The Condor 97:1057–1061
© The Cooper Ornithological Society 1995

USE OF ELISA FOR DETERMINATION OF PLASMA PROLACTIN LEVELS IN THE HOUSE WREN[1]

SHARON M. SINTICH AND MICHELLE K. KEAGLE
Department of Biology, Bradley University, Peoria, IL 61625

R. GIVEN HARPER
Department of Biology, Illinois Wesleyan University, Bloomington, IL 61702

IAN G. WELSFORD[2]
Department of Biology, Bradley University, Peoria, IL 61625

Key words: Prolactin; Troglodytes aedon; *House Wren; ELISA.*

Prolactin concentrations in avian plasma have been traditionally quantified using a radioimmunoassay (RIA) procedure. RIA is useful for such studies due to its sensitivity and specificity (e.g., Follett et al. 1972, Wingfield and Farner 1975, McNeilly et al. 1978, Burke and Papkoff 1980, Schwabl 1993). For example, the heterologous RIA for detecting prolactin in turkeys, developed by McNeilly et al. (1978), recovered 98% of a prolactin standard added to a turkey plasma sample and exhibited less than 0.1% cross reactivity with other plasma hormones. In an homologous RIA developed by Burke and Papkoff (1980), detection limits of approximately 0.42 ± 0.13 ng were reported. Despite these advantages, RIA has drawbacks for field ecologists, including the high cost of isotope and the necessity of working with radioactive ligands. We report here a non-radioactive alternative to RIA for measurement of plasma prolactin levels in the House Wren (*Troglodytes aedon* L.), namely, a commercially-available (Leinco Technologies Inc.) enzyme-linked immunosorbant assay (ELISA) kit. The ELISA kit used was based on capturing avian plasma prolactin with a murine monoclonal antibody (mAb) specific for prolactin, followed by detection of the captured prolactin with a polyclonal antibody directed against prolactin. The test may be ideal for usage by field ecologists since it is non-isotopic, portable, does not rely on specificity of the detection antibody, quick, involves little sample or standard preparation, can be used on small sample volumes and requires little specialized equipment.

METHODS

In 1991 and 1992, blood samples were withdrawn from the brachial vein of male and female House Wrens during the pre-egg laying, egg laying and incubation stages of the breeding cycle (n = 5 birds at each stage) at the Mackinaw study site located approximately 30 km northeast of Bloomington, IL (see Harper et al. 1994). Samples were placed on ice in sterile phosphate buffered saline (PBS^{++}:137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7\ H_2O$, 1.4 mM KH_2PO_4,pH 7.3–7.4, with 0.1 mM Phenylmethylsulfonylflouride (PMSF) and 0.1 mM 2-mercaptoethanol added to diminish plasma protein degradation and sample oxidation, respectively) until analyzed. Samples were centrifuged at 6,000 g in a Fisher micro-centrifuge (Model 235A) to remove blood cells and the supernatant was analyzed for the presence of prolactin using a prolactin-anti-prolactin ELISA kit from Leinco Technologies Inc. Rat anti-prolactin IgG antibody (murine monoclonal, i.e., the "capture antibody"; mAb) was coated onto sterile plastic wells (performed by the manufacturer) and an aliquot (25 μl) of plasma was added to the wells at 25°C. The plasma was removed from the wells which were washed with a detergent wash buffer supplied with the kit. Following this, 200 μl of goat anti-prolactin goat polyclonal antibody (primarily IgG, i.e., the "detection" antibody) which had been enzyme-labeled with horseradish peroxidase was added to each well. After a 45 min incubation, wells were washed with a detergent wash buffer supplied with the kit followed by doubly deionized, doubly autoclaved water to remove unbound labeled anti-prolactin. We added 200 μl of substrate chromogen (3,3′, 5,5′ tetramethylbenzidine (TMB)) to each well and incubated each for 15 min, producing a blue color. The reaction was stopped with 50 μl of 1 N H_2SO_4, which changed the color to yellow. The intensity of the color was proportional to the concentration of bound antibody present in each plasma sample. The wells were visually scored for intensity by comparing chromogen in unknown wells with 0, 25, 50, and 100 ng/ml prolactin standards, and quantified at 450 nm using a EL-311 microplate reader (Biotek Instruments Inc.). A standard concentration vs. absorbance curve was constructed using PSI-PLOT Program (PsIPlot Inc.). Since we were using an heterologous assay, it was important to determine if the plasma samples and the standards exhibited the same alteration in absorbance with changes in concentration. Five plasma samples (separate from those used in the analyses above) from each group of birds were diluted 1:2, 1:5, and 1:10 and the change in absorbance with dilution was compared with that of the known prolactin

