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Plasma Sex Steroid Binding Proteins (SSBP) in the Male Lizard, *Podarcis s. sicula*, during the Reproductive Cycle

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In male *Podarcis s. sicula* plasma, a sex steroid binding protein [SSBP(s)] binds testosterone (T) and estradiol-17β (E_2) with moderate affinity ($K_d = 0.23 \pm 0.08 \times 10^{-8}$ for $^3H-E_2$, and $0.24 \pm 0.07 \times 10^{-8}$ for ^{3}H-T) and high capacity. The SSBP binding affinity is unchanged throughout the sexual cycle, although its capacity is higher in nonreproductive males (winter and postreproductive period). This change may be related to changes in plasma T and E_2 levels, and is likely to be involved in mechanisms whereby free steroid is delivered to target organs. SSBP, under isoelectrofocusing, is distributed between pH 5.5–6.5 and pH 7.1–7.5. The concentration of these two forms varies during the annual cycle.

Plasma sex steroid binding proteins (SSBPs), with modest affinities and high capacities for their hormonal ligands, occur in all vertebrate types, except birds and a few mammals (Burton and Westphal, 1972; Corvol and Bardin, 1973; Anderson, 1974; Renoir *et al.*, 1980; Siiteri *et al.*, 1982; Wingfield *et al.*, 1984; Callard and Callard, 1987). There is species variation in the binding specificities of SSBPs: some simultaneously bind progesterone, testosterone, and estradiol-17β (Salhanick and Callard, 1980; Riley *et al.*, 1988), others testosterone and estradiol-17β (Fostier and Breton, 1975; Martin and Collenot, 1975; Burns and Rose, 1980; Santa-Coloma *et al.*, 1985; Pasmanick and Callard, 1986; Ho *et al.*, 1987), others progesterone and estradiol-17β (Boffa *et al.*, 1972), and others highly specifically bind only testosterone (Saboureau *et al.*, 1982; Audy *et al.*, 1982; Moore *et al.*, 1983) or progesterone (Heap *et al.*, 1981).

SSBP affinity for ligands remains unchanged throughout the year, whereas its plasma level (capacity) may vary within the sexual cycle (Audy *et al.*, 1982; Saboreau *et al.*, 1982; Ho *et al.*, 1987; Riley *et al.*, 1988).

A few studies in lower vertebrates suggest sexual variations in plasma SSBP levels. In male newts, *Taricha granulosa* (Moore *et al.*, 1983), and alligators, *Alligator mississippiensis* (Ho *et al.*, 1987), the plasma SSBP titer does not change significantly during the year. In contrast, in female alligators, plasma SSBP level is higher during the breeding period. A similar pattern occurs in female snakes, *Nerodia* (Riley *et al.*, 1988).

These findings suggest that sex-related patterns of the SSBP plasma titer exist in many lower vertebrates. This study investigates plasma SSBP in male lizards, *Podarcis s. sicula*, and assesses its variation during the sexual cycle.

MATERIALS AND METHODS

Animals and blood samples. Adult males of *Podarcis s. sicula* were captured on the outskirts of Naples from December 1989 to July 1990 during the main phases of the sexual cycle: i.e., nonreproductive period (winter); onset of genital recrudescence (March); breeding period (May); end of the breeding period (June); postreproductive period (nonreproductive, refractory lizards: July). The lizards were anesthetized by immersion in ice. Blood was collected by cardiac

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puncture into heparinized glass capillaries and centrifuged and plasma was stored at −30° until use. At autopsy, testes were excised and placed in Bouin's fixative fluid for later histology.

Measurement of [^{3}H]testosterone (^{3}H-T) and ^{3}H-estradiol-17β (^{3}H-E_2) binding to plasma. Radioactive steroids [2,4,6,7-^{3}H]estradiol (90/110 Ci/m*M*) and [1,2,6,7-^{3}H]testosterone (80/105 Ci/m*M*) were obtained from Amersham Radiochemical Centre (Amersham, Bucks, UK); unlabeled steroids were purchased from Sigma Chemical Co. (St. Louis, MO). Pure grade chemicals were used.

