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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).

COVER SHEET FOR PROPOSALS TO THE NATIONAL SCIENCE FOUNDATION

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Title Of Proposed Project

Hormones and avian life histories: exploring the mechanistic and functional bases of fecundity trade-offs in a male bird

Requested Amount	Proposed Duration (1-60 Months)	Requested Starting Date
$ 330,199	36 months	15 July 1994

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- ☒ Vertebrate Animals
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Biology	Department of Biology Indiana University Bloomington, IN 47405
PI/PD Fax Number 812-855-6705	

Names (Typed)	Social Security No.*	Highest Degree, Yr	Telephone Number	Electronic Mail Address
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TITLE

"Hormones and life histories: Exploring the Mechanistic and Functional Bases of Fecundity Trade-offs in a Male Bird"

I. PROJECT SUMMARY

Our primary objectives are to explore *in nature* the mechanistic bases for trade-offs in life-histories, as well as the evolutionary significance of phenotypic variation. We treat free-living male dark-eyed juncos (*Junco hyemalis*) with slow-release implants of testosterone (T) that maintain naturally occurring spring maximum levels of T for the entire breeding season. Controls receive empty implants. We then observe behavior, take physiological measurements, and monitor reproductive success and survival rates. We ask whether fitness of our experimental phenotype (T-males) is greater than, less than, or equal to that of controls (C-males).

We call this technique 'phenotypic engineering' and suggest that the engineering of hormonal phenotypes is a very useful way to determine when and how natural selection acts at the level of the organism. This is because hormones and their target tissues form an integrated whole. As the hormone increases or decreases, often in response to environmental stimulation, various targets are affected and traits appear and disappear as suites. Hence experimental treatment with hormones may increase fitness along some measures through its action on one or more traits, while simultaneously decreasing fitness by affecting other measures and other traits. It is onOnly by considering the net effects of treatment on an array of hormone-dependent traits *and* a variety of fitness measures that we can hope to understand what maintains existing distributions of hormonal phenotypes.

Another important feature of phenotypic engineering with hormones is that the phenotypes created, while they may be rare or even absent in nature, are *possible*. That is, they arise in response to our application of a hormone, but they could occur naturally simply by alterations in secretion or receptors. Thus, it is of real interest to know why such phenotypes are not more common, and their rarity provides possible support for three different views of the maintenance of phenotypic variation. 1) If the fitness of an experimental phenotype is greater than that of controls, this suggests that existing phenotypes must be maintained by constraints; otherwise the experimental phenotype should replace the norm. 2) If the control phenotype is more fit than the experimental one, this suggests that optimizing selection maintains the norm. 3) Finally, if there is little difference in the fitness of different phenotypes, this suggests neutrality and soft selection.

In the research described in this proposal we continue our exploration of the phenotypic consequences for male juncos of treatment with testosterone, placing special emphasis on the trade-off between mating effort and parental effort. Traits to be examined include mate-guarding, response to nest predators, and the indirect effect that treatment of the male has on begging behavior of the young. We will also explore temporal variation in fertility by counting sperm and measuring sperm. With respect to fitness, we will complete our effort to document the effect of T on within- and extra-pair matings and compare the frequency of extra-pair matings on our experimental study area with the frequency at control sites where no males are treated.

Our research will also address how testosterone affects the weight males attach to stimuli that ordinarily elicit parental effort as opposed to mating effort, and the potential adaptive importance of flexible (natural) as opposed to fixed (experimental) hormone profiles. We will compare the responses of T-treated and control males to environmental manipulations of brood size and the availability of extra-pair mating partners by measuring differences in mate-guarding, feeding of young, and vocal behavior, and we will relate responses to sperm counts and hormone levels.

Because the trade-offs between mating effort and parental effort underlie the reproductive patterns of many animals, this study will contribute to our basic understanding of the organismal and environmental factors that affect reproductive decision making.

II. TABLE OF CONTENTS

III. PROJECT DESCRIPTION

A. RESULTS FROM PRIOR NSF SUPPORT

1. Title, amount, and dates of prior support

"Hormones and Life Histories: an Integrative Approach," BSR 91-11498 to E.D. Ketterson and V. Nolan Jr., $250,000, July, 1991 - July, 1994.

2. Summary of results

a. Background

At the start of the current grant we knew that male juncos treated with testosterone sing more frequently and feed their offspring less frequently than do controls. We also knew that their plasma corticosterone level rises and that in spring, T-males lose their winter fat reserve earlier than controls do.

However, we had found that few of the direct or indirect correlates of fitness that we measured were significantly influenced by our hormone treatment, correlates that included number and quality (body mass) of offspring, physical condition of the mate (although she worked harder to feed nestlings to compensate for reduced feeding by the hormone-treated males), ability to retain a mate, and annual survival of males and their mates. We had found only one significant treatment difference: If testosterone implants are removed after breeding, males treated with T (T-males) exhibit *higher* overwinter survival than controls (C-males), an unexpected finding that suggested some delayed benefit from elevated T.

Accomplishments of the current funding period include (1) publication of these results (Ketterson et al. 1991 a, Ketterson and Nolan 1992, Ketterson et al. 1992), (2) discovery of previously unknown behavioral and physiological effects of treatment with T, (3) acquisition of additional information regarding the effect of the hormone on correlates of fitness, particularly survival of fledglings and the incidence of extra-pair fertilizations, and (4) establishment of a DNA fingerprinting lab at Indiana University.

b. Phenotypic consequences of treatment with testosterone

With the help of BSR 91-11498, we found that testosterone has the following phenotypic effects, all with potential consequences for fitness. *Molt*: For males whose implants are not removed, prebasic molt is suppressed or prevented (Nolan et al. 1992). Survivors return the next year with badly degraded plumage. *Home range size*: When T-males and C-males are radio-tracked at two stages of reproduction, while their females incubate eggs and when they have nestlings, T-males prove to have larger home ranges (Chandler et al., in press). During incubation, T-males range over areas three times larger than those of C-males (mean minimum convex polygon 6.36 ha for T-males, 1.96 ha for C-males). During both stages, T-males are found farther from their nests than controls (Fig.1 A,B, APPENDIX). *Mate choice*: Captive females show a preference for T-males over C-males in a setting designed to let females choose between them (Enstrom et al., in prep). In 1993, we matched 22 males for age, presented them as paired stimulus sets to 30 females, and videotaped male and female behavior. We are currently analyzing the videotapes and performing

RIAs on blood samples collected from all subjects, but the preference for T-males is clear, based on two a priori criteria, time spent with male and numbers of courtship displays (Fig. 2, APPENDIX). *Response to predators*: In a test of whether the tendency of T-males to roam farther from their nests would result in decreased effectiveness at defending the nest, we found (Cawthorn et al., in prep) in 1993, that T-males are significantly slower than C-males to detect a stuffed chipmunk placed near their nests (4.25 min. vs. 0.92 min, $p=0.05$)(Fig. 3, APPENDIX). Although fewer T-males detected the chipmunk (67% of 15 vs. 94% of 18), T-males, once present, were equally vigorous in defense of the nest, hitting the model 2.6 times per minute vs. 2.0 for C-males (ns). *Relative fertility*: To ascertain whether treatment with T influences fertility, we lavaged cloacas and collected ejaculates from free-living and captive males. We also sectioned testes of T- and C-males (five and four, respectively) to determine relative rates of spermatogenesis (in collaboration with Dale Sengelaub, Associate Professor of Psychology and Program in Neural Science, Indiana University). Results to date are contradictory (Fig. 4 A,B, APPENDIX), and we return to this subject in the PROJECT DESCRIPTION. *Correlated responses in captives*: Studying captive males brought from Virginia to Indiana, we sought to obtain hard-to-measure responses to testosterone by biweekly monitoring of food consumption, body mass, locomotor activity, vocal behavior, hormones, and blood proteins (Cawthorn et al. in prep., Christensen et al. in prep., Titus et al. in prep.). We found treatment differences in song quality (more syllables/unit time in T-males), in pattern of locomotor activity (migratory nocturnal restlessness occurred in C-males but not T-males), in corticosterone (elevated in T-males, as reported for free-living males in Ketterson et al. 1991, Fig. 5 A,B APPENDIX), and in induction of corticosteroid binding protein (CBP)(greater in T-males than controls, Fig. 5C, APPENDIX). We found no differences in sperm counts or food consumption.

c. **Fitness consequences of treatment with testosterone**

During the funding period we continued to monitor apparent reproductive success (apparent because it is without regard to genetic analysis of parentage) and minimum adult survival rates, in order to determine effects of testosterone on fitness. To date, with the exception of nest-predation rate, measures of reproductive success are similar across years. Nevertheless, our additional years of data have given more power to our statistical comparisons. When the years are viewed collectively, T-males produced significantly fewer hatchlings and, consequently, significantly fewer fledglings. However, when we combine across years data on the fates of fledglings during the two weeks after nest-leaving, a notoriously difficult stage during which to measure avian survivorship, the treatment difference disappears. Of 338 fledglings followed, 202 (60%) survived to independence, and fledglings of the T-males were more likely to survive (Ziegenfus et al., in prep). Thus, C-males produce more fledglings, but because these suffer greater mortality during the post-fledging period, the net result of treatment on the survival of young is that reproductive success to independence is nearly identical for the two treatments. These data are presented in more detail in the PROJECT DESCRIPTION (Fig. 6, APPENDIX).

With respect to adult survival, if implants are not removed, T-males are significantly **less** likely than C-males return to breed the following spring (Nolan et al. 1992). When implants are removed in late summer or autumn, T-males have significantly lower hematocrit levels than C-males but are nevertheless significantly **more** likely than C-males to survive. This is a result we still cannot explain, but the data suggest that testosterone can compromise adult survival when the birds are exposed to the hormone for a very long time (i.e., into winter) or at an 'inappropriate' time of the year (Nolan et al. 1992). When T is present for shorter periods of time, survival may actually be enhanced.

Finally, and perhaps most importantly, we have devoted great effort to determining the effect of the hormone on the frequency of extra-pair fertilizations, as measured by DNA fingerprinting (Parker et al., manuscript). The paternity analysis is still in progress on blood collected in 1992 and 1993. Results to date indicate that testosterone may decrease a male's effectiveness at preventing his social mate from being inseminated by other males, but increase his effectiveness at inseminating the mates of other males. Interestingly, as with the non-genetic findings based on correlates of apparent reproductive success, the net result is that the fertilization success of the two treatment groups is nearly identical. We return to these data in the PROJECT DESCRIPTION.

3. **Publications and manuscripts during the granting period, July 1991 - December, 1993.** All publications were written during the granting period, although some are based on data collected before the award.

a. Publications (including three in press)

Ketterson, E.D., Nolan, V. Jr., Wolf, L., Ziegenfus, C. Dufty, A.M. Jr., Ball, G.F. and T.S. Johnsen. 1991 a. Testosterone and avian life histories: the effect of experimentally elevated testosterone on corticosterone and body mass in dark-eyed juncos. *Hormones and Behavior* 25: 489-503.

Ketterson, E.D., Nolan, V. Jr., Ziegenfus, C., and D.P. Cullen, M.C. Cawthorn, and L. Wolf. 1991 b. Non-breeding season attributes of male dark-eyed juncos that acquired breeding territories in their first year. *Proceedings XX International Ornithological Congress*: 1229-1239.

Nolan, V. Jr., Ketterson, E.D., Ziegenfus, C., D.P. Cullen, and C.R. Chandler. 1992. Testosterone and avian life histories: effects of experimentally elevated testosterone on molt and survival in male dark-eyed juncos. *Condor* 94: 364-370.

Ketterson, E.D., and V. Nolan Jr. 1992. Hormones and life histories: an integrative approach. *American Naturalist* 140 (suppl.)S33-S62.

Ketterson, E.D., Nolan, V. Jr., Wolf, L., and C. Ziegenfus. 1992. Testosterone and avian life histories: the effect of experimentally elevated testosterone on behavior and correlates of fitness in the dark-eyed junco. *American Naturalist* 140:980-999.

