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The Effect of Exogenous Testosterone on Parental Behavior, Plasma Prolactin, and Prolactin Binding Sites in Dark-Eyed Juncos

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Numerous studies have shown that parental behaviors are mediated by prolactin (PRL), while testosterone (T) interferes with their full expression. The limited data available suggest that reduced parental behavior induced by T is not mediated by reduced concentrations of plasma PRL. We hypothesized that T reduces parental behaviors by reducing PRL receptor binding activity at central neural sites that promote the expression of parental behaviors. To test this hypothesis we implanted male dark-eyed juncos (*Junco hyemalis*) with testosterone-filled or empty implants and measured T and PRL levels, paternal behavior, and specific binding of radiolabeled PRL at selected brain regions that have been implicated in the mediation of parental behaviors. Our findings concurred with previous studies in that T-treated males reduced their parental contributions, had higher levels of T, and had equivalent levels of PRL compared with controls. We found no differences in the capacity to bind ^{125}I-oPRL in three brain regions previously implicated in the mediation of parental care in birds, i.e., the preoptic area, ventromedial nucleus of the hypothalamus, and paraventricular nucleus of the hypothalamus. Thus our findings do not support the hypothesis that T interferes with the expression of parental behavior by reducing PRL receptor binding activity at central sites. © 1998 Academic Press

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In the majority of temperate-zone avian species that have been studied, males, whether or not they exhibit parental behavior, have elevated levels of the sex steroid hormone testosterone (T) early in the reproductive season. Following the sexual stage when territory establishment, courtship, pair formation, and copulation take place and immediately prior to the parental stage when the behaviors mediated by T would be inappropriate, T declines and remains generally low while dependent young are provisioned. Indeed, in studies in which T has been artificially elevated during nestling care, males have responded by decreasing their parental effort (Silverin, 1980; Hegner and Wingfield, 1987; Ketterson, Nolan, Wolf, and Ziegenfus, 1992; Saino and Møller, 1995; Ketterson, Nolan, Cawthorn, Parker, and Ziegenfus, 1996; Hunt, Hahn, and Wingfield, in press).

Testosterone levels decline at the onset of the parental phase when nesting-related stimuli act to promote an increase in PRL (see reviews in Ball, 1991; Goldsmith, 1991; Buntin, 1996). Numerous correlative and experimental studies have demonstrated a role for prolactin (PRL) in the mediation of incubation and nestling care; however, the link between prolactin and care of nestlings is not as clear as the link with incubation (for review, see Buntin, 1996). Regardless, the preponderance of information suggests that full expression of paternal care occurs only if testosterone decreases and PRL becomes elevated in the correct temporal sequence.

Although several studies have noted that T treatment during the parental stage results in reduced paternal care, only one study that we are aware of has measured plasma PRL in males implanted with T at

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this time. Oring, Fivizzani, and El Halawani (1989) experimentally elevated testosterone in the sex-role reversed spotted sandpiper (*Actitis macularia*) during incubation (which is performed by males), and, although PRL levels in testosterone-treated males were equivalent to those of control males, some testosterone-implanted males abandoned their nests altogether while others reduced the time spent incubating. A study of song sparrows (*Melospiza melodia*), a species in which the typical sex roles are not reversed and male parental care consists of sharing in the feeding of nestlings, measured testosterone, PRL, and parental care in males whose mates were implanted with estradiol. Females responded to their treatment by soliciting copulations well beyond the normal period, and males reacted by maintaining endogenous T at high levels throughout the parental stage (i.e., beyond the sexual stage). Prolactin levels, however, were unaffected (Wingfield, Ronchi, Goldsmith, and Marler, 1989). These findings are similar to those of the spotted sandpiper study in that, despite the absence of an effect on PRL levels, males with elevated *endogenous* testosterone provide less parental care than males whose mates received no estradiol implant. These studies suggest that the mechanism by which T prevents parental behavior is independent of PRL.

