Pergamon

0960-0760(95)00149-2

*J. Steroid Biochem. Molec. Biol.* Vol. 55, No. 1, pp. 93–106, 1995

0960-0760/95 $9.50+0.00

# Reptilian (*Chrysemys picta*) Hepatic Progesterone Receptors: Relationship to Plasma Steroids and the Vitellogenic Cycle

**G. Giannoukos and I. P. Callard***

*Department of Biology, Boston University, Boston, MA 02215, U.S.A.*

**In non-mammals, estrogen-induced yolk precursors produced by the adult female liver are the main nutritional source for development. Evidence exists that progesterone exerts counter-regulatory effects on estrogen-induced vitellogenesis, and we have used the turtle model (*Chrysemys picta*) to study changes in hepatic progesterone receptor during the vitellogenic cycle. Using radioligand methods, we show that high and lower affinity binding sites are present in the cytosolic but not nuclear extracts. The lower affinity site is detectable at all times of the year; the high affinity site is mainly observed during non-vitellogenic periods and does not correlate with plasma estrogen. DNA-cellulose chromatography shows that PR-A is present in spring, summer, and winter, and that PR-B is down-regulated except in animals which recently laid eggs. Western blots confirm the presence of PR in all months, but PR-A (88 kDa) is the dominant isoform. PR-B (125 kDa) is well correlated with the luteal phase, winter and fall. Immunocytochemical studies show that PR is nuclear in location, and nuclear heat shock protein 90 (hsp90) is present. Competitive binding studies of both sites reveal that progesterone is the most effective ligand for both, followed by pregnenolone, deoxycorticosterone, and R5020. RU 486 does not bind to the high affinity site but binds moderately well to the lower affinity site. This study suggests that progesterone receptor isoforms are differentially expressed and may be involved as transcriptional regulators of hepatic function outside the periods of active vitellogenesis in the turtle.**

*J. Steroid Biochem. Molec. Biol.*, Vol. 55, No. 1, pp. 93–106, 1995

## INTRODUCTION

In non-mammalian species, the main nutritional supply to the developing embryo is yolk. The principal precursor of the yolk proteins, vitellogenin, is made in the liver, released into the blood stream and is transported into the developing oocytes via membrane receptors [1]. Vitellogenin, a sex differentiated hepatic protein, has been characterized in invertebrate, teleost fish, elasmobranch, amphibian, reptilian, and avian species [2], and can be induced by estradiol (E) in males (frogs [3]; turtles [4,5]; birds [6]) and immature chicks [7].

In the physiological context of the adult female, vitellogenesis is a cyclic process in temperate zone vertebrates. Despite the ubiquity of E, vitellogenin is not always expressed, or is expressed at reduced levels, in many female vertebrates. This suggests a counter-regulatory role for other hormones, and work from this laboratory has provided evidence that progesterone (P) inhibits E-induced vitellogenesis in reptiles [4,8,9] and elasmobranchs [10]. In order to pursue the possibility that P, through its receptor, inhibits vitellogenesis, either directly via the vitellogenin gene or indirectly (e.g. via estrogen receptor down-regulation) or both, changes in hepatic progesterone receptor (PR) isoforms during the annual cycle of ovarian growth and vitellogenesis need to be assessed. We have chosen the turtle, a seasonal breeder, in which hepatic PR has already been identified [11] for this study. Here we report radioligand studies of PR isoforms during the annual reproductive cycle, along with Western blot analysis of PR isoforms and cellular localization of PR by immunocytochemistry (ICC). The results suggest that hepatic PR is present at all times, but that there are significant seasonal changes in high affinity binding associated with non-vitellogenic periods. In addition, PR-A and PR-B isoforms are represented to differing degrees in different

*Correspondence to I. P. Callard.
Received 5 Jan. 1995; accepted 18 May 1995.

seasonal samples, suggesting a seasonal basis, PR-B being most labile.

## MATERIALS AND METHODS

### *Animals*

Animals were freshly caught during February ($n$=6), April ($n$=8), May ($n$=4), June (early luteal, $n$=4; late luteal, $n$=6), August ($n$=4), September ($n$=4), and October ($n$=3) (Lemberger, WI) and shipped to Boston. Experimental animals were maintained in fresh running water aquarium tanks at 24 C (water), 22 C (air) on a 12 h light–dark photoperiod. Animals were fed Wardley reptile "total essential nutrition floating food sticks" (Wardley Corp., Secaucus, NJ).

Reproductively active animals (April–October) were classified on the basis of ovarian and follicular size at autopsy and the presence/absence and condition of oviductal eggs as determined by this laboratory. April animals were considered preovulatory on the basis of follicular size (15–17 mm) and plasma E levels. May–August animals were segregated based on (a) absence of eggs in the oviduct and size of ovaries, (b) eggs in the oviduct, and (c) having laid eggs in the laboratory. These were referred to, respectively, as peri-ovulatory (maximal follicular size ~18 mm) (a), post-ovulatory/gravid (b), and post-oviposited (eggs laid) (c). For group b (gravid animals), several criteria were used in order to assign animals to one of two post-ovulatory, but pre-oviposited, groups: early and late luteal. Animals in these last two groups were initially assigned on the basis of hardness of egg shell (i.e. flexibility and thickness based on direct visual inspection) and subsequently on the basis of plasma P (high P being indicative of luteal function) and oviduct receptor content (positive or negative high affinity binding).

### *Chemicals and reagents*

Radioactive [1,2,6,7-$^3$H]progesterone (P) (sp. act. 80.2–111.1 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Radioactive [2,4,6,7-$^3$H]estradiol-17$\beta$ (E) (sp. act. 103–108 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Radioinert steroids used were P (Nutritional Biochemicals Corportation, Cleveland, OH); R5020 and mibolerone (New England Nuclear, Boston, MA); RU 486 (Roussel Uclaf, France); corticosterone and pregnenolone (Sigma, St Louis, MO); E, 5$\alpha$-dihydrotestosterone, testosterone and deoxycorticosterone (Steraloids, Inc., Wilton, NH). Radioactive P and E were repurified before use by thin layer chromatography using either a benzene–acetone (4:1, v/v) system for P or an ether–hexane (3:1, v/v) system for E. Stock solutions of hormones were prepared in absolute ethanol and stored at −20°C. Estradiol-17$\beta$ and progesterone antisera for radioimmunoassay (RIA) analysis were provided by Dr G. Niswender (Colorado State University, Fort Collins, CO). Monoclonal antibodies, anti-PR 22 raised against the chick PR isoforms (PR-A and B) and AC88 derived from *Achlya ambisexualis* which recognizes heat shock protein (hsp) 90 in a variety of animal species, were provided by Dr D. O. Toft (Mayo Clinic, MN). Vectastain ABC Kit (biotinylated mouse IgG) was purchased from Vector Laboratories, Inc. (Burlingame, CA). Sephadex LH-20 and Dextran T-70 were purchased from Pharmacia (Piscataway, NJ). Acrylamide, *N-N'*-methylene-bis-acrylamide and other electrophoretic materials were from Bio-Rad (Richmond, CA). Norit A charcoal, prestained protein molecular weight markers, heparin sodium salt from bovine intestinal mucosa, mouse IgG purified immunoglobulin from serum, goat anti-mouse IgG (whole molecule) peroxidase conjugate, and 3',5'-diaminobenzidine were from Sigma (St Louis, MO). Enhanced chemiluminescence (ECL) Western blot detection reagents were purchased from Amersham (Arlington Heights, IL). Optifluor scintillation cocktail was obtained from Packard (Meriden, CT). Kodak X-OMAT film was purchased form Eastman Kodak Company (Rochester, NY). Other chemicals were reagent grade or better.

The buffers used were as follows: TEMG buffer (10 mM Tris–Base, 1 mM EDTA, 1 mM 2-mercaptoethanol, 10% glycerol, pH 7.5); homogenizing buffer (50 mM Tris–HCl, 1 mM EDTA, 12 mM monothioglycerol, 30% glycerol, pH 7.5). The nuclear extraction buffer used was 0.7 M KCl in homogenizing buffer according to Chen and Leavitt [12]. The nuclear washing buffer consisted of 10 mM Tris–Base, 12 mM monothioglycerol, 3 mM $MgCl_2$, 0.25 M sucrose at pH 7.5. Buffers for DNA–cellulose were as follows: 200 mg/l BSA in TEMG (Buffer A); 0.5 M NaCl+200 mg/l BSA in TEMG (Buffer B). Dextran-coated charcoal (DCC) was made up in TEMG (0.5% washed Norit-A charcoal and 0.05% Dextran T-70). Western buffer consisted of 20 mM Tris–Base, 150 mM NaCl, 0.5% Tween 20, 1.0% BSA with a pH of 7.5. Turtle buffer for perfusion of liver consisted of 130 mM NaCl, 3 mM KCl, 10 mM HEPES, 1 mM $MgSO_47H_2O$, and 1 mM $CaCl_22H_2O$, pH 7.5. PBS buffer consisted of 154 mM NaCl, 9.15 mM sodium phosphate dibasic anhydrous, 33.3 mM sodium phosphate monobasic anhydrous, pH 7.2. RIA buffer contained 5.9 mM $K_2HPO_4$, 3.97 mM $KH_2PO_4$, 139.9 mM NaCl, 15.4 mM $NaN_3$, and 0.1% gelatin (g/vol), pH 7.4.

