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EFFECT OF SEX, STAGE OF REPRODUCTION, SEASON, AND MATE REMOVAL ON PROLACTIN IN DARK-EYED JUNCOS[1]

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Abstract. Prolactin has been associated with incubation and brooding in passerine birds, but its possible association with other parental behaviors remains unclear. We measured plasma concentrations of prolactin (prl) in Dark-eyed Juncos (*Junco hyemalis*), a species in which only females incubate and brood but both sexes feed nestlings. Breeding males and females were bled at the time their eggs hatched, and half the males were taken from their territories. Females and the remaining males were bled again when their young left the nest. Removed males were quickly replaced by new males, some of which we caught and bled. Replacement males courted the females but rarely fed their predecessors' young. Removed males were held in an aviary and bled again in late summer.

Prl concentrations were higher in females than males, both at hatching and at nest leaving. Female prl was higher at hatching than at nest leaving and did not vary seasonally. Male prl was higher at hatching than at nest leaving and higher earlier in the season than later.

Rearing young alone had no detectable effect on prl in females. Prl of replacement males was lower than that of fathers at hatching but not at nest leaving. Prl in removed males in the aviary was lower than in fathers at hatching but not at nest leaving. These patterns of prl secretion resemble those in other species that raise more than one brood per season and in which females provide the bulk of parental care. In addition, prl may be associated with male parental behavior in juncos.

Key words: Prolactin; Dark-eyed Junco; biparental care; parental behavior; incubation; brooding; nestlings; replacement males; male removal.

INTRODUCTION

Prolactin (prl) has frequently been associated with parental behavior in birds and mammals (Lehrman 1965, Goldsmith 1983, Rosenblatt 1984). However, because prl is important in a variety of other behavioral and physiological processes, the nature of the relationship between it and parental behavior remains unclear.

Studies of prl profiles from a variety of free-living birds in which the parental roles of males and females differ provide a comparative approach to the problem. During incubation, prl is higher in females than males in species (or populations) in which only females incubate (e.g., European Starling, *Sturnus vulgaris,* Dawson and Goldsmith 1982; Pied Flycatcher, *Ficedula hypoleuca,* Silverin and Goldsmith 1983; Song Sparrow, *Melospiza melodia,* Wingfield et al. 1989, Wingfield and Goldsmith 1990; White-crowned Sparrow, *Zonotrichia leucophrys,* Hiatt et al. 1987), and also in species in which both sexes incubate (European Starling; G. Ball, unpubl. observ.). When only males incubate or when male incubation predominates, male prl levels are higher than female levels (e.g., Wilson's Phalarope, *Phalaropus tricolor,* Oring et al. 1988; Spotted Sandpiper, *Actitis macularia,* Oring et al. 1986). When neither sex incubates, there is no sexual difference (Brown-headed Cowbird, *Molothrus ater,* Dufty et al. 1987). These observations clearly point toward a role for prl in incubation.

Brooding of young also appears to be associated with prl. In species with precocial young, prl secretion declines at hatching but nevertheless remains somewhat elevated during brooding (Goldsmith 1983 for review; also Hector and Goldsmith 1985; Oring et al. 1986, 1988). The

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same is apparently true for species with altricial young (Dawson and Goldsmith 1982, Silverin and Goldsmith 1983).

Beyond its role in the production of crop milk in columbiforms (Goldsmith et al. 1981, Lea 1987), much less is known about prl's potential involvement in forms of parental care other than incubation and brooding, e.g., in behaviors such as feeding nestlings or guarding young. It is known, however, that these activities, because they often persist beyond the stages of incubation and brooding of nestlings, can continue in the absence of peak levels of prl. In many species, males perform no incubation or brooding but do deliver food to nestlings. In these males is there a temporal correlation between feeding young and prl secretion? In the free-living passerines that have been studied so far, the data suggest that this may possibly be true, but they do not exclude other interpretations. Prl increases during the breeding season in male Pied Flycatchers (Silverin and Goldsmith 1983), European Starlings (Dawson and Goldsmith 1982), White-crowned Sparrows (Hiatt et al. 1987), and Song Sparrows (Wingfield et al. 1989, Wingfield and Goldsmith 1990), although these increases could simply reflect a response to long days (Dawson and Goldsmith 1985, Wingfield and Goldsmith 1990). Resolution of this question would be greatly aided by obtaining data on nonbreeding individuals. If prl were higher in males behaving parentally than in nonparental males at the same date, a role for prl in paternal behavior might be indicated (Dawson and Goldsmith 1985).

