REPRODUCTIVE ENDOCRINOLOGY OF THE OZARK HELLBENDER, *CRYPTOBRANCHUS ALLEGANIENSIS BISHOPI*

A Thesis

Presented to

the Graduate Faculty of the Department of Biology

Southwest Missouri State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Stephanie Gail Reed

July 1994

REPRODUCTIVE ENDOCRINOLOGY OF THE OZARK HELLBENDER, *CRYPTOBRANCHUS ALLEGANIENSIS BISHOPI*

Biology Department

Southwest Missouri State University, 15 July, 1994

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ABSTRACT

Seasonal changes in steroid concentrations in the serum of adult Ozark hellbenders, *Cryptobranchus alleganiensis bishopi*, were investigated from June, 1993 to December, 1993. These dates included the periods before, during and after the breeding season. Radioimmunoassays were performed to measure the concentrations of estradiol, progesterone and testosterone. Estradiol concentrations in females increased throughout July, when gonadal changes were first observed, and remained high during the time when oocytes were maturing and breeding took place. In males, estradiol levels were constant between June and December and had values one hundred times lower than the values of female estradiol. Progesterone concentrations in females peaked when gonadal changes were first observed and again just after the breeding season. Similar peaks in progesterone concentrations were seen in males. Testosterone concentrations in females peaked during the last two weeks of September when breeding was taking place; levels were low at all other times. Testosterone concentrations in males started increasing in July, when cloacal gland enlargement was first observed; levels further increased and remained high throughout the breeding season then decreased shortly thereafter. This study suggested that estradiol played an important role in oocyte maturation, that progesterone may have initiated gamete maturation and have terminated breeding processes in both females and males, and that testosterone functioned in mating behavior and in the development of secondary sexual characteristics, the reproductive tract, and spermatozoa.

This abstract is approved as to form and content

Chairman, Advisory Committee

Southwest Missouri State University

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July 1994

Approved:

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ACKNOWLEDGMENTS

I thank Drs. Robert Wilkinson, Thomas Tomasi, and Christopher Barnhart for their advice, aid, and patience throughout this study. I am grateful for the field assistance of Drs. Wilkinson and Peterson, and to many of the graduate students of SMSU. Without their help, the field component of this study would have been impossible. I thank Dr. Tomasi for his aid in and out of the laboratory. I also thank the Department of Biology, Southwest Missouri State University for financial support and equipment used in this study. I am particularly grateful to fellow graduate student Paul Schell. His friendship and constant devotion to this project gave me the strength to complete this sometimes arduous task.

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INTRODUCTION

The family Crytobranchidae consists of three species in two genera, *Andrias* and *Cryptobranchus*. *Andrias* includes the Chinese and Japanese giant salamanders (*A. davidianus* and *A. japonicus*, respectively) and is restricted to Asia (Nickerson and Mays, 1973b). *Cryptobranchus* is restricted to North America. Two subspecies, the eastern hellbender (*C. alleganiensis alleganiensis*) and the Ozark hellbender (*C. alleganiensis bishopi*) are recognized (Nickerson and Mays, 1973b). The eastern hellbender can be found in swift rivers and streams throughout the central Appalachian Mountains from southern New York to northern Georgia and in portions of the Ozarks (Williams et al., 1981). The Ozark hellbender inhabits south-flowing streams in the Ozarks of Missouri and Arkansas, specifically in the North Fork of the White River, the Spring River and drainages of the Black River (Nickerson and Mays, 1973a; Trauth et al., 1993).

Hellbenders are among the largest salamanders in North America. The Eastern hellbender reaches lengths up to 740 mm (Fitch, 1947), while the Ozark hellbender may reach 620 mm total length (Nickerson and Mays, 1973b). Sexual maturity is attained in female Ozark hellbenders over 238 mm snout-vent length (SVL) and 330 mm total length (TL) and in male Ozark hellbenders over 244 mm SVL and 338 mm TL (Dundee and Dundee, 1965).

Hellbenders breed for about two weeks from late August to early October throughout their range (Smith, 1907; Nickerson and Mays, 1973b), with the exception of the Spring River, AR population which breeds in January (Peterson et al., 1989). Ozark hellbenders in the North Fork of the White River breed between late September and early October (Nickerson and Mays, 1973b).

(Moore, 1978). In female *Triturus carnifex*, progesterone levels showed only minor changes during the annual reproductive cycle with the exception of a significant drop in levels (from 2.25 to 0.8 ng/ml) at the end of the reproductive period (Gobbetti et al. , 1991, 1992).