### *Preparation of cytosol and nuclear extracts*

All procedures were carried out at 0–4°C. After decapitation, the plastron was removed to expose the body cavity. A cannula was inserted into the inferior vena cava through which 150 ml of heparinized (150 units/ml) turtle buffer was pumped using a Masterflex perfusion pump (Barnant Company, Barrington, IL)

set at 2.5 ml/min. Small incisions were made on the surface of the liver to allow for outflow of perfused buffer. The livers were removed and washed three times in 0.65% NaCl TEMG buffer. Samples were weighed (approx. 5 g), minced and homogenized in 3 vol (vol/g) homogenizing buffer using a Potter–Elvehjem homogenizer using three up and down strokes. The homogenate was filtered through eight layers of cheese cloth and centrifuged at 1400 ***g*** for 20 min. The surface lipid was aspirated, and the supernatant was centrifuged at 100,000 ***g*** for 1 h (37,000 rpm, rotor Sorvall T-865.1, Sorvall OTD-65 centrifuge, Du Pont Instuments, Wilmington, DE) to obtain crude cytosol, which was then frozen at −70°C. The pellets were washed 3× in washing buffer with centrifugation at 1000 ***g*** for 15 min at 4°C between each wash. The pellet was then re-suspended in 3 vol (vol/g) of nuclear extraction buffer and incubated for 1 h on ice with vortexing every 15 min. The final nuclear suspension was centrifuged at 100,000 ***g*** for 60 min to yield a clear nuclear extract. Samples were stored at −70°C until analysis.

*Sephadex LH-20 chromatography*

Sephadex LH-20 was swollen in distilled water, packed into 5 × 105 mm acid washed columns, and equilibrated with TEMG buffer at 4°C. Chromatography was performed according to Mak *et al.* [13]. Briefly, 100 ml cytosol was applied and allowed to adsorb onto the equilibrated columns. A further 200 ml buffer was added to the columns and allowed to adsorb. The columns were sealed and incubated for 30 min at 4°C followed by elution of sample with 800 μl of TEMG buffer. Optifluor (4 ml) was added and radioactivity measured in a Delta Liquid Scintillation Counter (Tracor, Elk Grove, IL; efficiency for tritium, 60%). Values were expressed as fmoles steroid bound per mg protein in extract as determined by method of Lowry [14].

*Saturation analysis of [$^3$H]P binding*

To remove endogenous steroids, cytosol was charcoal stripped prior to analysis by incubating for 10 min at 0°C with a DCC pellet derived from a suspension equivalent to sample volume followed by centrifugation (2800 rpm × 5 min at 4°C). Cytosol was diluted 1:10–1:40 in TEMG buffer. Aliquots (100 μl) of diluted cytosol were incubated with 1–100 nM [$^3$H]P (total binding) and 1–100 nM [$^3$H]P plus 400-fold radioinert P (non-specific binding) for 16 h at 4°C. Samples contained 1 μM corticosterone to prevent P binding to the corticosterone binding globulin-like components [11,12]. Bound and free steroids were separated by LH-20 chromatography. Specific binding was calculated by subtracting non-specific binding from total binding. Appropriate cytosol dilution of each sample was determined prior to saturation analysis by a mini-Sephadex LH-20 assay as follows: cytosol was diluted 1:10–1:40 and 100 μl of each dilution was incubated with 8 nM [$^3$H]P (previously determined concentration for saturation of high affinity P binding component) plus 1 μM corticosterone in the presence (non-specific binding) or absence (total binding) of 400-fold radioinert P for 16 h at 4°C followed by Sephadex LH-20 chromatography. Dilution exhibiting the highest [$^3$H]P binding was chosen for subsequent saturation analysis. Data was analyzed by the Lundon ReceptorFit Saturation Two-Site program (Lundon Software, Inc., Chagrin Falls, OH).

*Competition analysis*

100 μl of diluted and DCC stripped cytosol was incubated with 8 or 80 nM [$^3$H]P with or without increasing concentrations (10-, 100-, and 1000-fold excess) of radioinert competitors for 16 h at 4°C. Bound and free steroids were separated by Sephadex LH-20 chromatography. Percent inhibition was calculated by subtracting the percent specific [$^3$H]P bound in the presence of each competitor from total [$^3$H]P (100%).

*DNA–cellulose affinity chromatography*

DNA–cellulose was prepared according to Alberts and Herrick [15], and procedures similar to those by Salhanick *et al.* [16] were used with modifications. The post-labelling method was used. Diluted cytosol (1 ml) was applied and allowed to adsorb onto DNA–cellulose columns (5 × 120 mm) equilibrated with TEMG+200 mg/l BSA buffer. The columns were incubated at 22°C for 30 min followed by 4°C for 10 min. and then washed with TEMG buffer for 3 h using a Rainin Rabbit peristaltic pump (Rainin Inst. Co., Inc., Boston, MA) set at a 4 ml/h rate. 20 or 80 nM [$^3$H]P plus 1 μM corticosterone in the presence (non-specific binding) or absence (total binding) of 400-fold radioinert P in 1 ml TEMG buffer was added and adsorbed onto the columns followed by a 16 h incubation at 4°C. The columns were then washed for 6.5 h with buffer A+10 mM sodium molybdate followed by a 45 min wash in buffer A (without sodium molybdate). 1 ml fractions were then eluted with a linear 0–0.5 NaCl gradient using buffers A and B. Salt concentration was determined by Conductivity Meter CDM3 (The London Co., Cleveland, OH) and radioactivity by scintillation counting.

*Immunocytochemistry (ICC)*

Livers were removed and infiltrated with 10% sucrose/PBS buffer for 6 h followed by freezing in Tissue Tek OCT Compound (Miles Inc., Elkhart, IN) and stored at −70°C. 10 micron fresh frozen sections were obtained on an AO Cryo-Cut Cryostat Microtome Model 840C (American Optical Corportation, Buffalo, NY) and air dried on gel

coated slides for 10 min. The sections were fixed in 4% paraformaldehyde/PBS buffer for 10 min, washed for 10 min in PBS, followed by 2 min in 0.05% $H_2O_2$/PBS. The sections were washed for 10 min in PBS followed by 20 min in blocking solution (Vector ABC Kit). Application of primary antibody [8.3 μg/ml anti-PR 22, specific for either the chick PR-A or B isoforms, or 6.25 μg/ml AC88 (hsp90)] for 24 h was terminated by a 15 min PBS wash followed by the application of secondary antibody labelled with biotinylated complexes (Vector ABC Kit) for 30 min. A 10 min PBS wash was followed by a 30 min application of avidin complexed with peroxidase. The sections were washed for 10 min in PBS followed by a 2 min exposure of 3′, 5′-diaminobenzidine (5 mg/ml in Tris–HCl, pH 7.2) in the presence of 0.025% $H_2O_2$ which resulted in the precipitation of a brown chromogen. The reaction was stopped by distilled water (10 min) followed by a series of 2 min ethanol washes: 70%, 2×; 95%, 2×; 100%, 2×. The sections were dehydrated with 2, 5 min washes in xylene and cover slips mounted with Permount (Fisher, Fair Lawn, NJ).

*Ammonium sulfate precipitation of PR*

Cytosol PR was precipitated with 50% ammonium sulfate incubated for 30 min at 4°C on a rocker platform. Extracts were centrifuged at 4000 ***g*** (IEC centra MP4R, Fisher Scientific, Pittsburg, PA). The pellet was washed with distilled water and reconstituted in 100 ml distilled water. Protein concentration was determined by the method of Lowry [14].

*Western blotting procedure*

Equal volumes of cytosol and nuclear extracts, to reflect changes in PR protein over the seasonal cycle, were precipitated and resolved by electrophoresis on discontinuous polyacrylamide gels according to the method of Laemmli [17] using 5–20% gradient gels. Western procedure was according to Sullivan *et al.* [18] with modifications as follows: the proteins were transfered onto nitrocellulose (Schleicher and Schuell, Keene, NH) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio Rad, Richmond, CA) for 45 min at 15V. The nitrocellulose was blocked for 30 min at 37°C in Western buffer. The nitrocellulose was incubated with primary antibody (anti-PR 22 or mouse IgG) at a concentration of 8.3–12.5 μg/ml Western buffer overnight at 4°C on a rocker platform. The nitrocellulose was washed 3 times (15 min, 1×; 5 min, 2×) in Western buffer and incubated with horse radish peroxidase conjugated goat anti-mouse IgG diluted 1:1000 in Western buffer at 22°C for 4 h on a rocker platform. The nitrocellulose was washed 3 times (15 min, 1×, 5 min, 2×) in Western buffer followed by a 1 min incubation in ECL Western blotting reagents. Bands were exposed on Kodak film.