Another question concerns not simply the temporal correlation between parental behavior and prl but the quantitative relation between plasma concentration of prl and the frequency or duration of the behaviors. Some female Pied Flycatchers are aided by their mates while tending young but other females are not. An early report indicated that unaided females, which must compensate for lack of male help, might have higher prl than aided females (Silverin and Goldsmith 1983), but a later study reported no difference between the two categories of females (Silverin and Goldsmith 1984). Similarly, number and duration of incubation shifts did not correlate with prl in albatrosses (*Diomedea* spp., Hector and Goldsmith 1985).

We measured prl in a passerine species, the Dark-eyed Junco (*Junco hyemalis*). Only females incubate and brood the altricial nestlings, but both sexes deliver food. Males do not engage in courtship feeding. The study was part of a larger one in which we measured the reproductive benefits that males gain by providing parental care (Wolf et al. 1988, 1990, unpubl.). We removed males of experimental pairs at hatching and monitored subsequent histories of their mates and of control pairs. The removed males were quickly replaced by new males, often of unknown origin but typically, we believe, unmated prior to our manipulations. Most replacement males did not feed the nestlings of their predecessors, and the unassisted females fed their young twice as frequently as control females (Wolf et al. 1990). Unassisted females also spent a higher proportion of their time brooding during the first two-thirds of the nestling interval (Wolf et al. 1990).

We compared prl of control pairs and of experimental birds according to sex, stage of reproduction, season, and treatment; we also compared replacement males with fathers. Based on the findings in other species, among controls we expected prl of females to be higher than that of males and to be higher at hatching than at nest leaving. If frequency of feeding behavior is correlated with plasma prl concentration, then we would also expect prl to be higher in experimental females, because they doubled their feeding rate. Finally, if male parental behaviors are associated with prl, we would expect (a) fathers to have higher prl than replacement males, (b) prl to increase in replacement males that later mated and bred with the females made available by our removals, and (c) prl to fall in removed males held in captivity. Tests of these predictions follow.

METHODS

SPECIES AND STUDY AREA

We studied juncos at Mountain Lake Biological Station near Pembroke, Virginia, during the breeding seasons of 1985 and 1986 (see Wolf 1987 for description of the study area). Birds in this population live in flocks during winter (pers. observ.) and are sedentary or make short altitudinal migrations (Nolan et al. 1986). During the breeding season, males are territorial; pairs form in March or April and some remain together until October or possibly later (pers. observ.). Females build the nest and do all the incubation and brooding; males and females feed the nestlings and fledglings. Females begin a sec-

ond brood while the males continue to care for the first brood. The season is long enough to permit the occasional raising of three broods, but predation is very common and many pairs fail to rear any young to independence (see Wolf et al. 1988, 1990, unpubl.).

BLOOD SAMPLING

This study is based on 137 blood samples taken from 95 individual birds. All except nine samples were taken from free-living juncos; the exceptions were taken from caged males. Free-living birds were caught in mist nets or (much less frequently) in Potter traps (7% of cases). Most (82%) of the samples were taken before noon. Birds were caught either as they approached or left their nests to incubate, brood, or deliver food (72%), or they were caught when they flew into nets in response to tape-recorded distress screams of nestlings (21% of samples) or male song (7% of samples). Because response to vocalizations could alter prl, we took samples from birds captured in this way only if capture was within 3 min of the time we began to play the tape. A comparison of females caught at nest leaving with and without the help of distress screams indicated no difference in their prl (screams: $n = 9$, $\bar{x} = 99.5$ ng/ml, SD = 77.44; no screams: $n = 6$, $\bar{x} = 83.4$ ng/ml, SD = 11.32, $t = 0.5$, $P = 0.626$). Whatever the method, bleeding was completed within 10 min of initial disturbance (e.g., opening a net) in 74% of cases and within 15 min in 97%.