Endogenous steroids were removed by adding the plasma samples to an equal volume of TEMG [10 m*M* Tris–HCl, 1 m*M* EDTA, 1 m*M* 2-mercaptoethanol, 10% glycerol (v/v), pH 7.5] containing 0.05% (w/v) dextran (Dextran T-70, Pharmacia Fine Chemicals, Piscataway, NJ) and 0.5% (w/v) coated charcoal (Norit A charcoal, Sigma). The mixtures were vortexed and incubated for 10 min at 4°, and charcoal was removed by centrifugation at 3000 rpm for 10 min. Unless specified, these steroid-free supernatants were utilized in all subsequent analyses.

For K_d determinations plasma, diluted with TEMG to a protein concentration of 2 mg/ml, was used. The protein content was determined (Lowry *et al.*, 1951) using BSA as a standard. In undiluted plasma, proteins ranged from 70 to 110 mg/ml. Sample aliquots (200 μl) were added to tubes containing increasing amounts (0.3–20 n*M*) of labeled testosterone or estradiol-17β, with and without a 100-fold excess of the respective unlabeled steroid. Tubes were incubated for 1 hr at 0° and 0.6 ml of dextran-coated charcoal in TEMG was then added. After being vortexed mixtures were incubated for 1 min at 0° and centrifuged and supernatants were assayed for radioactivity in 5-ml Maxifluor scintillation fluid (Maxifluor, Packard, Milan, Italy), using liquid scintillation spectrometry (Packard 1600-CA) at 60% counting efficiency. Specific binding data were examined by Scatchard analysis. Both K_d and K_a were calculated.

Binding specificity and association and dissociation kinetics of labeled testosterone and estradiol-17β in plasma were determined by incubating samples (200 μl) with 5 n*M* labeled testosterone or estradiol-17β with and without a 1000-fold excess of various unlabeled steroids. Incubation procedures and radioactivity evaluation were as above.

SSBP heat lability was assayed by incubating samples (200 μl) with 5 n*M* labeled testosterone or estradiol-17β with or without a 100-fold excess of the respective unlabeled steroid for 1 hr at 4, 20, 37, and 45°. Radioactivity was assayed as above.

Isoelectrofocusing. Isoelectrofocusing followed Matsumada and Goldman's (1974) method. Glass columns (31 × 0.5 cm) were filled with a mixture of 12.5% sucrose in water containing 0.01% Triton and 2% pH 3.5–10 Ampholine (Pharmacia, Sweden). After a 1-hr prerun at 4° (200 V), 200 μl of sample (preincubated with 5 n*M* labeled testosterone or estradiol-17β for 1 hr at 20°) was layered on the column. Electrofocusing was carried out for 18 hr at 4° (200 V). Thereafter, 400-μl aliquots were removed from the column base and used to determine pH and binding of labeled hormone as described above.

Measurement of plasma testosterone and estradiol-17β. Plasma sex hormone content was measured by a radioimmunoassay (RIA) previously validated for this reptile (Ciarcia *et al.*, 1986). The sensitivities were testosterone, 3 pg (intraassay variation, 5.7%; interassay variation, 12%); estradiol-17β 3 pg (intraassay variation 5.7%; interassay variation, 9%).

Statistical analysis. Numerical data were analyzed by a one-way ANOVA followed by the multiple range Duncan test.

RESULTS

Testicular histology of the *Podarcis s. sicula* used underwent a sexual cycle identical to that previously described (Angelini and Picariello, 1975).

Figure 1 shows a typical time course of specific binding of labeled testosterone and estradiol-17β to plasma samples at 4°. Binding equilibrium was reached after a 1-hr incubation (Fig. 1A). The plasma–steroid complex appeared to be stable for the subsequent 24 hr. The dissociation kinetics of the steroid–plasma complex was biphasic (Fig. 1B). The first component had a half-life of about 5 min, whereas the second underwent no dissociation.

The labeled hormone binding is thermolabile since, after 1 hr of incubation it was reduced to half at 37° and abolished at 45°.