Rogers, C.M., Nolan, V. Jr., and E.D. Ketterson. 1993. Geographic variation in winter fat of dark-eyed juncos: displacement to a common environment. *Ecology* 74:1183-1190.

Rogers, C.M., Ramenofsky, M., Ketterson, E.D., Nolan, V. Jr., and J.C. Wingfield. 1993. Plasma corticosterone, adrenal mass, winter weather, and season in nonbreeding populations of dark-eyed juncos (*Junco hyemalis hyemalis*). *Auk* 110:279-285.

Chandler, C.R., Ketterson, E.D., Nolan, V. Jr., and C. Ziegenfus. In press. Effects of testosterone on spatial activity in free-ranging male dark-eyed juncos. *Animal Behaviour.*

Rogers, C.M., Nolan, V. Jr., and E.D. Ketterson. In press. Winter fattening in dark-eyed juncos: plasticity and interaction with post-breeding migration. *Oecologia.*

Ketterson, E.D., and V. Nolan Jr. In press. Hormones and life histories: an integrative approach. *In*: *Behavioral Mechanisms in Evolutionary Ecology*, L.A. Real, ed. U. Chicago Press.

b. Manuscripts

Chandler, C.R., Ketterson, E.D., Nolan, V., Jr., and C. Ziegenfus. Cooperation between male and female dark-eyed juncos (*Junco hyemalis*) disrupted by testosterone. Submitted to *Behavioral Ecology*.

Parker, P.G., Ketterson, E.D., Nolan, V., Jr., Raouf, S., and C. Ziegenfus. Testosterone and extra-pair fertilization rates in apparently monogamous dark-eyed juncos. To be submitted to *Evolution*.

Ketterson, E.D., and V. Nolan Jr. Paternal behavior in birds. *Annual Review of Ecology and Systematics*, invited manuscript due February 15, 1994.

4. **Contribution to the development of human resources**: students receiving training with the help of BSR 91-11498, July 1991-December 1993

a. *Post-doctoral students:* **C. Ray Chandler**, 1989-92, now Assistant Professor of Biology, Ball State University; **Michelle Cawthorn**, 1989-92, now Assistant Professor of Biology, Ball State University; **Walter Piper**, 1991-1993, now researcher at the Smithsonian; **David Enstrom**, 1992-present, also Visiting Assistant Professor, Indiana University; **Elaina Tuttle**, 1993-present, post-doctoral fellow supported by NSF Research Training Group in Animal Behavior, Indiana University.

b. *Graduate students*: **Daniel A. Cristol**, completed Ph.D., 1993, now postdoctoral associate of John Krebs, Oxford University; **Torgeir S. Johnsen**, completed Ph.D., 1991, now post-doctoral associate of Marlene Zuk, University of California, Riverside; **Cynthia Ann Wolock Madej**, completed M.A., 1991, now laboratory technician, Indiana University; **Daniel Albrecht**, 1991-1993, now Ph.D. candidate at University of New Mexico; **Alex Buerkle**, Ph.D candidate, 1991-present; **Lori Christensen**, M.A. candidate, 1992-present; **Tracey Kast**, M.A. candidate, 1993-present; **Daniela Monk**, Ph.D. candidate, 1991-present; **Samrrah Raouf**, Ph.D. candidate, 1988-present. **Russell Titus**, Ph.D. candidate, 1990-present.

c. *Undergraduate students*: **Juan Carlos Martinez Sanchez**, Virginia Tech, summer field assistant, 1991, now graduate student at University of Washington; **Kevin Kimber**, Amherst College, summer field assistant, 1991, now research assistant Cornell University; **Holly Graef**, undergraduate research assistant, academic year and summer, 1991-92, now employed in private sector; **Lori Christensen**, undergraduate research assistant, academic year and 1992, 1993 field seasons, received B.S. with honors, Indiana University, now graduate student, Indiana University; **Margaret Chambers**, Lehigh University, 1992 and 1993 field assistant, 1992-93 academic year researcher, now graduate student at University of Alaska; **Michelle Watson**, Queen's University, field assistant, 1992; **Eric Snajdr**, Miami University (Ohio), field assistant 1993 and academic year assistant, 1993-94; **Jennifer Hill**, Purdue University, NSF/REU student and field assistant, 1993, still an undergraduate; **Dana Morris**, University of Missouri, NSF/REU student and field assistant, 1993, still an undergraduate.

B. INTRODUCTION AND RELATION OF PROPOSED WORK TO PRESENT STATE OF KNOWLEDGE

According to Stearns (1989), trade-offs are fitness costs that occur when a beneficial change in one trait is linked to a detrimental change in another. Thus fitness costs that occur when two traits depend upon the same hormone and are co-expressed but only one is beneficial, are costs that may be regarded as trade-offs. In this view, hormones are one of the important mechanistic links between organismal biology and evolutionary ecology.

Students of life history evolution have long been fascinated by trade-offs (see reviews in Dingle and Hegmann 1982, Reznick 1985, Partridge and Harvey 1988, Stearns 1989, Roff 1992), and much effort has been devoted to measuring the cost of reproduction as a basis for a trade-off between survival and reproduction (e.g., DeSteven 1980, Nur 1984). Less effort has been devoted to uncovering the physiological mechanisms that underlie trade-offs, particularly those associated with the endocrine system, although several workers have urged this approach (Stearns 1989, West-Eberhard 1989, Zuk et al. 1990, Ligon et al. 1990, Moore 1991). The dearth of hormonal studies is surprising because hormones produce correlated effects with potentially antagonistic fitness consequences, precisely the situation we seek when attempting to understand and to demonstrate the existence of trade-offs (Williams 1957). It is also surprising because, in terms of levels of organization, hormones lie between genes and life history traits, but hormones are much easier to manipulate than genes. Indeed, investigators have begun to employ hormones in an experimental approach to life history evolution (Fox, 1983, Wingfield, 1984, Marler and Moore 1988a, 1988b, 1989, 1991; Sinervo and Licht 1991 a,b, Sinervo 1993, Glickman et al. 1993).

The endocrine approach would seem particularly profitable when applied to the male's fecundity trade-offs, i.e., mating effort or parental effort, in which fitness may be achieved by increasing the number of females fertilized or increasing paternal care and the likelihood that young survive (Maynard Smith 1977, Clutton-Brock 1991). This is because a single hormone, testosterone (T), appears to play a pivotal role in regulating the balance of mating effort and parental effort (Balthazart 1983, Wingfield et al. 1987, 1990, Ball 1991, Balthezart and Ball 1993).

Testosterone: In birds, testosterone is intimately associated with reproduction. Fundamentally, it mediates copulatory behavior (summarized in Balthezart and Ball 1993), promotes sperm production (Lake 1981, Scanes 1986), enhances courtship vocalizations (Arnold 1982, Wada 1981, 1982, 1986, Harding et al. 1988, Gyger et al. 1988, Nowicki and Ball 1989, Ketterson et al. 1992), and increases aggressiveness (Balthazart, 1983, Wingfield et al. 1987, 1990, Archawaranon and Wiley 1988; Beletsky et al. 1990). However, it suppresses male incubation and feeding of the young (Silverin 1980; Hegner and Wingfield 1987, Oring et al. 1989, Ketterson et al. 1992).

Testosterone also influences survival (Dufty 1989), as indicated by its role in many physiological responses. For example, it increases locomotor activity (Wada 1982, 1986, Masa and Bottoni 1987) and metabolic rate (Hannsler and Prinzinger 1979, Feuerbacher and Prinzinger 1981), and suppresses lipid storage (Wingfield 1984, Ketterson et al. 1991 a) and the onset of postnuptial molt (Runfeldt and Wingfield 1985, Schleussner et al. 1985, Nolan et al. 1992). It has also been implicated in corticosterone metabolism (Ketterson et al. 1991) and in suppression of the immune system (Zuk 1990, Folstad and Karter 1992, but see Weatherhead et al. 1993). Despite the obvious importance of testosterone and survival, for this proposal we have elected to focus on the relationship between T and fecundity trade-offs.

Testosterone and avian mating systems: Wingfield and his associates' pioneering studies of testosterone and avian mating systems (1987, 1990) provide the comparative evidence demonstrating a link between testosterone and interspecific variability in allocation of effort between mating and parental behavior. When seasonal profiles of plasma testosterone of polygynous and monogamous species (as traditionally defined without reference to genetically determined paternity) are compared, males of polygynous species exhibit sustained secretion of T at peak levels for much of the breeding season, whereas monogamous males exhibit a single early peak (Wingfield et al. 1987). Males of monogamous species provide parental care, whereas polygynous species do so much less if at all (Oring 1982, Vehrencamp and Bradbury 1984). The ratio of summer to winter levels of T also correlates with mating system and parental behavior: the greater the seasonal change, the more likely a species is to be polygynous and the less likely it is to provide parental care (Wingfield et al. 1990). However, in monogamous species, an aggressive challenge (e.g., by an intruder) leads to a greater increase in T than in polygynous species (Wingfield et al. 1990), suggesting that polygynous males operate close to their physiological maximum of T for most of the breeding season, while monogamous species maintain lower levels of T and secrete it "on demand." Our study species, the junco, is occasionally polygynous (< 10% of cases), but in general it conforms to the monogamous pattern described in this paragraph (Ketterson and Nolan 1992).

If patterns of secretion of testosterone can account for so much of the variability in avian mating systems, it is obviously of interest to know how readily one pattern of mating effort and parental care can be converted to another. Genetic variation in testosterone-mediated traits has been demonstrated in a number of ways. Aggressiveness, for example, has been shown to be highly heritable (e.g., Moss et al. 1982, Boag 1982, Maxson et al. 1983), and selection on one of a suite of testosterone-dependent traits can lead to correlated responses in other such traits (e.g., mating frequency, aggressiveness, and size of the cloacal gland in the Japanese quail, Sefton and Siegal 1975, Cunningham and Siegal 1978). Phylogenetic studies of the evolution of male parental care in birds have shown that care can take many forms and has evolved and been lost in a variety of lineages (Silver et al. 1985, McKitrick 1992). Thus, within families, males belonging to different genera may differ widely in the kind care provided (e.g., Icterinae).

When we combine these observations of 1) within-species genetic variability for testosterone-related traits and 2) between-lineage lability in patterns of care, and 3) Wingfield et al.'s (1987,1990) link between profiles of T and mating systems, it seems likely to us that the physiology underlying fecundity trade-offs is highly conserved. But we also expect that at the levels of species, populations, and of individuals, male birds are likely -- within limits and on differing time scales -- to be able to match their physiologies to their environments to achieve an adaptive balance of mating and parental effort. This is a proposition that calls for experimental testing.

Experimental manipulations of T: Studies in which plasma testosterone has been experimentally increased have demonstrated that exposure to T during the parental phase shifts male effort away from offspring and towards other forms of reproductive effort (e.g., sexual/aggressive behavior)(Silverin 1980, Watson and Parr, 1981, Wingfield 1984, Hegner and Wingfield 1987, Dufty 1989, Oring et al. 1989, Ketterson and Nolan 1992, Ketterson and Nolan unpublished data, see below). With the exception of our own work, these studies have also shown a decrease in the number or quality of offspring produced when males invest less in parental care than is typical. Manipulative studies have also shown that treatment with T can increase the number of mates acquired by males in some ordinarily monogamous species (Wingfield 1984), and Hegner and Wingfield (1987) speculated that testosterone might increase fecundity by facilitating extra-pair fertilizations.

As indicated (PROJECT SUMMARY), our objectives are to explore the physiological and behavioral bases for fecundity trade-offs, as well as the evolutionary significance of phenotypic variation in nature. We ask what the fitness consequences for dark-eyed juncos are of phenotypes that we create and that deviate from the norm. Results of our study to date are summarized in RESULTS FROM PRIOR NSF SUPPORT, and this proposal describes where we would like to take this work in the future.