The caged males from which we took samples were birds that we had removed from their territories. These lived in large outdoor aviaries located on the study area. We bled them before noon on 20 July, or 22 July, or 3 August 1986. All were caught within one min of our arrival at the aviary, and bleeding was completed within 7 min.

Samples were taken by pricking the alar vein and collecting the blood in microhematocrit tubes. These were held on ice for up to 3 hr, usually much less, and were then spun down and the plasma drawn off. Plasma was stored in polyethylene microtubes at −20°C before being sent on dry ice to Bristol, United Kingdom, to be assayed.

THE ASSAY

We assayed for prl using the heterologous method of McNeilly et al. (1978), as modified by Goldsmith and Hall (1980). This method has recently been shown to provide results in close agreement with the homologous turkey assay of Burke and Papkoff (1980) (see Oring et al. 1986). Samples (10 or 20 μl) were assayed in duplicate and expressed in terms of ovine standard (NIADDK o-Prl ps-12). In order to validate the assay for the junco, a plasma pool at four doubling dilutions and three pituitary extracts at four tenfold dilutions were compared with the ovine standard. The parallel nature of the resulting curves is evident in Figure 1.

Two assays were performed, one on the 1985 samples and another on those from 1986. Inter- and intra-assay coefficients of variation for the prl assays are 12.3% ($n = 10$) and 5.2% ($n = 10$), respectively. The samples collected in 1985 and 1986 were, however, measured in assays conducted 18 months apart, and quality control plasma pools indicated approximately 50% higher values in the latter assay than in the first one. Rather than alter the assay results, we have presented the absolute prl levels as obtained (see figures) and have accounted for the difference between the 2 years in the statistical analysis, as described below.

DATA ANALYSIS

For analysis, samples were divided according to (1) stage of reproduction, (2) portion of the season (date), (3) treatment, and (4) sex, as follows:

Stage of reproduction. The nestling interval was divided into three stages according to age of nestlings (hatching day = day 0): stage 1, days 0–3 (47.6% of the stage 1 samples were taken on day 0); stage 2, days 4–7; and stage 3, days 8–11 (43.7% of the stage 3 samples were taken on day 11, i.e., at nest leaving).

Season or date. This variable was coded in two ways. Breeding begins in late April or early May, and we assigned samples a relative date, which was the number of days (inclusive) from 1 May to the day we collected samples. We also divided the season into approximate halves and quarters. May and June represent the first two quarters or the first half, and July and August the third and fourth quarters or the second half. The earliest sample was taken on 20 May, and the latest on 10 August.

Treatment. Females whose mates we removed at hatching are referred to as unaided females, and those whose mates were not removed as aided females. Because the treatment (male remov-

