasten evolution if attributes directly favored in one sex also benefit the other, or it may serve as a constraint when the attributes are disadvantageous to the second sex.

Proximate mechanisms underlying sex differences and resemblances include ontogenetic integration, steroid hormones, and cell-autonomous gene expression. Gonadal steroids can act early in development to organize tissues so that they are or are not responsive to activation by a steroid later in life. The organization/activation phenomenon can be viewed as an endocrine form of sex-limited expression. Gonadal steroids can also affect the sexes as adults and when their effects are similar, there is potential for correlated selection.

The focus of this proposal is a steroid hormone, testosterone, and its integrating effect on sex differences and resemblances in adult males and females of a songbird species, the dark-eyed junco. The research has four objectives:

- (1) to assess the role of testosterone in sexual integration by comparing natural male and female hormone profiles, hormonal response of females to stimuli known to increase T in males, and hormone levels in females that do and do not produce young by extra-pair fertilizations (EPFs). Resemblance between the sexes will reveal the potential for correlated selection; an androgen influence on the frequency of EPFs would reveal a role for testosterone in sexual selection on females.
- (2) to alter the level of circulating testosterone experimentally and compare effects of altered testosterone in females to previous results from males. Individual experiments will address whether elevated testosterone in females alters attractiveness to males (sexual selection) and whether it affects parental behavior, social status, or immune function (natural selection). They will also address interactions between testosterone, corticosterone, corticosteroid binding globulins.
- (3) to determine whether female responses to altered testosterone are beneficial, neutral, or detrimental in the field. If the sexes constrain one another, alterations that are beneficial in one sex should be detrimental in the other. If the sexes reinforce one another, alterations may be beneficial in both sexes. If the sexes are independent, then alterations already known to be beneficial or detrimental in males may have no fitness consequences for females.
- (4) to compare how degree of relatedness affects a sexually selected trait and two hormone-related traits. Juncos of known genetic relatedness (472 measured over 6 years) will be assessed for family resemblance in measures of body size and a plumage trait that affects attractiveness. Additional juncos will be reared and compared for three hormonally mediated traits. The presence of a genetic correlation would predict correlated selection, the absence of one would predict sexual independence.

Research described in this proposal will provide opportunities for training future scientists of diverse backgrounds in the conduct of laboratory and field experiments that address the integration of proximate and ultimate explanations for biological differences. It will also enrich graduate and undergraduate courses taught at Indiana University. Potential societal implications of the findings to be expected include (1) importance of hormonally active agents in the environment, (2) improved methods for breeding captive songbirds for conservation, and (3) a greater understanding of the relationship between sex and gender.

## PROJECT DESCRIPTION

### RESULTS FROM PRIOR NSF SUPPORT

**IBN-9728384 to Ellen D Ketterson and Val Nolan Jr., Indiana University, Bloomington, \$375,277, 03/01/98 – 02/28/02, “Using Hormones to Explore Adaptation and Constraint in a Male Bird.”**

Research supported by this award addressed effects of experimentally elevated testosterone on the behavior, physiology, and fitness of males of a socially monogamous songbird, the dark-eyed junco (*Junco hyemalis*: Emberizidae). We used hormone implants to raise plasma levels of testosterone to the maximum values that occur naturally at the time breeding begins and held them there, creating a seasonal pattern that typifies males of polygynous species (Wingfield et al., 1990). In controls, plasma levels of testosterone peaked in early spring, then declined to lower levels, as is true of juncos naturally. Prolonged experimental elevation of testosterone produced phenotypes (or *phenocopies*) that are rare in natural populations but that obviously lie within the “genomic capacity” of the organism. We characterized the experimental phenotype (*T-males*) in relation to controls (*C-males*) and asked why the experimental phenotype is not more common in nature. We had previously shown that testosterone mediates a trade-off between mating effort and parental effort: T-males court more, are more attractive to females, and provide less care for offspring. They are also more successful at siring young through extra-pair fertilizations, but less successful at rearing young with their social mates. During the period covered by this award we found that testosterone suppresses immune function and reduces survivorship. We also identified an array of traits that are and are not sensitive to hormonal manipulations. We believe the work has contributed significantly to understanding of the evolution of complex adaptations, particularly those mediated by hormones and involving trade-offs. Its impact can be assessed in terms of contribution to human resources, enhancement of education/outreach/potential society implications, and contributions to the scientific literature, as described immediately below.

**Contribution to the development of human resources.**—Numbers of students receiving training with the help of IBN-9728384, 1998-2001: 9 post-doctoral associates (4 male, 5 female), 8 graduate students (2 male, 6 female, 1 Hispanic), 19 undergraduate or post-undergraduate students (8 male, 11 female, 1 African-American, 3 first-generation; 11 supported by REU programs, 7 already gone on to graduate school, and 8 are co-authors on submitted manuscripts or publications). Other societal contributions are described at the end of the proposal under **BROADER IMPACTS**, p. 15.

*Post-doctoral associates:* **A Buerkle** (now visiting faculty, U of Wisconsin, Eau Claire), **E Clotfelter** (now Asst. Prof., Providence College), **D Monk** (now at Washington State U), **D Neudorf** (now Asst. Prof., Sam Houston State U), **M Sandell** (now researcher, Lund U, Sweden), **S Schoech** (now Asst. Prof., Memphis U), **W Reed** (now Asst. Prof., North Dakota State U), **J Casto** (still in training, SIT), **D. Duffy** (SiT)

*Graduate students:* **N Arguedas** (Ohio State U, now post-doc, Ohio State), **K Jones** (employed in industry), **J Lipar** (now post-doc, Washington State U), **T Smulders** (Cornell U, now post-doc, Duke U), **S Raouf** (now post-doc, U of Washington), **B VanRoo**, **J Grindstaff**, **B Heidinger** (all SiT)

*Undergraduate research students and field assistants:* **C Aron** (completed MS, Indiana U), **E Donnelly** (William and Mary, now grad student, Wake Forest), **B Elitzur** (U Cal, Berkeley), **M Faurot** (Washburn University, SiT) **W Garrison**, **J Hill** (now grad student, U of New Mexico), **D Hopenstand** (U Cal, Berkeley), **E Hagen** (Truman State U), **M Hill** (now grad student U of Vermont), **S Hudman** (now grad student, U of Vermont), **J Kleffner**, **S Lynn** (now grad student, U of Washington), **J McGlothlin** (now grad student, Indiana U), **D O'Neill** (Washington U, St. Louis), **I Parker-Renga** (Indiana U), **J Sanders** (Indiana U), **K Schubert** (William and Mary, now grad student, Queens U), **D Ziolkowski** (now grad student, U of Missouri, St. Louis), **W Wolf** (Indiana U, SiT).

**Publications resulting from the current award, 1998-2001.**—[total of 32, including 3 submitted journal articles, 17 journal articles, 2 book chapters, and 12 published abstracts (*Society for Neuroscience Abstracts, American Zoologist, Hormones and Behavior, titles not listed*)].

Wolf, W.L., Casto, J.M. Nolan, V. Jr., and E. D. Ketterson. 200x. The effect of female ornamentation on mate choice by male dark-eyed juncos. *Behavioral Ecology*, submitted.

