EXOGENOUS TESTOSTERONE AND THE ADRENOCORTICAL RESPONSE IN DARK-EYED JUNCOS

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ABSTRACT.—Hormonal manipulations with implants allow examination of the costs and benefits of behaviors and physiologic states mediated by a given hormone. As a part of ongoing research into the effects of the steroid hormone testosterone (T) in Dark-eyed Juncos (*Junco hyemalis*), we measured the corticosterone (B, a steroid hormone secreted by the adrenal in response to stress) response to the stress of capture and handling in males treated with T (T-males) and in control males (C-males). Although B may be essential for energy mobilization, chronic or repeated exposure to elevated levels of B can have many negative effects. Because T mediates many behaviors that may increase the likelihood that an individual will encounter stressors, we predicted that plasma B would rise more rapidly in T-males than in controls. In the first few minutes post-capture, the increase in B levels was significantly higher in T-males than in controls. B levels in samples collected 10, 30, and 60 min post-capture were consistently higher in T-males than in C-males; however, the difference was not statistically significant. Because previous work has shown that T-males reduce their parental contribution, we compared females that were mated to T-males and C-males (hereafter T- and C-females). B levels of T-females increased sharply in the first few minutes post-capture, whereas in C-females they did not; however, the responses were not statistically different. Males had higher initial levels and a greater B-response to stress than females when data were compared irrespective of treatment. Our results suggest that the behaviors or physiological changes induced by T are potentially costly and that such costs may in part be incurred through elevated B. *Received 25 November 1997, accepted 5 May 1998.*

IN MOST NORTH-TEMPERATE BIRDS, circulating levels of testosterone (T) in males are elevated during the sexual phase of the breeding season (i.e. territory establishment, pair formation, nest building, and copulation) and become lower during parental care when eggs or young are present (Wingfield 1984, Ball 1991). Although relatively high levels of T early in the breeding season may be essential for the expression of reproductive physiology and behaviors, it is thought that the behaviors promoted by T are incompatible with the care of young (see below). Accordingly, males with experimentally elevated levels of T (T-males) during the parental phase can be expected to reduce their parental effort compared with control males (C-males). Indeed, the few studies that have artificially elevated T during incubation and brood rearing have found that treated males decrease their parental effort (Silverin 1980; Hegner and Wingfield 1987; Oring et al. 1989; Ketterson et al. 1992, 1996; Saino and Møller 1995; Schoech et al. 1998).

Other studies of free-living animals with experimentally elevated T have demonstrated that additional costs may be associated with maintaining elevated testosterone levels after the time when levels typically peak. For example, in mountain spiny lizards (*Sceloporus jarrovi*; Marler and Moore 1988, 1989) and Brown-headed Cowbirds (*Molothrus ater*; Dufty 1989), T-implanted males suffered higher mortality than controls. Whereas Dufty (1989) attributed the mortality to wounds caused by increased aggression in T-treated cowbirds, Marler and Moore (1988, 1989) suspected that T-males: (1) starved due to a reduction in the amount of time they allocated to feeding, and (2) suffered higher predation because of increased exposure and/or decreased vigilance. Testosterone-treated lizards also expended more energy as a result of an increase in territorial behavior (Marler et al. 1995). In contrast, field studies of Dark-eyed Juncos (*Junco hyemalis*) revealed no differences in survival between T-implanted and control birds (Ketterson et al. 1996) except when implants were not removed prior to the winter so that the prebasic molt was suppressed (Nolan et al. 1992).

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Other research (Ketterson et al. 1991, 1992, 1996; Raouf et al. 1998) suggests that the maintenance of elevated levels of testosterone throughout the breeding season causes no marked disadvantages to male juncos. As has been found for other species (see above), T-males provision offspring less frequently than do C-males (Ketterson et al. 1996, Schoech et al. 1998). Possibly as a result of the reduction in care, the social mates of T-males fledge fewer young than those of C-males (Ketterson et al. 1996, Raouf et al. 1998). Thus, although one might expect T-males to have lower reproductive success than C-males, they do not because T-males sire more young through extrapair fertilizations (EPFs) and suffer fewer losses of paternity to EPFs in their social mates. As a result, the net reproductive success of T-males does not differ from that of C-males (Raouf et al. 1998). These findings suggest that the costs (if any) of experimentally elevated T in adult males are related more to their survival than to their reproductive success.

