EFFECT OF TEMPERATURE AND STORAGE DURATION ON THE STABILITY OF STEROID HORMONES IN BLOOD SAMPLES FROM WESTERN DIAMOND-BACKED RATTLESNAKES (*CROTALUS ATROX*)

EMILY N. TAYLOR AND GORDON W. SCHUETT

Made in United States of America
Reprinted from HERPETOLOGICAL REVIEW
Vol. 35, No. 1, March 2004

ARTICLES

Herpetological Review, 2004, 35(1), 14–17.

Effect of Temperature and Storage Duration on the Stability of Steroid Hormones in Blood Samples from Western Diamond-backed Rattlesnakes (*Crotalus atrox*)

EMILY N. TAYLOR
School of Life Sciences, Arizona State University
Tempe, Arizona 85287-4601, USA
e-mail: entaylor@asu.edu

and
GORDON W. SCHUETT
Department of Herpetology, Zoo Atlanta
800 Cherokee Avenue, S.E., Atlanta, Georgia 30315-1440, USA
Department of Biology, Center for Behavioral Neuroscience
Georgia State University, 33 Gilmer Street, S.E., Unit 8
Atlanta, Georgia 30303-3088, USA
e-mail: gschuett@zooatlanta.org

Studies of field behavioral endocrinology permit researchers to examine the relationship between hormones and an organism's behavior under natural conditions. For analyses of circulating steroid hormones, blood harvested in the laboratory is typically centrifuged immediately to obtain plasma, which subsequently is stored frozen (e.g., ultra-low freezing, -80°C). Collection of blood samples outside of a laboratory setting, however, poses potential problems with respect to proper short-term storage. When conducting research in remote field sites, several hours or even days may pass before samples can be centrifuged and the resultant plasma stored (e.g., -80°C). Prior to processing, steroids in blood samples may be metabolized (e.g., degraded, converted), leading to erroneous values derived from quantitative analyses such as radioimmunoassays. Alternately, hormones may remain stable under the above-mentioned conditions. There are few published data on the stability of steroids in blood samples collected under field conditions, and no studies are published on reptiles.

Levels of steroids in blood samples may be sensitive to temperature, and this sensitivity may differ among lineages (e.g., mammals versus reptiles). Circulating steroids associated with binding proteins degrade more slowly than free steroids (Pardridge and Mietus 1979), and at high temperatures the rate of steroid dissociation from their binding proteins increases (e.g., Ho et al. 1987). Consequently, metabolism of bound and free steroids may be accelerated at high temperatures. Reptiles have higher circulating concentrations of steroids and binding proteins than mammals, and steroid binding proteins from these two amniote lineages seem to be inherently different both in their specificity and affinity for steroids (e.g., Callard and Callard 1987; Jennings et al. 2000). These facts raise the possibility that blood samples from mammals and reptiles show differences in temperature-sensitive metabolism of steroids.

Research on stability of steroid hormones has been primarily conducted to evaluate the proper storage of mammalian blood and serum in veterinary clinics and hospitals. These results indicate that steroids in plasma and serum are relatively stable when frozen (Bolelli et al. 1995; Kley and Rick 1984; Kley et al. 1985). Refrigeration of plasma samples also yields relatively high stability of steroids (Key et al. 1996; Kley and Rick 1984; Olson et al. 1981; Reimers et al. 1982), although Behrend et al. (1998) reported a significant decline from baseline in cortisol levels in dog serum stored at 4°C for five days. Storage of plasma at higher temperatures (22–37°C) often leads to increased steroid hormone metabolism (Behrend et al. 1998; Olson et al. 1981; Reimers et al. 1982, 1983).