Neudorf, D.L., Ziolkowski, D.J. Jr., Nolan V. Jr., and E.D. Ketterson. 200x. Female home-range size and behavior during the fertile period is not affected by testosterone manipulations of male attractiveness. *Ethology*, submitted, under consideration after revision

Clotfelter, E.D. Schubert, K.A. Nolan, V. Jr., and E.D. Ketterson. 200x. Mouth colour signals thermal stress in nestling dark-eyed juncos (*Junco hyemalis*). *Animal Behaviour*, submitted, under consideration after revision.

Nolan, V. Jr., Cristol, D., Clotfelter, E. D., Ketterson, E. D., Schoech, S., Rogers, C., Snajdr, E. and R.C. Titus. 200x. Dark-eyed Junco (*Junco hyemalis*). In: *The Birds of North America*, No. xxx. (Ed. by Poole, A. & Gill, F.), The Birds of North America, Inc., Philadelphia, PA.

Ketterson, E.D., Nolan, V. Jr., Casto, J.M., Buerkle, C.A., Clotfelter, E., Grindstaff, J.L., Jones, K.J., Lipar, J.L., McNabb, F.M.A., Neudorf, D.L., Parker-Renga, I., Schoech, S.J. and E. Snajdr. 2001. Testosterone, phenotype, and fitness: a research program in evolutionary behavioral endocrinology. Pp. 19-40, in A. Dawson and C.M. Chaturvedi (eds.), *Avian Endocrinology*. Narosa Publishing House, New Delhi, India

Clotfelter, E.D., V. Nolan Jr., and E.D. Ketterson. 2001. Effects of elevated testosterone and food deprivation on food consumption and prey size preferences in male dark-eyed juncos (*Junco hyemalis*). *Ethology*, 107:439-449.

Grindstaff, J.L., Buerkle, C.M., Casto, J.M., Nolan, V. Jr., and E.D. Ketterson. 2001. Male attractiveness and offspring sex ratio in dark-eyed juncos (*Junco hyemalis*). *Behavioral Ecology and Sociobiology* 50:312-316.

Casto, J.M., Nolan, V. Jr., and E. D. Ketterson. 2001. Steroid hormones and immune function: experimental studies in wild and captive dark-eyed juncos (*Junco hyemalis*). *American Naturalist* 157:408-420.

Lipar, J.L., and E.D. Ketterson. 2000. Maternally derived yolk testosterone enhances the development of the hatching muscle in the red-winged blackbird, *Agelaius phoeniceus*. *Proceedings Royal Society, London B*, 267: 2005-2010.

Hudman, S.P., Ketterson, E.D., and V. Nolan Jr. 2000. Effects of time of sampling on oocyst detection and effects of age and experimentally elevated testosterone on prevalence of coccidia in male dark-eyed juncos *Auk*, 117: 1048-1051.

Lynn, S.E., Houtman, A.M. Weathers, W.W., Ketterson, E.D., and V. Nolan Jr. 2000. Testosterone increases activity but not daily energy expenditure in captive male dark-eyed juncos. *Animal Behaviour* 60:581-587.

Smulders, T.V., Casto, J.M., Nolan, V. Jr., Ketterson, E.D., and T.J. DeVogd. 2000. Effects of captivity and testosterone on the volumes of four brain regions in the dark-eyed junco (*Junco hyemalis*). *Journal of Neurobiology* 43: 244-253.

Lipar, J.L., Ketterson, E.D., Nolan, V. Jr., and J.M. Casto. 1999. Egg yolk layers vary in the concentration of steroid hormones in two avian species. *General and Comparative Endocrinology*, 115: 220-227.

Ketterson, E.D., and V. Nolan Jr. 1999. Adaptation, exaptation, and constraint: a hormonal perspective. *American Naturalist*, 153:S4-S25.

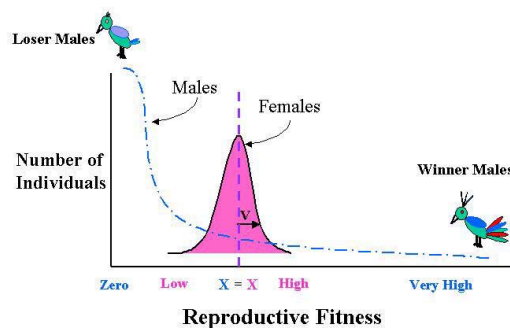
Hill, J.A., Enstrom, D.E., Ketterson, E.D., Nolan, V. Jr., and C. Ziegenfus. 1999. Mate choice based on static vs. dynamic secondary sexual traits in the dark-eyed junco. *Behavioral Ecology* 10 (1): 91-96.

Schwagmeyer, P.L. and E. D. Ketterson. 1999. Breeding Synchrony and EPF Rates: the Key to a Can of

- Worms? *Trends in Ecology and Evolution* 14:47-48.
- Lipar, J., Ketterson, E.D., and V. Nolan Jr. 1999. Intra-clutch variation in testosterone content of eggs of red-winged blackbirds. *Auk* 116:231-235.
- Schoech, S., Ketterson, E.D., and V. Nolan Jr. 1999. Exogenous testosterone and the adrenocortical response in the Dark-eyed Junco, *Junco hyemalis*. *Auk* 116:64-72.
- Schoech, S., Buntin, J., Sharp, P. Ketterson, E.D., and V. Nolan Jr. 1998. The effect of exogenous testosterone on parental behavior, plasma prolactin, and prolactin binding sites in dark-eyed juncos. *Hormones and Behavior* 34:1-10.
- Cawthorn, J.M., Morris, D., Ketterson, E.D., and Nolan, V. Jr. 1998. Influence of elevated testosterone on nest defence in dark-eyed juncos. *Animal Behaviour* 56:617-621.
- Kast, T.L., Ketterson, E.D., and V. Nolan Jr. 1998. Variation in ejaculate quality in dark-eyed juncos according to season, stage of reproduction, and testosterone treatment. *Auk* 115(3): 684-693.
- Ketterson, E.D., Parker, P.G., Raouf, S.A., Nolan, V. Jr., Ziegenfus, C., and C.R. Chandler. 1998. Relative importance of extra-pair fertilizations to male and female reproductive success in dark-eyed juncos. Pp. 81-101 in *Avian reproductive tactics: female and male perspectives* (P. G. Parker and N. T. Burley, eds.). *Ornithological Monographs* No. 49, Lawrence, KS.

## INTRODUCTION

This proposal addresses hormone-related sex differences and similarities in a songbird and asks why the sexes are not more different than they are. In particular, it addresses the activational role of testosterone in female behavior and physiology and attempts to assess the extent to which testosterone may affect the outcome of sexual selection on females and constrain it in males.



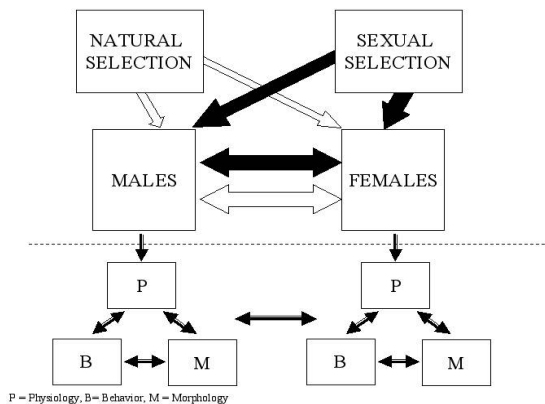
Evolutionary biologists have long recognized that traits that enhance fitness in males are not necessarily the same as those that enhance fitness in females (Darwin 1871, Andersson 1994). Males invest more time and energy in attracting or pursuing mates; females invest more in caring for offspring. Competition for mates (**sexual selection**) creates some highly successful males and some that sire no offspring at all (see Fig.1, dotted line is frequency distribution of male reproductive

success) (Schuster and Wade, 2002). Females vary less among themselves in reproductive success (Fig. 1, shaded distribution)(Schuster and Wade, 2002).