*Plasma steroid extraction procedure*

Animals were sacrificed by decapitation and blood was collected into heparinized test tubes. Blood was centrifuged at 2800 rpm for 10 min to obtain plasma and subsequently stored at −20°C until extraction. Steroids were extracted from plasma by methods routinely used in this laboratory [19]. Briefly, P and E were extracted from 0.5 ml turtle plasma with 2×10 vol diethyl ether. To check recovery of steroids from plasma, 1000 cpms of each radiolabelled tracer were extracted from an equal volume of DCC stripped turtle plasma [20]. Plasma samples were vortexed for 30 s and centrifuged for 15 min at 2800 rpm at 4°C. After a clear separation of the two phases, the lower aqueous phase was quick frozen by immersing the test tube in a dry-ice–methanol mixture. The organic layer was decanted into clean 16×100 mm test tubes and dried under a stream of nitrogen. The steroids were resuspended in the original 0.5 ml volume with RIA buffer.

*Steroid radioimmunoassay (RIA)*

P and E levels were determined by RIA as previously described [19] using antibodies obtained from Dr G. Niswender. Aliquots of extracted steroids (2–50 μl) diluted to final volume of 100 μl were incubated with 100 μl of the appropriate radiolabelled tracer (10,000–15,000 cpms) and 200 μl of diluted antisera for a final incubation volume of 400 μl, overnight at 4°C. The final incubation dilution of antisera was as follows: P 1:35–1:54, maximum binding 32–57%; and E 1:30–1:35, maximum binding 30–48%. Bound and free steroids were separated using 400 μl DCC suspension in RIA buffer. Bound radioactivity was measured in a Delta Liquid Scintillation Counter (Tracor, Elk Grove, IL; efficiency for tritium, 60%). Assay sensitivity was 3.9 pg for P and E. The interassay coefficient of variation was 13.5% for P and 13.75% for E.

## RESULTS

*Seasonal cycle of hepatic progesterone binding sites determined by Scatchard analysis: correlations with plasma steroids and ovarian growth (Figs 1 and 2)*

Saturation analysis for diluted cytosol (1:10–1:40) extracts ranging from 1–100 nM [$^3$H]P and 1 μM corticosterone was performed in the presence (non-specific binding) and absence (total binding) of 400-fold excess radioinert P. Scatchard analysis was done using the Lundon ReceptorFit Saturation Two Site program to determine PR values for the high and low affinity binding components over the seasonal cycle [Fig. 1(A)]. The molar values of the two binding components were converted into fmol/mg protein. Typical biphasic (high and lower affinity sites) and single site (low affinity only) Scatchard plots for samples where either both or only the lower affinity site were present are shown [Fig. 2(A,B)]. The $K_d$

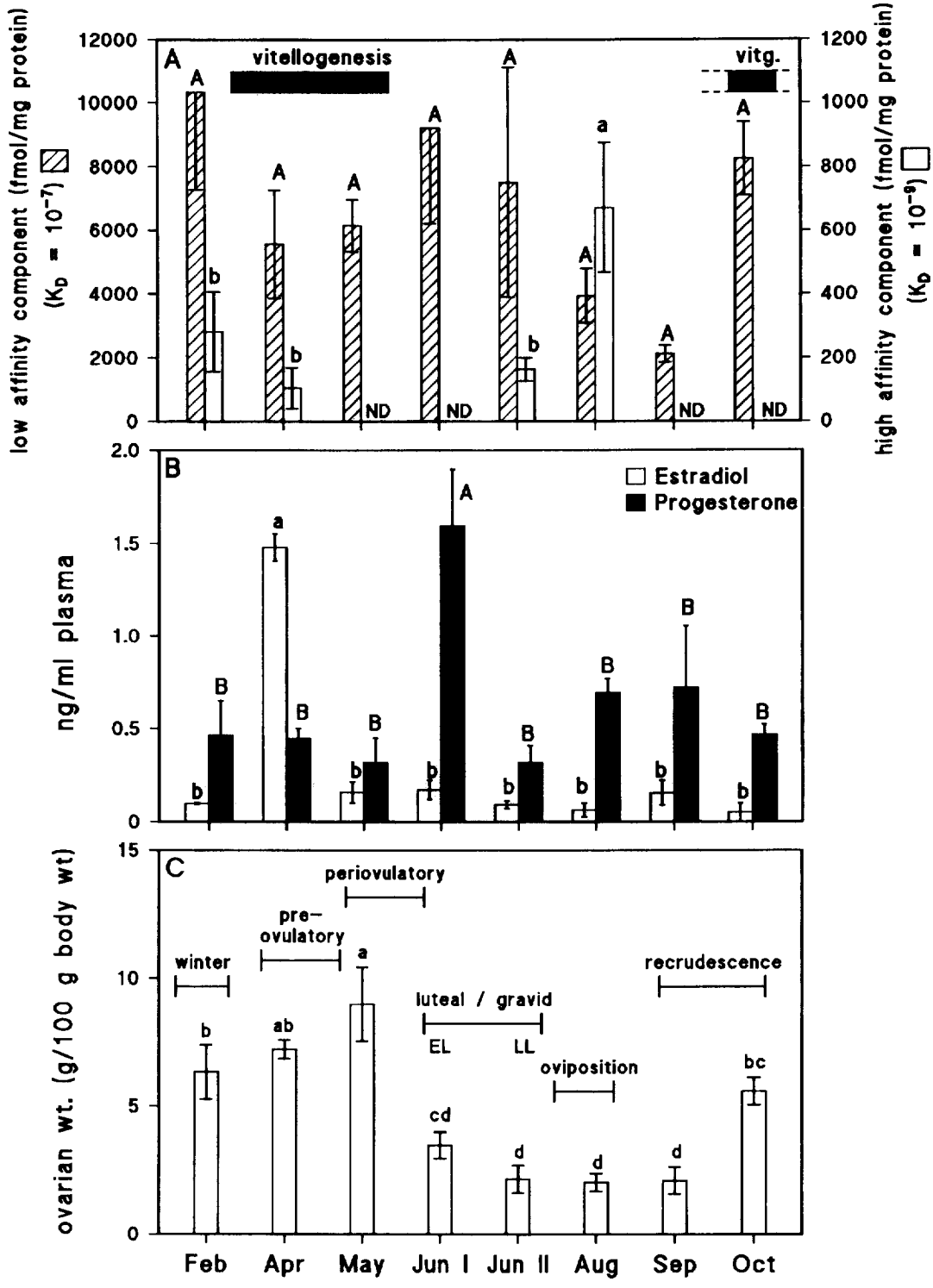


Fig. 1. Seasonal changes in hepatic cytosol PR levels (A), plasma steroid levels (B), and ovarian weight (C). Receptor binding studies were determined by LH-20 chromatography followed by Scatchard analysis using the Lundon ReceptorFit Saturation Two Site Program (Lundon Software) as described in Materials and Methods. Values are expressed as the mean ± the standard error of the mean. One-way ANOVA followed by Duncan's multiple range test ($P<0.05$) of seasonal changes in hepatic PR, plasma steroid levels, and ovarian weight. For the receptor (A) and steroid levels (B), statistical analysis was performed on each variable separately (capital letters were used for statistical results of the low affinity component and progesterone levels; lower case letters were used for the high affinity component and E levels). ND, non-detectable. February ($n$=6), April ($n$=8), May ($n$=4), June (early luteal, $n$=4; late luteal, $n$=6), August ($n$=4), September ($n$=4), October ($n$=3).

