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Effects of Vasoactive Intestinal Peptide on Prolactin Secretion in Three Species of Passerine Birds

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Previous work on domesticated species has indicated that vasoactive intestinal peptide (VIP) is an important prolactin-releasing factor in these birds, but no comparative work in passerine birds has been reported. This study showed that iv injections of VIP (50–100 μg/kg body mass) result in a dramatic, but transitory, rise in plasma prolactin in Mexican jays (*Aphelocoma ultramarina*). Significant increases in prolactin were also observed following VIP injection in blue jays (*Cyanocitta cristata*) and zebra finches (*Poephilla guttata*). At the dosage we used, maximum levels of prolactin attained were slightly lower (Mexican jays) or very similar (blue jay and zebra finch) to the maximum prolactin levels observed in other, breeding birds of the same species. In zebra finches that initially had low prolactin, VIP injection resulted in a greater than 10-fold increase in prolactin within 10 min, but those individuals that already had elevated prolactin showed no further increase in response to VIP. Slow-release pellets of VIP implanted subcutaneously in Mexican jays and releasing 10 or 15 μg VIP/day (two or three pellets) produced a significant increase in plasma prolactin (78 and 92% rise, respectively) compared to birds with placebo pellets or with with one pellet releasing only 5 μg/day.

Substantial correlative and manipulative experimentation in a large number of species supports the hypothesis that prolactin influences parental behavior in birds, specifically incubation of eggs and care of altricial young (reviewed in Buntin, 1996). Control of prolactin synthesis and release from the anterior pituitary, however, has been studied extensively only in a few domesticated species of economic importance in the Order Galliformes (chickens, *Gallus gallus domesticus*, and turkeys, *Meleagris gallopavo*) and in two species in the Order Columbiformes (ring dove, *Streptopelia risoria*, and pigeon, *Columba livia*). Work on such captive species simplifies the problems with obtaining adequate sample sizes and with controlling the behavioral stages of reproduction. On the other hand, artificial selection in domesticated species has led to precocial maturation, high egg production, reduced seasonality, and reduced broodiness (Sossinka, 1982), all traits that could affect the natural cycle of hormone release and its control. In addition, in contrast to most birds, male galliform birds play little or no role in parental care. Galliform birds produce precocial young, whereas most bird species produce altricial young that need considerable parental care. Essentially continuous tactile stimulation from the eggs is required to maintain elevated prolactin and broodiness in chickens, ducks, and turkey hens (El Halawani *et al.*, 1980; Hall *et al.*, 1986; Hall, 1987; Sharp *et al.*, 1988), whereas in many species in which both members of the pair incubate, one bird can be absent from the nest for hours to days (Hector and Goldsmith, 1985; Lea *et al.*, 1986; Vleck *et al.*, in press), but still maintain the urge to return to the nest and incubate. Members of the Columbiform family employ prolactin to stimulate maturation and secretion of the highly specialized crop

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gland, an adaptation for providing nutrition to the hatchlings that is not found in any other order of birds. Thus, control of prolactin secretion in domesticated galliform and columbiform species may not be identical to that in other bird species.

The majority of avian species belong to the Order Passeriformes (the perching birds). A rise in plasma prolactin is associated with the onset or maintenance of egg incubation and care of young in a number of free-living passerine species (Goldsmith, 1991; Buntin, 1996). A strong test of the hypothesis that these behaviors depend on elevated prolactin requires a method for manipulating the plasma levels of prolactin. Prolactin has been artificially elevated in several nonpasserine species via supplementation with prolactin of mammalian origin (Janik and Buntin, 1985; Lea *et al.*, 1986; Sharp *et al.*, 1988; Pedersen, 1989; Youngren *et al.*, 1991). Although these manipulations have, in many cases, resulted in an increase in display of parental behavior, the ability to alter the level of the native hormone would allow a more precise definition of the relationship between hormone and behavior.