**Natural selection can also generate sex differences.** If males and females differ in their ecology, then natural selection will favor attributes that suit each sex to its environment, and these attributes may differ (Karubian and Swaddle, 2001; Ligon, 1999; Selander, 1966). Conversely, if the sexes are quite similar in their ecology, natural selection may lead to character displacement, causing the sexes to diverge (Johnson and Macdonald, 2001).

**Evolution of sex differences may be constrained by correlated selection.** If features that would be advantageous to one sex have detrimental effects in the other sex, the sexes may be more similar than would be 'optimal' for each considered alone (Lande, 1980; Lande, 1987; Lande and Arnold, 1983; Price and Burley, 1994). The boxes and arrows above the dotted line in Fig. 2 represent the relationships among sexual (thick black arrows), natural (thick clear arrows) and correlated selection (thick black and white double-headed arrows connecting male and female boxes). The important point is that the sexes can

influence (hasten or delay) one another's evolution because they share the same genome, giving rise to intersexual pleiotropy and the potential for correlated selection that may be antagonistic or reinforcing



(Chippindale et al., 2001; Rice, 1996; Rice and Chippindale, 2001). The boxes and arrows below the line represent within-generation sex differences in physiology, behavior, and morphology and their interactions. Within sexes, the phenotype can be constrained by multivariate selection (selection on one trait affects outcome of selection on others because they are correlated in their expression). Further, each sex affects the phenotype that the other develops by acting as part of its biotic environment. Consequently, the sexes act as selective factors on one another.

**An important challenge** is to distinguish among these explanations for specific sex differences in particular systems, while seeking a general understanding. Sexual and natural selection on males have been extensively studied, as has natural selection in females, but sexual selection on females and correlational selection are relatively under-explored.

**Also under-explored are the proximate mechanisms on which natural, sexual, and correlated selection must act.** Mechanistically and developmentally, the sexes are joined and differentiated by variation in ontogenetic integration (Badyaev and Hill, 2000; Badyaev et al., 2000), steroid hormones (Nelson, 2000), and non-steroidal, cell autonomous features (Arnold, 1996; Wade et al., 1999). The emphasis in this proposal is on the gonadal steroid testosterone, which regulates many of the traits that give rise to sex differences. For some traits, gonadal steroids act early in development to organize tissues, including neural circuits, and thus to masculinize males or de-masculinize females (or de-feminize/feminize respectively) (Adkins-Regan, 1975; Balthazart and Ball, 1998). If **organization** is complete, the sexes will respond independently to exposure to gonadal steroids later in life. More often, the sexes differ quantitatively not qualitatively in their responses to steroids as adults, suggesting that the relevant circuitry develops in both sexes and differences between them reflect differences in the degree of **activation** by circulating steroids or available receptors. The focus of this proposal is on how selection might act on traits that can be activated by testosterone in adulthood in both males and females.

Critical to a steroid's effect are its rate of secretion, its half-life in the circulation, its attachment to steroid binding proteins, and the abundance and distribution of receptors at target tissues (Ketterson and Nolan, 1999; Nelson, 2000). Evolution of hormonally mediated traits requires co-evolution of signals (e.g., hormones) and responses (e.g., receptors, hormone response elements, coding regions, etc.) and can arise out of numerous combinatorial changes. Alteration in circulating level of a hormone is clearly only part of any explanatory account of the development, expression, or evolution of sex differences, but it can be surprisingly informative, as the next paragraph indicates.

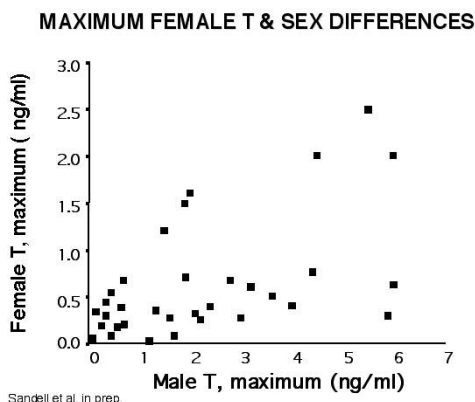
**Males are typically the more aggressive, noisy, and colorful sex, and variation in circulating levels of testosterone (T) is one key to understanding variation among males within and among species.** Past work on birds has shown that -

1. Bird species vary in patterns of testosterone secretion according to mating system, sexual dimorphism, and parental behavior (Wingfield et al., 1990; Wingfield et al., 2000).
2. Certain sexually selected or fitness-related traits co-vary with plasma testosterone at the level of the individual, e.g., individuals with naturally higher circulating levels of testosterone sing more (Johnsen,

1998), produce larger ornaments (Zuk et al., 1995), and possess stronger immune responses (Duffy et al., 2000).

3. Experimental elevation of testosterone in free-living birds has demonstrated that T-levels affect sexual selection (the balance between mating effort and parental effort)(Hegner and Wingfield, 1987; Ketterson and Nolan, 1999; Ketterson et al., 2001; Wingfield, 1984), and natural selection (the balance between reproductive effort and survival)(e.g. Folstad and Karter, 1992; as summarized in Ketterson and Nolan, 1999).
4. Comparisons of artificially selected lines demonstrate that selection on one testosterone-mediated, sexually selected trait can lead to positive and negative correlated responses to selection in other traits (e.g., comb and cloacal gland size in quail (Sefton and Siegel, 1975), comb and immunocompetence in chickens (Verhulst et al., 1999)).

**Far less is known about T in female birds.** Female birds also vary in seasonal secretion of testosterone and expression of male-like traits (Gowaty, 1996; Gowaty, 1997; Langmore, 1998; Sandell, 1998; Sandell and Smith, 1996; Sandell and Smith, 1997; Tella et al., 1997; Wingfield, 1994), but the significance of circulating T in female birds is still unclear. In a preliminary comparative analysis of sex differences in testosterone in socially monogamous species Wingfield and colleagues (Wingfield et al., 2000) concluded that sex differences in T are proportional to the degree of sexual dimorphism (behavior, body size and body size). Sandell et al. (in prep.) also compared seasonal maximum values of T in males and females (Fig. 3). The phylogenetic analysis is incomplete, but the scatter plot suggests two kinds of relationships. In one pattern, male and female T co-vary, in the other the sexes are independent (regardless of how high male T is, female T is low). Clearly the relationship between male and female T varies among bird species and future studies will reveal whether the differences are related to dimorphism.