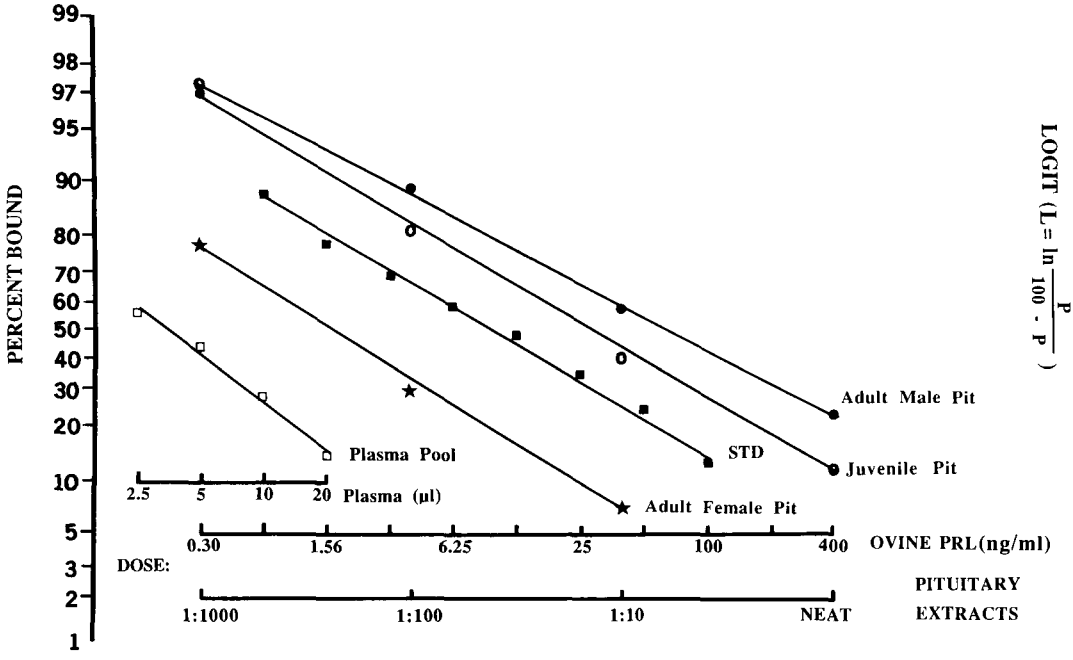

FIGURE 1. Comparison of dilution series of Dark-eyed Junco pituitaries and plasma to ovine standard, showing the parallel nature of the curves (see Methods).

al) was applied at hatching, there was no reason to expect females to differ at that time. If rearing young alone affects prl, then the female treatment groups would be expected to differ at nest leaving.

Males caught at hatching (stage 1) are referred to as fathers whether we removed them or returned them to their territories. At nest leaving (stage 3) the category fathers includes only males that were permitted to remain with their offspring and mates. Almost all removed males were replaced, and most replacement males appeared within 48 hr of removal of the original male. However, the time between their appearance and our taking of blood samples varied. Of 12 replacement males that we bled during the time when the females that they were associating with were feeding young, 10 ignored their predecessors' young (nonfeeding replacement males), and two delivered food to the nestlings (feeding replacement males).

Although we were primarily interested in differences in prl associated with stage of reproduction, date, treatment, and sex, variation with year/assay was a possible confounding factor (see above). Since all the samples from 1985 were run in one assay and all those from 1986 in another, any effects of year could not be distinguished from those of assay. As will be shown, males were more affected by year/assay than were females. In order to facilitate comparisons among analyses where it was and was not necessary to correct statistically for year and date, in our ANOVAs we consistently used year as a main effect in addition to the other variables of interest (e.g., stage of reproduction, sex, or treatment) and date as a covariate (SPSS, Nie et al. 1975). Such analyses permit multiple comparisons and generate means (but not standard errors) that are adjusted for other main effects. These adjusted means (adj. $\bar{x}$) are reported in the text where appropriate and may be compared to the unadjusted means and standard errors presented separately by year in Figure 2. All the comparisons reported below as statistically different when years were combined were also different in year-by-year comparisons except fathers at hatching vs. fathers at nest leaving in 1985 (t-test, $P = 0.062$) and males at nest leaving vs. females at nest leaving in 1986 (t-test, $P = 0.238$).

Two-tailed probabilities of less than 0.10 are reported, and those less than or equal to 0.05 are