These binding characteristics of plasma sex hormones did not change throughout the sexual cycle (not shown).

Specificity of SSBP was examined by competition analysis using several unlabeled steroids (data not shown). Testosterone and estradiol-17β competed for labeled testosterone and estradiol-17β binding sites in the same manner. Progesterone actively competed with labeled testosterone and, to a lesser extent, with labeled estradiol-17β. DES, corticosterone, and androstenedione

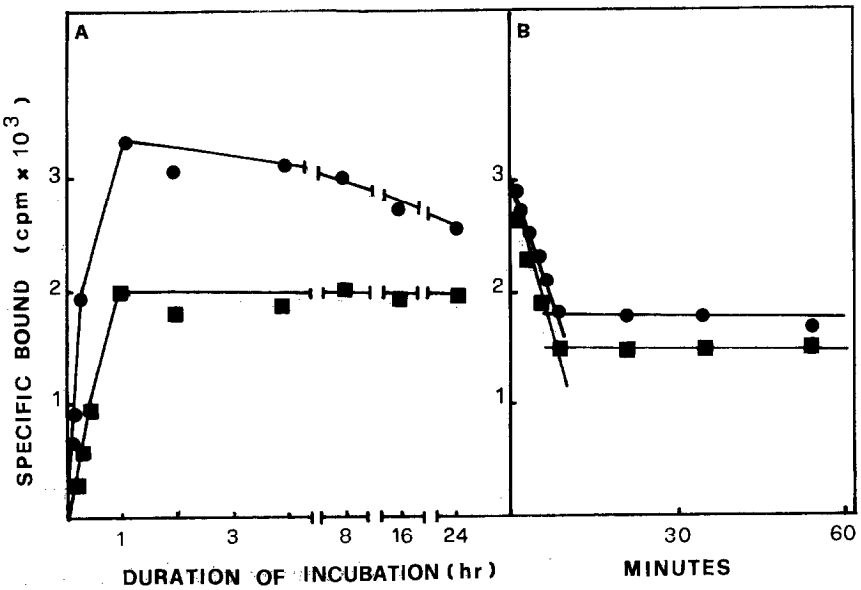

FIG. 1. (A) Association kinetics of ^{3}H-E_2 (●) and ^{3}H-T (■) to *Podarcis s. sicula* SSBP. Plasma samples were incubated with 5 n*M* ^{3}H-labeled steroid ± 100-fold excess unlabeled competitor at 4° for the times indicated. Specific binding was determined using DCC as described under Materials and Methods. (B) Dissociation kinetics of ^{3}H-E_2 (●) and ^{3}H-T (■) to *Podarcis s. sicula* SSBP. Plasma samples were incubated with 5 n*M* ^{3}H-labeled steroid to equilibrium. After addition of 100-fold excess unlabeled competitor, specific binding was measured using DCC at the times indicated. Each point is the mean of triplicate determination in two to three separate experiments. Both kinetics were determined during the reproductive period.

were poor competitors for both binding sites. Androstenedione did not compete with labeled testosterone, rather it produced a 50% displacement of labeled estradiol-17β binding.

Scatchard analysis gave very similar K_d values for plasma binding of the labeled steroid: 0.23 ± 0.08 × 10^{-8} *M* for ^{3}H-E_2 and 0.24 ± 0.07 × 10^{-8} *M* for ^{3}H-T. No significant modification of K_d values were seen during the sexual cycle (Table 1) although at the end of the reproductive period assessment of the K_d was hampered by an abnormal profile of the Scatchard plot (Fig. 2).

Table 2 gives the variations in plasma sex hormone binding during the sexual cycle. The binding capacity for both labeled testosterone and estradiol-17β was higher during winter ($P < 0.05$); it then began to decrease at the onset of the breeding season and reached its nadir at the end of this period ($P < 0.01$). An increase in binding capacity took place postreproductively ($P < 0.05$).