For example, T treatment can increase the frequency of aggressive interactions (Balthazart 1983; Wingfield et al. 1987, 1990) and locomotor activity (Wada 1982, 1986) while decreasing lipid storage (Wingfield 1984, Ketterson et al. 1991). Probably as a result of changes in behavior and physiology, male juncos treated with T increase the size of their territory or home range (Chandler et al. 1994). A recent study of captive juncos (S. Lynn unpubl. data) found that T-males increased the time spent foraging and moving and decreased the time allotted to self care compared with C-males. Clearly, reducing self maintenance while simultaneously increasing aggression, territorial defense, and other energetically costly behaviors might prove stressful.

The well-established link between stress and the secretion of the adrenal steroid hormone corticosterone (Harvey et al. 1974, Selye 1979, Siegel 1980, Greenberg and Wingfield 1987) led us to investigate whether circulating levels of corticosterone (B) are elevated in T-males. Two recent studies of Dark-eyed Juncos, one conducted with free-living birds and the other with captives, showed that basal levels of B are higher in T-males than in C-males (Ketterson et al. 1991, Klukowski et al. 1997). Although basal levels of B provide information about the current (or immediate past) state of the hypotha-lamic-pituitary-adrenal (HPA) axis, the capture-stress paradigm of Wingfield (1994) allows one to gain additional information about the condition of the HPA axis by sampling captured individuals repeatedly. For example, a T-male that allocates a disproportionate amount of time to sexual or aggressive behaviors while reducing self maintenance behaviors might have less stored energy, or its energy might be in a form that is not readily accessible. As a result, a T-male might exhibit a more pronounced B response to a stressor than would a C-male.

Here, we compare the stress response of T- and C-male Dark-eyed Juncos. Because females mated to T-males work harder at provisioning their offspring than females mated to C-males (Ketterson et al. 1992), we also compare the B responses of these two groups of females (referred to as T- and C-females, according to the treatment of their mates). Moreover, because of the relationship between body condition and B that has been found in other species (Wingfield et al. 1994b, Schoech et al. 1997), we examine the relationship between body mass and B.

METHODS

Study site and species.—The study population of Dark-eyed Juncos (*J. h. carolinensis*) was located in and around the University of Virginia's Mountain Lake Biological Station in western Virginia (37°22′N, 80°31′W) at 1,180m elevation. The reproductive biology of this population has been under study by Ketterson, Nolan, and colleagues since 1983. The data we report were collected during the breeding seasons of 1995, 1996, and 1997.

Most of the juncos in the study area have been captured and banded with a unique combination of U.S. Fish and Wildlife Service aluminum bands and plastic colored bands. Almost all nests are located each year, and the pair associated with each nest is identified by intensive field observations, allowing us to know the reproductive stage of all individuals.

Testosterone treatment and blood collection.—Males were captured and implanted between 15 April and 15 May before or shortly after pair formation. Individuals were anesthetized by inhalation of metophane and implanted with two 10-mm long Silastic tubes (1.44 mm inner diameter, 1.96 mm outer diameter) that were empty (C-males) or packed with crystalline testosterone (T-males; see Wingfield and Farner 1983, Ketterson et al. 1991, Schoech et al. 1996). Although we did not measure T, T-implants of these dimensions are known to elevate T to levels that are similar to the early season maximum of non-implanted males, after which it remains elevated

throughout the breeding season (Ketterson et al. 1991, 1996; Enstrom et al. 1997; Schoech et al. 1998). In the preceding studies, T levels were approximately 3-fold higher in T-males than in C-males. Implants were positioned subcutaneously along the left flank under the wing. Individuals that had been banded in earlier years were designated as treatment or control based on the previous year's treatment, i.e. birds were given the same treatment in subsequent years. Juncos that were new to the study tract were randomly designated as treatment or control. In a few cases, we failed to capture a male between the dates pre-established for implanting; these males were comparable to C-males and were incorporated into the control data set.