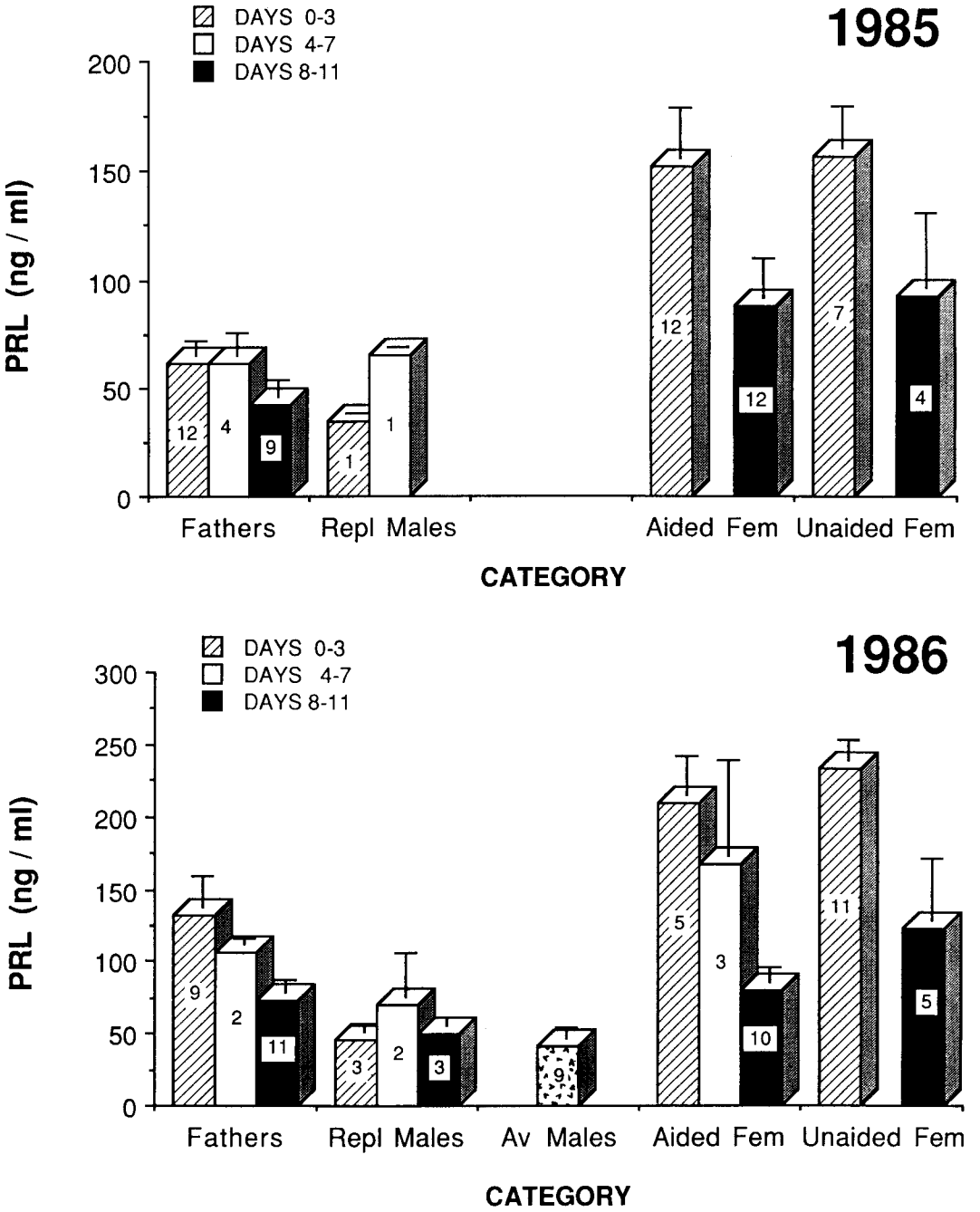

FIGURE 2. Prl (ng/ml ± SE) in male and female Dark-eyed Juncos during each of two breeding seasons (1985 and 1986). All but the aviary males were free living and were tending nestlings, except the replacement males were associating with a female that was tending nestlings but did not themselves feed young. Data are further subdivided according to the age of the nestlings (see legend). Sample sizes appear within the histogram bars.

considered statistically significant; values higher than 0.1 are reported simply as not significant (ns).

RESULTS

STAGE OF REPRODUCTION

Among fathers, after correcting for year and date, prl was higher at hatching than at nest leaving (Fig. 2; adj. $\bar{x}$, stage 1, 94.1 ng/ml, $n = 21$; adj. $\bar{x}$, stage 3, 56.7 ng/ml, $n = 20$) (ANOVA, stage, $P < 0.002$; year, $P < 0.001$; date, $P < 0.01$). During stage 2, the midnestling interval, prl was obtained from only six males, and the mean was intermediate between the values at hatching and nest leaving (adj. $\bar{x}$, 82.7 ng/ml, no statistical comparison made with stages 1 or 3).