Briefly, we suggest that our manipulative studies with hormones continue to provide the opportunity to determine the relative magnitude of both the costs and benefits of redirecting male effort from parental care to mating. To date, no study but ours has attempted to determine the net effect of treatment on both mating and parental behavior and on the fitness consequences of altered investment in mating and parenting. For example, we do not yet know whether testosterone increases or decreases success in fertilizing a male's social mate or the mates of neighbors, and we are not yet sure why socially monogamous species do not maintain testosterone at high levels throughout the breeding season.

We also think that our understanding of how, in a mechanistic sense, the male junco or any other bird pursues a mixed reproductive strategy (Trivers 1972) is woefully incomplete. We are now well aware that extra-pair fertilizations are common in apparently monogamous birds (Westneat et al. 1990, Birkhead and Moller 1992, Moller and Birkhead 1993). One likely expectation is that males have means for assessing the relative likelihood of success via mating as opposed to parenting and that their short-term hormonal and behavioral responses are plastic and adaptive. In circumstances where mating effort is likely to provide returns, e.g., because nearby females are fertile but probably guarded by their social mates, we might expect elevation in T. In other circumstances, e.g., when young are of high reproductive value or are numerous, we might expect suppressed secretion of T and reduced mating effort, despite the availability of fertile females in the neighborhood. However, very little is known about how males weigh stimuli associated with enhanced reproductive opportunities via mating effort and parental effort, how rapidly they can respond to changed circumstances, and what the effect of such stimuli is on the allocation of effort. Thus another goal of this proposal is to determine the physiological and behavioral responses of male juncos to natural circumstances that shift the prospective balance between mating and parental effort on a day-to-day basis.

C. GENERAL AND SPECIFIC OBJECTIVES

Our current goals are two. The first general goal is to complete work begun during the current funding period on (a) the phenotypic effects of testosterone and (b) their relation to relative fertilization success of T-males and controls, both with their social mates and with the mates of other males.

More specifically we propose to finish measuring the following phenotypic effects of treatment with T: (1) home range size of males when their females can be presumed to be fertile, (2) nest defense against predators during incubation, (3) temporal structure and other aspects of male feeding behavior directed towards young and any indirect effect of male treatment on the vocalizations of young, and (4) relative fertility, i.e., treatment-related differences in sperm density and morphology. We will also complete our analysis of the effect of T on extra-pair fertilizations. The components of fecundity that have received our greatest attention are monitoring production of fledglings and their survival through the period of dependence. We also need to determine the relative success of the treatment groups at defending paternity with their social mates and acquiring

paternity through extra-pair fertilizations, and this requires the analysis of more than 500 DNA samples that have already been collected.

Our second goal is study male responses to environmental cues that signal change in potential relative return on mating vs. parental effort. Here we will compare hormonal response of unmanipulated males to our experimental variations of the environment, and we will determine how treatment with T affects responses to the same altered environmental variables.

D. GENERAL PLAN OF WORK

1. The bird, the study area, and the scale of effort; basic methods

The dark-eyed junco is an abundant, socially monogamous, ground-nesting passerine bird in which females build the nest, and males and females help to rear nestlings and fledglings (e.g., Wolf et al 1988, 1990, 1991). Juncos are widely distributed in North America, and we study them at the University of Virginia's Mountain Lake Biological Station near Pembroke, Virginia. Since our work there began in 1983, we have banded over 4500 juncos with individually unique color markings.

Our field season runs from April-August, and each year we implant 80-110 males, treating half with T and half as controls. Each bird receives two 10-mm silastic implants, either packed with testosterone or empty (see Ketterson et al. 1991). Plasma levels of T induced by the hormone mimic the early season maxima of unmanipulated males, and levels remain elevated for the entire breeding season [Ketterson et al. 1991, Ketterson and Nolan 1992, unpublished data, RIAs performed using the methods of Wingfield (Wingfield and Farner 1975, Wingfield et al. 1982, Ketterson et al. 1991)].

Fifty to sixty of these males breed each year on our study area. Other areas are set aside as control study areas on which no males are manipulated. From all adults, we collect blood samples for DNA, and from most we collect plasma for hormones and hormone-binding proteins. We sample DNA from approximately 300 nestlings per year. We also monitor nest success, mate fidelity, and return rates (minimum survival) of males, females, and juveniles.

2. Effect of treatment with T on fitness-related phenotypic traits

a. Behavior

(1) *Relative home range size when females are fertile.* T-males with eggs or nestlings have larger home ranges and wander farther from their nests than C-males (Chandler et al. in press, Fig. 1 A,B, APPEBDIX), which conforms with the view that T promotes mating effort at the expense of parental effort, and can thus be both beneficial and costly. However, to complete this story we need to know whether T-males can adjust this tendency to roam when their mates are fertile. Do T-males guard their mates as effectively, more effectively, or less effectively than C-males?

To answer this question we will use the methods perfected by Chandler et al. (in press) to track 20 males (10 in each treatment group, transmitter from Wildlife Materials, Carbondale, IL) for periods of three to four days at times when their females are building nests and laying eggs. We will locate the males every half-hour, flag their positions, and later plot areas and calculate home ranges. We will focus on the early spring when most females are fertile, but we will also track later in the

season, when fertility will be induced by removing the eggs from the nests of incubating females, causing them to recycle. Nest-building will begin one or two days after removal, and we will track the males from the day following removal until the first egg is laid in the replacement clutch. See Fig. 1 A, B (APPENDIX, From Chandler et al., in press) for results to date, obtained when females were not fertile.

(2) *Response to predators.* To complete study of the effect of T on behavior related to nest defense, we will compare the response to T-males and C-males to stuffed chipmunks placed near the nest at the time that females are incubating (see Fig. 3, RESULTS FROM PRIOR NSF SUPPORT).

As before, a model chipmunk will be covered with camouflage cloth tied to a string. After 30-minutes, during which juncos resume normal behavior, the cloth is removed by pulling the string. One observer films the trial, while another records latency (time before adults arrive), behavior of adults towards the model (number of swoops and hits, closest approach), and total time spent in defense. Trials last for 10 minutes, the maximum duration of a normal predation event. We will perform trials at 10 nests of each treatment group during the incubation stage.

At the end of each trial we propose to capture and bleed the birds to determine their hormonal status (testosterone and corticosterone). We know of no previous efforts to do this. One possible result, of course, is no effect on hormonal profiles. Brief encounters with predators might not engender responses by steroid hormones, which are traditionally thought of as slow-acting. However, recent work on non-genomic actions of steroids may alter this expectation (Orchinik and McEwen 1993, Schumacher 1990). It would, then, be quite interesting to find that either T or corticosterone become elevated in males or females that have just behaved 'aggressively' towards a model predator.

(3) *Response to nestlings*. During the nestling stage, unmanipulated male and female juncos feed young at the same rate (Wolf et al. 1990, Ketterson et al. 1992a), whereas males treated with testosterone feed nestlings less frequently than do either females or control males (Ketterson et al. 1992). Contrary to theory (e.g., Winkler 1988), the mates of T-males increase their rate of feeding until it equals or exceeds that of a C-male and female working together (Ketterson et al. 1992a). Consequently, the growth rate (mass) of the young does not differ according to male treatment (Ketterson et al. 1992 a, unpublished data).

However, beyond these basic observations, we know very little about *how* testosterone disrupts male parental behavior or what leads to compensatory reactions in females. For example, we do not know whether T-males not only come to the nest less often but also come less predictably. We do not know the hormone's effect on the size of individual food items brought to the nest, total size of the load delivered, or allocation of time at the nest when delivering food (e.g., delivering food, removing feces, surveying surroundings). Finally, we do not know how treatment of the male indirectly affects the vocalizations of the young. Do T-males hear similar amounts of begging/visit as C-males but simply respond less strongly, i.e., wait longer before returning with food? We have spectral data (unpublished) showing that begging calls of young are not uniform, but we do not know whether T-and C-males are exposed to begging of similar 'quality'.

From a blind, we propose to observe the nests of 20 males of each treatment when the young are in the last third of nestling life. One of the most important dependent variables will be the intervals between feedings, whose distributions we compare using a Kolmogoroff-Smirnov test. Some behavior can best be studied on film, so we propose to film at nests of 10 males of each treatment,

using a camcorder (SONY handycam TR101). Simultaneously we will record vocalizations with a microphone on the nest rim, approximately 1 cm from the young (Sony WM D6C, professional Walkman and Sennheiser ME20 omnidirectional microphone). We know from experience that juncos tolerate this intrusion and that the equipment produces tapes of high quality (Andrew King, pers. comm.)

We will estimate load size from frames that give a perpendicular view of the adult's head (average junco bill length, 5.7mm). We will analyze vocalizations on a Mac Quadra using Canary 1.1 software, asking whether the young of T-males call longer or more loudly and whether there are differences in the spectral qualities of their vocalizations. Some recording sessions will be of the whole brood, because we are interested in the collective impact of the brood on adult response. In other sessions, we will temporarily remove all but one of the nestlings so as to compare the begging of individual nestlings of T-males and controls. Thus we hope to achieve a greater understanding of how T affects responsiveness to cues that ordinarily elicit male parental care.

b. Physiology

(1) *Fertility*: Recently, there has been an explosion of interest in sperm and sperm competition (e.g., Birkhead et al. 1993, Gomendio and Roldan 1993, Baker and Bellis 1993a, 1993b). Comparative studies have shown larger testes in the males of polygynous species and an inverse relationship between testes size and level of male parental care (Moller 1988, 1989, 1991). Other comparisons of interest are a trade-off between sperm size and sperm number (summarized in Gomendio and Roldan 1993). The cost to females of laying and incubating unfertilized eggs needs no comment, and support for the fertilization-insurance hypothesis to account for multiple mating by female birds is growing. Nevertheless, we know of almost no field studies of temporal variation in sperm density in birds, and of none on the effect of testosterone on sperm.

Because T is necessary to spermatogenesis, there are reasons to think that both natural elevation of T and treatment with T should increase sperm density. However, there is also reasoning that predicts suppression of spermatogenesis by T. Testosterone secretion in nature is self-regulating; as T increases, gonadotropins fall, and vice versa. This is relevant to sperm production because testosterone, while necessary to spermatogenesis, can also suppress secretion of the gonadotropin, follicle stimulating hormone (FSH), which is also necessary to spermatogenesis. To date, administration of exogenous testosterone has been reported to suppress, maintain, promote, or have no effect on avian spermatogenesis (summarized in Lofts and Murton 1973, see also Desjardin and Turek 1977, Hagen and Dzuik 1985).

Our paternity analysis (see below) has shown that T-males sire 67% of young in their nests, as compared to 77% for controls. Although our implants only prolong the maintenance of T at its natural maximum (i.e, do not induce unnaturally high levels), it seems essential to document the effect of our treatment on the junco's spermatogenesis. Furthermore, in the final section of this proposal, we ask whether environmental changes that signal the availability of a fertile female are reflected in sperm counts. The stimuli that trigger secretion of T are diverse, e.g. daylength, aggressive encounters with conspecifics, female sexual behavior (Wingfield and Moore 1988), but possible short-term effects of T on sperm counts have not been documented in birds.

Preliminary data on sperm counts have produced conflicting data. We first lavaged captive T- and C-males using the methods of Quay (1984 a,b 1985, 1986, 1987) and found equal numbers of

sperm, but the values obtained were too variable to inspire confidence (Cawthorn et al. in prep). More recently, our graduate student, Tracey Kast, collected ejaculates by cloacal manipulation of captives and of free-living males with late-stage nestlings, and these suggest that sperm densities are somewhat depressed in T-males. (Fig. 4 B, APPENDIX). However, cell counts from a small number of males whose testes we preserved revealed more sperm in the testes of T-males (Fig. 4 A, APPENDIX).

We propose to resolve these uncertainties and to produce a seasonal profile of the following measures of fertility, according to treatment: ejaculate size, sperm density, and sperm morphology (length of sperm, ratio of head to tail). Males are sampled in the morning to control for time of day. The ejaculate is collected in 5ul capillary tubes and diluted with Minnesota Turkey Extender (Ogasawara and Earnst 1970). The concentration of sperm is determined by a hemacytometer count. Slides are made and stained with Giemsa; the sperm are measured using an ocular micrometer and an Olympus phase contrast microscope. These procedures were learned from Elaina Tuttle, a postdoctoral fellow in our lab who has applied them to white-throated sparrows, and we know that they work well with the junco.