To assess stress responses, all birds were captured when their nestlings were between 8 and 11 days old. To control for possible diel fluctuations in corticosterone secretion, almost all samples were collected between 0700 and 1100. The few samples collected later in the day did not differ from the early samples, so all samples were combined for analysis.

Our protocol in measuring stress responses was as follows: (1) a timer was started when the bird entered the net; (2) the individual was removed from the net and an initial blood sample collected (to control for differences in the time required to remove individuals from the net, we noted the elapsed time between the bird's first entering the net and collection of the initial sample); (3) the bird was held in the hand until 10 min had passed from the time it had entered the net (during which time we took standard morphometric data); and (4) a second sample was then collected. In 1996 and 1997, in addition to taking the initial and 10-min samples, we placed birds in loosely woven cloth bags and collected samples at 30 and 60 min. All blood samples were collected in microhematocrit tubes (50μL of whole blood) following puncture of the brachial vein with a 26-gauge needle. Blood samples were kept on ice until transport to the laboratory, where they were centrifuged and the plasma fraction harvested and stored at −20°C until taken on dry ice to Bloomington, Indiana, for assay.

Radioimmunoassay.—Direct assay of corticosterone was conducted according to Wingfield et al. (1992), with the exception that we used anhydrous diethyl ether rather than dichloromethane to extract steroids from plasma. Prior to extraction, approximately 2,000 cpm of radio-labeled B were added to each sample and permitted to equilibrate overnight. This allowed calculation of the percentage of hormone recovered during the process. Samples were run in a single assay following each field season. Mean plasma volume in the 1995 samples ($n = 86$) was 26.37 ± SE of 0.64 μL; recovery was 91.0%, and intra-assay variation was 13.5%. Mean plasma volume in 1996 ($n = 104$) was 20.59 ± 0.46 μL; recovery was 80.11%, and intra-assay variation was 16.5%. Mean volume in

1997 ($n = 78$) was 20.13 ± 0.58 μL; recovery was 84.5%, and intra-assay variation was 12.0%. Inter-assay variation ($n = 3$) was 13.1%.

Statistical analyses.—Although our goal had been to collect the initial blood sample within one minute after an individual entered the net, the procedure often took longer ($\bar{x} = 2.45 ± 0.12$ min, $n = 81$). Consequently, we used regression analysis to relate B to handling time. As a preliminary analysis, however, we used analysis of covariance to test for homogeneity of slopes. When no year effects were noted in any category (T-males, $F = 0.25$, $P = 0.79$, $n = 17$; C-males, $F = 0.97$, $P = 0.40$, $n = 23$; T-females, $F = 0.83$, $P = 0.45$, $n = 24$; C-females, $F = 0.46$, $P = 0.64$, $n = 17$), we combined data from the three years. We then determined whether the B response in the first few minutes of handling differed between T-males and C-males and between T-females and C-females. Data from samples collected at 10, 30, and 60 minute postcapture in 1996 and 1997 were analyzed with repeated-measures analysis of variance.

To further examine the relationship between body mass and B, and to control for differences in the time required to obtain the initial plasma sample, we calculated the rate of change in B levels between the initial sample (B_1) and the 10-min sample (B_{10}) for each individual and used that rate to extrapolate what each junco's B level would have been at 1 min postcapture. We assumed a linear change in B between the times the initial sample (T_1) and the 10-min sample (T_{10}) were collected and between 1 min and the actual time of the initial sample. We calculated the rate of change in B levels between the initial and 10-min sample using the equation:

$$(B_{10} - B_1)/(T_{10} - T_1) = X \text{ ng/min.} \tag{1}$$

We then determined how much time an initial sample deviated from the desired collection time of 1 min (i.e. $T_1 - Y$ min = 1 min). Y was then multiplied by the above rate, giving the number of ng that B would have changed in the elapsed time. This value was then subtracted from B_1 to yield the extrapolated value at 1 min. The relationships between body mass and extrapolated B levels at 1 min were then examined with regression analysis. Data were transformed as necessary to ensure equal variance (Sokal and Rohlf 1981, Neter et al. 1985).