[1] Received 21 December 1994. Accepted 11 May 1995.

[2] Present address of corresponding author: Department of Biology, 229 Main St., Keene State College, Keene, NH 03435-2001.

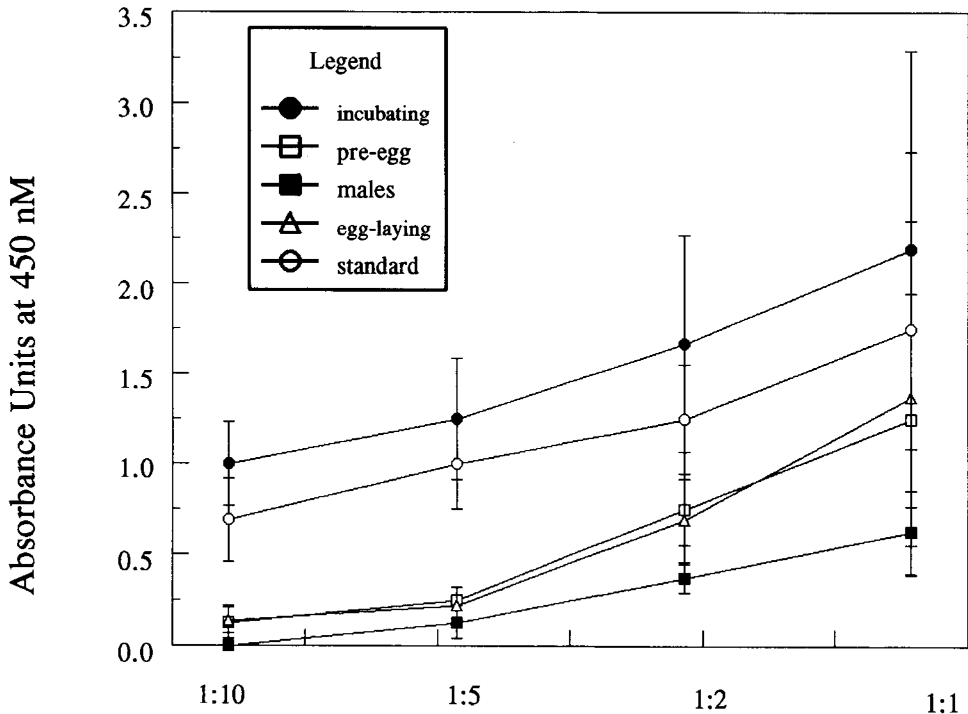

FIGURE 1. Serial dilution curve of plasma samples (n = 5 each) from incubating females ("incubating"), pre-egg-laying females ("pre-egg"), males (combined samples from all breeding stages; "males"), egg-laying females ("egg laying") and prolactin standards ("standard").

standards (i.e., 0, 25, 50 and 100 ng/ml). Due to low sample size and the fact that analyses revealed no significant difference between the plasma prolactin levels of males at different breeding stages, the plasma samples of males from varying breeding times were pooled for the serial dilution trials. Standards and plasma dilutions showed linear absorbance curves and their slopes were similar (Fig. 1), thus allowing us to use interpolation to estimate prolactin concentration of unknowns. The reported assay sensitivity of the ELISA kit was <1.0 ng/ml (Leinco, Inc.) but we determined the sensitivity of the assay in our hands by serial dilution of known concentrations of prolactin. To determine assay recovery (100 ± 1.25% for human serum; Leinco Technologies), 10 plasma samples (aliquots of the same samples used for the serial dilutions) were "spiked" with a known amount of prolactin (5, 10 and 50 ng/ml). Percent recovery was calculated by comparing expected concentration in spiked samples with the value actually obtained. In all instances, ELISA runs were performed "blind" as plasma samples were identified by band numbers only.