A number of chemicals are known to affect avian prolactin secretion, but again, these studies have been limited to galliform and columbiform species (reviewed in El Halawani *et al.*, 1997). Vasoactive intestinal peptide (VIP) is a potent prolactin-releasing factor in ring dove and pigeons (Lea and Vowles, 1986; Cloues *et al.*, 1990; Peczely, 1992), female turkeys (Knapp *et al.*, 1988; Opel and Proudman, 1988; Proudman and Opel, 1988; Mauro *et al.*, 1989; El Halawani *et al.*, 1990; Pitts *et al.*, 1994b), and bantam hens (Macnamee *et al.*, 1986; Talbot *et al.*, 1991). Recently VIP in portal blood in the turkey has been shown to vary in parallel with plasma prolactin at different stages in the reproductive cycle (Youngren *et al.*, 1996a). Dopamine is a prolactin-inhibiting factor in birds at high concentrations, but stimulates prolactin secretion at low concentrations, and its effectiveness may depend upon the stage of the breeding cycle (El Halawani *et al.*, 1991; Youngren *et al.*, 1995, 1996b). Serotonin (5-HT) is stimulatory for prolactin release (El Halawani *et al.*, 1988; Macnamee and Sharp, 1989; Pitts *et al.*, 1996).

El Halawani *et al.* (1991) suggested the following model for the neuroendocrine control of prolactin secretion in female turkeys. Serotonergic neurons augment VIP release from neurons in the infundibular nuclear complex-median eminence region although whether this effect is direct or not is unknown (El Halawani *et al.*, 1997). This hypothalamic prolactin-releasing peptide in turn stimulates prolactin release from the anterior pituitary. In laying hens, dopaminergic inputs via D2 dopamine receptors in the anterior pituitary limit the VIP-stimulated prolactin section. During incubation, there is diminished dopaminergic neurotransmission, increased serotonin turnover and VIP secretion, and increased anterior pituitary capacity to secrete prolactin in response to VIP. The cyclical nature of the control is thought to be controlled by a combination of feedback inhibition of prolactin, photoperiodic input, tactile stimuli from nest and eggs, and other brain influences (reviewed in Mauro *et al.*, 1989; Buntin, 1996).

In this study we examined the effect of VIP injections on prolactin level in two corvids (the Mexican jay, *Aphelocoma ultramarina*, and the blue jay, *Cyanocitta cristata*) and an estrildid finch (the zebra finch, *Poephila guttata*). We also tested the ability of subcutaneous pellets of VIP to effect a long-term increase in prolactin in Mexican jays.

MATERIALS AND METHODS

Animals

Sixteen adult Mexican jays were captured in traps at the Santa Rita Experimental Range headquarters in southern Arizona in March or in September–October. Six blue jays were trapped at McFarland County Park in central Iowa in June–July. All birds were trapped under appropriate USFWS and State permits. Jays were housed in individual cages with *ad libitum* food and water. The diet consisted of a smorgasbord of fruits, vegetables, canned dog food, and mixed bird seed including peanuts and sunflower seeds. Periodically birds were given meal worms and an oral dose of ABDEC vitamins. Masses of Mexican jays varied between about 105 and 135 g over the course of the experiments and blue jays weighed between 72 and 92 g. All birds maintained hematocrit throughout the experiments. Jays are sexually monomorphic, and we did not know the sexes of the birds we trapped. The zebra finches that we used (mass 10–12 g) were the direct descendants of a group of zebra finches collected in South Australia in 1980, so although these birds had never been free-living, they are closer to the wild type zebra finches than those zebra finches available commercially which have been reared for hundreds of generations in captivity. These birds were maintained in mixed-sex groups in small, indoor flight cages, with nest boxes and nesting material provided and on a 14:10 (LD) photoperiod. They were provided *ad libitum* finch seed, water, and occasional spinach, and chopped eggs.