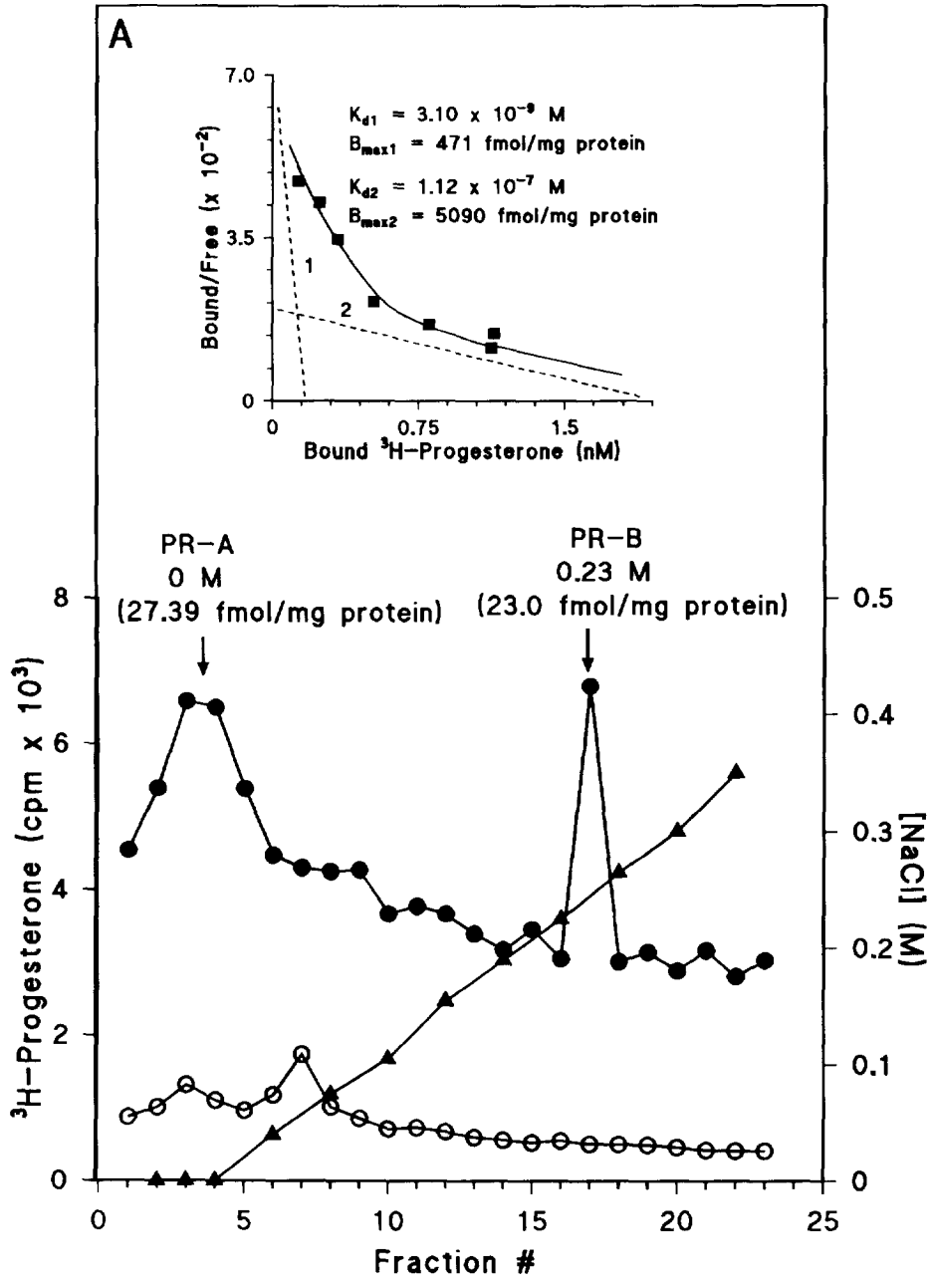


**Fig. 2(A)** *-legend on p. 100.*

values were: $K_{d1}=3.10\times10^{-9}$ M and $K_{d2}=1.12\times10^{-7}$ M [Fig. 2(A)]; and $K_{d2}=1.02\times10^{-7}$ M [Fig. 2(B)].

Both the high and lower affinity components were present in the cytosol extracts [Fig. 1(A)], and no detectable binding was found in nuclear extracts (data not shown). During the winter (February), high affinity binding was present; the binding decreased in the spring after emergence from hibernation concurrent with the spring peak in plasma E [1478 ± 208 pg/ml plasma, $P<0.05$ vs remaining periods of cycle; Fig. 1(B)] and ovarian growth [Fig. 1(C)] associated with vitellogenesis. By the peri-ovulatory period and into the early post-ovulatory (luteal) phase (May and June early luteal stage), high affinity binding was not detectable. The June-I (early luteal) phase is marked by a peak in plasma P [1595 ± 591 pg/ml plasma, $P<0.05$ vs remaining periods of the cycle; Fig. 1(B)]. High affinity PR levels began to increase during the June-II (late luteal) phase, following peak plasma P, and reached significant levels ($P<0.05$ vs February, April, and June late luteal) after egg laying in August. Ovarian weights remain low in June–September. By the onset of the second vitellogenic period in the autumn, high affinity PR was again undetectable.

Lower affinity PR binding was present throughout the cycle, but no significant changes were detected. The lower affinity binding sites were in excess of the high affinity binding sites by as much as 50-fold.

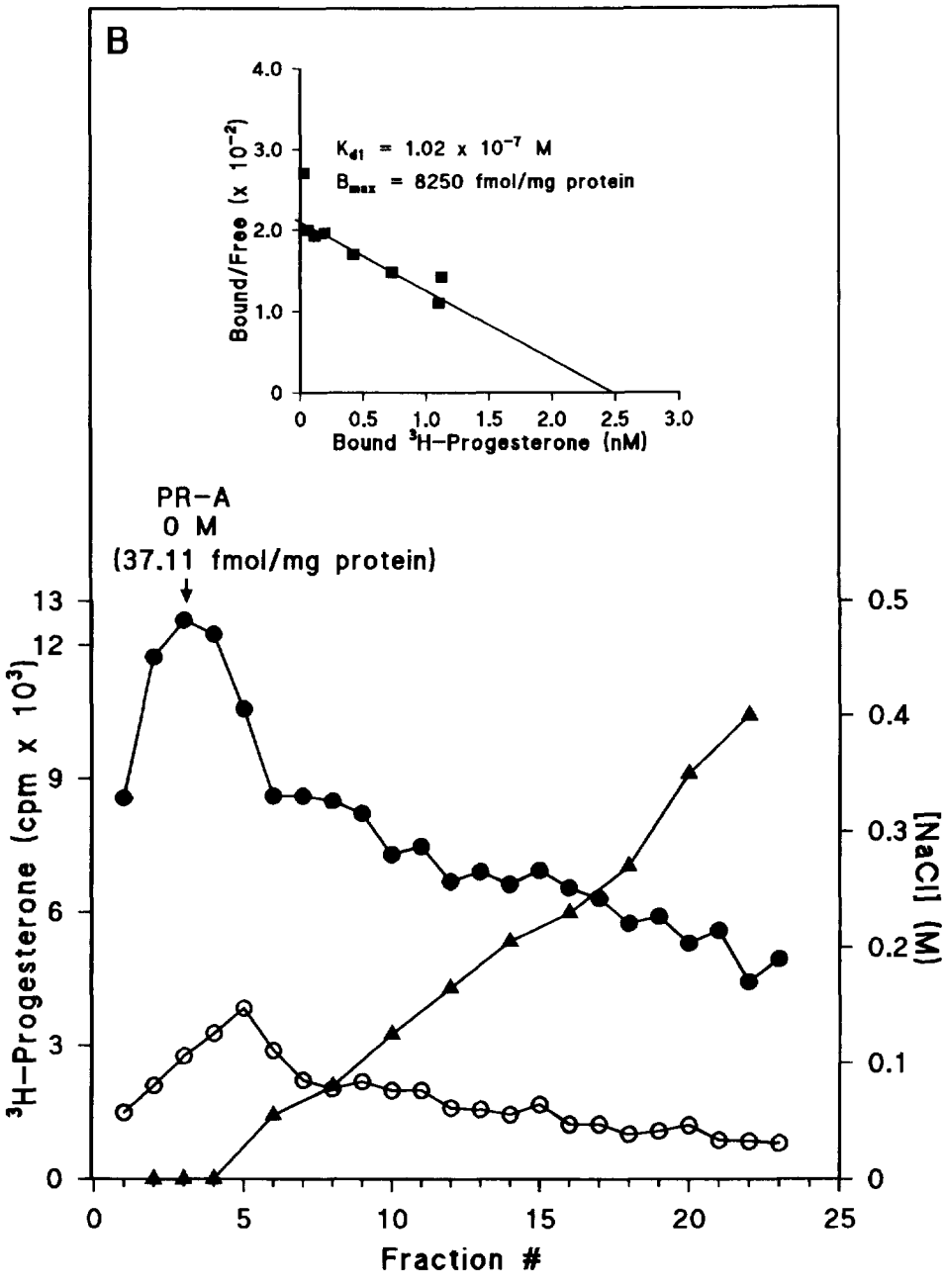


**Fig. 2(B)**—*legend overleaf.*

*Changes in PR determined by Scatchard analysis and DNA–cellulose affinity chromatography [Fig. 2(A–C)]*