To substantiate that the ELISA was specifically detecting prolactin in House Wren plasma rather than simply reacting non-specifically to plasma proteins, reverse-phase high pressure liquid chromatography (Waters RP-HPLC) was performed on five additional plasma samples. Plasma was prepared for RP-HPLC by boiling in 1N acetic acid/0.1% thiodyglycol, followed by sonication for 2 min and centrifugation at 20,000 g (IEC Model B-20A Centrifuge) at 0°C, and drying for 5 hr. Samples were resuspended in elution buffer (80% water/20% acetonitrile) and run on a selectosil C18 column. Over the course of a run (averaging 35 min/sample), the eluent concentration was changed from 80% water/20% acetonitrile to 50% water/50% acetonitrile. Elution peaks (read by absorbance at 260 nm) were collected every 4 min by an automatic sampler and assayed with the ELISA protocol described above.

Differences between the plasma prolactin concentrations of pre-egg laying, egg-laying, and incubating stages of males vs. females were tested with a two-way Analysis of Variance (ANOVA) followed by post-hoc comparisons (Fisher's LSD; Sokal and Rohlf 1981) where appropriate. Analyses were run using the DataDesk software package (Data Desk Inc.) and in all instances, a probability of below 0.05 was considered significant.

RESULTS

The calculated limit of sensitivity for the ELISA used was 2 ± 3.4 ng/ml ($\bar{x}$ ± SE; n = 5). The calculated

percentage recovery of prolactin was 93 ± 2.1 ($\bar{x}$ ± SE; n = 15). Serial dilution of plasma samples from each group of birds yielded linear curves (correlation coefficients of 0.988 for standards, 0.988 for incubating females, 0.97 for egg-laying females, 0.99 for pre-egg laying females and 0.95 for males) even though there was a difference in the absolute amount of prolactin present in each sample (Fig. 1). In fact, the 1:10 dilution of plasma from male birds gave no absorbance above background. The slopes of the lines for each group of birds were similar (0.17 for standards, 0.19 for incubating females, 0.19 egg-laying females, 0.175 for pre-egg laying females and 0.21 for males).

The ELISA reacted specifically with an HPLC elution peak (number 6 in Fig. 2) that corresponded to the elution profile of authentic prolactin (Fig. 3). There was no significant background reaction associated with other plasma fractions (Fig. 2 and 3). There was a significant interaction between sex and breeding stage for female birds ($F_{2,22}$ = 73.387, P < 0.0001). Post hoc comparisons revealed that pre-egg laying and egg-laying females had significantly lower plasma prolactin levels than did incubating birds (Fig. 4). There was no significant difference in the plasma prolactin levels in male birds at differing stages in the breeding cycle (Fig. 4).