Prolactin Response to VIP Injection

Eight adult Mexican jays that had been captured at the beginning of their normal reproductive season in March were transported to Iowa by air and kept on a photoperiod which matched that of their natural habitat, with increasing daylength through the spring. After 1 month in captivity (at the peak egg-laying period in nature), a blood sample was taken from the wing vein of each bird within 2–3 min of capture, and then they were given a 100 μl iv injection of either saline or porcine VIP (Peninsula Lab). Two birds received saline, and the other six birds received either 5 μg (∼50 μg/kg body mass) or 10 μg VIP (∼100 μg/kg body mass) dissolved in 100 μl saline. These dosages were chosen because they were the two highest dosages tested by Macnamee *et al.* (1986) in bantam hens. Blood samples were taken from the jays at 3, 10, and 20 min postinjection. One bird was bled 36 min after the VIP injection. We held the birds in a net bag between sample times.

In the blue jays and zebra finches we measured the response of prolactin secretion to VIP injection by taking an initial blood sample within 2–3 min of capture, injecting porcine VIP iv and taking one subsequent blood sample approximately 6 min (blue jays) or 10 min (zebra finches) later. Blue jays were tested during the normal nesting season for this species and were maintained on a 14:10 (LD) photoperiod. Three blue jays received 4.55 μg VIP (∼60 μg/kg body mass) in 100 μl saline. The other three received saline as a control. Three male and three female zebra finches were given an iv injection of 0.75 μg VIP (∼75 μg/kg body mass) in 40 μl saline. Four control finches (two males, two females) received 40 μl of saline. To catch the finches from the flight cage, we turned off the lights, entered the flight cage, and using a flashlight, picked up the bird we wanted, a process requiring <15 seconds.

Prolactin Response to VIP Implants

Eight Mexican jays, captured during the fall, nonbreeding season were kept on a 12:12 (LD) photoperiod at the University of Arizona. In mid-November, a 100-μl blood sample was taken from a wing vein and then each bird was given a subcutaneous implant containing VIP (Bachem Chemical) or vehicle. Implant pellets were prepared by Innovative Research. Each VIP pellet contained 100 μg VIP/pellet, and was designed to release 5 μg VIP/day for 20 days. Each bird received either one placebo pellet containing the vehicle but no VIP (two birds), one subcutaneous VIP pellet (two birds), two VIP pellets (two birds), or three VIP pellets (two birds). Blood samples were taken from each bird on Days 1 through 4 after pellet implantation and then subsequently every other day, at the same time of day, until 18 days after implantation. The order in which birds were sampled was randomized each day. Birds were then moved to an outdoor aviary on Day 19 in preparation for release. A final blood sample was taken on the 25th day after implantation at which time the birds were released at their site of capture. The implant vehicle was still visible under the skin and was not removed.

Prolactin Radioimmunoassay

The blood samples for each experiment were centrifuged and plasma was stored at −20°C until assayed for prolactin. We measured plasma levels of prolactin with a postprecipitation, double antibody using purified chicken prolactin as a standard and a rabbit antiserum raised against prolactin (both obtained from Dr. A. F. Parlow, Director, Pituitary Hormones and Antisera Center, Harbor-UCLA Medical Center, Torrance, CA). We radiolabeled the prolactin with I^{125} using chloramine-T. Dilutions of Mexican jay and zebra finch plasma bind to the antibody in a manner relatively parallel to dilution of the standard chicken prolactin (Fig. 1), indicating that this heterologous RIA can be used to assess relative levels of plasma prolactin in these species. We did not have enough plasma from the blue jays to test dilutions; however, plasma from

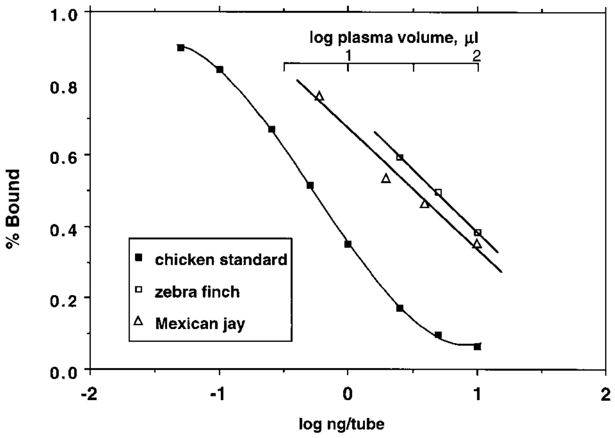

FIG. 1. Representative standard curve from an avian prolactin radioimmunoassay. This graph shows the relationship between percentage binding of radiolabeled chicken prolactin to prolactin antiserum and the logarithm of the nanograms of chicken prolactin reference preparation per tube. Also shown are the results of binding using various dilutions of zebra finch plasma and Mexican jay plasma.