RESULTS

Initial levels of corticosterone.—Initial samples from T-males showed a marked increase in B with time since capture ($F = 17.63$, $n = 17$, $P = 0.001$, $r^2 = 0.54$; $y = 10.66x + 0.05$; Fig. 1). In contrast, the relationship between B and the time required to collect the first sample was not significant in C-males ($F = 0.08$, $n = 23$, $P = 0.79$, $r^2 = 0.004$; $y = 0.65x + 16.46$). The slopes
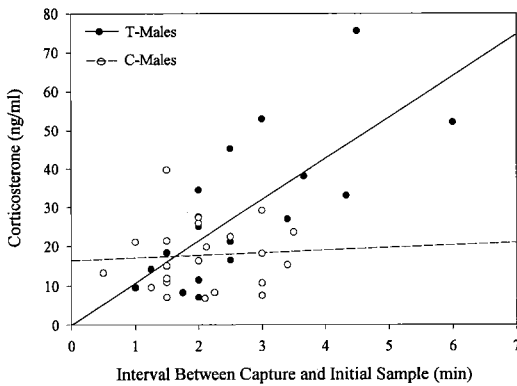
FIG. 1. Corticosterone levels as a function of the interval between capture and collection of initial blood samples in testosterone-implanted (T-males) and control (C-males) Dark-eyed Juncos.
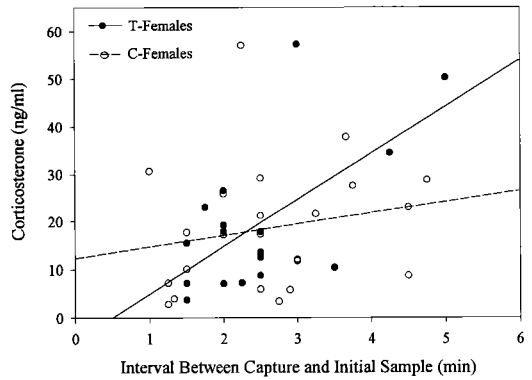

FIG. 2. Corticosterone levels as a function of the interval between capture and collection of initial blood samples in Dark-eyed Juncos mated to T-males (T-females) and mated to C-males (C-females).

of the regression lines for T- and C-males differed significantly ($F = 7.76$, $P = 0.008$, Fig. 1). Inspection of Figure 1 suggests that the time required to collect the initial samples differed between groups, but this difference was not significant ($t = -1.75$, $P = 0.09$). To further address the possibility that differences in B were due to differences in sampling time, we compared B levels of a subset of the two groups whose blood samples were collected within 4 min of capture. Again, T-males reacted rapidly to capture and handling ($F = 8.28$, $n = 14$, $P = 0.01$, $r^2 = 0.41$; $y = 11.97x - 3.02$), whereas C-males did not ($F = 0.08$, $n = 23$, $P = 0.79$, $r^2 = 0.004$; $y = 0.65x + 16.46$); the slopes of the regression lines differed significantly ($F = 6.53$, $P = 0.02$).

T-females also showed an increase in B levels with initial sampling time ($F = 9.99$, $n = 17$, $P = 0.006$, $r^2 = 0.40$; $y = 9.83x - 4.89$), whereas C-females did not ($F = 0.88$, $n = 24$, $P = 0.36$, $r^2 = 0.04$; $y = 2.36x + 12.36$). However, the slopes of the regression lines were not significantly different for T- and C-females ($F = 3.40$, $P = 0.07$; Fig. 2).

B levels extrapolated to 1 min post-capture should approximate basal levels. Extrapolated basal levels of B were higher in T-males (24.18 ± 4.1 ng) than in C-males (14.65 ± 1.8 ng; $t = -2.13$, $P = 0.04$) but were not significantly different between T-females (16.41 ± 3.5 ng) and C-females (15.9 ± 2.6 ng; $t = -0.13$, $P = 0.91$).