Turtle oviduct PR isoforms have previously been separated and identified by DNA–cellulose chromatography [21]. To determine potential seasonal hepatic changes and whether high or lower affinity sites were preferentially associated with PR-A or B isoforms, diluted cytosol extracts containing only the lower affinity binding component [Fig. 2(B), May] or both the high and lower affinity binding components [Fig. 2(A), August] and winter samples [Fig. 2(C); February, high and lower affinity binding] were chosen for DNA–cellulose affinity chromatography on the basis of Scatchard analysis. In all extracts, specific binding to DNA–cellulose was observed. Elution profiles differed slightly depending on the presence or absence of sodium molybdate in the elution buffer. In the absence of molybdate, elution of A and B isoforms was at 0 and 0.23 M NaCl, respectively [see Fig. 2(A–C)]; in the presence of molybdate in the elution buffer, A and B isoforms were eluted from DNA–cellulose at a slightly higher salt concentration (0.095 and 0.305 M NaCl, respectively; data not shown). In the May sample, in which only the lower affinity binding component was detectable [Fig. 2(B)], only PR-A (37.11 fmol/mg protein) binding was

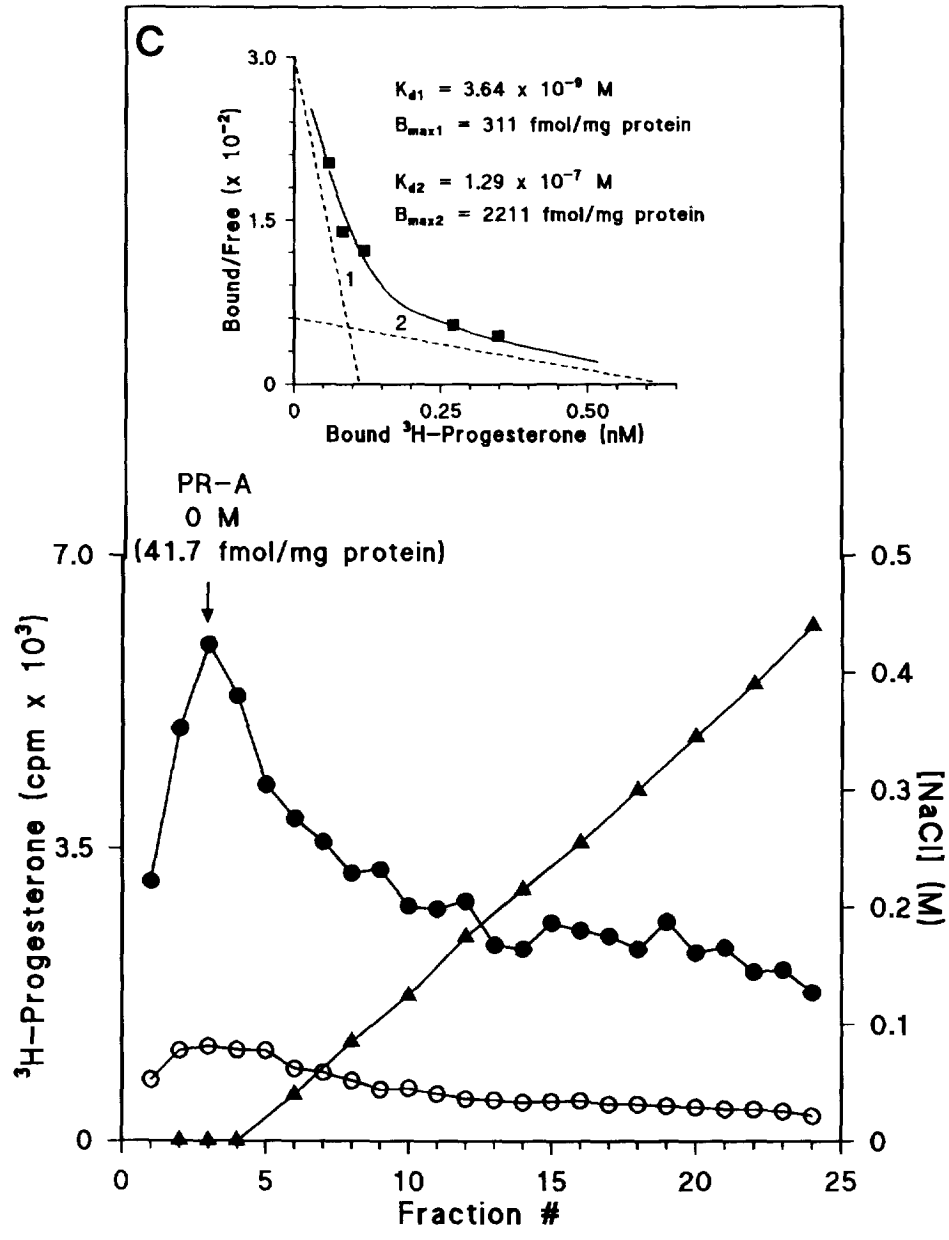


Fig. 2(C).

**Fig. 2. DNA–cellulose affinity chromatography of hepatic PR using cytosol extracts containing high and lower affinity sites [(A), August animal; and (C), February animal] or extracts containing only the lower affinity sites [(B), May animal], as determined by Scatchard analysis (A–C, insets). The Lundon ReceptorFit Saturation Two-Site program revealed a Scatchard plot with either one parameter as seen in May (insert B: $K_d$=1.02 × $10^{-7}$ fmol/mg protein, $B_{max}$=8250 fmol/mg protein), or two parameters as seen in August (insert A, dashed lines: $K_{d1}$=3.10 × $10^{-9}$ M, $B_{max1}$=471 fmol/mg protein; $K_{d2}$=1.12 × $10^{-7}$ M , $B_{max2}$=5090 fmol/mg protein) and February (inset C, dashed lines: $K_{d1}$=3.64 × $10^{-9}$ M, $B_{max1}$=311 fmol/mg protein; $K_{d2}$=1.29 × $10^{-7}$ M , $B_{max2}$=2211 fmol/mg protein). In May extracts containing only one binding site (B), one specific peak eluted at 0 M NaCl (37.11 fmol/mg protein). In August extracts containing two binding sites (B), two specific peaks eluted from DNA-cellulose at 0 M NaCl (27.39 fmol/mg protein) and 0.23 M NaCl (23.0 fmol/mg protein). In February extracts (C), one specific peak eluted from DNA–cellulose at 0M NaCl (41.7 fmol/mg protein). Total binding (●); non-specific binding (○); NaCl gradient (▲).**

detectable by DNA–cellulose chromatography. In August samples containing both high and lower affinity binding components [Fig. 2(A)], both PR-A and B isoforms were present in approximately equal values (27.39 and 23.0 fmol/mg protein, respectively). In winter animals [February, Fig. 2(C)], cytosol extracts contain both high and lower affinity binding components but only the PR-A isoform (41.7 fmol/mg protein) was observed, as in peri-ovulatory [May, Fig. 2(B)] animals.

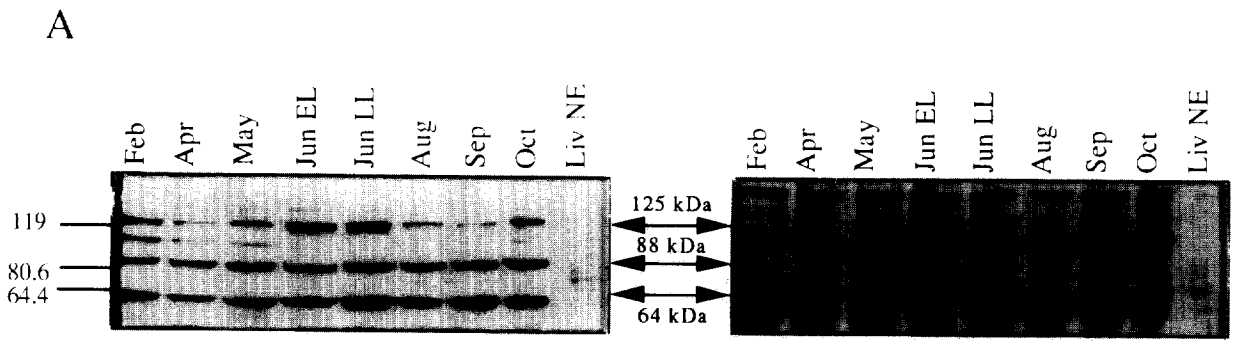


**Fig. 3. Western blot analysis of hepatic PR in cytosol extracts during the seasonal cycle. 1 ml cytosol and nuclear (used as negative control) extracts were precipitated with 50% ammonium sulfate and the protein pellet was dissolved in distilled water and added to 2 × loading buffer and boiled for 2 min. Equal volumes (30 μl) of each cytosol and a nuclear sample were run on 5–20% gradient gels. Samples were transferred onto nitrocellulose, incubated with 12.5 μg/ml of either PR-22 antibody (A) or mouse IgG (B). Early luteal (EL), late luteal (LL).**

*Seasonal changes in hepatic PR determined by Western blot analysis [Fig. 3(A,B)]*

Western blot analysis of cytosol extracts was conducted throughout the seasonal cycle using the anti-PR 22 antibody. In cytosol extracts [Fig. 3(A)], three specific bands of 125 (PR-B), 88 kDa (PR-A), and 64 kDa (putative PR-C) were seen throughout the cycle. Two bands, the 88 kDa band representing the PR-A and the 64 kDa band representing the putative PR-C, were intensely labelled throughout the seasonal cycle [Fig. 3(A)]. The 125 kDa band representing the PR-B varied throughout the cycle and strongest labelling was observed during the early and late luteal stages in June. No labelling was observed in nuclear liver extracts confirming radioligand studies which showed no P binding. In addition, no reactive bands were seen at the corresponding molecular weights in the mouse IgG controls [Fig. 3(B)].