Isoelectrofocusing showed sex hormone binding molecules to be distributed in two pH ranges 5.5–6.5 and 7.1–7.5 (Fig. 3). Fig-

TABLE 1
SSBP DISSOCIATION CONSTANT IN THE PLASMA OF MALE *Podarcis s. sicula*, DURING THE REPRODUCTIVE CYCLE

Period of the cycle	(*n*)[a]	^{3}H-E_2 (×10^{-8})	^{3}H-T (×10^{-8})
Winter	3	0.20 ± 0.07	0.20 ± 0.05
Early reproductive	3	0.28 ± 0.09	0.33 ± 0.08
Reproductive	3	0.50 ± 0.05	0.28 ± 0.06
Late reproductive	3	—[b]	—[b]
Postreproductive	3	0.20 ± 0.06	0.20 ± 0.05

[a] Each determination was conducted on a pool of 10 animals.

[b] Abnormal profile of Scatchard plot (see text). Mean ± SE.

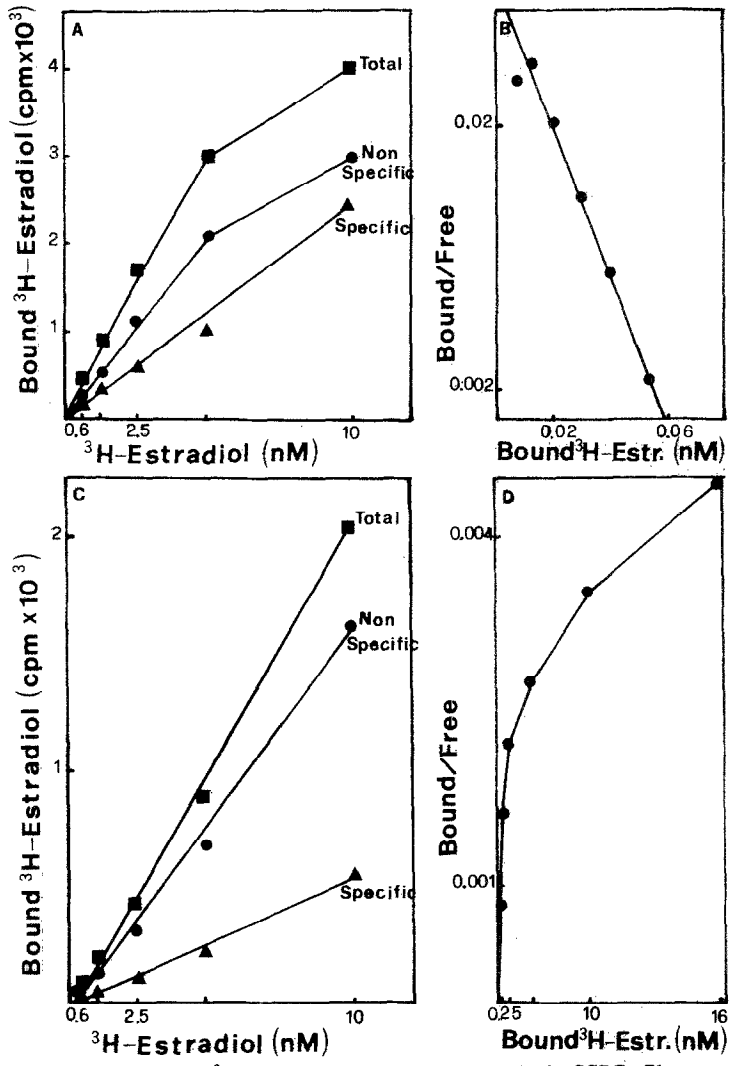

FIG. 2. Scatchard analysis of ^{3}H-E_2 binding to *Podarcis s. sicula* SSBP. Plasma samples were incubated with [^{3}H]estradiol ± 100-fold excess unlabeled competitor. Specific binding was determined using DCC. (A) Saturation curve and (B) Scatchard analysis of reproductive period. (C) Saturation curve and (D) Scatchard analysis of late reproductive period.

ure 4 shows the pattern of isoelectrofocused binding molecules during the reproductive cycle. In nonreproductive animals (both winter and postreproductive refractory lizards), and in those undergoing spring recrudescence of the reproductive system, the binding molecules were focusing predominantely at pH 5.5–6.5 ($P < 0.05$). During the breeding period these molecules decreased ($P < 0.01$) whereas those focusing at pH 7.1–7.5 slightly increased.