*Body mass and initial levels of corticosterone.—*In T-males, lighter individuals had higher extrapolated basal levels of B than did heavier in-

dividuals ($F = 16.42$, $n = 16$, $P = 0.001$, $r^2 = 0.54$; $y = -8.93x + 209.09$), whereas the two variables were unrelated in C-males ($F = 1.24$, $n = 23$, $P = 0.28$, $r^2 = 0.056$; $y = -1.78x + 52.0$; Fig. 3). The slopes of the regression lines differed significantly ($F = 7.28$, $P = 0.011$).

Surprisingly, the same analysis revealed a significant negative relationship between body mass and extrapolated basal levels of B in C-females ($F = 5.38$, $n = 23$, $P = 0.03$, $r^2 = 0.20$; $y = -5.91x + 134.86$) but not in T-females ($F = 0.22$, $n = 16$, $P = 0.65$, $r^2 = 0.02$; $y = -1.89x + 54.03$; Fig. 4). The slopes of the regression lines were not significantly different ($F = 0.79$, $P = 0.38$).

*Serial sampling of corticosterone.—*B increased significantly with handling time in males sam-
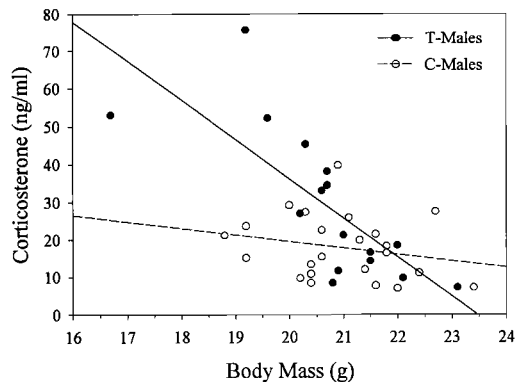

FIG. 3. Corticosterone levels extrapolated to 1 min post-capture as a function of body mass in T-male and C-male Dark-eyed Juncos.
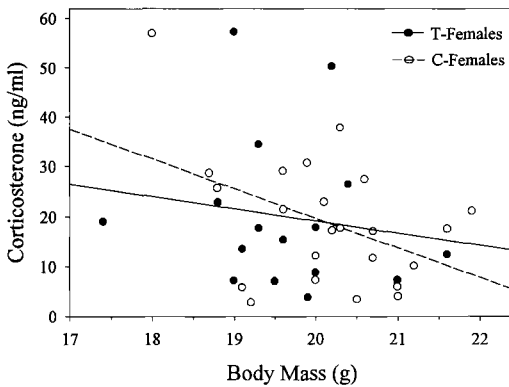
FIG. 4. Corticosterone levels extrapolated to 1 min post-capture as a function of body mass in T-female and C-female Dark-eyed Juncos.

pled at 10, 30, and 60 min post-capture (repeated-measures ANOVA, $F = 28.12$, $P < 0.001$). Levels appeared to be higher in T-males, but treatment had no significant effect on B ($F = 3.51$, $P = 0.08$, Fig. 5). In contrast, the B response of females showed neither an effect of handling time ($F = 2.51$, $P = 0.10$) nor an effect of the treatment of their mates ($F < 0.001$, $P = 0.99$).

The apparent lack of an adrenal response to capture and handling in females led us to incorporate the extrapolated B values into the repeated-measures model. This analysis revealed a marked B response to capture and handling in females ($F = 43.46$, $P < 0.001$) and males ($F = 80.15$, $P < 0.001$). However, there were no significant differences attributable to treatment (males, $F = 3.43$, $P = 0.08$; females, $F < 0.001$, $P = 0.99$).

Inspection of these data led us to compare males and females irrespective of treatment. Males had significantly higher B than females ($F = 43.09$, $P < 0.001$; Fig. 6), and B increased significantly with handling time ($F = 15.56$, $P < 0.001$). Furthermore, when the extrapolated B data were incorporated, significant differences again were attributable to sex ($F = 22.67$, $P < 0.001$) and handling time ($F = 131.40$, $P < 0.001$).