*ICC analysis of PR and hsp90 distribution in turtle liver (Fig. 4)*

The same monoclonal antibody (anti-PR 22) was used to monitor the localization and changes in hepatic PR throughout the seasonal cycle. ICC results revealed PR localization in the nuclei of the cells [Fig. 4(B–I)]. Progesterone receptor staining in nuclei was observed in tissue sections taken from every stage of the cycle. This staining was specific for the PR since parallel sections incubated with an anti-mouse IgG monoclonal antibody showed clear nuclei [Fig. 4(A)].

Using the monoclonal antibody for hsp90 (AC88), peri-ovulatory and June (late luteal) hepatic sections were examined. hsp90 staining was observed in the cytoplasm and nuclei of liver sections in peri-ovulatory animals [Fig. 4(J)] where PR high affinity binding is not detectable [Fig. 1(A)], however, staining was not observed in June-II (late luteal animals; data not shown) where PR high affinity binding was present [Fig. 1(A)].

*Binding specificity of the lower affinity binding site (Table 1).*

The specificity of [$^3$H]P binding activity of the cytosolic lower affinity component was determined by competition assay in the presence of 10-, 100-, and 1000-fold radioinert competitor. Using 100-fold

*Table 1. Competition study on hepatic low affinity PR sites*

| | % Inhibition lower affinity site | | | % Inhibition high affinity site | | |
|---|---|---|---|---|---|---|
| Cold steroid | 10 × | 100 × | 1000 × | 10 × | 100 × | 1000 × |
| Progesterone | 24.1 | 81.4 | 94.5 | 67.3* | 95.0* | 100* |
| Promegestone (5020) | 4.8 | 56.5 | 83.5 | 33.4* | 69.9* | 92.9* |
| RU 486 | 0.5 | 22.9 | 73.1 | 3.2 | 0 | 17.8 |
| Pregnenolone | 13 | 61.8 | 88.3* | 31.4* | 45.9* | 91.8* |
| Deoxycorticosterone | 0 | 36.8 | 89.9 | 39.7* | 89.1* | 95.4* |
| Dihydrotestosterone | 0 | 12.9 | 75.9 | 5.2* | 11.9* | 60.9* |
| Testosterone | 0 | 25.5 | 80.4 | 13.4 | 14 | 71.4 |
| Mibolerone | 0 | 0 | 39.9 | 18 | 43 | 40 |
| Estradiol | 0.6 | 37.3 | 68.2 | 0 | 14.6 | 34.9 |
| Corticosterone | 0 | 31.9 | 66.7 | 5.7* | 17.9* | 42.8* |

100 μl of DCC stripped cytosol extracts, diluted 1: 30, were incubated overnight at 4 °C with 80 mM [$^3$H]P in the presence of 10 ×, 100 ×, 1000 × competitors. Data is presented at % inhibition of [$^3$H]P by competitor. In addition, a competition study for high anity site as previously published [11], using 30 nN [$^3$H]P, with additional data from this study, using 8 nM [$^3$H]P, is shown for comparison (*).

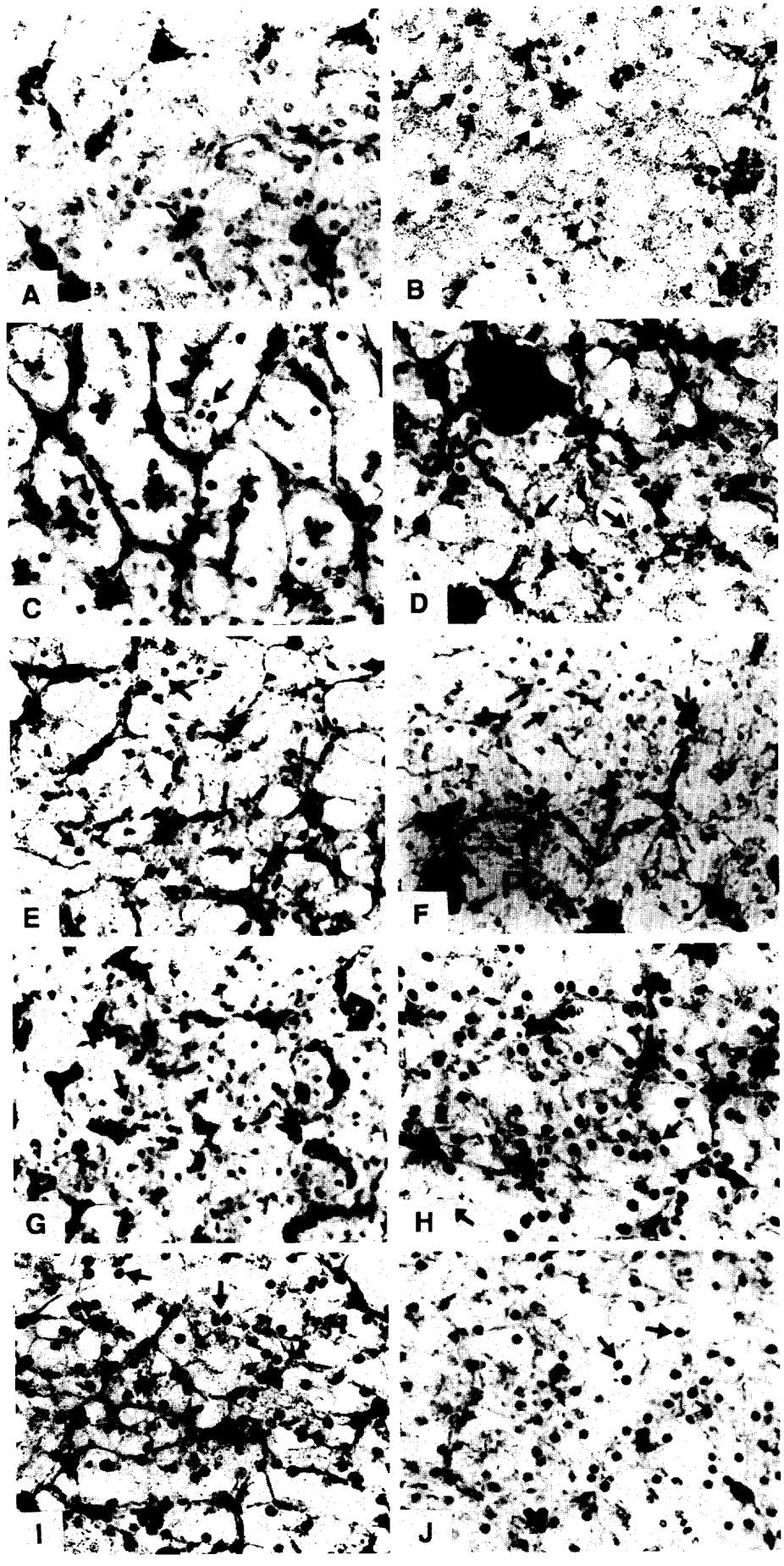


**Fig. 4** — *legend opposite.*

competitor, P competed the best followed by pregnenolone and R5020. Other steroids competed less well (estradiol = deoxycorticosterone > corticosterone > testosterone = RU 486 > dihydrotestosterone > mibolerone). The binding specificity of the high affinity site as previously published [11] is shown for comparison with additional data provided from this study.

## DISCUSSION

In previous studies, we characterized the first vertebrate hepatic PR in the liver of *Chrysemys picta* [11], and we suggested that PR is physiologically important in shutting off vitellogenesis in response to E after ovulation and oviposition in this and other species. In the current study, we investigated seasonal changes in the high and lower affinity PR sites, which have previously been described [11], and the distribution of the receptor and its different isoforms in liver by immunochemical means. The results show that hepatic lower affinity PR sites are present throughout the reproductive cycle with no significant variation. High affinity sites, measured by Scatchard analysis, are present in the late winter and early spring, disappear in May/June early luteal animals during the vitellogenic and early post-vitellogenic phase, and reappear just before (June, late luteal) and remain until after oviposition (early August). Neither high nor lower affinity sites were found in the nuclear compartment using radioligand binding techniques, suggesting a lower affinity of hepatic PR for DNA than turtle oviduct PR (discussed below). The observed changes in high affinity binding sites in liver suggest a function outside of the active vitellogenic phase [i.e. late winter/early spring; and June/August (post vitellogenic and pre-recrudescence)]. While ovarian weights are high in February, vitellogenin synthesis is arrested [22]; ovarian weights begin to increase in April when E levels and E-induced vitellogenesis are highest, even though some high affinity PR remains in the liver. High affinity PR levels are not particularly well correlated with plasma E, declining during high E (April) and undetectable in May/June (early luteal phase) as E declines associated with ovulation. High affinity PR returns before oviposition (June late luteal) and increases maximally in August (following oviposition), a period when ovarian weight is at a nadir (June late luteal–September); high affinity PR disappears as vitellogenesis begins in the autumn, associated with a small increment in plasma E (September/October). Thus, detection of high affinity PR by radioligand methods is associated with periods during which vitellogenesis is not occurring (winter, early spring, and summer).