Females resembled males: prl was higher at hatching than at nest leaving (Fig. 2; aided and unaided females combined, adj. $\bar{x}$, stage 1, 190.3 ng/ml, $n = 35$; adj. $\bar{x}$, stage 3, 88.5 ng/ml, $n = 31$) (ANOVA, stage, $P < 0.001$; year, $P < 0.005$; date, ns). Only three females, all aided, were bled at stage 2, and their mean value was intermediate between that of stages 1 and 3 (adj. $\bar{x}$, 142.5 ng/ml; no statistical comparison of stage 2 with stages 1 or 3).

Twelve females were bled at both stages 1 and 3, thus providing serial samples. In 11 of the 12, the stage-3 sample was taken later in the season than the stage-1 sample. In all cases prl was higher in the stage-1 sample and the ratio (level at stage 1/level at stage 3) averaged 2.56 and showed very little variation (SE = 0.22, $n = 12$, extremes 1.19 to 4.38).

SEASON

Focusing first on fathers, male prl declined between the first and second half of the season (May–June vs. July–August) at both hatching (adj. first-half $\bar{x}$, 108.8 ng/ml, $n = 10$; adj. second-half $\bar{x}$, 75.8 ng/ml, $n = 11$) and nest leaving (adj. first-half $\bar{x}$, 79.2 ng/ml, $n = 8$; adj. second-half $\bar{x}$, 49.8 ng/ml, $n = 21$). The difference was significant only at nest leaving (ANOVA, prl at hatching, $0.05 < P < 0.1$; year, $P < 0.01$; prl at nest leaving, $P < 0.02$; year, ns).

Because prl did not differ between experimental and control females (see below), we combined these groups before determining the effect of season on female prl. Samples were therefore adequate to let us compare season by quarters. Seasonal quarter had no effect on prl in females either at hatching (Fig. 3; ANOVA, prl and year, ns) or at nest leaving (Fig. 3, ANOVA, prl and year, ns). When we divided season into halves as we did for males, the answer was the same: there was no significant variation in prl with date.

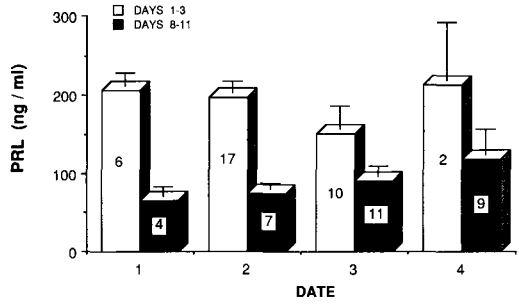

FIGURE 3. Prl (ng/ml ± SE) in female Dark-eyed Juncos according to quarter of the breeding season. Open bars represent females bled during stage 1 (nestlings 0–3 days old) and solid bars represent females bled during stage 3 (nestlings 8–11 days old). Quarters 1–4 represent birds bled during May, June, July, and August, respectively.

TREATMENT

Among males prl was significantly greater in fathers than in nonfeeding replacement males at stage 1 (Fig. 2; adj. $\bar{x}$, fathers, 96.0 ng/ml, $n = 21$; adj. $\bar{x}$, replacement males, 19.5 ng/ml, $n = 4$) (ANOVA, treatment, $P < 0.01$; year, $P < 0.01$; date, ns). The same was true when we combined data taken at all three stages of nestling age (adj. $\bar{x}$, fathers, 78.7 ng/ml, $n = 47$; adj. $\bar{x}$, replacement males, 39.8 ng/ml, $n = 10$) (ANOVA, treatment, $P < 0.01$; year, $P < 0.001$; date, $0.05 < P < 0.1$), but there were no significant differences at stage 2 alone or stage 3 alone.