It is apparent that the method of choice for storing plasma samples for later hormone analysis is ultra-low freezing (-80°C), and that storage at higher temperatures for protracted periods can have negative effects on the concentrations of steroid hormones. The conditions to which the samples were exposed in the aforementioned experiments were representative of the various storage methods available in veterinary or clinical laboratories (e.g., ultra-low freezing, refrigeration) rather than the conditions typically encountered in the field, especially in deserts. Because researchers may have no choice but to delay processing blood for 24 h or more, it is critical to determine the effects of sample storage under those temperature and duration conditions.

We were motivated to address the above question for our own studies of seasonal steroid hormone levels of rattlesnakes (*Crotalus* spp.) in the Sonoran Desert (Arizona). Typically, we obtain blood samples and immediately store them in a cooler filled with ice (0°C), but other times we are forced to carry them on our person (25–40°C) in the course of sampling animals until we can store them on ice. Centrifugation, collection of plasma, and ultra-low freezing (-80°C) of these blood samples is done in a laboratory within 6–24 h. In this experiment, we tested whether steroid hormone levels are stable in blood samples from rattlesnakes stored at two temperatures (0°C and 40°C) for two durations (6 h and 24 h).

Materials and Methods

Subjects.—Twenty (10 male, 10 female) adult Western Diamond-backed Rattlesnakes (*Crotalus atrox*) were used in this study. All subjects were long-term (3–4 years), healthy captives (housed in the Animal Care Facility, Department of Life Sciences, Arizona State University West) that originated from several areas in central Maricopa County, AZ, near the vicinity of Phoenix. Snout–vent length and mass of males (SVL: mean = 89.1 ± SE 2.39 cm, range 74.0–98.0 cm; mass: mean = 480.45 ± 32.81 g, range 278.80–639.3 g, N = 10) and females (SVL: mean = 86.4 ± 1.65 cm, range 78.0–92 cm; mass: mean = 534.65 ± 47 g, range 323.0–773.80 g, N = 10) were similar (SVL: $F_{1,18} = 0.864$, $p = 0.365$; mass: $F_{1,18} = 0.891$, $p = 0.358$). Subjects were housed individually in glass enclosures, and offered laboratory rodents weekly during the active season (March through October). Water was available in glass bowls *ad libitum*. Blood collection occurred on a single day (late morning) in March 2000.

Study design.—Blood harvested from each subject (see below) was partitioned into five treatment groups (split-plot design; Quinn and Keough 2002):

T_0: Blood was immediately centrifuged and plasma stored in an ultra-low freezer (-80°C). This treatment served as the control.
T_1: Whole blood was placed on ice in a cooler (0°C) for 6 h, then

centrifuged and stored (-80°C).
T_2: Whole blood was placed in an incubator (40°C) for 6 h, then centrifuged and stored (-80°C).
T_3: Whole blood was placed on ice in a cooler (0°C) for 24 h, then centrifuged and stored (-80°C).
T_4: Whole blood was placed in an incubator (40°C) for 24 h, then centrifuged and stored (-80°C).

Collection of blood and plasma.—Subjects were gently removed from their individual enclosures using hooks and/or tongs, then quickly restrained in a standard squeeze-box. After snakes were secured (1–3 min), 3.0 ml of blood was harvested from their tails (within 1 min) using heparin-treated syringes. All snakes were returned to their enclosures within several minutes of restraint. The blood from each subject was immediately partitioned (0.6 ml) into five 1.5 ml sterile centrifuge tubes, and placed in one of the five treatment groups. Following the respective treatment, blood samples were centrifuged for 4 min at 1200 g, and plasma was collected and placed into 1.5 ml sterile centrifuge tubes and immediately stored at -80°C until radioimmunoassays could be performed (<6 months).

Radioimmunoassay of plasma sex steroids.—Radioimmunoassays (RIAs) of sex steroids examined in this study [testosterone (T) and 17β-estradiol (E2)] were performed on plasma using commercial kits.

RIAs of T followed Schuett et al. (1997), including validation (quantitative recovery and parallelism). Samples in the present study were run in duplicate (N = 200) in two RIAs. The intra-assay coefficients of variation (CV) for T was 9.1% and 11.1%, and the inter-assay CV was 11.9%. The T values are presented as arithmetic means ± SE (ng/ml).