Although a case can be made for E up-regulation of PR in the oviduct [23], this may not be the case for the liver. In fact, E down-regulation of PR appears more likely. This may involve E-induction of epidermal growth factor (EGF) which down-regulates PR binding in T47D breast cancer cells [24]. Although only limited studies of hsp90 ICC were done, the key groups were considered to be peri-ovulatory and post-ovulatory because of the increase in plasma progesterone associated with ovulation. Whether the non-detectability of hsp90 in post-ovulatory animals is due to increased P cannot be determined without further studies. However, since hsp90 is inducible by E in rodents [25], changes in hsp90 may be steroid regulated and could play a role in regulating availability of PR and the physiological effects of P on hepatic vitellogenesis.

It does not appear that P down-regulates its own receptor in the turtle liver. Although high affinity binding can only be demonstrated outside of the vitellogenic phase, the receptor is present at all times as shown by ICC and Western blots. Furthermore, in P-injected animals, PR is not down-regulated [26]. High plasma P levels in the early luteal phase may suppress E related growth factor(s) or hsp90 changes discussed above, releasing the inhibition of P action.

Previous studies of the turtle hepatic PR [11] showed that after photoaffinity labelling with R5020 and elution from DEAE–Sepharose columns, SDS–PAGE of the high and low salt peaks revealed molecular weights of 123 and 95 kDa, respectively. This is similar to the chick in which the high salt form (equivalent to PR-B) is 115 kDa, and the low salt form (equivalent to PR-A) is 79 kDa [27]. However, it was previously determined that the affinity of the B and A isoforms of the turtle PR for DNA–cellulose [21] is the reverse of the chick (i.e. chick PR-B=low salt, high molecular weight; PR-A=high salt, low molecular weight) [27]. We have used molecular weight as the primary determinant of terminology (i.e. 123 kDa form=PR-B, 95 kDa form=PR-A). These differences in DNA–cellulose affinity may relate to inherent differences in amino acid sequences, phosphorylation state, or other inherent properties of the molecule.

As in the oviduct, both A and B isoforms produce biphasic Scatchard plots after elution from DEAE-columns [11] demonstrating the presence of both high and lower affinity PR sites which cannot be separated

**Fig. 4** (*opposite*). **ICC analysis of hepatic PR over the seasonal cycle. 10 micron frozen sections were incubated with 8.33 μg/ml (B–I) of either PR-22 or mouse IgG (A) antibodies and 6.25 μg/ml AC88 (J). The DAB reaction resulted in the precipitation of a brown chromagen at the sight of the reaction. Control sections showed clear nuclei. Photos were taken at high magnification (40×). The months for each hepatic cross-section are as follows: (B), Feb.; (C), Apr.; (D), May; (E), June early luteal; (F), June late luteal; (G), Aug.; (H), Sept.; (I), Oct.; (J), May. PC, pigmented cells. Arrows indicate stained nuclei.**

by affinity chromatography or DEAE–Sepharose. The binding capacity of the lower affinity site exceeds that of the high affinity site by 10-fold. A second lower affinity site has also been described for the chick oviduct [28], T47D cells [29] and turtle oviduct [21], and the two sites appear to be present on one molecule [28]. Unlike the oviduct, where the high affinity site could only be detected in the nuclear compartment [23], no binding activity (high or lower affinity) is detectable in the hepatic nuclear compartment, all of the activity being in the cytosol. This was confirmed by the Western blot analysis which failed to show PR isoforms present in hepatic nuclear extracts. This suggests that the binding characteristics of hepatic and oviduct receptor for the nucleus may be different. Whether this is of physiologic importance and related to tissue specific factors or due to methodological problems remains to be determined. It is clear from ICC, however, that immunodetectable PR resides in the nucleus and can be seen at all times of the year. Again, as with studies of the turtle oviduct PR, even though a high affinity form is not detected by radioligand studies (i.e. May, June early luteal, September, and October), the PR-A isoform can be eluted from DNA–cellulose columns. Since high and low salt forms of the hepatic PR exhibit high and lower affinity sites [11], PR must be present even when high affinity sites are undetectable by Scatchard analysis.

Although Scatchard analysis provides information on the seasonal variation in high and lower affinity binding sites, changes in PR isoforms can only be determined by Western blotting and affinity chromatography. The results of DNA–cellulose experiments suggest that PR-A is present in spring, summer, and winter, and that the B isoform is down-regulated except in animals which have recently laid eggs (August). Western blot analysis suggest that although the A form (~88 kDa) is dominant throughout the year, a less distinct band representing the putative B form (~125 kDa) varies during the year. A potential PR-C (~64 kDa) is also seen on Western blots [30; see below]. The apparent absence of the B form in winter liver tissue corresponds to similar findings in the turtle oviduct [21] where, as determined by DNA–cellulose, PR-B is absent in winter animals but may be induced by E. The results suggest that seasonal changes in binding site availability, as opposed to receptor protein availability, may be primary determinants of hormone effect.

In agreement with radioligand studies, PR could be detected by Western blotting in cytosol extracts. Although the 64 kDa band does not correspond to any specific protein eluted from DNA–cellulose (where only PR-A and B are detected by radioligand studies) it may represent a third PR isoform. In this regard, two new amino terminal truncated human PR mRNAs, which are induced by estrogen, have been isolated and hybridize with probes complementary to DNA- and hormone-binding domains of the PR [30]. The predicted molecular weights of the proteins derived from the RNAs are in the 45–50 kDa range, and the receptor protein, tentatively PR-C, would lack the first DNA binding zinc finger. In a separate study, monoclonal antibodies to rabbit PR were used to purify the PR from human uteri, and it was discovered that several of the antibodies recognized three different proteins with molecular weights corresponding to 110, 79 and 65 kDa, all of which have truncated amino-termini [31]. The 64 kDa protein labelled in our blots may represent a PR-C isoform. Since the amino-terminus is important in the trans-activation of transcription, truncation of the N-terminus could affect initiation of transcription of P-dependent genes depending on the ratio of PR isoforms during the cycle. It is also possible that this band (~64 kDa) may represent proteolytic degradation of both the A and B isoforms, since protease inhibitors were not added to the extracts. Therefore, studies need to be performed to further characterize this putative PR isoform.

In contrast to both radioligand studies and Western blotting, the use of PR-22 antibody (PR-A/B specific) in ICC localized hepatic PR exclusively in the nuclei. PR immunostaining was observed throughout the seasonal cycle. However, as with Western blots, it is difficult to make any quantitative judgments about seasonal changes in hepatic PR from ICC studies. There appear to be some differences in the density of immunostained nuclei which may reflect hypertrophy of the liver during vitellogenesis in spring and autumn. Immunostained nuclei are consistent with the primary location of PR [32], and as suggested above, radioligand studies imply that hepatic PR is weakly bound to nuclear DNA, tissue homogenization resulting in the dissociation of PR from DNA. Milgrom and co-workers [33] have also shown that PR exchange between the cytoplasmic and nuclear compartments is dictated by two nuclear localization signals, one of which is in the hinge region and the other (hormone dependent signal) is localized in the second zinc finger of the receptor. Further, since the shuttling of the receptor into the nucleus is an ATP-dependent process, the release of ATP from ruptured cells may result in PR release into the cytosol. In this regard, however, the liver appears to be different from the oviduct of the turtle.

Steroid binding affinities for both the lower and high affinity sites are very similar to those observed in the oviduct of the same species [23]. At all concentrations, P is the most effective competitor for the lower affinity as well as the high affinity sites. The other C-21 steroids tested (pregnenolone, deoxycorticosterone) and R5020 were also effective. Affinity for the androgens (testosterone and dihydrotestosterone) was low at 100× but moderate at 1000× for both the low and high affinity sites. Mibolerone, a synthetic

androgen, does not compete well for the lower affinity sites, whereas moderate binding is evident for the high affinity sites. E and corticosterone are weak competitors for the high affinity sites, however they compete similarly or somewhat better than the androgens (testosterone and dihydrotestosterone) for the lower affinity sites. Finally, RU 486 does not bind to the high affinity site to any significant degree, although its binding to the lower affinity site is in the same range for the androgens, E, and corticosterone, as seen in the oviduct.