When the two feeding replacement males (data not in Fig. 2) were compared to the 10 nonfeeding replacement males, no difference in prl was indicated (adj. $\bar{x}$, feeding replacement males, 48.8 ng/ml, $n = 2$; adj. $\bar{x}$, nonfeeding replacement males, 52.5 ng/ml, $n = 10$; year and date, ns). However, the two replacement males that fed were not caught until stage 3, and, as shown above, fathers at stage 3 did not differ from nonfeeding replacement males.

Some replacement males paired with unaided females whose mates we had removed when these females attempted subsequent broods. In these cases, replacement males behaved like fathers when the eggs hatched, feeding and guarding their

presumptive offspring. We might expect prl levels in these males to be higher upon recapture, but there was no difference in prl from samples taken shortly after three replacement males appeared on the territory ($\bar{x}$ = 53.8 ng/ml, SE = 3.18, n = 3) and later while these same individuals were rearing young of their own broods ($\bar{x}$ = 47.2 ng/ml, SE = 1.49, n = 3). However, the latter samples were necessarily collected later in the season when male prl was lower and were too few to permit statistical adjustment for date.

Finally, prl of fathers at stage 1 was significantly higher than that of removed males when these were bled as captives (Fig. 2, 1986; adj. $\bar{x}$, fathers, stage 1, 99.9 ng/ml, n = 21; adj. $\bar{x}$, removed males in captivity, 22.44 ng/ml, n = 9) (ANOVA, treatment, $P < 0.002$; year, $P < 0.001$; date, $P < 0.002$). Three fathers bled at hatching (stage 1) were among the removed males bled in captivity. In serial samples from these three (at hatching and in captivity), prl increased in one case (62.5 ng/ml vs. 68.4 ng/ml) and decreased in the other two (149.0 ng/ml vs. 24.0 ng/ml and 226 ng/ml vs. 42.3 ng/ml). Prl in fathers during stage 2 was also significantly greater than in captive males (ANOVA, $P < 0.004$; year, $P < 0.021$; date, $P < 0.005$), but the two groups did not differ at stage 3 (ns).

Among females, as expected, there was no difference in prl between treatment groups at hatching (Fig. 2; adj. aided female $\bar{x}$, 180.7 ng/ml, n = 17; adj. unaided female $\bar{x}$, 193.2 ng/ml, n = 18) (ANOVA, treatment, ns; year, $P < 0.01$; date, ns). Neither did they differ at nest leaving (stage 3) (Fig. 2; adj. aided female $\bar{x}$, 82.4 ng/ml, n = 22; adj. unaided female $\bar{x}$, 115.4, n = 9) (ANOVA, treatment and year, ns; date, $P < 0.05$).

SEX

Females had higher prl than males (fathers) both at hatching (Fig. 2; adj. male $\bar{x}$, 88.0 ng/ml, adj. female $\bar{x}$, 173.3 ng/ml) (ANOVA, sex, $P < 0.03$; year, $P < 0.01$; date, $0.05 < P < 0.10$) and at nest leaving (Fig. 2; adj. male $\bar{x}$, 58.9 ng/ml, adj. female $\bar{x}$, 85.3 ng/ml) (ANOVA, sex, $P < 0.05$; year and date, ns). Females also had higher prl during the midnestling period, but the difference was nonsignificant (ANOVA, sex, year, and date, ns).

DISCUSSION

When Goldsmith (1983) reviewed the literature on plasma prl in avian species, only three passerines had been studied, one in captivity (Canary, *Serinus canarius,* Goldsmith 1982) and two in the wild (European Starling, Dawson and Goldsmith 1982; Pied Flycatcher, Silverin and Goldsmith 1983). Since then there have been reports for three additional free-living passerines (Song Sparrow, Wingfield et al. 1989, Wingfield and Goldsmith 1990; Brown-headed Cowbird, Dufty et al. 1987; White-crowned Sparrow, Hiatt et al. 1987); and additional information has accumulated on the starling, the Canary, and the Pied Flycatcher (Dawson and Goldsmith 1985; Goldsmith et al. 1984; Silverin and Goldsmith 1984, 1990; G. Ball, unpubl.). Our results may most appropriately be compared to those of other passerines because of similarities in taxonomy and life history, and we emphasize these comparisons in the following paragraphs.