Androgens are synthesized under LH stimulation by Leydig cells of the testicular interstitial tissue in males and by thecal cells of the ovary in females (Jameson, 1988). As in other vertebrates, testosterone in male amphibians plays a major role in development of the reproductive tract, secondary sexual characteristics, and mating behavior (Moore, 1987). The role of testosterone in females is uncertain. A study by Specker and Moore (1980) temporally correlated changes in androgen concentrations (testosterone and dihydrotestosterone) in male *Taricha granulosa* with alterations in testis composition, nuptial characteristics and sexual behaviors. Increasing androgen levels (54.7 ng/ml) were associated with an increase in testicular weight, increasing numbers of mature spermatozoa, swollen cloacae, enlarged caudal fins, keratinized nuptial pads and amplectic clasping. Androgen implants in both sexes of *T. granulosa* resulted in amplectic clasping (Moore et al., 1992). Studies on *T. carnifex* have shown that androgen levels in both males and females increased prior to reproduction, reached their highest levels during the breeding period (50 ng/ml in males and 4.8 ng/ml in females), then dropped quickly thereafter (Gobbetti et al., 1991; Gobbetti and Zerani 1992a, 1992b; Gobbetti et al. , 1992). In the tiger salamander, *Ambystoma tigrinum*, testosterone was shown to inhibit the proliferation of secondary spermatogonia in larvae treated for 20 days with FSH, yet it stimulated spermatogenesis in larvae treated for 20 days with testosterone and then given a combined treatment of FSH and testosterone for another 20 days

Laboratory (Nickerson and Mays, 1973b) and field (Topping and Ingersol, 1981) studies revealed that the average number of eggs deposited by female Ozark hellbenders was 270. These eggs are deposited in depressions under flat rocks (Smith, 1907). The male moves in a position above the eggs and deposits a white ropy or cloudy mass that consists of seminal fluid mixed with cloacal gland secretions. Males guard nests that may contain eggs from more than one female until hatching occurs in four to six weeks (Smith, 1907). All females apparently produce a clutch of ova each year (Peterson et al., 1989; Ingersol et al., 1991), although Topping and Ingersol (1981) and Ingersol et al. (1991) found that nearly one-fourth of gravid females retained and reabsorbed their eggs even though the females did not appear to be physically impaired reproductively.

In vertebrates, reproductive events are controlled by gonadal hormones, most of which are steroids. Gonadal hormones are controlled in turn by pituitary and hypothalamic hormones. All steroids are ultimately derived from cholesterol and are chemically similar. Progesterone is directly derived from cholesterol. It can be converted to androgens and estrogens, and androgens in both sexes can be converted to estrogens (Jameson, 1988). Progesterones, androgens and estrogens are synthesized in the gonads and in the adrenal cortex (Jameson, 1988). These hormones have physiological effects and also play roles in behavioral patterns of both sexes.

Several studies have described the reproductive endocrinology of amphibians. Most of the data come from studies describing anurans while only a few describe urodeles. As with other vertebrates, spermatogenesis, oocyte maturation and ovulation are controlled by the pituitary gonadotropic hormones, luteinizing hormone, LH, and follicle-stimulating hormone, FSH, (reviewed by Dodd and Wiebe, 1968; Lofts, 1974; Licht et al., 1975), whereas gonadal hormones appear to mediate this control.

Estrogen is formed and secreted by granulosa cells of the mature follicle and is responsible for oocyte maturation and the development of secondary sexual characteristics (Jameson, 1988). Plasma estradiol levels in both genders of *Triturus carnifex*, the crested newt, reached their highest levels (12 ng/ml) at the end of the reproductive cycle and this was interpreted as a mechanism responsible for terminating breeding processes (Gobbetti et al., 1991; Gobbetti and Zerani, 1992a). This peak could initiate a negative feed-back mechanism on the hypothalamo-pituitary-gonadal axis, although the mechanism for this phenomenon is still unclear (Zerani et al., 1991). Estradiol implants, but not control implants, resulted in egg-laying behavior and oviposition in *Taricha granulosa*, the rough-skinned newt, in females that were ready to oviposit (Moore et al., 1992). There is no known response to estrogen in male amphibians.

Progesterone is produced in ovarian granulosa cells and testicular Leydig cells (Jameson, 1988). It is also secreted by the abdominal gland of male *T. carnifex*, which suggests a role of this steroid as a pheromone (Gobbetti and Zerani, 1992c). Progesterone concentrations in female *T. granulosa* were highest (0.75 ng/ml) at the onset of the breeding season, which was a time when female newts were most sexually attractive to a male, and were about twice as high in those females that possessed oviductal eggs (and were also courted) as those that did not possess oviductal eggs (Moore et al., 1979). Progesterone injections increased the sexual attractiveness of *T. granulosa* females; all progesterone-injected females were clasped during a 30-hour test, but none of the controls were clasped during similar trials