**How independent is the evolution of the sexes?**— This paragraph addresses three evolutionary scenarios to describe co-evolution of the sexes in hormone-mediated traits. The first ‘evolutionary constraint owing to correlated selection and antagonistic pleiotropy,’ notes that females tend to prefer T-mediated traits in males, as revealed by studies of natural (Alatalo et al., 1996) and experimentally induced variation in testosterone (Wingfield, 1984) (Enstrom et al., 1997; Fusani et al., 1997; Hill et al., 1999; Raouf et al., 1997; Zuk et al., 1995). In the presence of genetic (hormonal) correlations between the sexes, sexual selection on males would engender a correlated response in females (Holland and Rice, 1998; Holland and Rice, 1999). If the response in females were detrimental to them – e.g., if selection were to masculinize females rendering them less attractive to males - further evolution of sexual dimorphism would be slowed (Lande and Arnold, 1983)(Price, 1993; Bakker, 1999; Chippindale et al., 2001; Price and Burley, 1994). The second proposes that if T-mediated traits are regulated in the same way mechanistically in males and females and are beneficial to both, then selection on one sex could reinforce adaptation in the other (‘evolutionary reinforcement through correlated selection and concordant pleiotropy’) Finally, selection could favor sexual independence via the evolution of sex-limited expression that may be organism-wide or tissue-specific in nature (‘evolutionary independence owing to correlated selection having given rise to epigenetic isolating mechanisms’). Independent evolution of hormone-mediated traits could occur when target tissues are hormone-responsive in one sex and not the other (e.g., organization during early development leads to sex-limited expression, see above).

## RESEARCH OBJECTIVES, STRATEGIES, AND PREDICTIONS

**Overall research objective.**—The research described in this proposal addresses the ultimate and proximate bases of sex differences in a weakly dimorphic songbird, the dark-eyed junco. It will document natural variation in T in response to environmental cues and employ hormonal manipulations to reveal the degree of hormonal correlation between the sexes and to simulate selection. Rather than select artificially for sexual divergence or similarity (Delph, 1999) or use selection experiments to detect possible intersexual conflict (Rice, 1996), we propose to elevate hormone concentrations experimentally, document the consequences, and relate these to fitness. Finally, we will assess the potential for correlated selection by measuring genetic correlations between the sexes.

- **Objective #1:** to determine whether or not T mediates traits in females that it mediates in males. We will compare hormone profiles in the sexes and assess how females respond to stimuli known to increase T in males. Sex similarities will suggest a hormonal correlation in stimulus control of behavior, while sex differences will not. A hormonal correlation would allow correlated selection, while the absence of one would predict independent evolution.
- **Objective #2:** to experimentally alter testosterone in females, determine traits that respond to altered testosterone, and compare results to previous findings from males. The extent of resemblance between the sexes in mechanism will predict the potential for correlated selection: greater resemblance makes a correlated response to selection possible, the absence of a resemblance makes a correlated response to selection less likely.
- **Objective #3:** to determine whether traits that are T-mediated in females are beneficial, neutral or detrimental when experimentally enhanced. If the sexes constrain one another, then alterations that are beneficial in one sex should be detrimental in the other. If the sexes reinforce one another, alterations may be beneficial in both sexes. If the sexes are independent, then alterations already known to be beneficial or detrimental in males may have no fitness consequences in females.
- **Objective #4:** to compare how degree of relatedness affects a sexually selected trait and two hormone-related traits. We will employ long-term data to compare similarity in related and unrelated individuals in a plumage trait and we will collect new data comparing siblings for testosterone and corticosterone. In the presence of a genetic correlation, we would expect the sexes to evolve inter-dependently; in the absence of one, we would predict independence.

## RELATION TO PRESENT STATE OF KNOWLEDGE IN THE FIELD

**T and natural selection in male songbirds.**— Time and energy diverted to reproduction from self-maintenance may shorten life, and seasonal elevation in testosterone may be a proximate mechanism underlying this trade-off (Casto et al., 2001; Deviche et al., 2001b; Folstad and Karter, 1992; Ligon et al., 1990; Verhulst et al., 1999; Zuk, 1996), but see (Hasselquist et al., 1999; Hillgarth and Wingfield, 1997; Owens and Short, 1995; Peters, 2000). Current thinking indicates that when testosterone plays this role, it may act indirectly by elevating corticosterone, i.e., it is corticosterone that is immunosuppressive but it and T are linked (Casto et al., 200x; Casto et al., 2001; Evans et al., 2000).

**T and sexual selection in male and female songbirds.**— Arnold (1994) and Arnold and Duvall (1994) define sexual selection as the statistical dependence of reproductive success on mating success, and they show that sexual selection is stronger in males of polygynous than monogamous species, and, within monogamous bird species, that sexual selection is stronger in males than females, owing to extra-pair fertilizations (e.g., Webster et al., 1995). In dark-eyed juncos, males with experimentally elevated T are more successful at extra-pair fertilizations (Raouf et al., 1997), but a comparison of males and females (when males were not treated with T) showed that reproductive success increased with mating success in both sexes (Ketterson et al., 1998). It is not clear why females that mate with more males produce more



offspring. Perhaps they are naturally more fecund (i.e., would produce more offspring with or without extra mates), which makes them more attractive. One objective of this proposal (see Objectives #1 and #2) is to determine attributes of females that contribute to mating success (number of mates) and whether they are androgen-dependent.

**T and correlated selection in songbirds.**— Most studies contrasting sexual and natural selection on the sexes with correlated selection have focused on plumage traits or other traits of the integument. In general, such studies have assumed that expression of male-like traits by females is the result of correlated selection, and researchers have examined whether such traits are beneficial, neutral, or detrimental to females (Amundsen et al., 1997; Hill, 1993; Cuervo, 1996; Muma and Weatherhead, 1989; Potti and Merino, 1996; Rohde et al., 1999). In some cases females benefit from being more male-like (Ruusila et al., 2001), in others masculine traits are costly (Price and Burley, 1994) or neutral (Wolf et al., 200x). Amundsen (2000) concludes that female ornaments are frequently acted upon directly by sexual selection (mate choice). The connection between testosterone and plumage traits is in dispute. Some studies indicate that T influences plumage-related traits (Eens et al., 2000; Evans et al., 2000; Stoehr and Hill, 2001); others indicate that it does not (Kimball and Ligon, 1999; Owens and Short, 1995). Much remains to be learned in this area.

**Proximate considerations: androgens in females.**— There are two issues of particular relevance: organization/activation and the distinction between the circulating form of a hormone and the active form it takes at its target. Much of what is known of T in females has been learned by studying early development (organization) of sexually differentiated traits that appear in one sex but not the other, e.g. mounting in quail (Astiningsih and Rogers, 1996; Henry and Burke, 1999) (for summary see Balthazart and Ball, 1996). At issue here, however, is whether testosterone elicits male-like behavior in adult females, and numerous studies suggest that it does (Adkins-Regan, 1999; Hausberger et al., 1996; Kern and King, 1972; Nottebohm, 1980; Rudd et al., 1996; Stacey and Kobayaski, 1996); but see (Deviche and Gullledge, 2000). Regarding circulating vs. active forms of a hormone, when aggression and sexual behavior are studied in females, available evidence suggests that hormones other than T (e.g., estradiol, AVT) initiate a behavioral response from target tissues (Maney et al., 1997; Woodley and Moore, 1999), but recalling that the emphasis in this proposal is on behaviors elicited by *circulating* T in adult females, there is also evidence to suggest a role for testosterone (e.g. Myerson et al., 1973; Woodley and Moore, 1999) but see (Elekonich and Wingfield, 2000). Again much remains to be learned.