two other jay species also provides similar dilution curves (Schoech *et al.*, 1996; Brown and Vleck, 1998).

Jay samples were assayed in triplicate and finch samples in duplicate in aliquots of 20 µl of plasma. The first antibody was used at a final dilution of 1:300,000, and the second antibody (goat-anti-rabbit γ-globulin) was prepared by diluting at a 1:10 ratio in assay buffer. The blood samples from each separate experiment were measured in a single radioimmunoassay. The average intraassay coefficient of variation was 7.3%, the interassay coefficient of variation was 6.2%, and the least detectable plasma concentration was about 2 ng/ml.

Statistics

For experiments with Mexican jays that included more than one pair of measurements on a single bird, we used a repeated measures design and tested significance levels with analyses of variance using JMP 3.2.1 software (SAS Institute, Inc. 1995). In both cases, the data met the sphericity condition (Mauchly Criterion) and therefore we used univariate *F* tests. For experiments with blue jays and zebra finches we used nonparametric Wilcoxen rank sums tests to test treatment effects and report the χ^2 test statistic for one-way tests. Values reported are means ± standard errors.

RESULTS

VIP Injections

Prolactin levels increased in all three species in response to VIP injection. In the Mexican jays, prolactin levels were generally low (<10 ng/ml) in all birds before the VIP injections, even though samples were taken during the normal nesting season for this species. This may have been due to their transport from Arizona to Iowa and their time in captivity (1 month). The two saline-injected birds and one of the three low-dose VIP birds showed no response to the VIP injection. In the other five birds, prolactin levels increased an average of 97% within the first 5 min postinjection, reached an average of 142% of preinjection levels within 10 min, and had begun to decline by 20 min postinjection (Fig. 2). Repeated measures ANOVA could not distinguish a treatment effect in this data set ($F_{(2,5)} = 2.64$, $P > 0.16$), because of the small sample size and the fact that one VIP-injected bird did not respond. On five other occasions, however, this same bird responded to injections of 6.25 µg VIP with prolactin level increases ranging from 61 to 150%, measured 5–7 min later. It seems likely that the lack of response in this individual in this experiment was due to a faulty iv injection. If we leave out the data for this one bird, then the treatment effect becomes highly significant ($F_{(2,4)} = 13.05$, $P < 0.018$).

In the six blue jays, prolactin levels were generally similar to levels obtained from birds at capture in the field (mean field level = 31.9 ± 1.6 ng/ml). Plasma prolactin in the blue jays increased only slightly or not at all with saline injection, and increased by an average of 40% by 6 min post-VIP injection. Prolactin in the saline and VIP groups did not differ before treatment ($\chi^2 = 0.048$, $df = 1$, $P = 0.827$), but prolactin was higher in the VIP group than in the control group after the injections ($\chi^2 = 3.857$, $df = 1$, $P = 0.049$).