3. Effect of treatment with T on relative fitness

Determination of the effect of T on fitness becomes especially interesting because of the evidence that a major effect of experimentally elevated testosterone is to shift male reproductive effort away from parental care and towards mating effort. The observations that T-males range more widely, sing more, are more attractive to females, and feed their young less often than controls predict lower success for young in the nests of T-male's mates (hereafter apparent reproductive success) but potentially greater success at extra-pair fertilizations.

Our results on apparent reproductive success show no treatment difference in numbers of offspring surviving to independence from parental care, but they do show differences in relative success along the way to independence (Fig. 6, APPENDIX). Briefly, in 141 T-broods and 136 C-broods observed over four years (1989-1992), mean clutch size was very similar (3.49 vs. 3.32 eggs, T-males first in all comparisons, ns); percentage of nests escaping total loss to predation before hatching was slightly higher in T-males (64% vs. 57%, ns); and mean number of eggs hatching was significantly lower (3.06 vs. 3.36 nestlings, $p < 0.03$). The percentage of nests escaping predation during the nestling stage was very similar (64% vs. 68%, ns), but mean number of fledglings from such nests was significantly lower (2.72 vs 3.16, $p < 0.02$). Nevertheless, the mean number of young reaching independence (1.69 vs. 1.61, ns, based on daily censuses during the two weeks following fledging, methods as in Wolf et al. 1989) was almost identical, because the young of T-males were more likely to survive this last stage of parental care. (It is worth noting that most studies of avian reproductive success are based only on number of fledglings. If we had stopped monitoring reproductive success when the young left the nest, our conclusion about the effect of treatment with T on male reproductive success would have been different and incorrect.)

In sum, the net effect of testosterone-induced phenotypic alterations on traditional measures of reproductive success appears to be nil. We find this somewhat surprising result extremely interesting, but it emphasizes the important of a genetic analysis of paternity. To date, in collaboration with Patricia G. Parker (formerly Rabenold) of Ohio State University, we have analyzed DNA from the blood of 46 males (21 T-males and 25 C-males), 43 females, and 194 nestlings belonging to 69 broods.

Methods were standard: Putative fathers and mothers of nestlings were assigned by observing behavior of color-banded adults at the nest. Nestlings were bled when they were 6 days old. The DNA was extracted using proteinase K and phenol, digested with Hae III, run on an agarose gel, and transferred to a nylon membrane, which was probed with Jeffreys' 33.6 and 33.15 (Jeffreys et al. 1985, Rabenold et al. 1990). When young that could not have been sired by the putative father were identified , they were run on gels with potential extra-pair sires that lived in the neighborhood until a match was found.

Results to date are as follows. In 1990, the average number of bands in offspring that were unattributable to either putative parent was 0.2308, whereas the average number of bands on which exclusions were based was 10 (range 5-16). Without regard to treatment, 10 of 46 (22%) males sired young through extra-pair matings, while 17 of 43 (40%) females were fertilized by more than one male. Of the 194 offspring, 53 (27%) were sired by males other than the putative father. All but one excluded young was assignable to a sire, and almost all extra-pair sires were immediate neighbors. All nestlings, with the possible exception of one, were assigned to their putative mothers.

Broken down by treatment, more T-males than C-males lost paternity of at least one nestling (52.% vs. 32%, ns), and more broods of T-males contained at least one young fathered by a neighboring male (45% vs. 26%, ns). The percentage of excluded young was 33% from broods of T-males and 23% from brood of C-males (ns)(Table 1 A, APPENDIX).

The effect of testosterone on **net season-long relative reproductive success** is summarized in Table 1, APPENDIX). In the years 1990 and 1991, T-males had lower apparent reproductive success (3.76 vs. 4.60 per male, ns), greater gains arising from extra-pair fertilizations (nestlings sired in other nests, 1.0 vs. 0.44, ns), and slightly higher losses to extra-pair fertilizations (excluded nestlings in own nest, 1.08 vs. 1.24). The net effect was slightly lower success by T-males (3.52 vs. 3.96, ns)(Table 1B, APPENDIX). Recall that DNA analysis of young is based on those that reached age 6 days.

Although the differences just presented are not significant statistically, they fit so cleanly with our observations of behavioral differences (less parental care by T-males, more time spent away from the nest, greater attractiveness to females), that we think it essential to devote added effort to this approach. We also note that this is one of the few studies to determine the net effect of extra-pair fertilizations on male reproductive success, and the first to do it in the context of hormonally engineered phenotypes. To determine how much more effort is required, we computed a) the power of our current comparisons and b) the sample sizes that would be necessary to detect differences at the 0.05 level 75% of the time (Cohen 1988). Taking the three basic contingency tables (# males with exclusions, # broods with exclusions, # young excluded), the power of our current comparisons is, respectively, $<50\%$ (#males), 56% (#broods), and 50% (#young). If we assume the observed percentages of exclusions are the 'real' population values, then the sample sizes required for a power of 75% are 93, 100, and 330, as compared with the 46 males, 69 broods, and 194 young already analyzed.

Blood samples collected during the breeding seasons of 1992 and 1993 (methods as in 1990 and 1991) but not analyzed provide DNA from an additional 76 males and their mates and 316 young from 105 broods. Our total potential sample sizes thus exceed those indicated by the power analysis by at least 25%. The 1992 samples have been extracted while the 1993 samples await extraction.

Finally, we have collected blood from two additional study sites occupied only by untreated males, in order to determine whether the rate of extra-pair fertilizations in our experimental population is influenced by the fact that half the males are implanted with testosterone. Adding to the importance of determining these background rates of EPFs is the current interest in the relationship between level of male parental care and frequency of extra-pair fertilizations (e.g., Moller and Birkhead 1993). To date we have samples from 59 males and their mates and 222 young. Half of these have been extracted, and of those extracted, half have been run on gels.

Our advanced graduate student, Samrrah Raouf, regularly produces highly readable auto-radiographs (Fig. 7, produced at Indiana), and we are asking for funds to support Ms. Raouf and to pay for the supplies necessary to analyze the samples we have described. Patricia Parker has agreed to continue providing any needed assistance (see LETTERS OF SUPPORT).

4. The mechanistic basis of mixed reproductive strategies in male birds: how testosterone influences environmental assessment and allocation of effort to mating and parenting.

Two important generalizations about hormones and behavior are as follows: First, the causal connection between hormones and behavior is bipolar. Hormones influence behavior, and behavior can influence hormonal state. Second, an important way in which hormones affect behavior is to modify how an organism responds to stimuli (Feder 1984, Moore 1991).

Few studies of a single species have addressed the quantitative relationship between natural variation in levels of T and behavior, but a recent study of male red-winged blackbirds (Agelaius phoeniceus) showed that individual variation in T correlated positively with both song rate and with frequency of aggressive interactions (Johnsen 1991). In this final portion of the proposal, we ask how treatment with testosterone alters responses of male juncos to stimuli that predict that fitness would be improved by increasing either mating effort or parental effort. We also ask how the internal state responds to exposure to these same stimuli.

We propose two environmental manipulations. In one we will increase or decrease the size of the brood, which normally is three or four, so that males will have two or six nestlings. In the other we will create new mating opportunities for males by inducing fertility in the females mated to their neighbors. Each manipulation will be performed in two locations, our usual experimental study site occupied by T- and C-males, and a second nearby site where none of the males will be treated.

The dependent variables will be plasma levels of testosterone, corticosterone, and prolactin, time allocated to feeding nestlings, vocal behavior, and sperm production.

If male juncos can assess short-term environmental changes that predict changed reproductive opportunities, and if their responses are hormonally mediated, then we would expect hormonal state and behavioral adjustments to reflect environmental changes. For example, simulated territorial intrusions elevate T in male birds species (Wingfield's challenge hypothesis, see INTRODUCTION, also Wingfield and Wada 1989), but, as far as we know, it has not been asked whether this also leads to a short-term decrease in parental behavior or temporary increase in song rate or sperm count. Neither are we aware of any studies of the effect of increased mating opportunities on T in free-living males. This information would obviously be important to understanding of the adaptive significance of a flexible hormonal control system. Similarly, as brood size increases, we might expect

suppression of testosterone and tuning down of associated traits, e.g., song rate, and possibly sperm production; feeding rate should increase, and perhaps load size per delivery.

These are predictions we would make for unmanipulated males. But what of males treated with testosterone? Season-long sampling of the plasma of these has confirmed that our implants were working and that T was still near its natural peak throughout the breeding season (Ketterson et al. 1991). It seems likely that these males will be less flexible than C-males. We expect them to be more prompt and effective in responding to signals predicting an increase in rewards from mating effort and less effective in responding to signals predicting an increase in the value of parental effort. Comparison of the responses of treatment groups to changes in value of the brood and availability of fertile females should test these predictions. Details of the protocol follow.

a. **Response to enhanced parental cues**

Procedures: At each site (treated males, untreated males), we will match broods for age and size, take baseline measurements, then switch nestlings between nests to create broods of 2 and 6 young, equally assigned to T-males and C-males. All our behavioral measures vary with nestling age; further, older nestlings are fed more frequently, so measures at that age are less subject to error. We will focus on nests with young 8-11 days old and match pairs to the day when possible.

On the morning prior to manipulation, we will observe from a blind for two-hour periods, beginning one hour after dawn (when feeding of young is at its peak) and counting male and female feeding trips and time between trips. We will also measure time the parents spend with the nest in view and count male song rates (see Wolf et al. 1990). In the late afternoon, we will manipulate brood size, then repeat this day's observations on the following day.

It is not possible to obtain enough plasma from the same bird to compare testosterone at 24 hour intervals, so before-and-after tests of T must be replaced with sampling half the males of each treatment prior to manipulations and half after. When we bleed the males after an observation period, we will also take a sperm sample.

We will observe 20 nests of controls and 20 of T-males, half the broods enhanced and half diminished. Data on rates of behavior will be analyzed with a repeated measures ANOVA, and on distributions of intervals between feedings with a Kolmogoroff-Smirnov test. Sperm counts will be as described above under Relative Fertility, and hormone assays will follow the methods of Wingfield (Wingfield and Farner 1975, Wingfield et al. 1982, Ketterson et al. 1991).

b. **Response to enhanced opportunity for fertilization success**

From each treatment we will select 15 focal males whose mates are tending nestlings, then remove the eggs from the nest of a neighboring female. As stated repeatedly, this will induce building of a replacement nest; the female will be presumed fertile until she lays egg 1. We can then determine the male's response to the conflicting opportunities.

We will again compare the effect of treatment on allocation of effort and will monitor any changes in male physiology. We predict increases in song rates, testosterone, and sperm counts, and decreases in feeding rate; equally important, we predict that the mating-effort response to the availability of a fertile female will be greater in T-males. Here again, we will measure the behavioral

and physiological responses to conflict on a study area where none of the males, which will also be asked to confront conflicting opportunities, has been treated with hormones.

E. SIGNIFICANCE AND LONG-TERM GOALS

The importance of this work lies in its attempt to connect the study of mechanisms and the study of function. If we are to understand how patterns of social organization evolve, including mating systems and species differences in the level of male parental effort, we must have a firm grasp of the means by which animals assess their environments and mobilize appropriate behavioral and physiological responses. If we are to understand what maintains existing patterns in nature, we must know the consequences, in terms of fitness, for animals that deviate from the prevailing norm.