Both PRL and T act peripherally and centrally to influence physiology and behavior (Balthazart, 1983; Buntin, Ruzycki, and Witebsky, 1993; Buntin, 1996). In those species that have been examined, specific binding of PRL is generally high in the preoptic area (POA) and in regions within the hypothalamus (see review in Buntin, 1996). Additionally, results of infusion of PRL directly into the POA or, alternately, of lesioning of this area support the hypothesis that PRL binds at sites in the POA and mediates parental behavior in both birds and mammals (Youngren, El Halawani, Silsby, and Phillips, 1989; Youngren, El Halawani, Phillips, and Silsby, 1991; Rosenblatt, 1992; Buntin *et al.*, 1993; Hnasko and Buntin, 1993; Slawaski and Buntin, 1995; Buntin, 1996).

One mechanism whereby testosterone might interfere with the expression of parental behavior is by reducing PRL receptor binding activity at neural sites that ordinarily lead to parental behavior. Steroid hormones are known to regulate populations of their own receptors (homospecific regulation) and of others (heterospecific regulation) at both peripheral and central sites (review in Hollenberg, 1985). While their homospecific actions are generally down-regulatory, their heterospecific actions are primarily up-regulatory. However, there are enough instances of heterospecific down-regulation of receptors by steroids (or steroid mimics) to merit examination of the effect of T on PRL receptors. Of particular interest is the report that the up-regulation of PRL receptors in mouse mammary glands is inhibited by progesterone *in vivo* and *in vitro* (Nishikawa, Moore, Nonomura, and Oka, 1994). If the hypothesis that T reduces PRL receptor binding activity is correct, then even in the presence of an adequate hormonal signal (i.e., elevated PRL) the mechanism(s) needed to transduce the hormonal message appropriately may be absent or compromised.

As part of ongoing investigations of the effects of T on male parental behavior in dark-eyed juncos (*Junco hyemalis*) and as a continuation of earlier work that found that parental male juncos had higher PRL levels than nonparental males (Ketterson, Nolan, Wolf, and Goldsmith, 1990), we compared testosterone-implanted males (T-males) with control males (C-males) with respect to: (1) brain PRL receptor populations, (2) PRL levels, (3) T levels, and (4) paternal behavior.

METHODS

General Methods

The study population of Carolina dark-eyed juncos (*J. h. carolinensis*) that we study inhabits the immediate vicinity of Mountain Lake Biological Station, in western Virginia (37° 22′ 30″ N, 80° 31′ W). This facility of the University of Virginia is in the Appalachian Mountains, at 1180 m elevation. The reproductive biology of an immediately adjacent population of juncos has been under study by Ketterson, Nolan, colleagues, and students since 1983.

Carolina juncos are attitudinal migrants that summer at high elevation. During the breeding season males are territorial and share their territory with a single female. Females do most of the nest building and are the sole incubators of four egg clutches. Both members of a pair defend the nest and feed and protect nestlings and fledglings. Data were collected during the spring and summer of 1996.

All procedures described herein were in compliance with and authorized by the Animal Care Committee of Indiana University.

Testosterone Implants

Almost all males were captured and implanted early in the season, either before or just after pairs initiated nest building; however, in a few cases im-

plantation did not take place until after incubation was initiated. Individuals were anesthetized by inhalation of metophane and implanted with two 10-mm-long Silastic tubes (Dow Corning; 1.473 mm i.d., 1.956 mm o.d.) that were either empty or packed with crystalline testosterone (Sigma Chemical Co.; see Wingfield and Farner, 1976; Ketterson, Nolan, Wolf, Ziegenfus, Ball, and Johnsen, 1991; Schoech, Mumme, and Wingfield, 1996). T implants of these dimensions are known to elevate T to levels that are similar to the early season natural maximum of unimplanted males, and implants cause levels to remain elevated throughout the breeding season (Ketterson *et al.*, 1991, 1996; Enstrom *et al.*, 1997). These studies report that mean T levels in T-males are approximately threefold higher than those in C-males (see Fig. 1 in Ketterson *et al.*, 1991, and Fig. 2 in Enstrom *et al.*, 1997). Implants were inserted subcutaneously along the left flank beneath the wing. The initial male captured was given a T-filled implant, the next was given an empty implant, and treatment of subsequent males was alternated accordingly. The data presented here are from seven T-males and seven C-males (see below for exceptions).