In the zebra finches, some birds initially had elevated levels of prolactin and others did not. Some females in the flock were laying eggs and some birds of both sexes were occasionally visiting the nest boxes. These differences may correlate with individual variation in prolactin, but we cannot test this hypothesis. Plasma prolactin did not differ between males and females either before or after VIP injection ($P > 0.5$), so

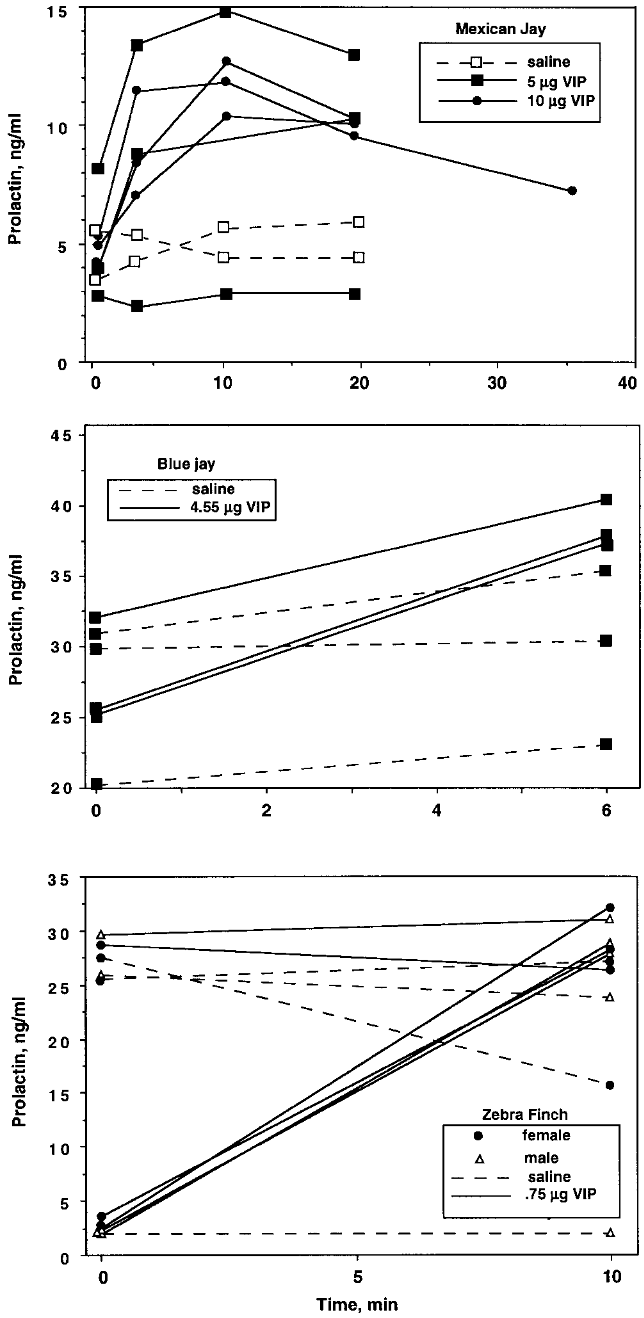

FIG. 2. The relationship between plasma prolactin and time following a single iv injection of either saline or VIP in saline. Serial samples following injection for the Mexican jays (50 or 100 µg/kg) are shown in the top panel. The middle panel (blue jay) and bottom panel (zebra finch) show the change in prolactin measured either 6 or 10 min following the VIP injection. Dosages used were 60 µg/kg for blue jays and 75 µg/kg for zebra finches. The first value shown for each animal is for plasma samples immediately before the VIP injection.

data from both sexes were combined for examining treatment effects. There was no difference in prolactin levels between the control and the experimental groups before treatment ($\chi^2 = 0.011$, $df = 1$, $P = 0.915$), but following treatment, the VIP-injected birds had significantly higher levels of prolactin than the saline-injected birds ($\chi^2 = 5.533$, $df = 1$, $P = 0.019$). VIP injection resulted in a mean prolactin increase >10 fold in those birds which did not initially have elevated prolactin, but birds which already had elevated prolactin showed little or no further increase (Fig. 2).