In this proposal, we describe how we create a novel phenotype with a hormone and compare its attributes and fitness to unmanipulated animals. The apparent effect of the hormone is to shift the natural balance of mating and parental effort away from parenting and toward mating. From an adaptationist perspective, animals that deviate from the norm should be selected against, but only by testing that perspective can we learn how robust it is. Thus far, our deviant phenotype performs similarly to controls on most measures, although there appear to be alternative routes to reproductive success. The work described in this proposal should tell us 1) whether the tendency of testosterone to alter the way males use space will have a detrimental effect on their ability to guard their mates against extra-pair fertilizations, 2) whether that same tendency to wander will have a detrimental effect on nest defence during incubation, 3) whether testosterone has an enhancing or suppressing effect on male fertility, 4) whether testosterone promotes extra-pair paternity at the cost of within-pair paternity, and 5), whether or not males are treated with testosterone, the means they emply to pursue a mixed reproductive strategy.

Our long-term goals include 1) expanding our approach to other species in which male parental effort is greater and less than that of the junco, 2) taking a developmental approach to the role of testosterone (e.g., if males were treated earlier in life, would the effects of treatment be greater than those we have documented to date?), 3) focussing on the impact of testosterone on life span (e.g., testosterone, corticosterone, stress, and disease), and, finally, 4) placing more emphasis on the female and her 'decisions' regarding fecundity (e.g., the role of hormones in determining clutch size).

Effect of Testosterone on Spatial Activity in Free-ranging Juncos

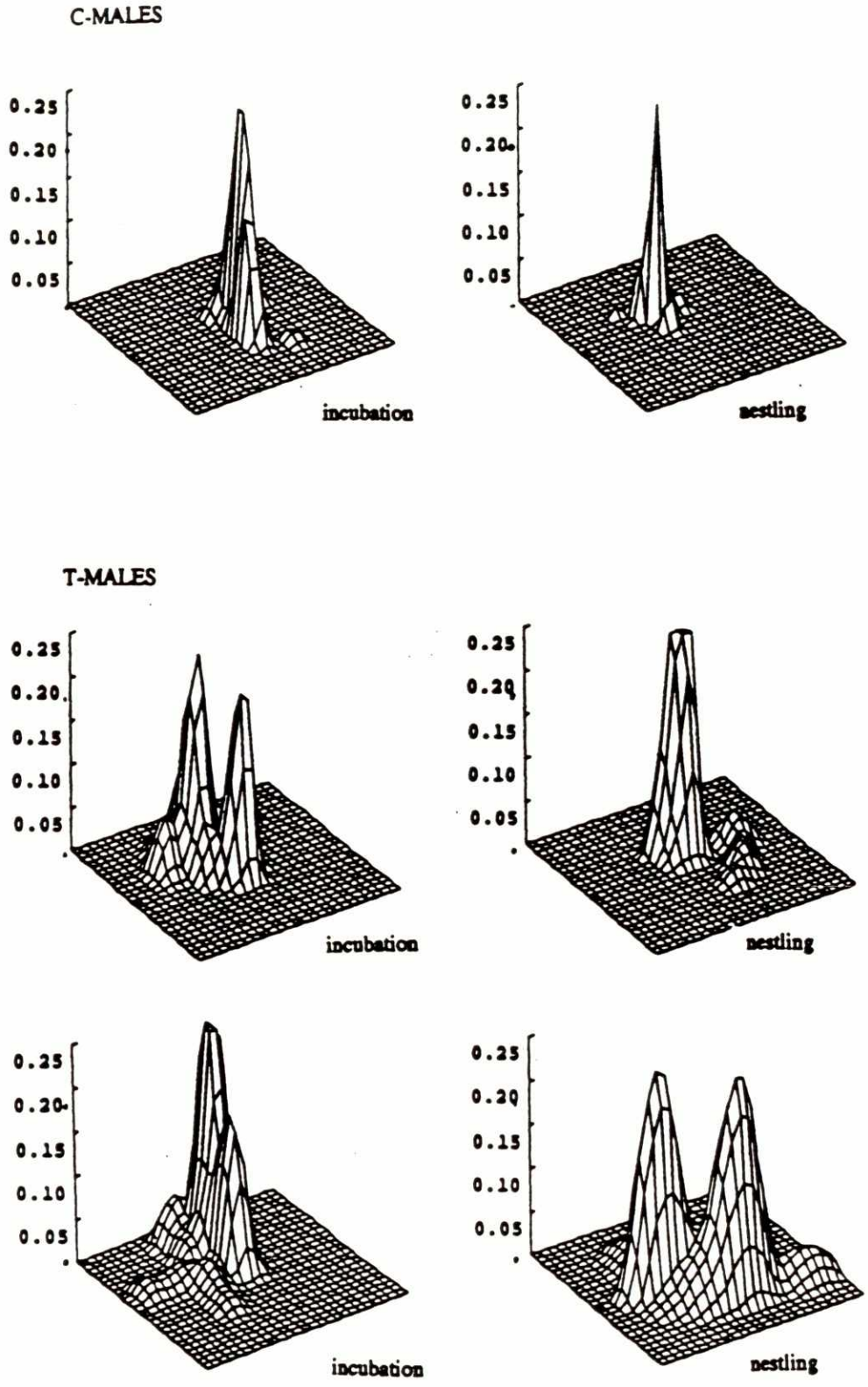


Figure 1A. Examples of spatial activity in two control (C-males) and four testosterone-treated (T-males) male juncos. The grids are 600m on each side and the height of the surface describes relative frequency of activity at that coordinate.

Effect of Testosterone on Spatial Activity in Free-ranging Juncos

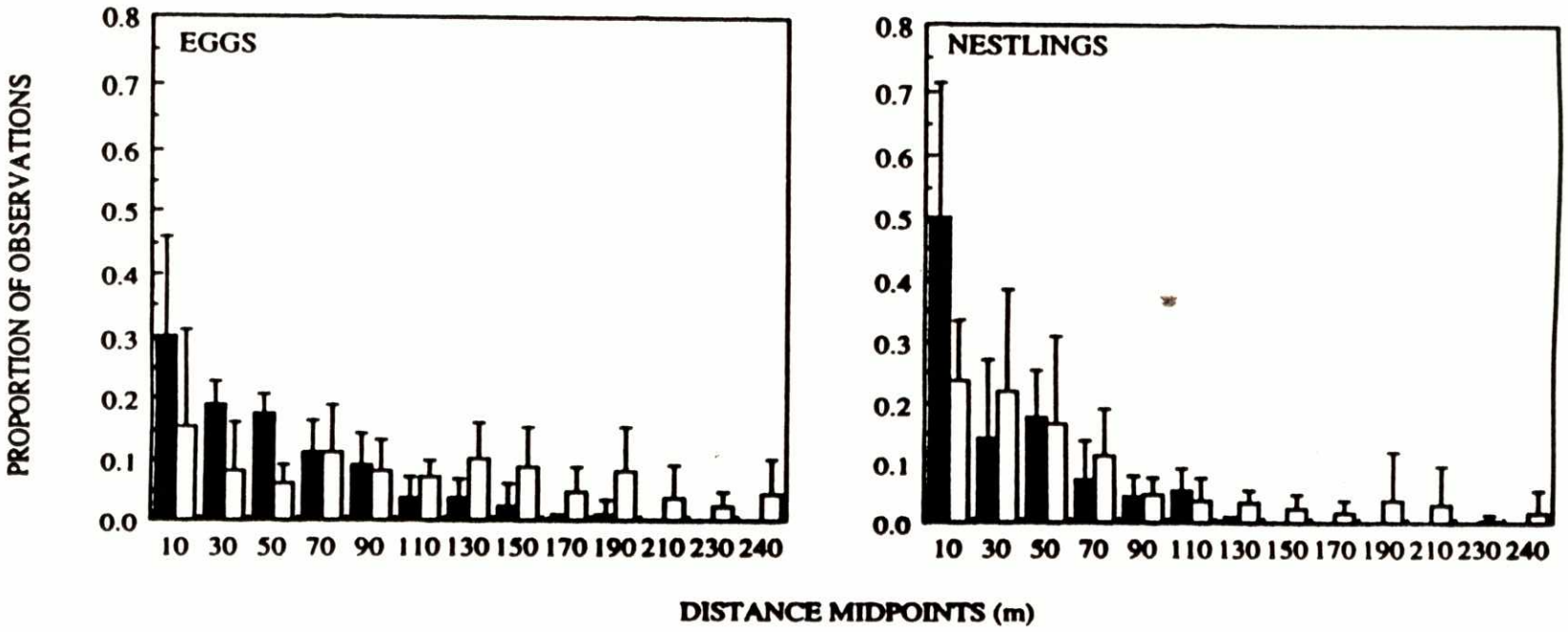


Figure 1B. Proportion of tracking locations at varying distances (m) from the nest for control (solid bars) and testosterone-treated (empty bars) male juncos during the egg and nestling stages. Values are means (+/- s.e.) for five individuals from each treatment group.

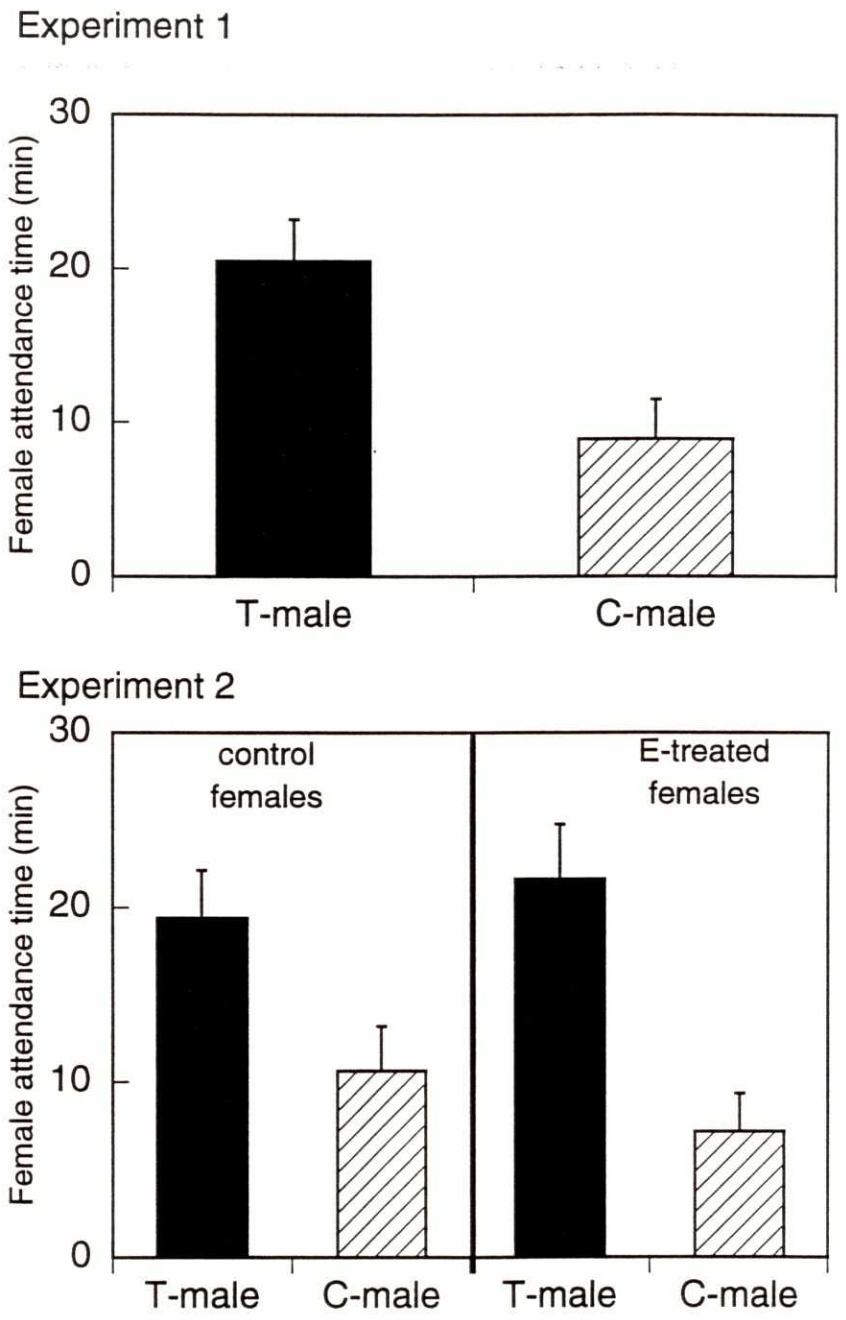


Figure 2: Time spent with T-treated vs control males during female mate-choice experiments. Two mate-choice experiments were conducted in the summer of 1993. In each trial, females were given a choice between a testosterone treated male (T-male) and a control implanted male (C-male). Males were presented in a Y-maze, visually isolated from each other. Females were confined in full view of both males for 20 min at the start of each trial, and then released for a 40 min assessment period during which they were free to interact with both males through 1.3 cm hardware cloth barriers. In the first experiment (Experiment 1), female hormone levels were not manipulated. In the second experiment (Experiment 2), half of the females were treated with estradiol (E). Females spent significantly more time with the T-males in both experiments (Experiment 1: $N = 28$, $P = 0.01$; Experiment 2: $N = 24$, $P = 0.001$). In addition, more female sexual displays were directed toward the T-males in both Experiments (experiment 1: 18 of 22; Experiment 2: 52 of 60). The preferences of E-treated and control females did not differ significantly.