Plasma testosterone concentrations increased until the onset of the spring recrudescence ($P < 0.01$) and thereafter decreased to reach a nadir at the end of breeding period ($P < 0.01$; Table 2). Plasma titers of estradiol-17β were higher at the begin-

TABLE 2
CONCENTRATIONS OF ANDROGENS, ESTRADIOL 17-β, AND SSBP BINDING CAPACITY IN THE PLASMA OF MALE LIZARDS *Podarcis s. sicula* DURING THE REPRODUCTIVE PERIOD

Period of the cycle	*n*	Androgen concentration ($M/1 \times 10^{-9}$)	Estradiol-17β concentration ($M/1 \times 10^{-9}$)	SSBP binding capacity ($M/1 \times 10^{-9}$)
Winter	10	97 ± 6.0	n.d.[a]	180 ± 23
Early reproductive	10	652 ± 45	0.7 ± 0.2	70 ± 11
Reproductive	10	173 ± 20	0.3 ± 0.05	20 ± 8
Late reproductive	10	17 ± 2.5	6.6 ± 1.4	0 ± 0
Postreproductive	10	35 ± 4.5	6.2 ± 1.1	90 ± 12

[a] Not dosable. Each figure represents the average ± SE value.

ning of reproductive period ($P < 0.01$), and, again, at the end of this period and in postreproductive lizards ($P < 0.01$).

DISCUSSION

This study reveals the presence of a SSBP(s) in the plasma of male lizard *Podarcis s. sicula*. This protein has a relatively low specificity since labeled testosterone and estradiol-17β bindings are readily displaced by both unlabeled testosterone and estradiol-17β. Although binding to labeled progesterone was not tested, the high progesterone competition for the binding of both labeled steroids suggests that lizard SSBP can also bind progesterone. These characteristics resemble those of other reptilian SSBP including turtle, *Chrysemys picta* (Salhanick and Callard, 1980), alligator, *Alligator mississipiensis* (Ho *et al.*, 1987), and snake, *Nerodia* (Riley *et al.*, 1988).

The binding affinities of lizard SSBP ($K_d = 0.24 \pm 0.07 \times 10^{-8}$ *M* for testosterone and $0.23 \pm 0.08 \times 10^{-8}$ *M* for estradiol-17β; $K_a = 4.6 \pm 0.07 \times 10^{8}\ M^{-1}$ for testosterone and $4.4 \pm 0.08 \times 10^{8}\ M^{-1}$ for estradiol-17β) are similar to those of the turtle, *Chrysemys picta* SSBP (Salhanick and Callard, 1980) and of the alligator, *Alligator mississipiensis* (Ho *et al.*, 1987). They, however, differ from those of the snake, *Nerodia* (10 times lower; Riley *et al.*, 1988), and the lizard, *Lacerta vivipara* (10 times higher; Martin and Xavier, 1981). The significance of these differences is unclear.

Podarcis s. sicula SSBP is thermolabile since its binding capacity is reduced by 50% at 37° and destroyed at 45°.

The kinetic of dissociation resolved into two components, the former readily dissociates whereas the latter does not. The dissociated component behaves as the goldfish SSBP (Pasmanik and Callard, 1986), *Alligator mississippiensis* (Ho *et al.*, 1987), and *Nerodia* (Riley *et al.*, 1988). The undissociated component could result from the presence of other lower affinity binders.

Podarcis s. sicula SSBP had the same order of affinity for both labeled testosterone and estradiol-17β. A similar property has been described for SSBP of the teleosts, *Salmo gairdneri* (Fostier and Breton, 1975), and *Carassius auratus* (Pasmanik and Callard, 1986), and the amphibians, *Bufo arenarum* (Santa Coloma *et al.*, 1985) and *Ambystoma tigrinum* (Burns and Rose, 1980). In other vertebrates SSBP has a higher affinity for estradiol-17β (*Scyllhiorinus canicula*) (Martin, 1975) or for testosterone (some amphibian species and the badger; Martin and Ozon, 1975; Audy *et al.*, 1982).