Slow-Release VIP Pellets

There was no obvious change in plasma prolactin in the Mexican jays following implantation with either the subcutaneous placebo pellet or with one VIP pellet (100 µg). Prolactin increased following implantation with two VIP pellets (200 µg) or with three VIP pellets (300 µg) (Fig. 3). Compared to the initial prolactin values (Day 0), prolactin measured over the next 18 days was elevated by an average of 78% in the two-pellet group and 92% in the three-pellet group, but increased only an average of 12% in the placebo group and decreased by 2% in the one-pellet group. On the day of release (25 days after implantation), prolactin was only 90% of the Day-0 level in the placebo and one-pellet birds, but was still 150% of the initial prolactin value in the two- and three-pellet birds. In this experiment, sample sizes were too small to permit statistical testing (two animals per group). However, if we combine the data for two- and three-pellet groups and compare them to the combined data from the placebo and one-pellet group, then there is a significant treatment effect over the first 8 days ($F_{(1,6)} = 7.00$, $P < 0.04$).

DISCUSSION

These experiments demonstrate that VIP acts as a potent prolactin-releasing agent in three passerine species, similar to its action in other birds that have been tested. Injections of VIP result in short-term surges of prolactin release that can elevate prolactin by up to 10-fold. The maximum levels of plasma prolactin following VIP injection in the blue jays were similar to

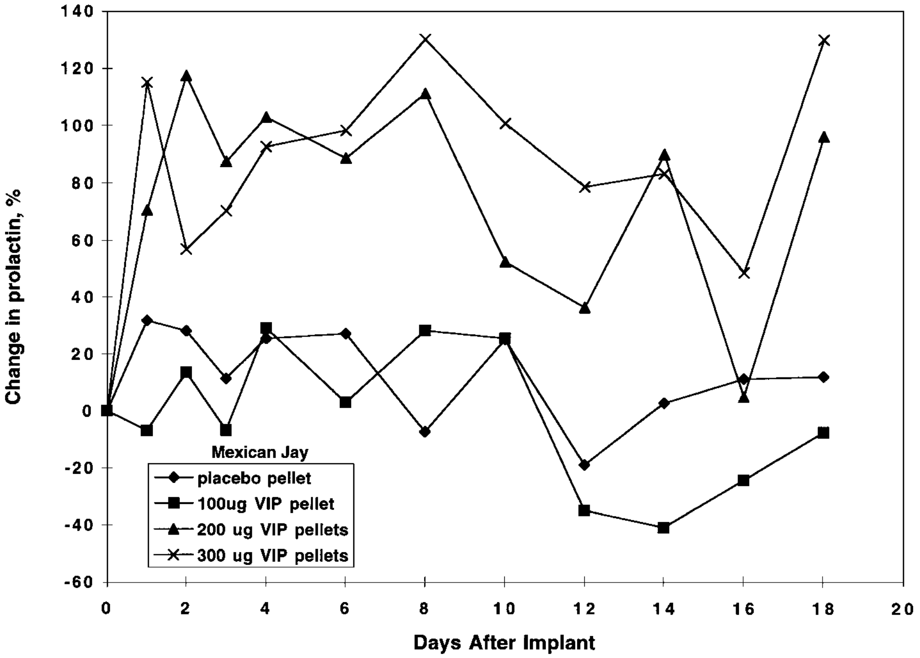

FIG. 3. Change in plasma prolactin following VIP implants in captive Mexican jays tested during late fall. Immediately after the reference sample of plasma was taken on Day 0, animals were given a subcutaneous implant of a placebo pellet or one to three VIP pellets, each containing 100 µg VIP and releasing at a rate of 5 µg/day. Points shown are the means for two animals per group.

those measured in field-caught birds during the breeding season. The maximum levels we measured in the captive Mexican jays following VIP injection at two different dosages were about 30% lower than the maximum levels measured in free-living Mexican jays during the breeding season (Brown and Vleck, 1998), although it is possible that higher dosages of VIP could have elevated prolactin more. VIP injections elevated prolactin in zebra finches that initially had low prolactin, but only to levels that were typical of the maximum found in other birds in the population (Fig. 2).

Complete clarification of the prolactin response to VIP would require careful generation of a dose-response relationship in birds in different reproductive stages. Given our data here, however, it is clear that VIP can elevate prolactin, but possibly only to within limits set by the bird's reproductive condition. Results from a number of studies suggest that pituitary response to VIP varies over different stages of the reproductive cycle (El Halawani *et al.,* 1990; Wong *et al.,* 1991; Mauro *et al.,* 1992; Rozenboim and El Halawani, 1993; Rozenboim *et al.,* 1993).