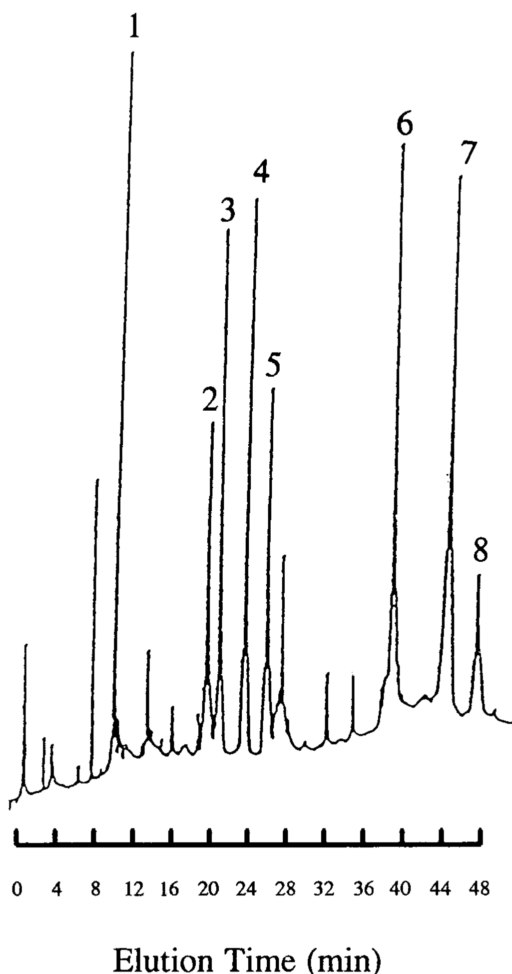

FIGURE 2. Sample RP-HPLC elution run of House Wren plasma. Collected peaks are numbered for identification purposes. Peak number 6 corresponded to authentic prolactin.

DISCUSSION

Our results indicate that a human prolactin ELISA test kit is effective in detecting avian prolactin concentrations. Replicate ELISA runs are recommended when using our protocol since color intensity degrades over time due to the continued reaction of sulfuric acid with the substrate chromogen. The sensitivity of the present ELISA was undoubtedly affected by the fact that it was used against avian, rather than human, serum prolactin. This is supported by the lower sensitivity estimate in the present study versus human prolactin as well as by the decrease in percentage recovery of prolactin from avian plasma versus human. However, both the recovery and sensitivity values obtained compare favorably with those reported for RIA (e.g., Burke and Papkoff 1980; McNeilly et al. 1978). Note that sensitivity estimates using capture antibodies may be skewed by the fact that the binding affinity of capture mAbs appears to be sensitive to the method used for mAb immobilization (e.g. Pesce and Gabriel Michael 1992, Joshi et al. 1992, Butler et al. 1992, Schwab and Bosshard 1992, Goldberg and Djavadi-Ohaniance 1993). Competitive binding studies are underway in an attempt to determine the apparent affinity of the immobilized antibody for avian plasma prolactin. Sensitivity may be improved by using an homologous ELISA (i.e., by using an anti-avian prolactin capture mAb; Pesce and Gabriel Michael 1992).

The sensitivity of the ELISA was also probably affected by the use of a chromogenic, rather than a flourogenic, substrate tag. It has been reported that the affinity values obtained in ELISA using chromogenic substrates are about 10^{-9} to 10^{-10} M, whereas the use of flourogenic conjugates increases the assay sensitivity by a factor of approximately 100 (e.g., Goldberg and Djavadi-Ohaniance 1993). For field ecologists, however, the cost differential between an optical wavelength microplate reader versus a flourescent-capable reader may outweigh this potential loss of sensitivity.

That ELISA can determine plasma prolactin differences in females during different breeding stages suggests that this technique may be useful in studies such as hormonal control of incubation behavior since it is known that prolactin plays a role in determining incubation onset or maintenance in a number of avian species (e.g., Mead and Morton 1985). The hormonal studies reported herein must be considered preliminary due to the small number of birds sampled and the fact that samples were taken at broadly defined stages in the breeding cycle. Assessment of prolactin's role in the breeding biology of the House Wren will undoubtably require sampling over varying times of the day throughout the breeding cycle (e.g., Dawson et al. 1985, Dufty and Wingfield 1987, Wingfield and Goldsmith 1990). However, the fact that plasma prolactin con-