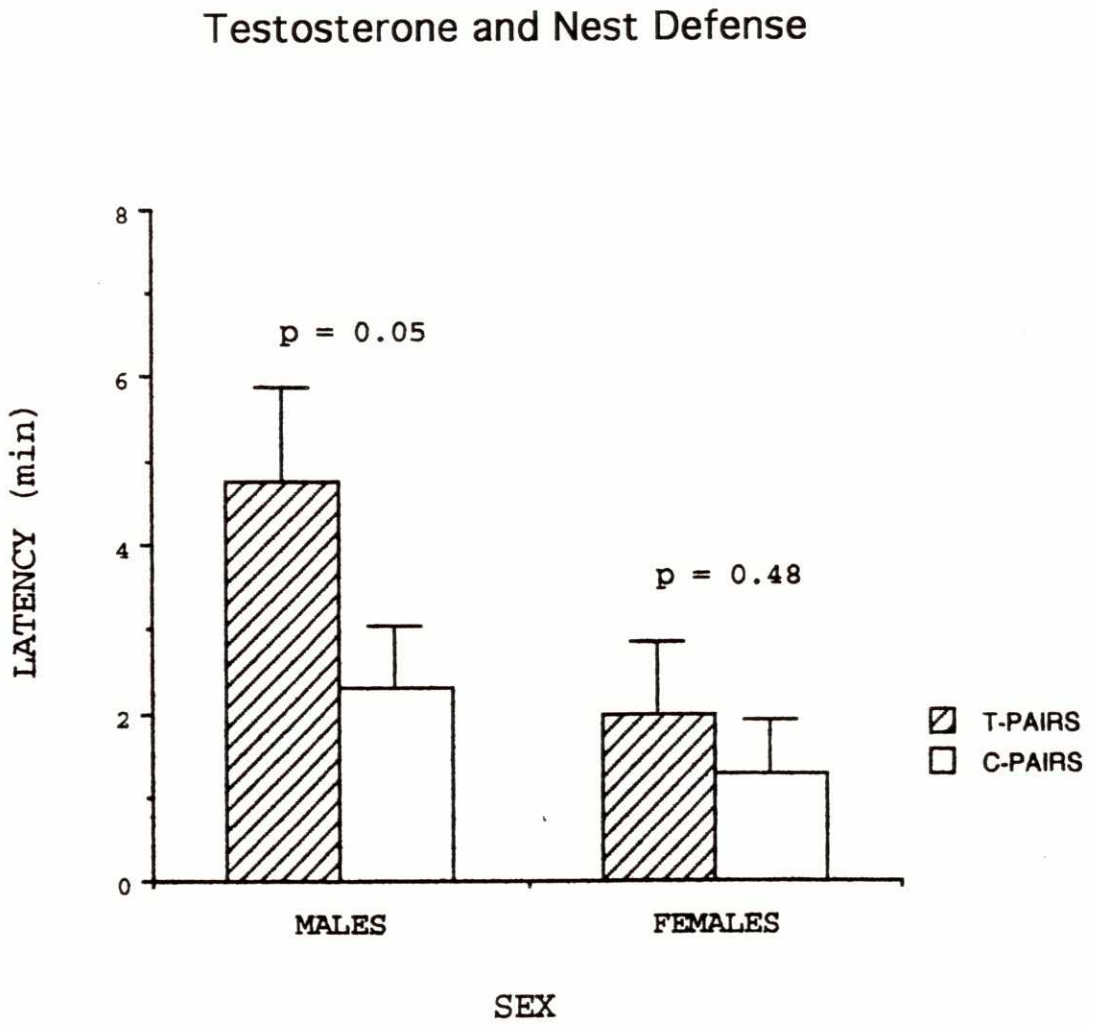


Figure 3. The effect of testosterone on nest defense. Time required for males and their mates to discover a model chipmunk placed near the nest. [No shows (3 T-males and 1 control) were assigned a value of ten minutes.]

The Effects of Elevated Testosterone on Spermatogenesis

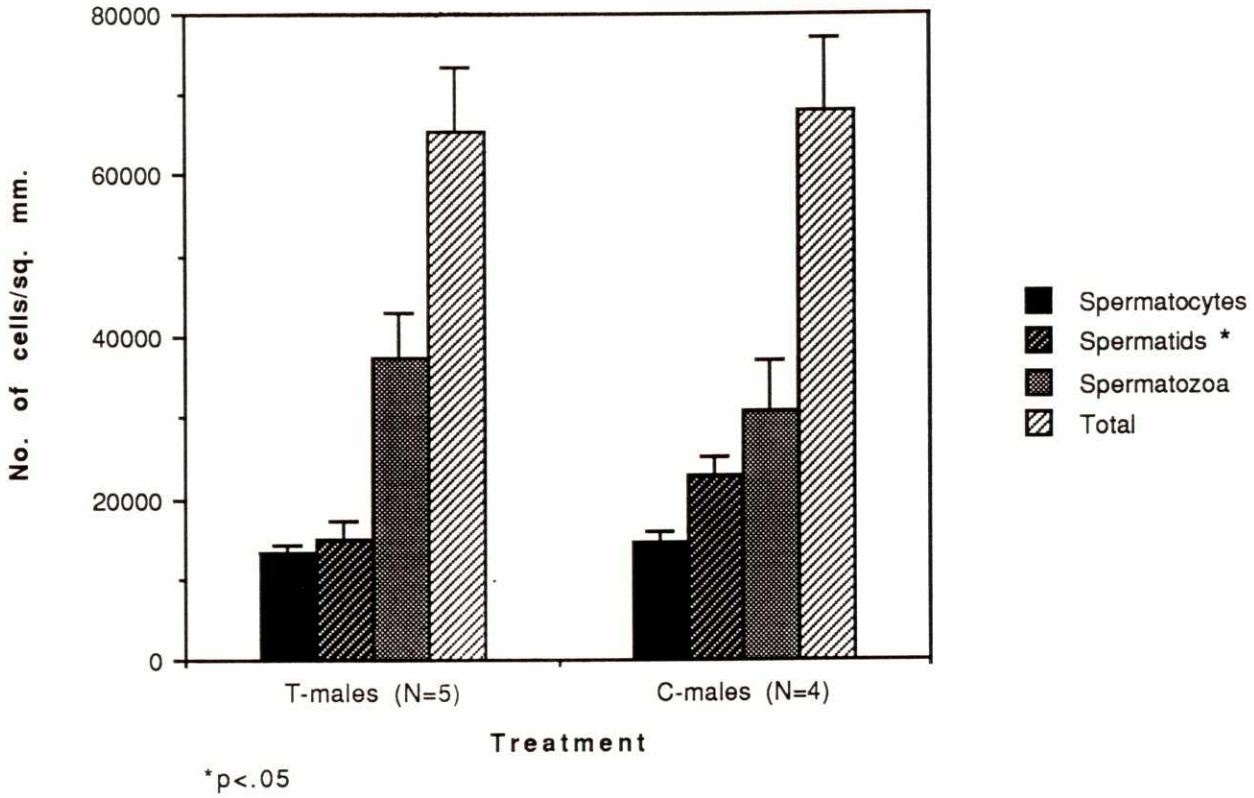


Figure 4a. The effects of testosterone on spermatogenesis. Captive males were sacrificed in early July, approximately twelve weeks after implantation with testosterone. Testes were fixed in Bouin's solution for 24 h, then transferred to 70% ethanol and embedded in Paraplast. The tissues were cut into 6 μm sections and stained with Weigert's iron hematoxlyin and eosin. Three sections, two fields from each section, were analyzed for each individual using a micrometer (field measured 0.0144 sq. mm). Shown are the means ($\pm$SE) for the number of cells found in three different spermatogenic stages. Total represents the sum of all cell types. There were no significant differences between treatment groups for spermatocytes (p=0.43), spermatozoa (p=0.45) or total (p=0.83). Analysis did show a significant difference between treatment groups for the number of spermatids/sq. mm. (p=0.04).

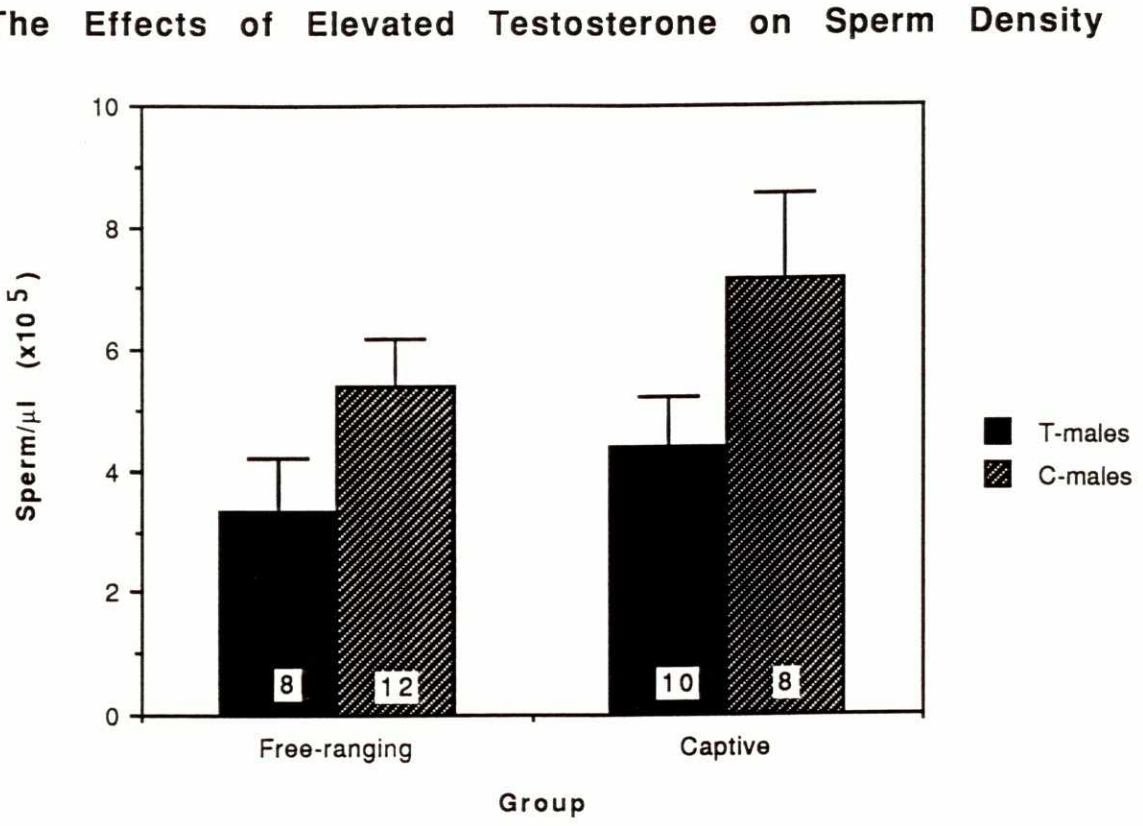


Figure 4b. The effects of elevated testosterone on sperm density. Shown are sperm densities for testosterone-treated and control males from two groups, captive and free-ranging. All males were sampled from 6/15/93 to 7/17/93. Sperm was collected from the free-ranging males when young were between six and eleven days of age. Captive males were housed individually and isolated from females. Males were massaged for a time not exceeding 3 minutes. Sperm samples were diluted 100-fold and counted using a hemacytometer. Two hemacytometer readings were counted per individual. Shown are the mean (±SE) for each treatment group. There were no significant differences between treatment groups for the free-ranging ($p=0.099$) or the captive ($p=0.102$) group.