There may be species differences in whether the control system for prolactin is seasonally sensitive or not, that vary with whether a species is photoperiodic. If we assume that the initial prolactin levels measured in the zebra finches allow us to distinguish two groups of birds—breeding birds with high prolactin and non-breeding birds with low prolactin (see Fig. 2), then VIP treatment of the presumed nonbreeding zebra finches resulted in prolactin levels for this species similar to that in the presumed breeding birds. These desert-adapted, Australian zebra finches are not photoperiodic and will breed whenever conditions (generally access to food and water) are permissive (Vleck and Priedkalns, 1985). There is also evidence that the pituitary in zebra finches displays a tonic secretion of gonadotropins (Farner and Serventy, 1960) rather than the large seasonal adjustments in secretion rate that are found in most birds. It could be that in nonphotoperiodic species the anterior pituitary may be maximally sensitive to VIP at all times rather than having a sensitivity that depends on a breeding season.

Elevated prolactin secretion in response to a single injection of VIP is probably short-lived. In the Mexican jays prolactin levels were declining from the peak in most individuals by 20 min postinjection, similar to the results reported by Macnamee *et al.* (1986) in bantam hens. This may occur either because the VIP is quickly inactivated and/or the pituitary prolactin stores are depleted. The latter has been found following acute infusion of VIP in turkeys (Pitts *et al.,* 1994a). The variance among individuals in the extent to which plasma prolactin is elevated following VIP injection may reflect the releasable pool in the anterior pituitary, which probably varies with the reproductive stage of the birds (Macnamee *et al.,* 1986).

Subcutaneous implants of slow-release VIP pellets in Mexican jays led to chronic increase in plasma prolactin in a dose-response manner, as has been found with different injection volumes of VIP in chickens (Macnamee *et al.,* 1986) and in turkeys (Opel and Proudman, 1988). In these fall-season, nonbreeding Mexican jays, the levels achieved were significantly below those found in breeding, free-living birds, although we do not know whether a higher dosage of VIP would have elevated prolactin to a greater extent than we report here. The mean prolactin level in the VIP-pellet implanted birds before implantation was only 5.9 ± 0.6 ng/ml, and the mean maximum level found after pellet implants releasing 10–15 µg/day was 11.0 ± 0.3 ng/ml, well below the average of 22–24 ng/ml found in free-living birds in the breeding season (Brown and Vleck, 1998). It was also below the values achieved in spring-time captive Mexican jays in response to a single 5 or 10 µg VIP injection (Fig. 2).

Pitts *et al.* (1994b) studied the long-term effects of VIP infusion into the third ventricle of turkey brains. At an infusion rate of 30 ng/min (43 µg/day), plasma prolactin was elevated for at least 9 days compared to controls and then declined, possibly due to downregulation of VIP receptors in the anterior pituitary. Surprisingly, nest attentiveness in these turkeys did not increase even though plasma prolactin was elevated to levels typically found in incubating turkey hens. This is in contrast to other studies in which intracranial infusion of ovine prolactin did lead to an increase in nesting behavior (Youngren *et al.,* 1991).

VIP has now been demonstrated to be a prolactin-releasing factor in at least three orders of birds, including three species in the most abundant order of birds, the Passeriformes. Whether or not VIP is the natural hypothalamic releasing for prolactin in passerines, as it appears to be in Galliformes (El Halawani *et al.,* 1997), remains to be determined. Subcutaneously implanted pellets of VIP are a viable method for increasing prolactin secretion for up to at least a week (see Fig. 3). Such pellet implants may be preferred over infusion pumps in free-living birds because the birds do not have to be retrapped to remove the implant, and the pellets can be inserted under the skin in a matter of minutes in the field. Increasing VIP in this manner should elevate plasma prolactin, but possibly within limits set by the species and season.

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