**Inheritance of T-mediated traits, response to selection.**— Most efforts to determine whether hormonal traits are heritable have been made in agricultural settings or in studies of humans or rats. In beef cattle, testis size and elevation of T in response to stimulation by gonadotropin releasing hormone (GnRH) is heritable (0.52) (Davis, 1993), and in boars, selection on plasma T leads to a correlated response in litter size (Robinson et al., 1994). In human females, plasma T is heritable (Harris et al., 1998), but not highly concordant in male and female dizygotic twins (Harris et al., 1998). In birds, recall earlier references to correlated responses in crowing and cloacal gland size in quail in lines selected for copulation frequency and in immunocompetence in chickens selected for comb size (Sefton and Siegel, 1975; Verhulst et al., 1999). Also in Japanese quail, female dominance rank is “highly heritable” (Nol et al., 1996) but whether that reflects androgen-mediation is not known. The state of knowledge regarding inheritance of hormone-mediated traits in natural populations of birds is rudimentary.

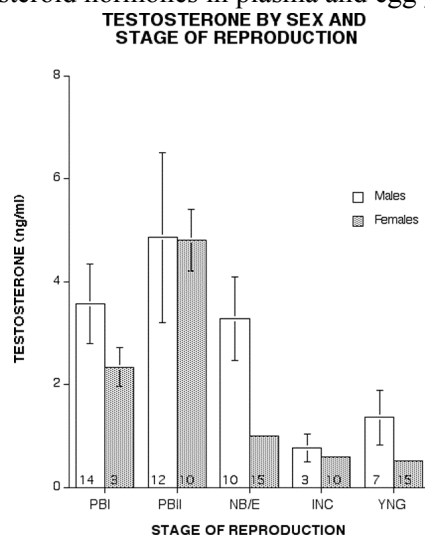
## **BASIC METHODS AND PRELIMINARY FINDINGS**

**Study species.**—The dark-eyed junco is a weakly dimorphic bird species in which males have been well studied (e.g., Deviche et al., 2001b; Deviche et al., 2000; Nolan et al., 200x), but females have received less attention. Juncos differ quantitatively in body size, tendency to migrate, and amount of white in the

outer tail feathers (tail white, which enhances male attractiveness)(Hill et al., 1999). They differ qualitatively in vocal behavior (only males typically sing) and parental behavior (only females incubate). The species is abundant throughout North America and *Junco hyemalis carolinensis* breeds in the Appalachian Mountains of eastern North America where it is an altitudinal migrant (Ketterson et al., 1991b). Carolina juncos are territorial during breeding and form socially monogamous bonds while frequently producing young via extra-pair fertilizations (varies annually, ~35%)(Ketterson et al., 1998; Raouf et al., 1997). Females build the nest and incubate eggs; both males and females care for nestlings and fledglings (e.g., Wolf et al., 1988; Wolf et al., 1990). Pairs can produce two, even three broods per season, but rarely have the opportunity because nest predation is common in most years. Fidelity to previous year's breeding sites is nearly complete among males, less so among females. Offspring of both sexes also exhibit site fidelity, although the proportion returning (~12%) is less than adults (Ketterson et al., 1991b). Each year the study area supports individuals of varying degrees of relatedness (grandparents, parents, sibs, half-sibs, etc.).

**Study sites.**—When studying juncos in the wild, we work at Mountain Lake Biological Station (University of Virginia), near Pembroke VA. When studying juncos as captives, we house them either at the biological station in Virginia or in Indiana at a temperature- and day-length-regulated 11-room indoor aviary (2400 sq. ft) or an outdoor aviary (1920 sq. ft.) that can be divided into 30 breeding compartments or serve as a single enclosed space in which small populations of juncos can live semi-naturally. Juncos thrive in captivity as evidenced by high survivorship and excellent physical condition. When held indoors they readily initiate reproduction and exhibit normal behavior during courtship, nest building, egg-laying and incubation. They are less reliable when it comes to rearing nestlings (50%).

**Determining circulating levels of plasma and yolk hormones.**— To characterize concentrations of steroid hormones in plasma and egg yolk [progesterone (P), testosterone (T), di-hydrotestosterone (DHT),



estradiol (E2), and corticosterone (CORT)], we use tritium-based radio-immunoassays (RIAs) as introduced by Wingfield and Farner (Wingfield and Farner, 1975) (see also Ketterson et al., 1991a; Lipar and Ketterson, 1998; Schoech et al., 1999). With help from M. Hau (Princeton) and T Van't Hoff (Wright State), we (J. Casto) have adapted the T assay to lower concentrations of T through use of a more specific antibody (Endocrine Sciences T3-125). For DHEA, we use a modified version of the Wingfield protocol ("double water trap," no ethylene glycol) as recently described by Soma and Wingfield (2001).

**Sex differences in plasma T levels in vary seasonally and levels in females can be high.**— A comparison of the seasonal profile of plasma testosterone in free-living male and female juncos shows that the sexes resemble one another (see Fig.4)(Ketterson et al., 200x). Based on 101 blood samples collected during one breeding

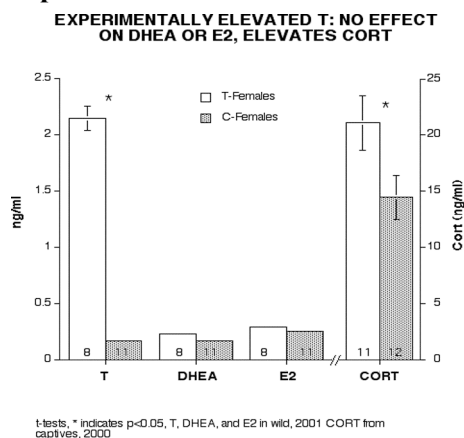
season and classified according to 5 stages of reproduction, (1) pre-breeding I (< 1 April), (2) pre-breeding II (> 1 April, individuals not known to be breeding) (3) females building nests or laying eggs and males presumably mate-guarding, (4) females incubating, and (5) males and females tending nestlings, we see that T was highest during stage (2) and prior to breeding in both sexes. Later it declined, faster in females than in males, and rebounded slightly during the nestling stage in males only. Nest-building/egg-laying is the only stage of reproduction in which T differed significantly between the sexes. Maximum values observed in individual females and males during each stage of reproduction were >10ng/ml (data not shown) and also did not differ according to sex (Ketterson et al., 2001). The high levels of T observed in

female juncos are unusual.

**Manipulating T in females.**— To prolong the natural early-season peak values of T in females, we will place silastic implants (1.5 mm i.d., 2.0 mm o.d.) subcutaneously along the flank, but employ smaller doses than previously used with males (one 7-mm implant packed with 5 mm T, as opposed to two 12-mm implants in males). Whether working in the field or with captives, we will assign treatment at random, blocking by age and, where relevant, keeping treatment constant in females that return in successive years. After implanting, we will recapture females with minimal disturbance, verify that implants are in place, and obtain plasma samples for RIAs. Implants will be removed at the end of the breeding season. We can readily implant and follow 50 females per breeding season.

**Silastic implants elevate T to natural early season peak levels**—To explore how female juncos respond hormonally and behaviorally to elevated T, we conducted 3 preliminary experiments, 2 with captives and 1 in the field. T-implants (again at 1/4 the dose given to males) induced levels of circulating T that closely resembled field-collected, early season, natural peak levels of 4.81 ng/ml (s.e. = 1.90, n = 10)(see Fig.4 above). The induced levels were 5.5 ng/ml (s.e. =3.17, n=3), 3.1 ng/ml (+/-1.66, n=13), and 2.1 ng/ml (s.e. =0.459, n=11) (McGlothlin et al., 200x; Parker-Renga et al., 200x)(Ketterson et al. unpubl.) for an average of 3.6 ng/ml. The field-collected sample (2.1 ng/ml) was obtained late in the season and represents a subset of 32 females implanted this past summer (2001) at Mountain Lake Biological Station (13 T and 16 C) as part of a preliminary study. Twelve of these (5 T and 7 C) are known to have built nests and incubated eggs after they were implanted. All observations to date indicate that females accept T-implants, induce physiological levels, and do not interfere with reproduction.