For RIAs of E2, radio-labeled E2, antibody, and a precipitating solution were purchased from Diagnostic Products Corporation (Los Angeles, California; catalog numbers E2D1, E2D2, and N6). Standards were prepared by serial dilutions in methanol of a stock solution. The anti-estradiol antibody was diluted 1:3 in phosphate buffered saline (PBS) containing 1:400 rabbit serum. One hundred microliters of snake plasma (with 300 µl of PBS) was extracted in 5.0 ml of diethyl ether (Fisher Scientific). After removing and saving the diethyl ether layer, the sample was heated to 90°C for 5 min, extracted with an additional 5.0 ml of diethyl ether and 200 µl of PBS. Gelatin (1%) was added to the extract following evaporation of the diethyl ether. Extraction recovery of ^{3}H-estradiol (New England Nuclear, Boston, Massachusetts; NET-381) was 78%. For the RIA, 100 µl of diluted antibody, 100 µl of ^{125}I-E2, and 1.0 ml of precipitating solution were used. A 24 h incubation (4°C) period followed each step. Antibody-bound ^{125}I was separated by centrifugation at 1600 g. Validation involved quantitative recovery and parallelism. Quantitative recovery of E2 added to snake plasma was 100%, and parallelism was demonstrated between the inhibition curve for the standards and dilutions. Samples were run in duplicate (N = 200) in two RIAs. The intra-assay CVs were 7.9% and 12.5%, and the inter-assay CV was 11.9%. The E2 values are presented as arithmetic means ± SE (pg/ml).

Statistical analyses.—Data were subjected to inspection for outliers, normality (skewness and kurtosis), and equality of variance prior to performing statistical tests. Outliers were not detected, and conditions for normality and equality of variance were met. To determine whether or not body size influenced mean hormone levels, we ran simple regressions of body mass versus steroid levels (dependent variable). In those tests we detected a significant relationship between body mass and plasma steroids in males (T: $r^2 = 0.084$, $F_{1,48} = 5.502$, $p = 0.023$; E: $r^2 = 0.241$, $F_{1,48} = 15.218$, $p = 0.0003$); in