STAGE AND SEASON

Prl of juncos in both sexes was lower at nest leaving than at hatching. Female levels were intermediate during stage 2, about midway through the nestling stage, and therefore these observations agree with other studies in suggesting an association between prl and incubation/brooding in females. The explanation for higher prl in males at stage 1 than at stage 3 is not obvious, because male juncos do not incubate or brood offspring. It does not seem likely that this finding was simply a reflection of the facts that feeding follows hatching and prl declines with date, because the difference between stages 1 and 3 was significant even after correcting for the effect of date. Whatever its cause, the pattern is reminiscent of that of starlings and to a lesser extent Canaries, in which males exhibit their highest levels of prl during incubation, prior to the time they make their major contribution to care of their offspring (Dawson and Goldsmith 1982, 1985; Goldsmith 1982).

In male juncos as the season progressed, when variation associated with stage of reproduction and year/assay was corrected for, prl declined. Females, on the other hand, did not show this decline; throughout the breeding season female prl continued to be high at hatching and lower at nest leaving. Female Song Sparrows, White-crowned Sparrows, and European Starlings resemble female juncos: if they reproduce more than once, prl is high during the subsequent bouts of incubation/brooding (Hiatt et al. 1987; Wingfield et al. 1989; Wingfield and Goldsmith 1990;

G. Ball, unpubl.). However, female Song Sparrows may differ from female juncos in not exhibiting a decline in prl between broods (Wingfield and Goldsmith 1990). Prl in male Song Sparrows appears to increase briefly after laying but does not show a close correspondence with particular stages of reproduction (Wingfield et al. 1989, Wingfield and Goldsmith 1990). Male starlings, like male juncos, also appear to have higher prl values early in the season; in any case there is no second peak of prl at the time of the second brood (G. Ball, unpubl.).

SEX AND TREATMENT

The fact that prl is higher in female than in male juncos conforms to the pattern in other avian species in which females provide more parental care than males, particularly more (or all) incubation. The recent reports that male prl exceeds female prl in species in which the parental roles are reversed, such as the Spotted Sandpiper (Oring et al. 1986) and the Wilson's Phalarope (Oring et al. 1988), provide strong comparative evidence that prl and incubation go hand in hand.

Our finding that at hatching, the time when males begin to deliver food to nestlings, fathers had higher levels of prl than both replacement males and removed captive males is evidence that tending offspring elevates prl in males. On the other hand, fathers differed from replacement and captive males in more ways than merely parental behavior. Fathers were members of pairs and had been with females for several weeks or more, and they had been in possession of a territory for at least that long. In contrast, replacement males were courting unaided females and were in the process of acquiring a territory (sometimes fighting neighbors); and captive males were caged with other males and were without access to females. Thus the difference in prl between fathers and these other categories of males may not reflect the fact that fathers were caring for offspring. Furthermore, prl was no higher in replacement males that fed their predecessors' young than in those that did not, although the stage at which the feeding replacement males were bled (stage 3) was not a stage at which fathers differed from nonfeeding replacement males. Finally, prl did not increase in replacement males that later bred, but again, because of the seasonal decline in prl in fathers, we would not have expected high levels in replacement males at the time they bred. More data are needed to determine whether breeding and behaving paternally elevates prl in male juncos (or vice versa), but two of our three predictions stemming from the hypothesis of a causal connection (a and c, see Introduction) were fulfilled.

The failure to find a difference in prl of aided and unaided females, despite the greater time spent brooding by unaided females and the fact that their rate of feeding nestlings is twice that of aided females (Wolf et al. 1990), indicates that frequency or duration of female parental behaviors is unrelated to circulating levels of prl in female Dark-eyed Juncos. In this they resemble female Pied Flycatchers, in which prl levels of females that are assisted by males do not differ from those of females that are not assisted (Silverin and Goldsmith 1984). Female juncos also resemble albatrosses where the duration of incubation is independent of circulating levels of prl (Hector and Goldsmith 1985).

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