**Experimental enhancement of T has no effect on DHEA or E2, elevates CORT.** — Ten of the field-



implanted females given 5 mm implants of T were compared for plasma levels of DHEA and E2, and we found no effect (Fig. 5), which suggests that any phenotypic consequences of T-implants are not likely to represent conversion (at the level of the circulating signal) to estradiol or DHEA. T-implants administered to 13 females studied as captives significantly elevated corticosterone (CORT)(T-females = 21.08 ng/ml, s.e. =2.43; C-females 14.45 ng/ml, s.e. =1.99, F=9.486, df=1, 46 p=0.004) (McGlothlin et al., 200x). CORT in males responds in the same way to T-implants (Casto et al., 2001; Klukowski et al., 1997; Schoech et al., 1998) and may be reflect elevation in corticosteroid binding globulin (CBG) (Deviche et al., 2001a) and relate to T's suppressive effect on immune function in males

(Casto et al., 2001; Evans et al., 2000). We shall examine whether T and its effect on CORT are also immunosuppressive in females (see experiment proposed below).

**Other methods.**—Our group has used all the methods proposed for the experiments described below successfully. They include bleeding nestlings and adults for hormones and DNA, performing steroid RIAs on plasma (Schoech et al., 1998) and egg yolks (Casto et al., 1999; Lipar and Ketterson, 1998), measuring immune function (cell-mediated and humoral measures)(Casto et al., 2001), assessing adrenocortical response to handling (Schoech et al., 1999), video taping at nests (Clotfelter et al., 200x-b), using a reflectometer (Ocean Optics) and software (NIH image) to quantify variation in feather color (Casto et al. in prep.), assessing attractiveness in mate choice trials (Enstrom et al., 1997; Hill et al., 1999; McGlothlin et al., 200x; Parker-Renga et al., 200x), conducting computer analysis of avian vocalizations (Canary)(Kennedy et al., 200x), and monitoring nests and egg-nestling condition (egg size/body

mass/linear dimensions), as well as documenting nest success, year-to-year site fidelity of young and adults, and mate fidelity (Reed et al., 200x). Since 1990, we have routinely collected blood samples for paternity analysis (first mini-satellites, more recently microsatellites, work performed in collaboration with P. G. Parker).

**In sum, preliminary data reveal** that males and females resemble one another naturally in their testosterone profiles except when females are building nests and laying eggs and males are presumably mate-guarding. Manipulations of T induce levels seen naturally in early spring. Females are not harmed by implants and the implants do not elevate circulating levels of estradiol, although they do elevate corticosterone. The methods we propose to use in this research are known to work.

## **EXPERIMENTS PLANNED**

### **Objective #1: Natural variation in T**

**Natural co-variation between T and behavior/stage of reproduction**—If high levels of T in females during the early spring (Fig.4) relate to resource competition (natural selection) prior to territory establishment, they should be higher in unmated as opposed to mated individuals. If they relate to reproductive competition with other females over mates or territories (sexual selection) or attractiveness to multiple males (sexual selection), they should be highest during a female's fertile period. If T relates to resource competition while rearing offspring, when resource demands are presumably greatest (natural selection), it should be highest at that time. To distinguish among these possibilities, we will use focal individual sampling to relate the behavior/status of individual females (Neudorf et al., 200x) to their hormone concentrations on the day when blood samples are collected. We will collect a minimum of 15 samples from males and females prior to and during pair formation, during nest-building, and during the rearing of young. Birds will be caught at baited traps or nets or while flying to or from their nests and bled within five minutes of capture. We will analyze variation in T in relation to behavior by repeated measures ANOVA including co-variables known to be important (e.g., date, handling time).

**Natural variation in T in relation to EPFs**—Female juncos that mate with multiple males have higher reproductive success than females that are genetically monogamous (Ketterson et al., 1998), evidence that sexual selection acts on female juncos (Arnold, 1994; Arnold and Duvall, 1994). To determine whether T or T-mediated traits are favored by sexual selection, we will monitor female reproductive attempts and collect blood from offspring and mates to determine relatedness and ask whether females that ultimately produce young sired by EPFs have higher T during the fertile period than females that do not. A positive result would be consistent with a role for T in affecting traits favored by sexual selection. T will be compared according to stage of reproduction (before and after pair formation) and presence/absence of EPFs using ANOVA.

**Response to intruders/predators.**—Male juncos defend territories against conspecifics and protect offspring against nest predators. Females defend the nest site against intruding males (pers. obs.) and defend nestlings against predators even more vigorously than males (Cawthorn et al., 1998). To determine whether similar situations induce similar alterations in T in males and females (stimulus control), we will compare male and female responses to the presence of a conspecific male, female, or predator. An experimental trial will begin with the capture of the mate not under study on that day. We will place a caged conspecific of the same or opposite sex (or a caged nest predator, chipmunk) within 1 m of a focal pair's nest at 2 stages of reproduction, nest-building and when young are present. Controls will be presented with a cage containing a similar-sized bird of another species, a female indigo bunting. We will observe behavior for 30 min prior to the introduction and for 20 min while the stimulus is present, then

capture the focal bird, and collect a plasma sample. A difference in the response to the control and experimental stimuli will demonstrate the salience of the stimulus. A similar hormonal response by males and females to the same stimulus in the same context will be evidence of a hormonal correlation in stimulus sensitivity and would be consistent with a prediction that selection on one sex could be expected to elicit a response in the other. Different responses from the sexes will provide evidence of independence.

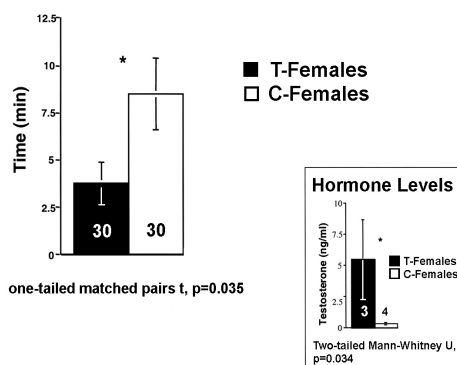
Parallel sets of experiments will be conducted on captive males and females during the breeding (long-day) and non-breeding (short-day) season in Indiana to assess the impact of a number of social contexts on androgen response, e.g., the effect of being housed with and without the opposite sex, held at high and low densities, or being of high or low status. Space does not allow us to provide detail but the objective will be the same. Do females respond like males to a stimulus (social situation) and does the answer vary depending on whether the context relates to natural selection or reproductive competition?

## Objective #2: Documenting phenotypic effects of experimentally elevated T

This set of experiments will simulate selection through altered T and document effects on female

attractiveness, social status, parental behavior, and immune status in order to assess how natural and sexual selection might act on T-mediated traits in females and whether correlated selection would be expected to give rise to antagonistic or reinforcing effects on fitness.