Behavioral Observations

We found nests of the 14 pairs and conducted focal nest observations when nestlings were 5 or 6 days of age; all nests contained either three or four nestlings. Observations were made with a spotting scope from a blind placed as far from the nest as possible or from a parked automobile with its windows covered. All watches lasted 1 h and began between 0700 and 1100 AM unless the parents indicated that our presence disturbed them; in these unusual cases we waited until they stopped calling before we began the observation period. We noted the following: (1) each parent's visits to the nest; (2) quantity of food delivered (see below); (3) time spent delivering food, inspecting the nest and/or its contents, and brooding (females only); and (4) number of fecal sacs (the feces of nestlings contained within a membrane) removed. The amount of food delivered during each visit was scored from 1–3, with 1 representing a single food item, 3 representing a bill-full, and 2 representing an intermediate quantity. All focal watches were conducted by S.J.S., which standardized the scores assigned. In one case, although both parents were present and regularly fed their young, their positions made it impossible to determine how much food was delivered. In another case, the location of the nest made it impossible to conduct focal observations. Unfortunately, both of these exceptions were control males.

Blood Sample and Brain Collection

Immediately following focal watches, males were captured in mist nets (to which they were attracted either in response to seed used as bait, tape playback of male junco song, or a combination of song playback and a male junco in a cage used as a lure), and a blood sample was taken. Samples were collected in microhematocrit tubes from a puncture in the brachial vein (26-gauge needle) and kept cool on ice until transport to the laboratory. To control for possible diel fluctuations in hormone levels all but 2 of the 22 samples were collected between 0700 and 1200. Samples were then centrifuged; the plasma fraction was harvested, stored at −20°C, and then transported on dry ice to Indiana University for later assay. Subsequently, a small volume of each plasma sample was shipped on dry ice to Scotland for PRL assay in the laboratory of Peter Sharp (see below). Preliminary analysis found no effect of the different capture methods employed or the hour of collection upon levels of either T or PRL, and therefore all samples were combined for statistical analyses.

Immediately after blood samples were taken, birds were decapitated and their brains harvested, frozen immediately on powdered dry ice, wrapped in parafilm and aluminum foil, transported to the laboratory on dry ice, and stored at −80°C. Brains were later shipped to the University of Wisconsin–Milwaukee for sectioning and assay of receptors.

Tissue Preparation and in Vitro Binding Assay

Frozen brains were sectioned coronally at 20 μm in a Lipshaw cryostat that maintained a temperature of −20°C throughout. Adjacent sections were collected on sequential glass slides, air-dried overnight, and then stored at −80°C until assay.

Ovine PRL (NIADDK-oPRL-I-3) was radiolabeled to a specific activity of 82 μCi/μg with ^{125}I (New England Nuclear, Boston, MA) using a lactoperoxidase method. Briefly, 5 μg of oPRL in phosphate buffer was combined with 0.5 mCi ^{125}I in 10 μl of 0.5 M phosphate buffer, 2.5 ng hydrogen peroxide, and 0.1 μg lactoperoxidase (Sigma) for 3 min. The reaction was quenched with 300 μl of 25 mM Tris–HCl buffer. The labeled hormone was separated on a Sephadex G-50 column that used 25 mM Tris with 0.2% bovine serum albumin (BSA) as the elution buffer (pH 7.4).