In summary, this study provides additional support for the role of the hepatic PR in hepatic function associated with lipid mobilization and with vitellogenin synthesis. Based on Western blots and ICC, several isoforms of hepatic PR are present throughout the annual cycle; further, detection of high affinity sites correlated well with periods of declining or inactive vitellogenesis, vitellogenic activity being confined to spring and autumn in this species. Since both E and P are present during the full annual cycle, with a significant E peak during the follicular phase and a P peak during the luteal phase, it is possible that the vitellogenic cycle is regulated by changes in the P/E ratio coupled with activation of PR. We believe that P may act to down regulate the vitellogenin gene after ovulation, and we have recently shown a decrease in vitellogenin mRNA transcripts in P treated vitellogenic animals (unpublished). It is also possible that P/PR act to depress hepatic estrogen receptor (ER). Prior studies from this laboratory have defined the hepatic ER cycle in this species which shows a significant increase in nuclear ER levels during the pre-/peri-ovulatory (April/May) and October months [11]. The immunocytochemical studies suggest the presence of PR even when high affinity sites cannot be detected by radioligand studies. The functional significance of tissue specific expression patterns of different PR isoforms associated with lipid mobilization and vitellogenesis remains to be clarified; however, we believe it is part of a dual regulatory system in which estrogen and P play opposing roles.

*Acknowledgement*—Supported by NIHRR 06633 to IPC.

## REFERENCES


1. Wahli W.: Evolution and expression of vitellogenin genes. *Trends Genet.* **4** (1988) 227–232.
2. Wallace R. A.: Vitellogenesis and oocyte growth in nonmammalian vertebrates. In *Developmental Biology. A Comprehensive Synthesis* (Edited by L. W. Browder). Plenum Publishing Corp., NY (1985) Vol.1, pp. 127–177.
3. Clemens M. J. R., Lofthouse R. and Tata J. R.: Estrogen-induced secretion of egg yolk proteins by male *Xenopus* liver in organ culture. *Biochem. J.* **128** (1975) 97–98.
4. Ho S-M., Danko D. and Callard I. P.: Effects of exogenous estradiol-17$\beta$ on plasma vitellogenin levels in male and female *Chrysemys* and its modulation by testosterone and progesterone. *Gen. Comp. Endocr.* **43** (1981) 413–421.
5. Magliola L.: The effects of estrogen on skeletal calcium muscle metabolism and on plasma parameters of vitellogenesis in the male, three toed turtle (*Terrapene carolina triunguis*). *Gen. Comp. Endocr.* **54** (1984) 162–170.
6. Deeley R. G., Mullinix D. P., Wetekam W., Kronenberg H. M., Meyers M., Eldridge J. D. and Goldberger R. F.: Vitellogenin synthesis in the avian liver. Vitellogenin is the precursor of egg yolk phosphoproteins. *J. Biol. Chem.* **250** (1975) 9060–9066.
7. Jost J. P. and Pehling G.: Immunochemical isolation and characterization of vitellogenin mRNA from liver estradiol-treated chicks. *Eur. J. Biochem.* **66** (1976) 339–346.
8. Callard I. P. and Zeigler H.: Inhibitory effects of prolactin upon gonadotropin-stimulated ovarian growth in the iguanid lizard, *Dipsosarus dorsalis*. *J. Endocr.* **47** (1970) 131–132.
9. Callard I. P., Bayne C. G. and McConnell W. F.: Hormones and reproduction in the female lizard, *Sceloporus cyanogenys*. *Gen. Comp. Endocr.* **18** (1972) 175–194.
10. Perez L. E. and Callard I. P.: Regulation of hepatic vitellogenin synthesis in the little skate (*Raja erinacea*); use of homologous enzyme linked immunoabsorbent assay. *J. Exp. Zool.* **266** (1993) 31–39.
11. Riley D., Reese J.C. and Callard I. P.: Hepatic progesterone receptors: characterization in the turtle *Chrysemys picta*. *Endocrinology* **123** (1988) 1195–1201.
12. Chen T. J. and Leavitt W. W.: Nuclear progesterone receptor in hamster uterus: measurement by $^3$H-progesterone exchange during the estrous cycle. *Endocrinology* **104** (1979) 1588–1597.
13. Mak P., Ho S. M. and Callard I. P.: Characterization of an estrogen receptor in the turtle testes. *Gen. Comp. Endocr.* **53** (1983) 182–89.
14. Lowry O. H., Roseborough N. J., Farr A. L. and Randall R. J.: Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193** (1951) 265–275.
15. Alberts B. M. and Herrick G.: DNA-cellulose chromatography. In *Methods of Enzymology* (Edited by S. Grossman and K. Moldare). Academic Press, NY (1970) Vol.XXI, pp.198–217.
16. Salhanick A. R., Vito C. C., Fox T. O. and Callard I. P.: Estrogen-binding proteins in the oviduct of the turtle, Chrysemys picta: evidence for a receptor species. *Endocrinology* **105** (1979) 1388–1394.
17. Laemmli U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** (1970) 680–685.
18. Sullivan W. P., Smith D. F., Beito T. G., Kreo C.J. and Toft D. O.: Hormone-dependent processing of the avian progesterone receptor. *J. Cell Biochem.* **36** (1988) 103–119.
19. Callard I. P., Lance V., Salhanick A. R. and Barad D.: The annual ovarian cycle of *Chrysemys picta*: correlated changes in plasma steroids and parameters of vitellogenesis. *Gen. Comp. Endocr.* **35** (1978) 245–257.
20. Venkatesh B., Tan C. H. and Lam T. T.: Blood steroid levels in the goldfish: measurement of six ovarian steroids in small volumes of serum by reverse-phase high performance liquid chromatography and radioimmunoassay. *Gen. Comp. Endocr.* **76** (1989) 398–407.
21. Reese J. C. and Callard I. P.: Two progesterone receptors in the oviduct of the freshwater turtle *Chrysemys picta*. Possible homology to mammalian and avian progesterone receptor systems. *J. Steroid Biochem.* **33** (1989) 297–310.
22. Gapp D. A., Ho S-M. and Callard I. P.: Plasma levels of vitellogenin in *Chrysemys picta* during the annual gonadal cycle: measurement by specific radioimmunoassay. *Endocrinology* **104** (1979) 784–790.
23. Giannoukos G., Coho D. W. and Callard I. P.: Turtle oviduct progesterone receptor: radioligand and immunocytochemical studies of changes during the seasonal cycle. *Endocrine* **3** (1995) 429–437.
24. Sarup J. C., Rao K. V. S. and Fox C. F.: Decreased progesterone binding and attenuated progesterone action in cultured human breast carcinoma cells treated with epidermal growth factor. *Cancer Res.* **48** (1988) 5071–5078.
25. Ramachandran C., Catelli M. G., Schneider W. and Shyamala G.: Estrogenic regulation of uterine 90-kilodalton heat shock protein. *Endocrinology* **123** (1988) 956–961.
26. Giannoukos G.: Progesterone receptor regulation in turtle liver and oviduct: immunochemical and radioligand studies. Ph.D. thesis, Boston University (1995).

27. Schrader W. T., Seleznev T., Vedeckis W. V. and O'Malley B. W.: Steroid receptor subunit structure. In *Gene Regulation by Steroid Hormones* (Edited by A. K. Roy and J. H. Clark). Springer-Verlag, NY (1978) pp.78–88.
28. Maggi A., Schrader W. T. and O'Malley B. W.: Progesterone binding sites of the chick oviduct receptor. *J. Biol. Chem.* **259** (1984) 10,956–10,966.
29. Sarup J. C., Rao K. V. S., Williams R. E. and Fox C. F.: Resolution of high and low affinity progesterone receptors from human breast carcinoma T47D cells. *J. Biol. Chem. 263* (1988) 5624–5633.
30. Wei L. L., Gonzalez-Aller C., Wood W. M., Miller L. A. and Horwitz K. B.: 5′-Heterogeneity in human progesterone receptor transcripts predicts a new amino-terminal truncated "C"-receptor and unique A-receptor messages. *Molec. Endocr.* **4** (1990) 1833–1840.
31. Vu-Hai M. T., Jolivet A., Ravet V., Lorenzo F., Perrot-Applanat M., Citerne M. and Milgrom E.: Novel monoclonal antibodies against human uterine progesterone receptor. *Biochem. J.* **260** (1989) 371–376.
32. Ennis B. W., Stumpf W. E., Gasc J. M. and Baulieu E. E.: Nuclear localization of progesterone receptor before and after exposure of progestin at low and high temperatures: autoradiographic and immunohistochemical studies in the chick oviduct. *Endocrinology* **119** (1986) 2066–2075.
33. Guiochon-Mantel A., Lescop P., Christin-Maitre S., Loosfelt H., Perrot-Applanat M. and Milgrom E.: Nucleocytoplasmic shuttling of the progesterone receptor. *EMBO J.* **12** (1991) 3851–3859.