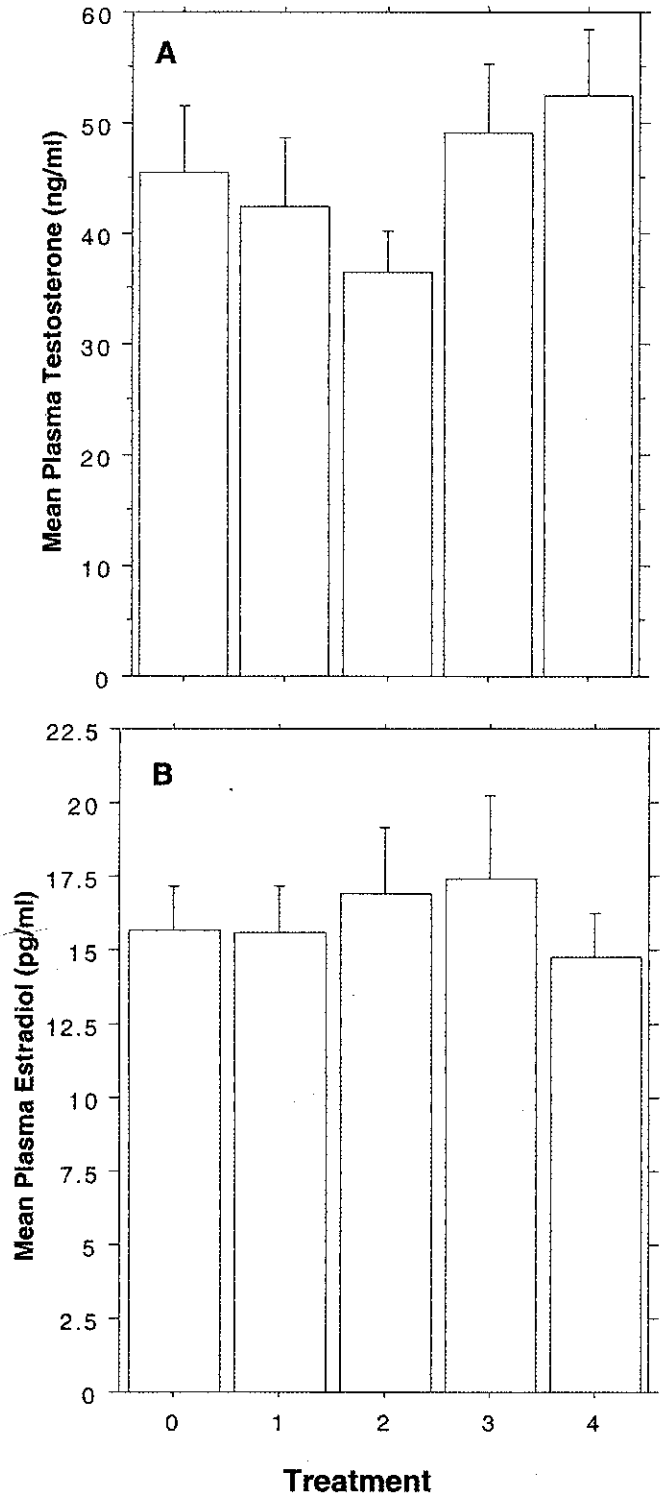

FIG. 1. Mean (± 1 SE) plasma levels of (A) testosterone and (B) 17β-estradiol for adult male *Crotalus atrox* (N = 10) resulting from blood exposed to five treatments (split-plot design). Treatments: 0 = control; 1 = 0°C for 6 h; 2 = 40°C for 6 h; 3 = 0°C for 24 h; and 4 = 40°C for 24 h. See text for details of the statistical analysis.