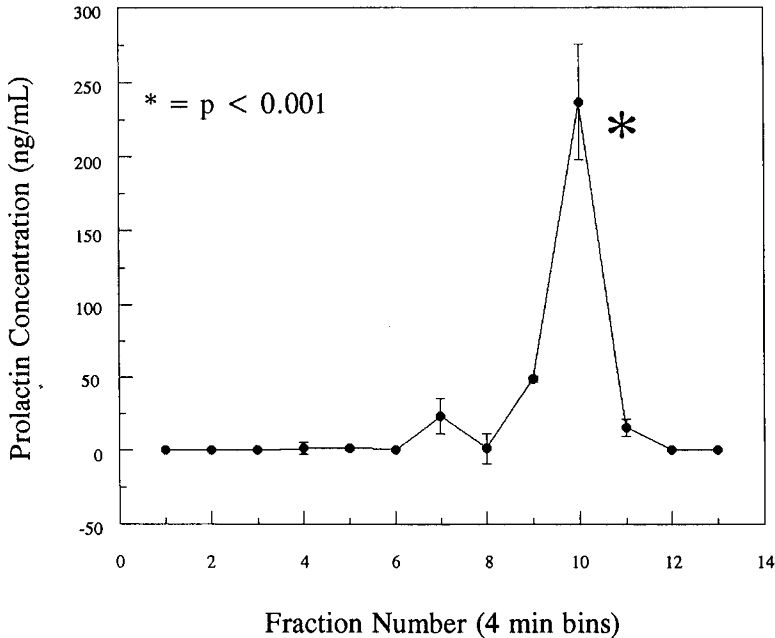

FIGURE 3. ELISA results on individual RP-HPLC peaks. Note that only the peak which eluted at 40 min (i.e., Peak 6; see Fig. 2) coeluted with authentic prolactin and demonstrated significant chromogen reaction in the ELISA. Each symbol represents the mean (±SD) value of five separate plasma trials.

centration of male birds did not exhibit any significant alteration over the breeding stages sampled suggests that the hormonal differences detected may be related to breeding behavior rather than some other phenomenon such as photorefractoriness (e.g., Dawson et al. 1985). Work is underway to evaluate the efficacy of the ELISA protocol for determination of other plasma reproductive hormones such as LH.

This work was supported in part by the OTEFD at Bradley Univ., a Whitehall Foundation GIAR and NSF DUE-9252732 awarded to IGW. The authors would like to thank Dr. S. Fan for assistance with the microplate reader and for helpful comments on the manuscript.

LITERATURE CITED

Burke, W. H., and H. Papkoff. 1980. Purification of turkey prolactin and the development of a homologous radioimmunoassay for its measurement. Gen. Comp. Endocrinol. 40:297–307.

Butler, J. E., L. Ni, R. Nessler, K. S. Joshi, M. Suter, B. Rosenberg, J. Chang, W. R. Brown, and L. A. Cantarero. 1992. The physical and functional behavior of capture antibodies adsorbed on polystyrene. J. Immunol. Methods 150:77–90.

Dawson, A., A. R. Goldsmith, and T. J. Nicholls. 1985. Development of photorefractoriness in intact and castrated male starlings (*Sturnus vulgaris*) exposed to different periods of long-day lengths. Physiol. Zool. 58:253–261.

Dufty, A. M., and J. C. Wingfield. 1987. Temporal patterns of prolactin secretion in a brood parasite, the Brown-headed Cowbird, *Molothrus ater*. J. Zool. (Lond.) 212:669–675.

Follett, B. K., C. G. Scanes, and F. J. Cunningham. 1972. A radioimmunoassay for avian luteinizing hormone. J. Endocr. 52:359–378.

Goldberg, M. E., and L. Djavadi-Ohaniance. 1993. Methods for measurement of antibody/antigen affinity based on ELISA and RIA. Curr. Opinion Immunol. 5:278–281.

Harper, R. G., S. A. Juliano, and Charles F. Thompson. 1994. Intrapopulation variation in hatching synchrony in House Wrens: test of the individual-optimization hypothesis. Auk 111:516–524.

Joshi, K. S., L. G. Hoffmann, and J. E. Butler. 1992. The immunochemistry of sandwich ELISAs V. the capture antibody performance of polyclonal antibody-enriched fractions prepared by various methods. Mol. Immunol. 29:971–981.