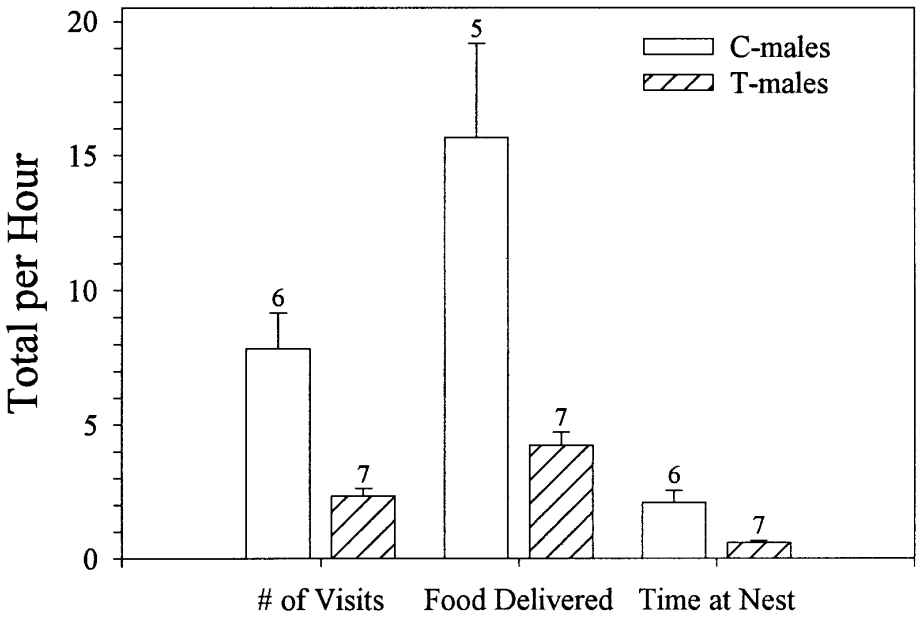

FIG. 1. Results of male behaviors during 1-h watches at nests of dark-eyed juncos. T-males (see text) visited the nest less often, delivered less food, and spent less time (min) than C-males. Note that because quantity of food delivered by one C-male could not be determined, and the nest of another C-male was in a location that precluded observation (see Methods), sample sizes (shown above error bars) differ among measures.

Prior to use, the labeled hormone was repurified on a Sephadex G-100 column (for further details, see Buntin *et al.*, 1993; Li *et al.*, 1995). The radiolabeled oPRL that was used for the binding assay was pooled and repurified from six separate iodinations.

All slide-mounted brain sections were initially preincubated at 4°C for 4 h in plastic Coplin jars filled with 25 mM Tris assay buffer with 0.2% bacitracin, 0.2% BSA, and 10 mM $CaCl_2$. They were then incubated at 4°C in buffer containing ^{125}I-labeled PRL (1.5×10^5 cpm/ml). After 88 h, the incubation buffer was discarded, and brain sections were rinsed in cold (4°C) assay buffer three times for 20 min. Each slide was then dipped three times in cold (4°C) dH_2O, after which the slides were placed on absorbent paper and allowed to dry overnight. The slide-mounted sections and ^{125}I polymer standards (Autoradiographic [^{125}I] Microscales, Amersham Corp., Arlington Heights, IL) were then placed in X-ray cassettes and exposed to Hyperfilm-βmax (Amersham) for 3 days.

Densitometric analysis of autoradiograms was conducted using the MCIDM4 imaging software from Imaging Research Inc. (St. Catharines, Ontario, Canada). We compared specific binding in the POA, the paraventricular nucleus of the hypothalamus (PVN), and the ventromedial nucleus of the hypothalamus (VMN) using the pigeon brain atlas of Kartan and Hodos (1967) and the chick brain atlas of Kuenzel and Masson (1988) for anatomical reference. We focused on these nuclei because they contain PRL binding sites in other species and because they have been implicated in mediating parental and feeding behavior (see Buntin, 1996).

As a control for possible variations in tissue thickness and background density, as well to control for nonspecific binding of ^{125}I-PRL, we subtracted binding values for the neostriatum (an area with few or no PRL receptors) from the brain region of interest in each section. All specific binding values are expressed as disintegrations per minute per milligram (dpm/mg) of the ^{125}I polymer standard. For the POA, specific binding readings were obtained from a minimum of three separate brain sections in each animal. For the PVN and VMN, a minimum of two readings were obtained.

Hormone Measurement

Plasma testosterone was measured by radioimmunoassay following separation from other steroid hormones by column chromatography (for details of procedures and reliability criteria see Wingfield and Farner, 1975; Ball and Wingfield, 1987). One notable exception to the methodology cited is that for sample

TABLE 1

Mean Time (min) Spent at the Nest and Mean Score for Quantity of Food Delivered to Nestlings per Visit (i.e., Total Time or Amount, Divided by Number of Visits per Hour)

	Males		Females	
	T-males	C-males	T-females	C-females
Time / visit	0.29 ± 0.07 (7)	0.24 ± 0.01 (6)	0.36 ± 0.07 (7)	0.38 ± 0.09 (6)
Comparisons	$U = 17.5$, $P = 0.62$		$U = 23.0$, $P = 0.78$	
Amount / visit	1.85 ± 0.24 (7)	1.81 ± 0.14 (5)	1.88 ± 0.20 (7)	1.76 ± 0.17 (5)
Comparisons	$U = 19.5$, $P = 0.74$		$U = 16.0$, $P = 0.81$	