DISCUSSION

Initial levels of corticosterone.—Our findings that T-males have higher baseline levels of B than do C-males is consistent with results of other studies of Dark-eyed Juncos (Ketterson et
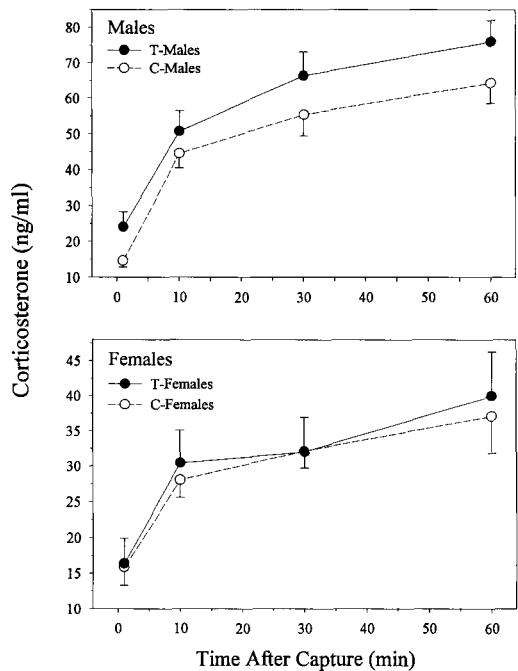

FIG. 5. Corticosterone levels from serially collected blood samples in T- and C-male (upper panel) and T- and C-female (lower panel) Dark-eyed Juncos. Values are $\bar{x} \pm$ SE. Data at 1 min post-capture are extrapolated for each individual using the rate of increase from initial sample to sample at 10 min.

al. 1991, Klukowski et al. 1997). Interestingly, our finding that the HPA axis of T-males responds more rapidly to capture and handling than that of C-males (Fig. 1) does not agree
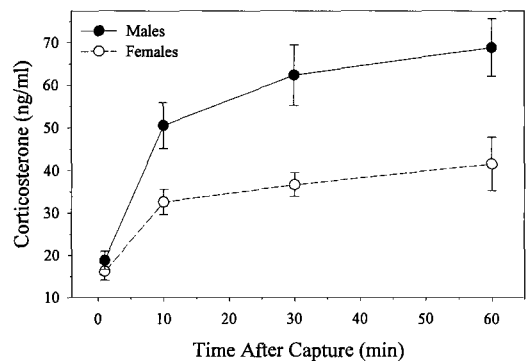

FIG. 6. Capture-stress responses of male and female Dark-eyed Juncos irrespective of treatment. Values are $\bar{x} \pm$ SE. Data at 1 min post-capture are extrapolated for each individual using the rate of increase from initial sample to sample at 10 min.

with the study by Klukowski et al. (1997), who found no difference in the rate of B increase with handling time, despite T-males having higher basal B levels. However, Dark-eyed Juncos housed in individual cages with access to *ad libitum* food are not exposed to the full spectrum of environmental conditions and social interactions that free-living individuals would experience. Consequently, the absence of an effect of T on the stress responses of captive juncos observed by Klukowski et al. (1997) is not surprising.

Our data support the hypothesis that the HPA axis is stimulated by chronically elevated testosterone to have an increased sensitivity to stressors and/or an increased secretory ability. If the response induced by the capture-stress protocol is comparable to the natural response to acute stressors encountered by juncos, then the highly responsive adrenocortical reaction in T-males may reflect a cost of elevated T that has not been documented before. Compared with controls, T-males are known to: (1) increase the size of their home range (Chandler et al. 1994); (2) increase their song rate (Ketterson et al. 1992); and (3) decrease the amount of time spent preening and sleeping (S. Lynn unpubl. data). It seems that increasing activity while reducing self-care results in increased adrenocortical sensitivity to stressors.

Our data on females are less consistent and, therefore, more difficult to interpret. The extrapolated B data, which revealed no differences in baseline B between T- and C-females, agree with an earlier report by Ketterson et al. (1991). However, the increase in initial B with time required for sample collection in T-females suggests that T-females are more sensitive to stressors than are C-females (Fig. 2). In Dark-eyed Juncos, T-females must feed their young more frequently than C-females because their mates come to the nest less often and bring less food (Ketterson et al. 1992). Similar compensatory feeding by mates of T-males has been reported for House Sparrows (*Passer domesticus*; Hegner and Wingfield 1987), Pied Flycatchers (*Ficedula hypoleuca*; Silverin 1980), Barn Swallows (*Hirundo rustica*; Saino and Møller 1995), and Lapland Longspurs (*Calcarius lapponicus*; Hunt et al. 1998). Furthermore, when Wolf et al. (1990) removed males from pairs of juncos, females compensated for the absence of their mates by increasing their own parental ef-

fort. If female juncos work harder to compensate for their mates' reduced paternal care, they may have less time to devote to self maintenance and, as a result, may be more sensitive to stressors. This suggestion is supported by Silverin (1983), who found that female Pied Flycatchers that raised nestlings unaided by their mates had higher levels of B than did females that were assisted by their mates.