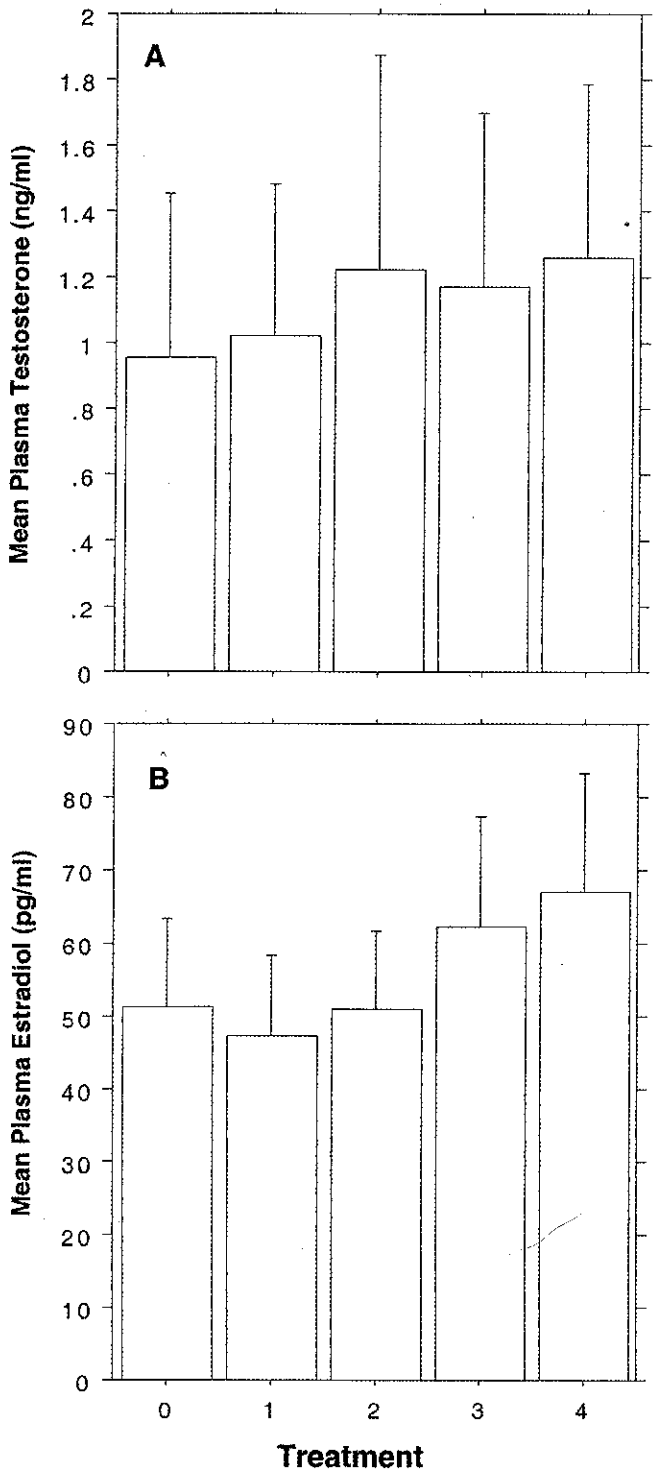

FIG. 2. Mean (± 1 SE) plasma levels of (A) testosterone and (B) 17β-estradiol for adult female *Crotalus atrox* (N = 10) resulting from blood exposed to five treatments (split-plot design). Treatment: 0 = control; 1 = 0°C for 6 h; 2 = 40°C for 6 h; 3 = 0°C for 24 h; and 4 = 40°C for 24 h. See text for details of the statistical analysis.

females, that relationship was significant for T ($r^2 = 0.267$, $F_{1, 48} = 17.506$, $p = 0.0001$), but not for E2 ($r^2 = 0.267$, $F_{1, 48} = 2.928$, $p = 0.0936$). We thus chose to use body mass as a covariate in subsequent statistical analyses.

Data were analyzed with a three factor split-plot multivariate analysis of covariance without replication, where sex and treatment are crossed fixed factors, subject is a random factor nested within sex, and body mass is the covariate. We achieved an unsaturated model by omitting the test for the treatment*subject (sex) interaction. The α-level of significance was set at 0.05. All tests were performed using SAS (SAS Institute 1999).

RESULTS AND DISCUSSION

We found that steroid hormone levels were significantly different in samples from males and females (sex main effect: $F_{2, 63} = 6.03$, $p = 0.004$) (Figs. 1 and 2). There were, however, no differences in hormone levels among treatments (treatment main effect: $F_{8, 32} = 0.05$, $p = 0.999$). Also, there was no significant interaction between treatment and sex (treatment*sex effect: $F_{8, 128} = 0.74$, $p = 0.659$), indicating that samples from males and females do not respond differently to the treatments.

Our results show that exposure of blood samples from adult male and female *C. atrox* to 0°C or 40°C, for two different durations (6 h or 24 h), does not change concentrations of plasma T and E2 (Figs. 1 and 2). Our results agree with the few studies that have investigated the stability of E2 and T in blood and plasma (serum) samples from mammals. Prior to our study, and to the best of our knowledge, the stability of T and E2 in samples from any animal had not been reported at temperatures higher than room temperature (22–25°C). Because our blood samples can potentially be exposed to temperatures in excess of 30°C, it was important for us to show that these high temperatures do not lead to steroid hormone degradation/reduction or conversion.

In this study, we tested the effects of storage temperature and duration on the stability of levels of two sex steroids, T and E2, known to be important in the reproductive biology of a range of reptilian species. Although all steroid hormones share a common cholesterol-based backbone and thus may exhibit similar responses to temperature, it is possible that other steroid hormones (e.g., corticosterone, progesterone) could respond differently to storage conditions. There is, moreover, the possibility that desert-adapted reptiles have plasma (and plasma components) that are more durable to conditions of high temperatures for protracted periods. Although we do not have data on other species of reptiles to compare our results, these ideas are important and need to be tested in a range of species, especially in those that are cold-adapted.