Note. U value from Mann–Whitney U test. Scores used to quantify food delivered to young were based on observations of food items carried to the nests in the bills of parents (see Methods for details).

extraction we used anhydrous diethyl ether rather than dichloromethane. Prior to extraction, 2000 cpm of radiolabeled T was added to each sample to permit calculation of the percentage of hormone recovered (70.4%). All samples were run in a single assay after the end of the field season (intra-assay coefficient of variation was 12.4%). The mean plasma volume (μl) plus or minus the standard error was 76.8 ± 2.8. Antibody was purchased from Wien Laboratories, standard was from Sigma, and radiolabeled T was from New England Nuclear.

Plasma PRL was measured using a radioimmunoassay developed for passerine PRL using recombinant-derived European starling (*Sturnus vulgaris*) PRL (for assay detail see Bentley, Goldsmith, Dawson, Glennie, Talbot, and Sharp, 1997). The antibody reared against starling PRL showed good cross-reactivity with junco PRL as determined by the high degree of parallelism between dilutions from a pool of junco plasma and the standard curve. Duplicate aliquots (10 μl) were measured in a single assay. The intra-assay coefficient of variation was 6.2%.

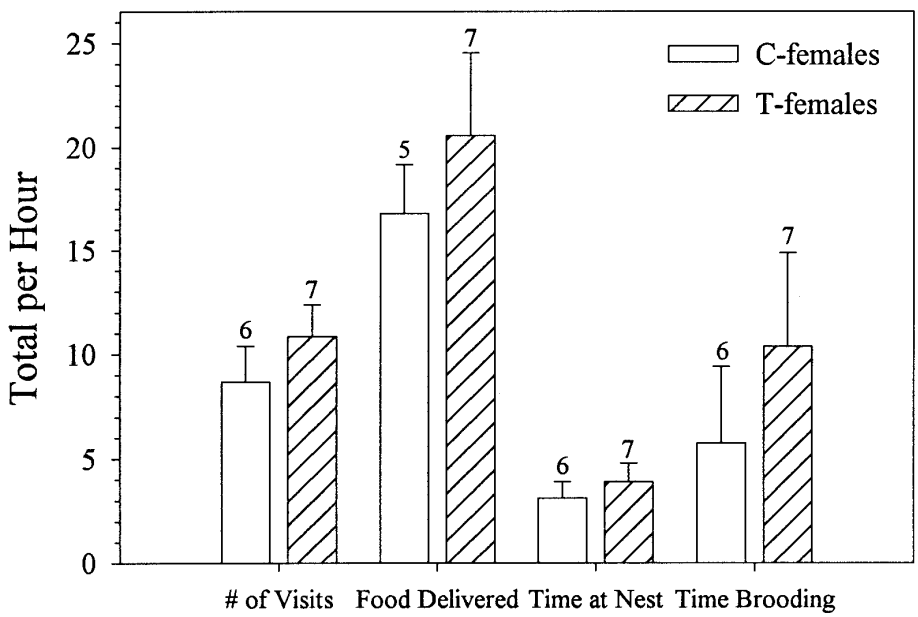

FIG. 2. Results of female behaviors during 1-h watches at nests of dark-eyed juncos. T-females and C-females (note that females are designated according to treatment of their mates and did not themselves receive implants; see text) did not differ in number of visits, quantity of food delivered, total time (min) feeding and inspecting, or total time brooding nestlings. Because quantity of food delivered by one C-female could not be determined, and the nest of another C-female was in a location that precluded observation (see Methods), sample sizes (shown above error bars) differ among measures.