McNeilly, A. S., R. J. Etches, and H. G. Friesen. 1978. A heterologous radioimmunoassay for avian prolactin: Application to the measurement of prolactin in the turkey. Acta Endocrinol. 89:60–69.

Mead, P. S., and M. L. Morton. 1985. Hatching asynchrony in the mountain White-crowned Sparrow (*Zonotrichia leucophyrs oriantha*): A selected or incidental trait? Auk 102:781–792.

Pesce, A. J., and J. Gabriel Michael. 1992. Artifacts and limitations of enzyme immunoassay. J. of Immunol. Methods 150:111–119.

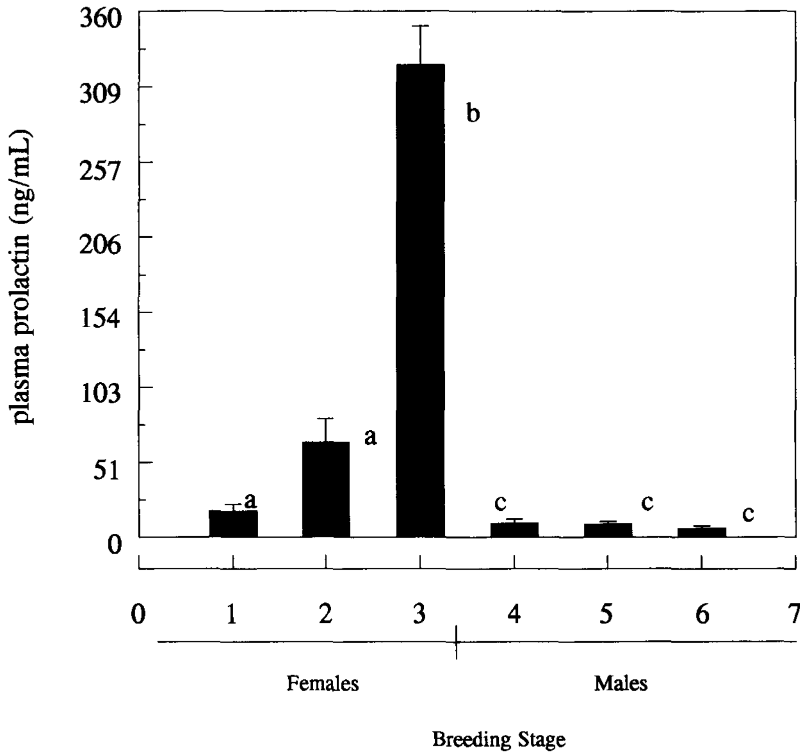

FIGURE 4. The amount of prolactin present in blood samples for females and males at three reproductive stages. Stage 1 = female pre-egg laying, Stage 2 = female egg laying, Stage 3 = female incubation, Stage 4 = male during pre-egg laying stage of the female, Stage 5 = male during egg laying stage of the female and Stage 6 = male during incubation stage of the female. Different letters indicate significant differences between stages. Each bar represents the mean ± SE of five plasma samples from separate birds.

SCHWAB, C., AND H. R. BOSSHARD. 1992. Caveats for the use of surface-adsorbed protein antigen to test the specificity of antibodies. J. Immunol. Methods 147:125–134.

SCHWABL, H. 1993. Yolk is a source of maternal testosterone for developing birds. Proc. Nat. Acad. Sci. 90:11446–11450.

SOKAL, R. R., AND F. J. ROHLF. 1981. Biometry. W. H. Freeman, New York.

WINGFIELD, J. C., AND D. S. FARNER. 1975. The determination of five steroids in avian plasma by radioimmunoassay and competitive protein binding. Steroids 26:311–327.

WINGFIELD, J. C., AND A. R. GOLDSMITH. 1990. Plasma levels of prolactin and gonadal steroids in relation to multiple brooding and renesting in free-living populations of the Song Sparrow, *Melospiza melodia*. Hormones and Behavior 24:89–103.