In conclusion, we have demonstrated that in Western Diamondbacked Rattlesnakes (*C. atrox*) from Arizona, blood samples exposed to high temperatures for short time periods (≤ 24 h) do not show significant changes in levels of plasma T and E2. Although we recommend that blood samples collected in the field be immediately placed on ice (0°C) and processed in ≤ 24 h, our data show that negative consequences of short-term storage of blood under the above-discussed conditions are at most minimal.

Acknowledgments.—Numerous people participated in field work, and we are especially appreciative of the help of Laura, Laudon, and Emory Schuett, Matthew Grober, Jack O'Leile, and Ryan Sawby. Edward Van Kirk and William Murdoch, Department of Animal Sciences, University of Wyoming skillfully performed all steroid analyses, and we are grateful for this favor. Dale DeNardo helped in sharpening our focus with respect to experimental design. Ryan Earley assisted with statistical analyses, and we appreciate his help. The manuscript benefited from the comments of two anonymous reviewers. Funding for this research was by a Re-

search Incentive Award, and a Scholarship, Research Creative Activities Award (Arizona State University) to GWS. This research was approved by the IACUC at Arizona State University (98-429R to GWS).

Literature Cited

Behrend, E. N., R. J. Kemppainen, and D. W. Young. 1998. Effect of storage conditions on cortisol, total thyroxine, and free thryroxine concentrations in serum and plasma of dogs. J. Amer. Vet. Med. Assoc. 212:1564–1568.

Bolelli, G., P. Muti, A. Micheli, R. Sciajno, F. Franceschetti, V. Krogh, P. Pisani, and F. Berrino. 1995. Validity for epidemiological studies of long-term cryconservation of steroid and protein hormones in serum and plasma. Cancer Epidemiol. Biomark. Prevent. 4:509–513.

Callard, I. P., and G. V. Callard. 1987. Sex steroid receptors and non-receptor binding proteins. *In* D. O. Norris and R. E. Jones (eds.), Hormones and Reproduction in Fishes, Amphibians, and Reptiles, pp. 355–384. Plenum Press, New York.

Ho, S. M., V. Lance, and M. Megaloudis. 1987. Plasma sex-steroid binding protein in a seasonally breeding reptile, *Alligator mississippiensis*. Gen. Comp. Endocrinol. 65:121–132.

Jennings, D. H., M. C. Moore, R. Knapp, L. Matthews, and M. Orchinik. 2000. Plasma steroid-binding globulin mediation of differences in stress reactivity in alternative male phenotypes in tree lizards, *Urosaurus ornatus*. Gen. Comp. Endocrinol. 120:289–299.

Key, T., S. Oakes, G. Davey, J. Moore, L. M. Edmond, U. J. McLoone, and D. I. Thurnham. 1996. Stability of vitamins A, C, and E, carotenoids, lipids, and testosterone in whole blood stored at 4°C for 6 and 24 hours before separation of serum and plasma. Cancer Epidemiol. Biomark. Prevent. 5:811–814.

Kley, H. K., and W. Rick. 1984. Einfluss von lagerung und temperatur auf die analyse von steroiden in plasma und blut. J. Clinic. Chem. Biochem. 22:371–378.

———, R. Sclaghecke, and H. L. Krüskemper. 1985. Stabilität von steroiden im plasma über einen zeitraum con 10 jahren. J. Clinic. Chem. Biochem. 23:875–878.

Olson, P. N., R. A. Bowen, P. W. Husted, and T. M. Nett. 1981. Effects of storage on concentrations of hydrocortisone (cortisol) in canine serum and plasma. Amer. J. Vet. Res. 42:1618–1620.