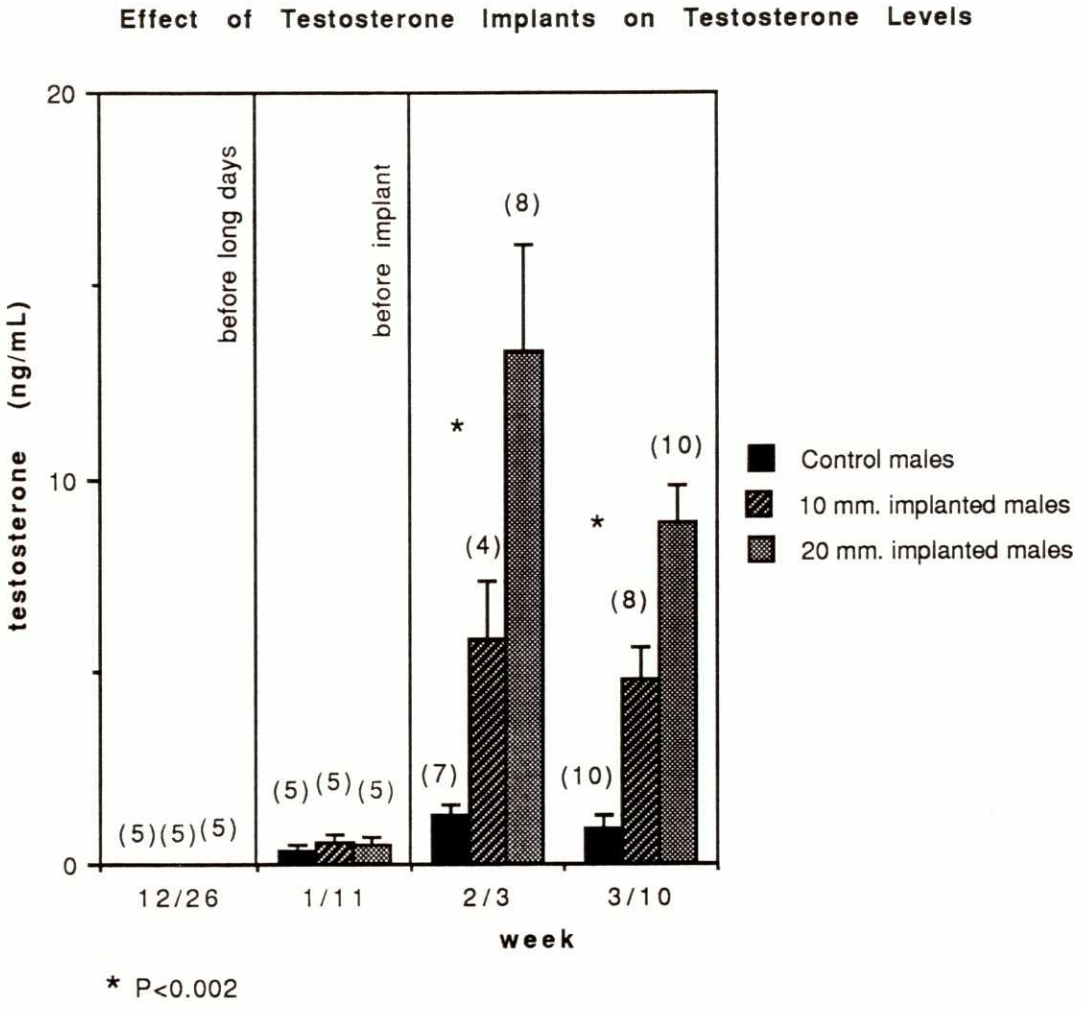


Figure 5A: Effect of testosterone implants on testosterone levels. Captive dark-eyed juncos were subjected to long days (14L:10D) for 16 days and then implanted with testosterone (see Ketterson et al. 1991). Blood samples were taken at 2-3 week intervals. Testosterone levels were measured by radio-immuno assay (see Ketterson et al. 1991). On 12/26 no testosterone could be detected. Sample sizes are displayed over the error bars.

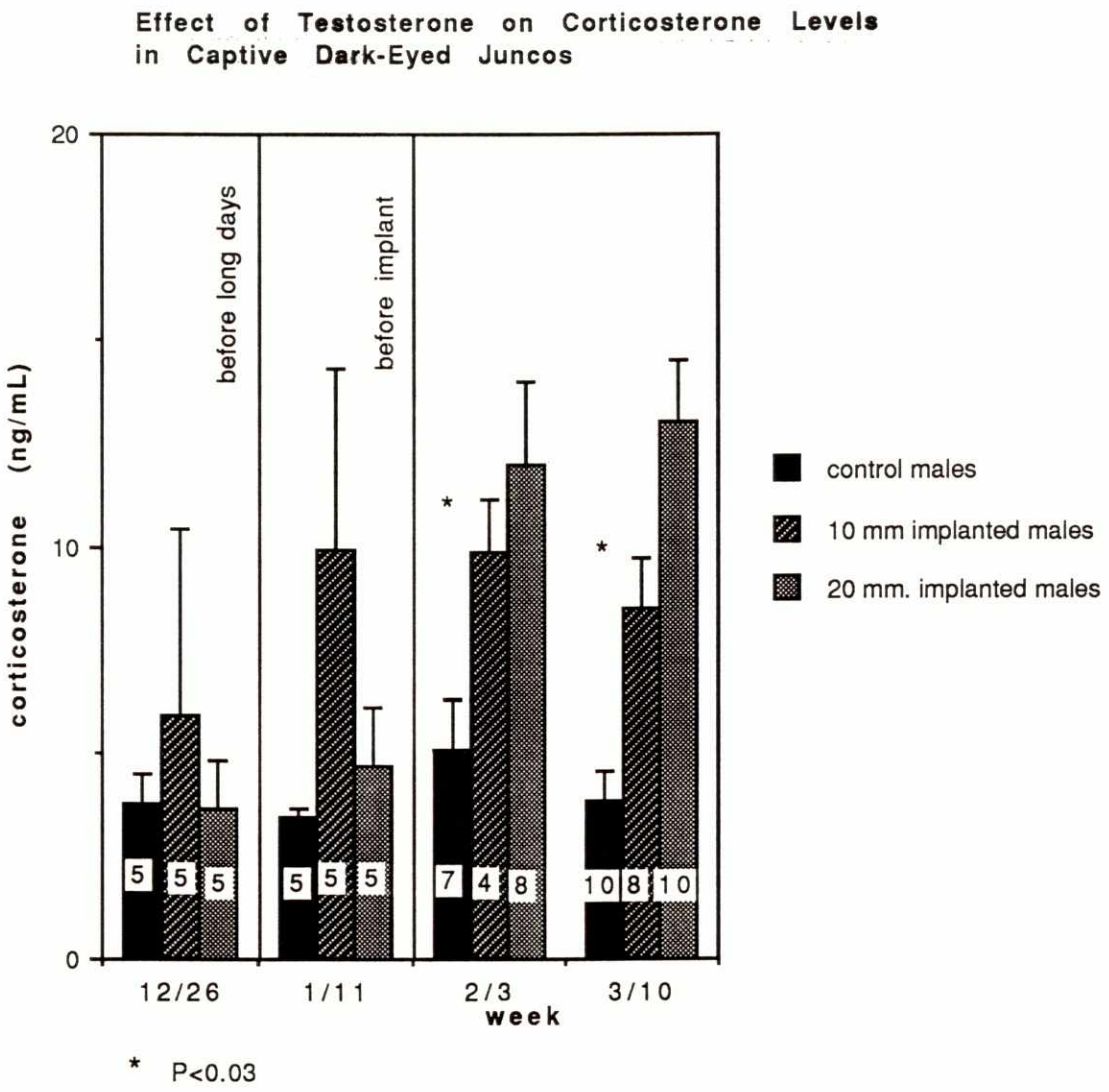


Figure 5B: Effect of testosterone on corticosterone levels in captive dark-eyed juncos. Captive dark-eyed juncos were subjected to long days (14L:10D) for 16 days and then implanted with testosterone (see Ketterson et al. 1991). Blood samples were taken at 2-3 week intervals. Corticosterone levels were measured by radio-immuno assay (see Ketterson et al. 1991).

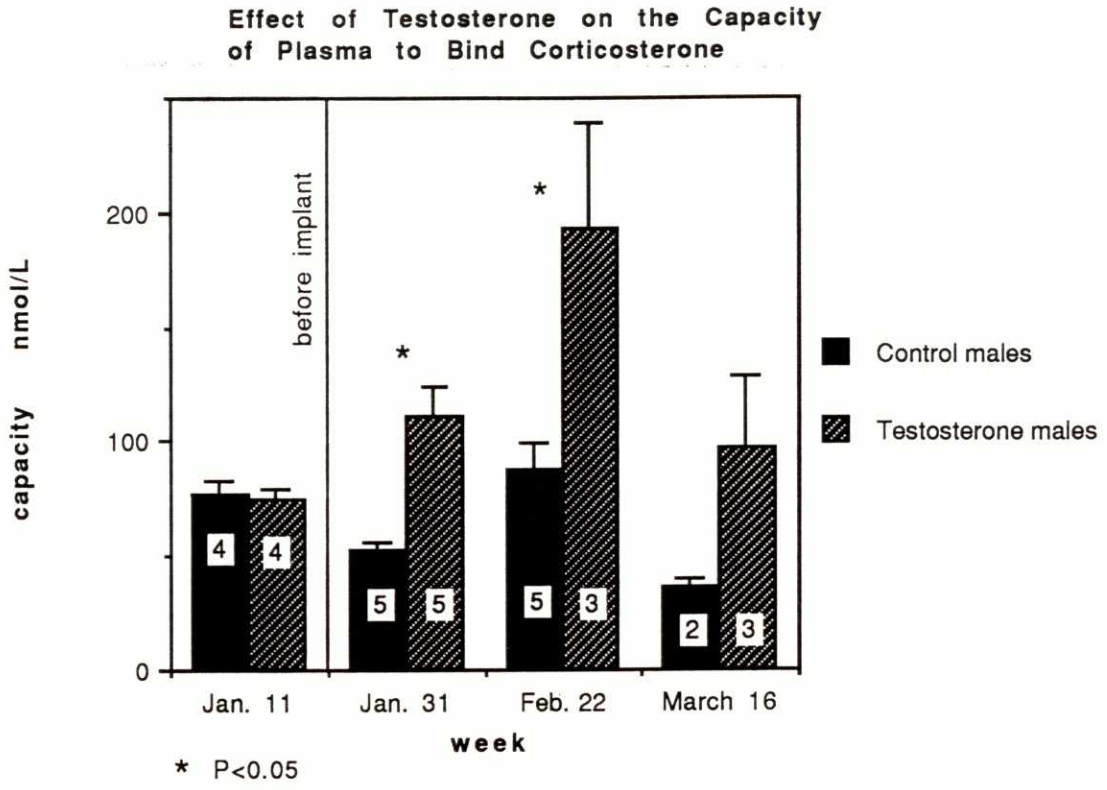
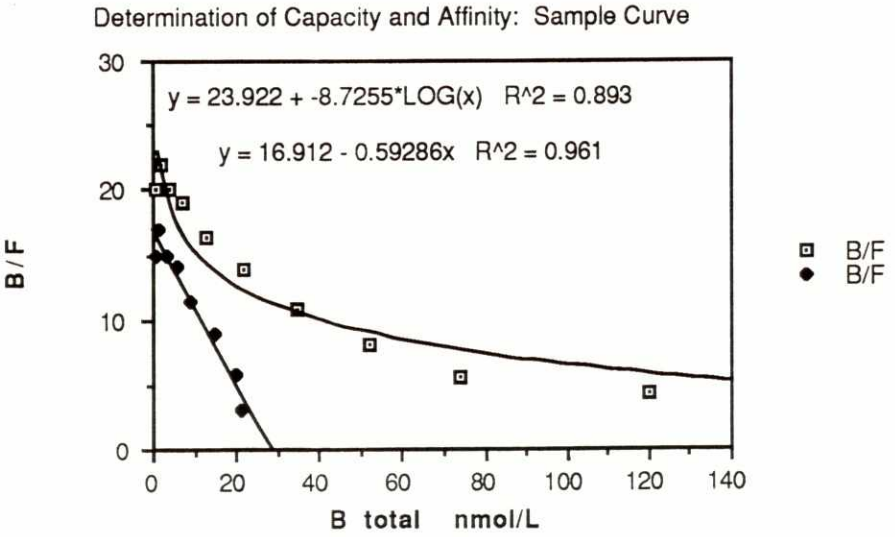