**Time Spent with T- and C-Females**



### Does experimental enhancement of T affect female attractiveness (sexual selection)?

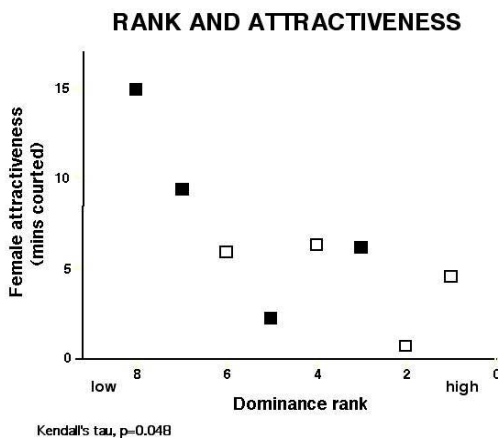
—T enhances attractiveness in male juncos, and the purpose of this experiment is to determine whether it also enhances attractiveness in females, has no effect, or reduces attractiveness. To test these alternatives, we will give males a choice between dyads of females, one member of which has experimentally elevated T (T-female) and one a control (C-female)(implants as already described).

Female dyads will be matched for age, body size, and appearance and presented to males using the protocol of Enstrom et al. (1997)(observe females in absence of males for 30min, introduce male and observe female behavior for 20 min, release male into choice apparatus and record his behavior and amount of time he spends courting each female). Direct observation and videotapes will reveal differences in attendance time (time spent with T- or C-female) and female and male behavior.

In preliminary trials based on 30 males choosing between 5 sets of females (each male saw one of 5 possible female dyads constructed from 8 females and matched for age and body size), we found that males tended to direct more courtship towards C- than T-females (matched pairs, t-test,  $p=0.07$ , see Fig. 6)(Parker-Renga et al., 200x). These trials were based on too few females to be conclusive or to properly quantify the effect of T-implants on female behavior, but preliminary observations indicated that T-females tended to be more male-like in their behavior: they consumed more food, were more active, and some sang (ordinarily only males sing) (Parker-Renga et al., 200x). If T suppresses female attractiveness, we will conclude that enhanced T in females would be deleterious with respect to this component fitness. If T has no effect, we will conclude that male attractiveness is not constrained by consequences that correlated selection might have for female attractiveness. If T enhances female attractiveness, we will conclude that there is potential for reinforcement.

**Female social status, does attractiveness trade-off with priority of access to resources?**— An extremely interesting outcome of this preliminary experiment (Parker-Renga et al., 200x) was the finding that females that were more attractive to males achieved lower social status when housed as a group with other females, while females that were unattractive were dominant (Fig. 7). The finding was based on only 8 females for which we correlated the amount of courtship time they received in the mate choice trials with the status they achieved when housed together after the mate choice trials were complete. The more attractive the female, the lower the status she achieved. In addition females treated with T appeared to be of higher status, though there were exceptions. Obviously this is based on a sample of 1 (1 flock), and we propose to test this further.

We will present a male with a dyad of two females and quantify the amount of courtship he directs toward



each member of the dyad. We will then introduce the females into a neutral arena and determine which is dominant. The females will not have had prior social encounters with one another. We will sample the females for hormones as we complete the trials. We will assess members of 25 dyads of females as preferred/non-preferred by males and dominant/subordinate. A test of independence will reveal whether attractiveness and status are related. We will also compare mean minutes courted for dominant and subordinate individuals. An inverse relationship will suggest a within-sex trade-off between traits related to natural and sexual selection.

**Does experimentally elevated T suppress parental behavior in females as it does in males (natural selection)?**—In the field we will observe behavior at the nests of C- and T-females during incubation and after hatching. Behavior will be videotaped in 2.5 hr sessions, one each in the morning and afternoon, at 3 regularly spaced occasions (Ketterson et al., 1992; Schoech et al., 1999)(Clotfelter et al., 200x-a). Tapes will be scored for time spent incubating (total time, length of bouts on the nest and off the nest) or feeding offspring (feedings/nestling/hr). At both stages we will compare treatments (repeated measures ANOVA after appropriate transformations). *Preliminary data* from videotapes, summer 2001 (4 T-females and 8 C-females), showed no difference in proportion of time T- and C-females spent on the nest, duration of bouts off of the nest, or rates of egg- turning. If T reduces incubation and feeding of nestlings, we expect it to reduce female reproductive success, but if T differs in its effect on parental behavior in females and males, we may find no effect. Sex -independence in this regard would suggest androgen-insensitivity in females in this behavior and reveal limits to correlative selection.

**Does experimentally elevated T alter female physiology in ways that might affect life span (natural selection)?**—Preliminary data indicate that T-implants elevate CORT in females as they do in males (see above\McGlothlin et al. 200x). To determine whether the implants also induce an increase in CBG as they do in males (Breuner and Orchinik, 2001; Klukowski et al., 1997) and a steeper rise in CORT with handling (compare males, Klukowski et al., 1997; Schoech et al., 1999), we will capture females at the nest and compare levels of CORT and CBG immediately after capture and at 15-min intervals thereafter for 1 hr (i.e., assess the “stress response”). Field tests will be preceded by validation in the laboratory in collaboration with Creagh Breuner (see letter of support). Similarly, we will compare female treatment groups for immune function, using the three tests we have already applied to males (Casto et al., 2001) (Casto et al., 200x). T- and C-females will be captured at the nest mid-way through the nestling period and tested for cell-mediated immunity by injecting a plant protein, PHA and measuring subsequent swelling of

the wing web (24 hrs later)(procedures modified from Lochmiller et al., 1993). Females will also be injected IP with sheep red blood cells (SRBC), a test of humoral immunity (IgM) ((procedures of Hay and Hudson, 1989; Ros et al., 1997) and re-captured 6 days later when the young leave the nest and bled again. As a second (or alternative) test of humoral immunity, we will measure (IgG), antibody production after injection with KLH (Casto et al., 200x; Duffy et al., 2000) in order to determine whether T has the same immunosuppressive effects in females that it has in males.

### **Objective #3: Relating effects of experimentally prolonged peak testosterone to measures of female fitness**

**Fitness measures, field.**—We will compare T- and C-females for performance in the field on various measures of fitness, including number and quality of young produced and proportion of young sired by extra-pair males (Ketterson et al., 1998; Raouf et al., 1997)(Ketterson, Nolan, Arguedas, Monk, and Parker, in prep.)(see letter of support from PG Parker, dated 1 July, but confirmed in January 2002). If T interferes with parental behavior and males do not compensate, then offspring production should be lower in T- than C-females. If T enhances sexual behavior, EPFs may be more frequent among T-females, if it reduces attractiveness, EPFs may be more frequent among C-females. We will also estimate female survivorship in the field by calculating simple return rates (percentage of females nesting in one year that nest in the next), and using the software MARK to estimate survival/failure to disperse in ways that account for inefficiency of detection (Reed et al., 200x). As indicated, we can monitor approximately 50 females per year, each of which will initiate reproduction repeatedly and 50% of which we expect to return.