In *Podarcis s. sicula* SSBP affinity was unchanged throughout the sexual cycle whereas its binding capacity decreases during the breeding period, when plasma

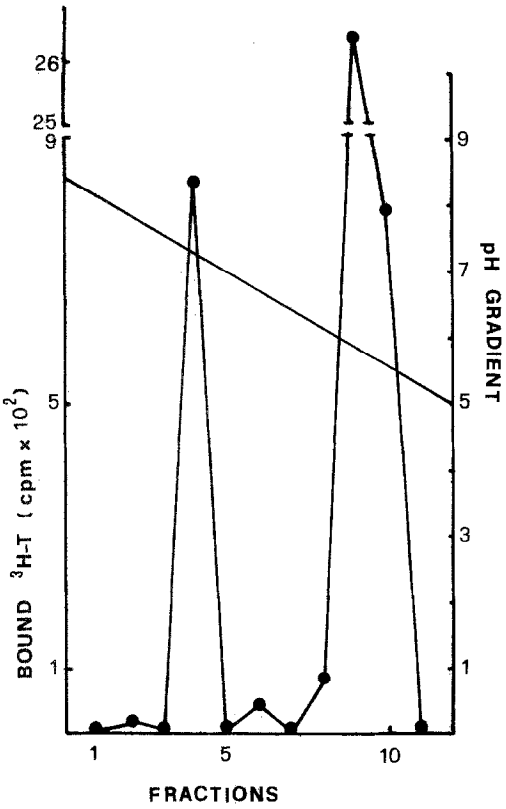

FIG. 3. Isoelectrofocusing of ^{3}H-T binding molecules in male lizard *Podarcis s. sicula* plasma, during the winter period. The experiment is representative of three different separations. Oblique line on the graph represents the pH gradient. Only specific binding is shown. Incubation with ^{3}H-E_2 gave similar results.

testosterone is higher. This SSBP–testosterone relationship may be physiologically significant since it permits greater availability of free-testosterone during the breeding season when the hormone maintains reproductive integrity and sexual drive (Licht, 1984; Ciarcia *et al.*, 1986).

SSBP variations during the sexual cycle have not been found in male newts, *Taricha granulosa* (Moore *et al.*, 1983), and alligators, *Alligator mississipiensis* (Ho *et al.*, 1987), whereas they occur in females of the same species and in male hedgehogs and badgers (Audy *et al.*, 1982; Saboureau *et al.*, 1982).

In male *Podarcis s. sicula* plasma estradiol-17β increases toward the end of the breeding season and is associated with a significantly decreased SSBP binding. This pattern renders the hormone readily available to its target organs, supporting the contention that estradiol-17β is involved in the induction of postreproductive refractoriness (Botte and Angelini, 1980). In several species estradiol-17β is known to inhibit gonadal function by systemic and/or local negative feedback (Botte and Delrio, 1967; Botte and Angelini, 1980; Polzonetti *et al.*, 1984; Varriale *et al.*, 1986).

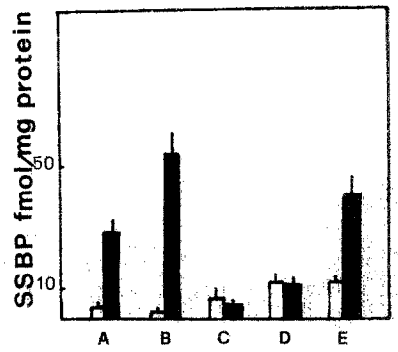

FIG. 4. *Podarcis s. sicula* plasma SSBP, subjected to isoelectrofocusing, during the following periods; A, winter; B, early reproductive; C, reproductive; D, late reproductive; E, postreproductive. (■) pH 5.5–6.5 fraction; (□) pH 7.1–7.5 fraction.

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