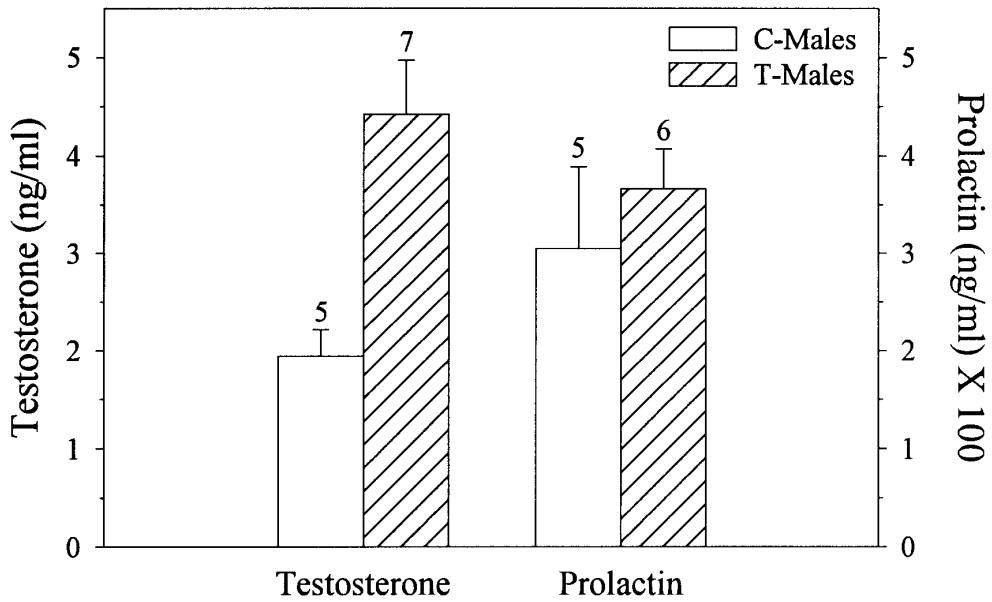

FIG. 3. Plasma levels of testosterone and prolactin in T-males and in C-males. T-males had higher T levels than C-males but there were no differences in prolactin levels.

Statistical Analyses

Behavioral comparisons were made with Mann–Whitney *U* tests. Hormonal and brain region comparisons were made with *T* tests. Systat 6.0 for Windows was used for all statistical tests.

RESULTS

Behavioral Observations

Testosterone-implanted males visited the nest fewer times per hour (Mann–Whitney two-tailed $U = 41.5$, $P = 0.003$), delivered less total food to dependent young ($U = 34.5$, $P = 0.005$), and spent less total time at the nest than C-males ($U = 42.0$, $P = 0.003$; Fig. 1). Interestingly in light of these differences, T- and C-males did not differ in number of fecal sacs removed from the nest ($U = 32.5$, $P = 0.075$). T- and C-males spent similar amounts of time at the nest during a nest visit, and the mean quantity of food delivered per trip to the nest did not differ between treatment groups (see Table 1).

Although females mated to T-males (T-females) tended to contribute more than females mated to C-males (C-females) for all measures considered (see Fig. 2 and Table 1), no differences were significant, e.g., number of visits ($U = 15.0$, $P = 0.75$), amount of food delivered ($U = 14.5$, $P = 0.24$), time spent at the nest (feeding and inspecting) ($U = 18.0$, $P = 0.69$), time spent brooding nestlings ($U = 18.0$, $P = 0.65$), or number of fecal sacs removed ($U = 12.0$, $P = 0.19$).

To determine whether nestlings received less total care (parents combined) as a result of T treatment of males, we pooled data for each pair and compared some of the above measures. Despite the reductions in care by T-males, nestlings of the treatment groups did not differ in total visits per hour ($U = 106.0$, $P = 0.26$), total food received per brood per hour ($U = 93.0$, $P = 0.18$), or total time adults were at the nest feeding or inspecting ($U = 110.0$, $P = 0.18$).

Hormone Comparisons

T-males had significantly higher T levels than C-males ($T = -4.04$, $P = 0.003$; Fig. 3). Prolactin levels, however, did not differ between treatments ($T = -0.66$, $P = 0.54$; Fig. 3).

Prolactin Receptors

We found no differences between T- and C-males in specific binding of radiolabeled PRL in any of the brain regions examined: the POA ($T = 0.77$, $P = 0.45$), the PVN ($T = -0.58$, $P = 0.57$), or the VMN ($T = -0.94$, $P = 0.36$; Fig. 4).