### **Objective #4: Assessing potential for correlated selection by quantifying similarity among relatives in a sexually selected and a hormone-related trait**

A final goal will be to compare a sexually selected trait and three hormone-related traits in unmanipulated males and females as a function of genetic relatedness (Lynch and Walsh, 1998; Merila and Sheldon, 2001) by asking the most basic question: Do male and female kin resemble one another more than non-kin? Our long-term studies have accumulated measurements of tail-white (a plumage trait that enhances male attractiveness)(Hill et al., 1999) from 472 juncos of *known* degrees of *genetic* relatedness (parent-offspring, full-sibs, half-sibs, uncle-nephew, grandparent-parent-offspring) studied during 6 years. For example in 1993, we know relatedness and tail white of 43 belonging to 17 different pedigrees involving an average of 3.18 individuals (e.g., 20 cases of male parent, female parent and at least one offspring). Graduate student J McGlothlin has begun data analysis, and our colleague Mike Wade has agreed to serve as a consultant. With respect to hormone-mediated traits, the most feasible approach will be to transport fledgling juncos taken from the nest to Indiana and to rear them to adulthood here (something we know we can do successfully). Plasma testosterone would seem the obvious measure, and we will make it, but we have more confidence in the repeatability of three less direct measures, each of which will require perfecting during the first year of the project. We will compare related and unrelated individuals for increase in T following a GnRH challenge [following methods of Schoech (1996)], the strength of the adrenocortical in response to handling (difference between basal level and level induced by 45 min of handling), a trait that we know to be repeatable in males juncos (unpubl. data), and, for females, the level of T in egg yolk (also known to be repeatable across eggs within a clutch)(Lipar et al. 1998, unpubl. data). Sample sizes will be smaller than for tail white, but we can readily rear 100 juncos from 25 families in one year, and, if the initial results are promising, repeat this in a second year.

With respect to analysis, full sibling heritabilities are the most common estimate of additive genetic variance in natural populations of vertebrates, although they are imperfect because the estimate includes

dominance, epistatic, and, most importantly, common-family environmental variance. Maternal and rearing effects are known to be important in birds, so heritability measures obtained in this way are an overestimate. With Wade's help, McGlothlin will calculate (1) full sibling heritability (twice the intraclass correlation of full sibling families, where the intraclass correlation is calculated as the among-family component of variance divided by the sum of the among-family component of variance plus the within-family component of variance, Becker, 1992) and compare it with (2) estimates obtained from restricted maximum likelihood analysis (REML) using the program DFREML that is especially useful with complex pedigrees involving multiple generations and half-sibs generated by extra-pair fertilizations and employs less restrictive assumptions than ANOVA (Lynch and Walsh, 1998).

### Overall interpretation of results

If elevated T acts to increase EPF-related behavior or reduce female attractiveness, we will conclude that it can play a direct role in sexual selection in females and that correlated selection could influence evolution of the male. If it acts to suppress immune function and reduce parental behavior, we will conclude that it can play a direct role in natural selection in females and again conclude that correlated selection could influence evolution of the male. The absence of effects would indicate independent evolution of the sexes for the attributes studied here.

### RESPONSE TO EARLIER REVIEW

An earlier version of this proposal was reviewed in Autumn 2001. The Animal Behavior panel recommended funding, Ecological and Evolutionary Physiology did not. The criticisms were extremely helpful, and we believe that the revised proposal addresses them successfully. Specific responses to criticisms follow:

- (1) **Criticism:** The proposal is based on a simplistic premise because circulating levels are only part of the regulation of any hormone-mediated trait; also important are binding proteins, receptors, metabolic conversions to active form of hormone at target, and interactions with other hormones. **Response:** We agree - not that the premise is simplistic - but that any hormone-mediated trait is incredibly complex when atomized. The focus of this proposal, however, is the integration of the organism and its interactions with other organisms over evolutionary time. The approach is inter-disciplinary, drawing inspiration from evolutionary biology, behavioral ecology, and behavioral endocrinology. The goal is not to determine how any one trait, e.g. aggression, is controlled from stimulus to expression, although we have the greatest respect for work done in that framework. Rather, we have selected one ancient and integrating signal, testosterone, which undoubtedly has its effects directly and indirectly, and we ask how a change in the strength of that signal ramifies in terms of the organism's phenotype and how such a change might be expected influence future evolution under natural, sexual, and correlative selective pressures. Our work is experimental in nature and largely conducted in the field.
- (2) **Criticism:** Females will not respond to testosterone in the way as males do because T acts organizationally, not activationally. **Response:** We have tried to be clearer that our goal is to assess when the sexes are linked in expression and when they are not in order to identify (1) traits are likely to be susceptible to correlated selection and (2) traits that are apparently insulated from such selection. We have now presented preliminary data identifying traits in females that do (e.g., corticosterone, vocal behavior, dominance, activity levels, attractiveness to males) and do not (may not) (e.g., parental behavior), respond to elevated testosterone. A major outcome of the research will be knowledge of which traits are and are not susceptible to activation in adulthood. Those that are not may indeed be organized early in development, which would be highly revealing about the extent of integration of the sexes and form the basis for future research.



- (3) **Criticism:** The implants will induce pharmacological or unnatural levels in females; **Response:** Based on a more complete analysis of the natural seasonal profile in females and additional measurements of the doses induced, we find that (1) the sexes are even more similar than we had realized and (2) that the implants accurately simulate levels exhibited by females in early spring. Further, we have found no detectable effect of implants on circulating estradiol or DHEA, and we did find an effect on CORT.
- (4) **Criticism:** Proposal made too few predictions, provided too little detail on experiments. **Response:** Agreed. We have tried to be far more explicit with respect to predictions and interpretations and have added detail on each experiment. We acknowledge that we are asking for the reviewer's confidence that if we have used a technique properly in the past, we will do so in the future and that we will update our methods as new ones become available (e.g., immune tests).
- (5) **Criticism:** The proposal presumes a genetic correlation between the sexes but there is no direct test. **Response:** Agreed. We now address this issue, we hope not at our peril. If this were easy, it would have been done already. Nevertheless we are excited at the opportunity to make use of our long-term data in a new way and also begin to assess family effects in repeatable, hormone-mediated traits and a sexually selected trait. The junco is not a hole-nesting bird and nest predation is high, so cross fostering is not an option, but even if it were, maternal effects, now known to be important, at least in part because of our own work (Lipar and Ketterson, 2000) could inflate measures of resemblance. Nevertheless site fidelity may permit field-based measures of similarity in the future. In the meantime, the absence of a family effect would be highly informative and variation in the presence of an effect is key to predictions of when to expect correlated selection.

### **BROADER IMPACTS OF THE PROPOSED RESEARCH**

**Enhancement of education/outreach/societal implications.**—Research proposed here would provide opportunities to train future scientists, some of whom will be recruited from Indiana University's REU program in Animal Behavior, which is devoted to members of groups underrepresented in science <http://www.indiana.edu/~animal/academics/reu.html>. It will also enhance the quality of three university classes taught by Ketterson: an undergraduate class in Biology of Birds and two graduate classes, Behavioral Ecology and Professional Ethics for the Bio-behavioral Sciences. Past NSF support has allowed me to bring personal experience to classroom, field and lab exercises, and discussions of ethical issues. My role as Co-Director of the NSF-supported Center for Integrative Study of Animal Behavior has provided the opportunity to promote research in animal behavior. The Center's website (developed by Martha Holder, Ph.D. and Center employee) offers advice on careers, help with homework for K-12 students, and information on research opportunities for graduate students. The site has had >250,000 visits in past 12 months, and past research by our group is described at <http://www.indiana.edu/~animal/research/ketterson.html>. Potential societal implications of research that would be supported by this proposal include (1) impact of endocrine disrupting chemicals in the environment, (2) improved methods for breeding captive songbirds for conservation, and (3) a greater understanding of the relationship between sex and gender.