Figure 5C: Effect of testosterone on the capacity of plasma to bind to corticosterone, a measure of corticosterone binding protein (CBP). Captive dark-eyed juncos were subjected to long days (14L:10D) for 16 days and then implanted with testosterone (see Ketterson et al. 1991). Blood samples were taken at 2-3 week intervals. The plasma capacity and affinity for corticosterone were assessed according to a procedure described by Wingfield, Matt, and Farner (1984). Briefly, we pooled equal volumes of plasma from 2 individuals, removed endogenous steroids with charcoal, and performed a competitive binding assay by adding a constant amount of tritiated corticosterone and varying amounts of unlabeled corticosterone (range: 0.2-100 ng). Bound and unbound hormone were separated and the bound fraction was counted in a scintillation counter. To determine the total mass of hormone bound, we calculated the bound/unbound ratio (B/F) and plotted it against total hormone bound (Scatchard 1949). The inverse of the slope of the line fitted to the curve by least squares regression is the dissociation constant (affinity). The intersection of the line with the abcissa is the binding capacity in nmol/L. (see below for example curve).



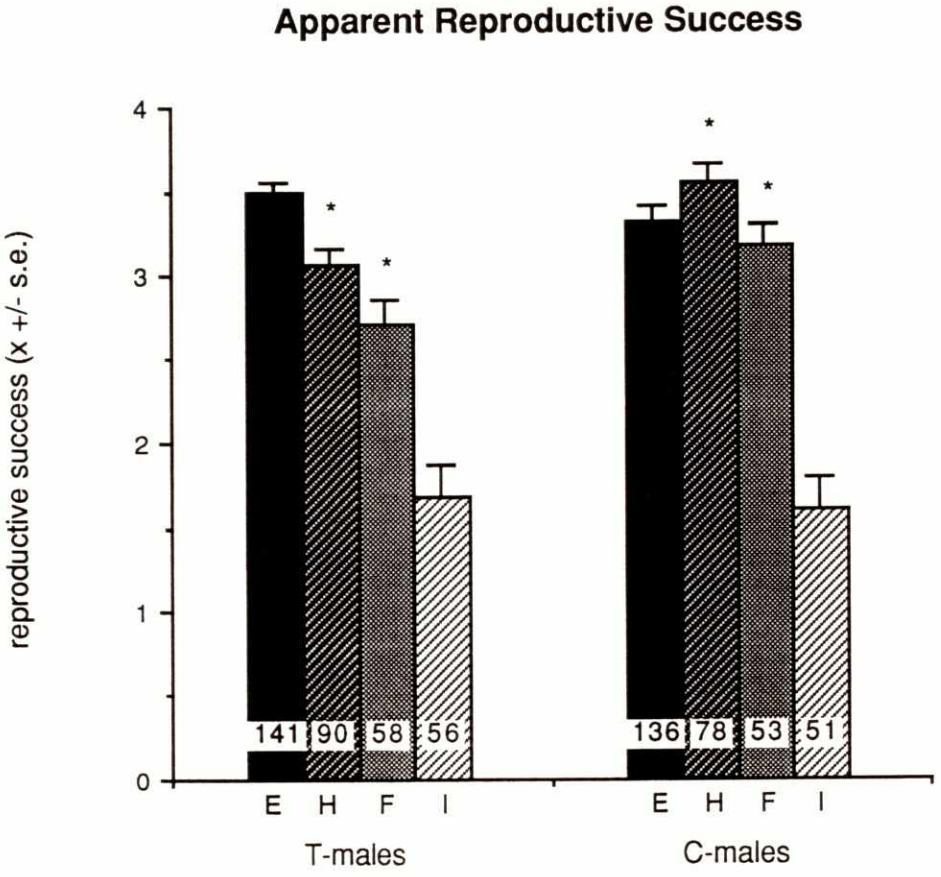


Fig. 6. Mean number of offspring of various ages produced by T-males and C-males (E=eggs, H=hatchlings, F=fledglings, I=independent young). Data from 1989-1992 combined (in prep).

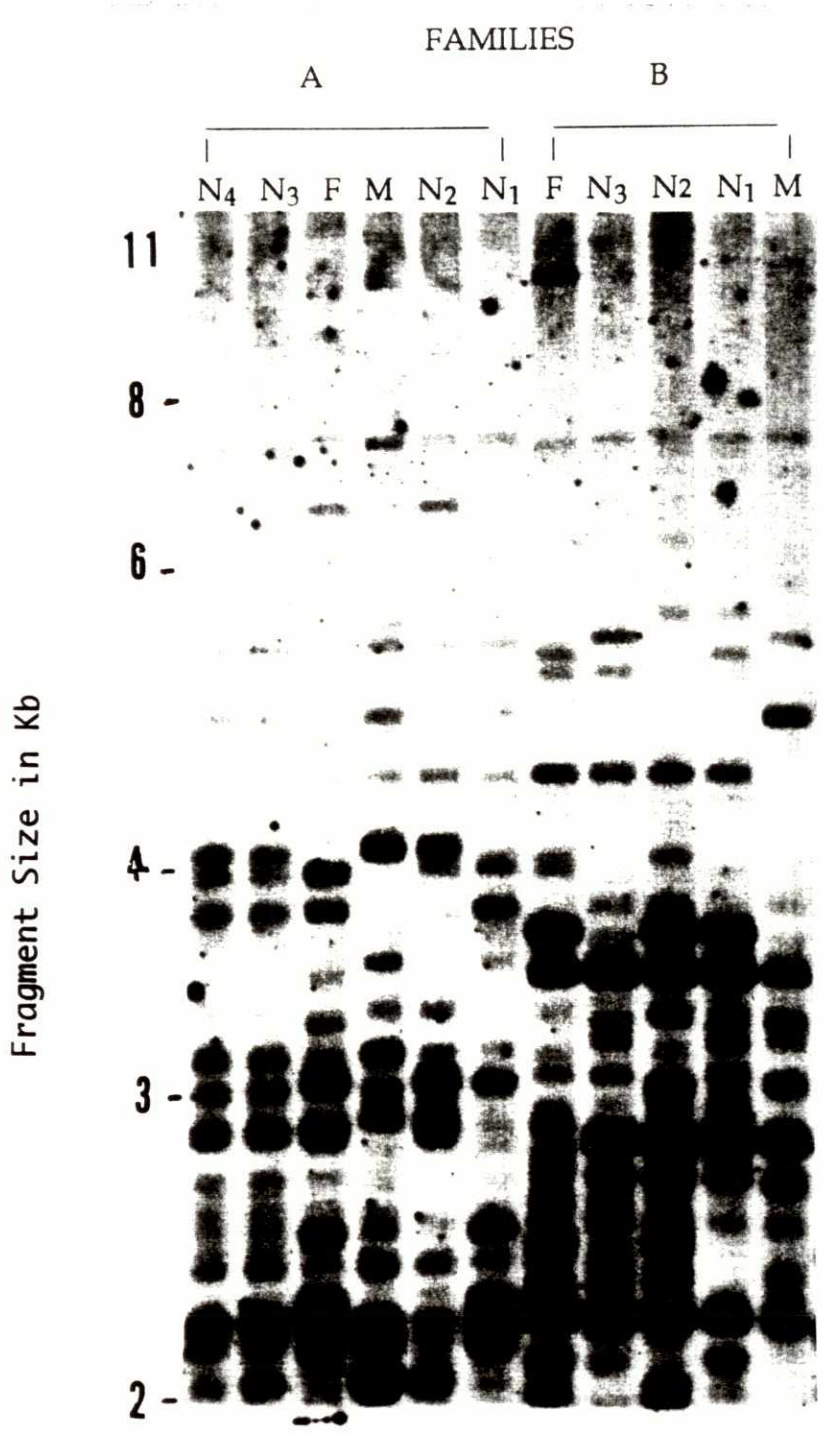


Fig. 7
This figure shows a typical fingerprint banding pattern from two junco families. The letter "M" refers to putative male parent and "F" to the putative female parent. The letter "N" refers to the nestlings. The size of the DNA fragments range from about 11 kb to 2 kb. The DNA was cut with the enzyme Hae III and probed with Jeffrey's 33.16. The probe was radio-labeled using the random priming technique. The membrane was hybridized over-night and exposed at -20 °C for two weeks with one intensifying screen. All the offspring belong to the putative parents, there are no young excluded.

To demonstrate scoring technique using band sharing: In family "B" the female has a band at about 4.5 kb which all three offspring inherited. The male has no band in that size range. Conversely, the band at 2.8 kb that also appears in all three offspring came from the father and not the mother.

TABLE 1. SUMMARY STATISTICS ON GENETIC ANALYSIS OF PATERNITY: EXCLUSIONS AND RELATIVE NET SEASON-LONG REPRODUCTIVE SUCCESS

C-MALES

YEAR	MALES	BRDS	YNG	#MAL EXC	#BR EXC	#YG EXC	%ML EXC	%BR EXC	%YG EXC
1990	13	18	58	4	5	15	30.8	27.8	25.9
1991	12	20	57	4	5	12	33.3	25.0	21.1
1990-1991	25	38	115	8	10	27	32.0	26.3	23.4

T-MALES

YEAR	MALES	BRDS	YNG	#MAL EXC	#BR EXC	#YG EXC	%ML EXC	%BR EXC	%YG EXC
90	6	8	19	3	3	4	50.0	37.5	21.1
91	15	23	60	8	11	22	53.3	47.8	36.6
90 91	21	31	79	11	14	26	52.4	45.2	32.9

Where BRDS signifies number of broods, YNG signifies number of young, #MAL EXC signifies numbers of males for which at least one young was excluded, i.e., the putative male could not have been the sire, #BR EXC signifies number of broods where putative male did not actually sire at least one young in the brood, and # YG EXC signifies the number of young that could not be assigned to their putative sires. The last three columns express these variables as percentages.

SUMMARY, NET RELATIVE REPRODUCTIVE SUCCESS

	APPARENT RS	GAINS VIA EPFS	LOSSES TO EPFS	NET RELATIVE RS
C-MALES (25)	4.60 (115/25)	0.44 (11/25)	1.08 (27/25)	3.96
T-MALES (21)	3.76 (79/21)	1.00 (21/21)	1.24 (26/21)	3.52
ADVANTAGE CONTROLS	+ 0.84	- 0.56	+ 0.15	+ .43

PHENOTYPIC EFFECTS

BEHAVIOR	PHYSIOLOGY	MORPHOLOGY
Song	Corticosterone	Brain nuclei
Feeding nestlings	Body Mass	Syrinx
Home range size	Molt	Gonads
Attractiveness	Sperm density	Sperm morphology
Nest defense	Blood carrying capacity	

FITNESS

SURVIVAL
- Summer
- Winter

REPRODUCTION
- Offspring
  - #, quality
  - home and away
- Mate
  - acquisition
  - retention

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