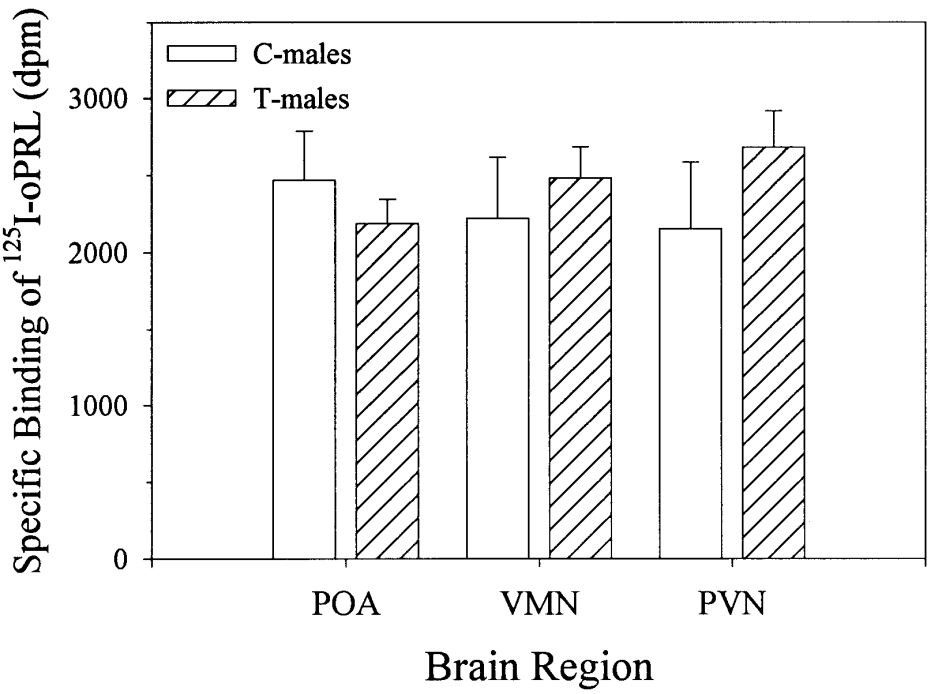

FIG. 4. Specific binding of ^{125}I-oPRL in the three brain regions tested. There were no differences due to treatment in specific binding of radiolabeled prolactin in the preoptic area (POA), ventromedial nucleus of the hypothalamus (VMN), or paraventricular nucleus of the hypothalamus (PVN). Sample sizes equal 7 in all cases.

DISCUSSION

Our findings provide further evidence (see introduction) that prevention of the decline in plasma T that ordinarily occurs prior to the onset of the parental phase interferes with the expression of parental behavior (Fig. 1). Such interference occurs irrespective of whether the T is exogenous (spotted sandpipers, Oring *et al.*, 1989; dark-eyed juncos, present paper) or endogenous (male song sparrows exposed to estradiol-treated females, Wingfield *et al.*, 1989). Moreover, elevated T does not achieve its effect by suppressing plasma PRL: T-male juncos did not differ from C-males in plasma PRL, which also confirms earlier findings on spotted sandpipers (Oring *et al.*, 1989) and song sparrows (Wingfield *et al.*, 1989).

While T led to a reduced number of visits to the nest by males and, as a result, the amount of food T-males delivered to young, we did not find that females compensated for their mates' reduced care (Fig. 2). This contrasts with previous findings in this species (Ketterson *et al.*, 1992). Paradoxically, when the total amount of food provided by both parents is considered, nestlings received statistically equivalent care irrespective of treatment of males.

Results of the binding assay, which revealed no treatment-related difference in the ability of the POA to bind PRL, failed to support the hypothesis that T interferes with parental behavior by altering PRL receptor density. However, given that more than 20 brain regions in ring doves (*Streptopelia risoria*) are known to specifically bind PRL (Buntin *et al.*, 1993), we cannot rule out the possibility that T might reduce PRL receptor binding activity in brain regions that we did not examine. Nevertheless, there is considerable evidence that the POA plays an important role in mediating parental care. Intracerebroventricular infusion of PRL induces feeding of young by ring doves (Buntin, Becker, and Ruzycki, 1991) and incubation by female turkeys (*Meleagris gallopavo;* Youngren *et al.*, 1991). However, infusion studies can be criticized because they may deliver the hormone of interest to multiple brain nuclei, making it difficult to determine precisely which areas are responsible for any behavior elicited. Therefore, lesioning studies perhaps provide more compelling evidence that the POA (and possibly the VMN and lateral hypothalamus, LHy) is essential to the expression of parental behavior. In hen turkeys, for example, lesions of the POA, VMN, and LHy block incubation behavior (Youngren *et al.*, 1989). These au-