The higher baseline levels of B in T-males than in controls may reflect chronically elevated B. The well-documented gluconeogenic actions of B are thought to be essential for the mobilization of energy stores, and this function may be especially important in facilitating short-term responses to stressors by increasing available glucose to fuel the fight-or-flight response. However, because B preferentially mediates the breakdown of proteins, in the long-term it can cause severe muscle catabolism (Selye 1979). Sapolsky (1992) listed several components of the stress response, in addition to gluconeogenesis, that can result in pathology, e.g. suppression of digestion, growth, immunity, and reproduction. In addition, Wingfield (1994) postulated that elevated B can induce behaviors inconsistent with the completion of reproductive effort (see below). If, as our data suggest, experimentally elevated T sensitizes the HPA axis and causes chronically elevated B, then it follows that a bird with elevated T is more likely to suffer from one or more of the above pathologies. If so, maintaining high T for a prolonged period may be disadvantageous and, thus, could be expected to be selected against.

Serial sampling of corticosterone.—Although our data from repeated sampling of B did not differ significantly according to treatment, T-males tended to have higher B than did C-males (Fig. 5). Furthermore, the levels of B extrapolated to 1 min were higher in T-males, and B rose more rapidly in T-males than in C-males (Fig. 1). Despite the fact that T-females showed a significant early increase in B in response to capture and handling, whereas C-females did not (Fig. 2), B levels of the two treatment groups did not differ (Fig. 5). Given the potential negative effects of B described above, the data from serial sampling further suggest that elevated T has costs, especially for males.

The clear difference in the B responses of male and female juncos to the capture-stress

paradigm (see Fig. 6) may reflect a sex differ- ence in the ability to modulate the adrenocor- tical response to stress. Mounting evidence suggests that some species of birds can dampen their stress response during the breeding sea- son (see below). This ability may minimize the negative effects of B upon the reproductive axis. Alternatively, it may suppress foraging or irruptive behavior, both of which are promoted by B, and both of which would interfere with parental care (Silverin 1986, Wingfield and Sil- verin 1986, Wingfield et al. 1990, Astheimer et al. 1992). Wingfield (1994) posited that the abil- ity to reduce the stress response is an adapta- tion to minimize nest abandonment during pe- riods of environmental perturbations. Indeed, desert species such as Black-throated Sparrows (*Amphispiza bilineata*), Cactus Wrens (*Campylo- rhynchus brunneicapillus*), Curve-billed Thrash- ers (*Toxostoma curvirostre*), and Abert's Towhees (*Pipilo aberti*) have significantly lower B re- sponses to capture and handling during the breeding season than during the nonbreeding season (Wingfield et al. 1992). Additionally, arctic-living Common Redpolls (*Carduelis flam- mea*) also modulate their stress response during breeding (Wingfield et al. 1994a). Unfortunate- ly, our data do not permit us to determine whether female juncos vary seasonally in their adrenocortical response. Interestingly, both adult and hatching-year female White-throated Sparrows (*Zonotrichia albicollis*), which are eco- logically comparable to juncos, had a greater stress response than males when tested during the fall (Schwabl 1995).

In summary, relative to controls, male juncos treated with testosterone have: (1) higher base- line levels of corticosterone; (2) a more rapid corticosterone response; and (3) a tendency to- ward higher levels of corticosterone in repeated samples, all of which suggest possible costs to the maintenance of high levels of testosterone. Additionally, differences in the adrenocortical response of males and females that we docu- mented are provocative, especially in light of seasonal and sexual differences reported for other species.

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