Pardridge, W. M., and L. J. Mietus. 1979. Transport of protein-bound steroid hormones into liver *in vivo*. Amer. J. Physiol. 237:E367–E372.

Quinn, G. P., and M. J. Keough. 2002. Experimental Design and Data Analysis for Biologists. Cambridge University Press, Cambridge.

Reimers, T. J., J. P. McCann, and R. G. Cowan. 1983. Effects of storage times and temperatures on T_3, T_4, LH, prolactin, insulin, cortisol, and progesterone concentrations in blood samples from cows. J. Anim. Sci. 57:683–691.

———, ———, ———, and P. W. Concannon. 1982. Effects of storage, hemolysis, and freezing and thawing on concentrations of thyroxine, cortisol, and insulin in blood samples. Proc. Soc. Exp. Biol. Med. 170:509–516.

Schuett, G. W., H. J. Harlow, J. D. Rose, E. A. Van Kirk, and W. J. Murdoch. 1997. Annual cycle of plasma testosterone in male copperheads, *Agkistrodon contortrix* (Serpentes, Viperidae): relationship to timing of spermatogenesis, mating, and agonistic behavior. Gen. Comp. Endocrinol. 105:417–424.

Herpetological Review, 2004, 35(1), 17–23.

Morphological Variation, Diet, and Vocalization of *Eleutherodactylus eugeniae* (Anura: Leptodactylidae) with Notes on its Reproduction and Ecology

JUAN M. GUAYASAMIN*
Museo de Zoología, Centro de Biodiversidad y Ambiente
Departamento de Ciencias Biológicas
Pontificia Universidad Católica del Ecuador
Apartado Postal 17-01-2184, Quito, Ecuador

ELISA BONACCORSO
Natural History Museum & Biodiversity Research Center
Department of Ecology and Evolutionary Biology
The University of Kansas, Lawrence, Kansas 66045-7561, USA
e-mail: elisab@ku.edu

and

PABLO A. MENÉNDEZ
MARTÍN R. BUSTAMANTE
Museo de Zoología, Centro de Biodiversidad y Ambiente
Departamento de Ciencias Biológicas
Pontificia Universidad Católica del Ecuador
Apartado Postal 17-01-2184, Quito, Ecuador

**Present address:*
Natural History Museum & Biodiversity Research Center
Department of Ecology and Evolutionary Biology
The University of Kansas, Lawrence, Kansas 66045-7561, USA
e-mail: juanm@ku.edu

Eleutherodactylus eugeniae was described by Lynch and Duellman (1997) from a small area of cloud forest, at elevations of 1700–2010 m in Provincia Pichincha in northwestern Ecuador. These authors examined very few specimens from the type locality. We collected 29 new individuals (7 juveniles, 9 females, 13 males), 7 from the type locality, and the rest from two new localities (see Appendix I). Herein, we describe aspects of the diet, vocalization, morphological variation, ecology, and reproductive biology of this species.

Materials and Methods

Ecological, reproductive, and morphological data were gathered on *Eleutherodactylus eugeniae* from three localities of cloud forests in northwestern Ecuador between 1994 and 2001. This region has an annual precipitation of 2600 mm, with a rainy season from December to May, and moderate rains from July to November; mean annual temperature is 15.6°C (Cañadaz-Cruz 1983). The localities and specimens examined are listed in Appendix I.

We recorded 12 individuals and collected four of them (Appendix I). Calls were recorded from 14–28 February and 10–24 September 1998, and from 27 February–11 March and 13–17 August 1999 in Bosque Integral Otonga reserve (BIO) with a Sony WM D6C Professional Walkman tape recorder and an Optimus Unidirectional Condenser Microphone. Calls were digitized at a sampling rate of 22 kHz at 8-bit precision with the built-in A/D converter on Power Macintosh 7100/80 AV computer and the software Canary 1.1 (Charif et al. 1993). Waveforms and spectra were analyzed with Canary 1.1. Acoustic terminology follows that de-