thors concluded, however, that loss of the POA was the primary cause of the deficit in behavior and that lesions of the VMN and LHy were disruptive simply because they interfered with a neural pathway connecting the POA and median eminence. Further evidence of the importance of the POA comes from a study of ring doves in which Slawski and Buntin (1995) found that lesioning the POA caused profound reduction in PRL-induced parental feeding in nonbreeding birds of both sexes. (For studies using lesions and PRL infusion that support a role for the POA in parental behavior in rats, see review in Numan, 1994; Bridges, Numan, Ronsheim, Mann, and Lupini, 1990).

If, as this evidence from the ring dove and turkey suggests, PRL binding in the POA is instrumental in the expression of parental behavior, then the results of our study are somewhat puzzling. T-males fed their offspring significantly less often than C-males, despite the absence of an effect of T treatment on either receptor populations in the POA or circulating levels of PRL. Three possible explanations come to mind. First is the possibility, already mentioned, that T affects PRL receptor populations in regions of the brain that we did not examine and that these regions are essential to transducing plasma PRL to parental behavior. Second is the possibility that while PRL may promote parental responses, it is not necessary for the display of these behaviors in juncos if other stimulatory cues are present during the posthatching period. In rats, there is evidence for a transition from hormonal to nonhormonal regulation of parental behavior in lactating females during the postpartum period (Fleming and Rosenblatt, 1974). Similarly, there is evidence in several avian species for a persistence of parental behaviors in the face of declining PRL levels as the posthatching period proceeds (see Buntin, 1996 for review). In addition, immunosuppression of PRL by passive immunization against the PRL-releasing factor vasoactive intestinal peptide (see Macnamee, Sharp, Lea, Sterling, and Harvey, 1986; Opel and Proudman, 1988, Sharp, Sterling, Talbot, and Huskisson, 1989) reportedly disrupts PRL-induced crop sac development in doves but did not disrupt incubation or squab feeding behavior (Lea, Talbot, and Sharp, 1991). Thus despite evidence from several avian species that PRL administration facilitates parental responses, we cannot rule out the possibility that parental behaviors can be expressed in the absence of the hormone.

A third possibility is that T and PRL act independently to promote competing behaviors. Upon coming to the nest to feed, T-males, like C-males, are stimulated by their young to collect more food. Presumably, PRL promotes this response to the young. While foraging, however, males of both treatments may encounter stimuli that could trigger behaviors that are incompatable with foraging and provisioning young, but T-males are more likely than C-males to be ''distracted'' by these stimuli and to be diverted from parental behavior. If, for example, T-males are more responsive than C-males to stimuli emanating from conspecific male neighbors [the link between territorial aggression and T is well established (see Balthazart, 1983; Wingfield, Ball, Dufty, Hegner, and Ramenofsky, 1987; Wingfield, Hegner, Dufty, and Ball, 1990)], they might be more likely to engage in territorial behavior. Other related testosterone-mediated behaviors may also contribute to the reduction of parental behavior by T-males. For example, it is our impression from the focal nest watches that T-males were far more likely to sing than were C-males, as has been demonstrated by other work on juncos. T-males sing more often (Ketterson *et al.*, 1992; Chandler, Ketterson, Nolan, and Ziegenfus, 1994) and have larger home ranges than C-males (Chandler *et al.*, 1994; Chandler, Ketterson, and Nolan, 1997), both of which might increase exposure to rival males or neighboring females and thus increase the amount of time spent away from parental duties.

In summary, our original hypothesis that T reduces PRL receptor binding activity or PRL receptor populations is not supported by our data. Our study concurs with other studies (see above) that have noted reductions in paternal behavior due to elevated T levels. Seemingly T and PRL facilitate conflicting behavioral responses which can be fully expressed only in temporal isolation.

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