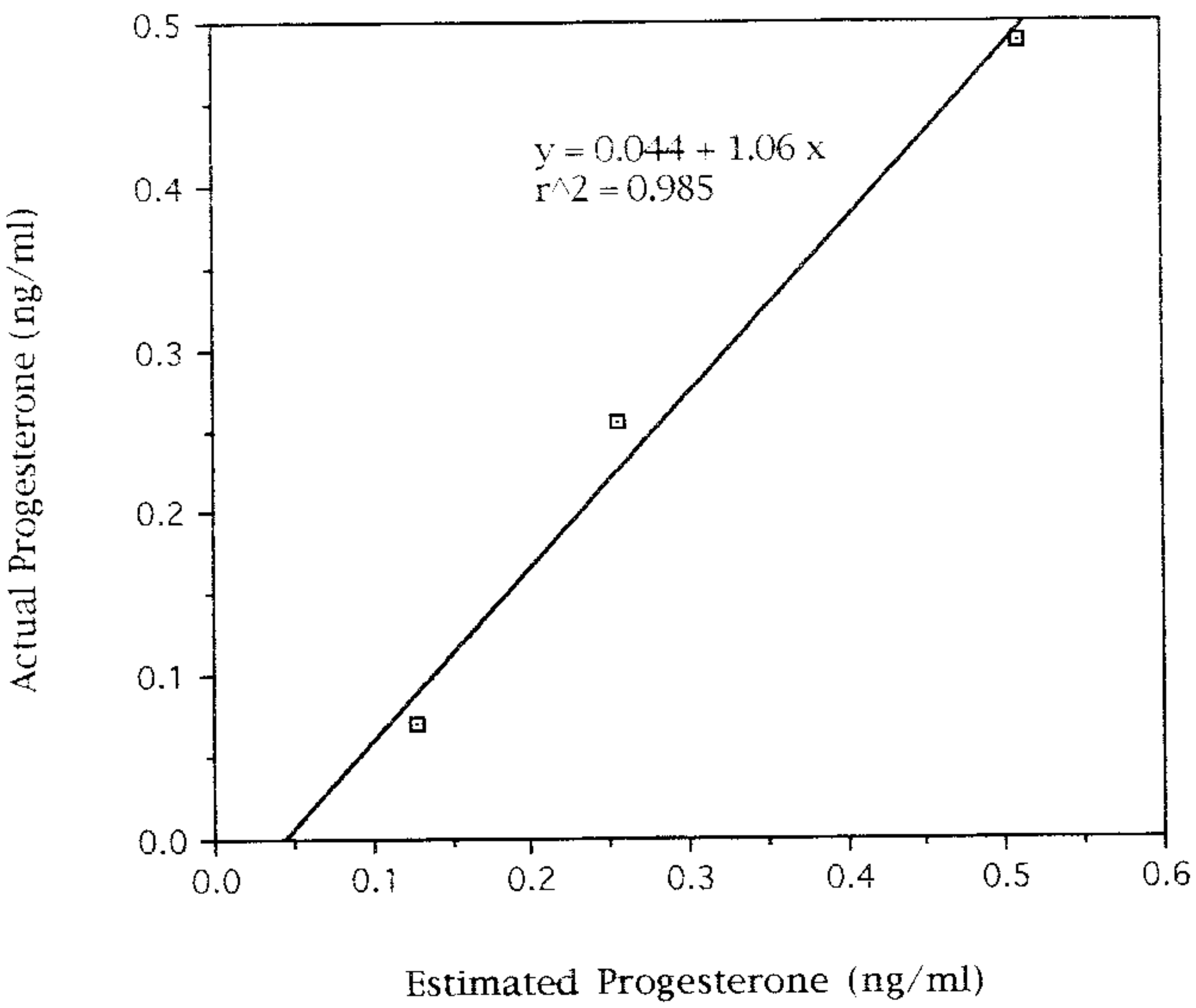

Fig. 4. A regression analysis of Ozark hellbender serum pool (0.255 ng/ml progesterone) at a dilution of 50%, 100%, and 200% was performed for assay validation.

(Moore, 1975). Andreoletti et al. (1983) showed that in male *Triturus carnifex* sex behavior is depressed by castration and partially reinstated by replacement therapy with testosterone.

While there is considerable information regarding the reproductive endocrinology of some salamanders, there are no data available specifically in cryptobranchids. The purpose of this study was to examine the levels of three steroid hormones in males and females throughout gamete maturation and the reproductive season in *Cryptobranchus alleganiensis bishopi.* Based on studies of other species, it was hypothesized that estradiol concentrations would be elevated in females during the maturation of oocytes and would remain elevated throughout the breeding season with a peak at the end of the reproductive cycle; estradiol concentrations in males were hypothesized to remain low throughout the maturation of spermatogonia and the breeding season, although elevated levels might be seen at the end of the reproductive cycle. It was further hypothesized that progesterone concentrations would be high at the start of the breeding season in females. Since the effects of progesterone in male amphibians is unclear, no hypothesis was made regarding the concentrations of progesterone in males. It also was hypothesized that testosterone levels in males would be high throughout the maturation of spermatogonia and the breeding season, whereas the concentrations of testosterone in females might remain low and unchanged.

MATERIALS AND METHODS

Ozark hellbenders were collected with permisstion from the Missouri Department of Conservation primarily from two large riffles and adjacent pools of a 90 m section of the North Fork of the White River, Ozark County, Missouri, approximately 45 km upstream from Norfork Lake (Fig. 1). Five hellbenders were collected from other areas nearby. Between one and six hellbenders of each gender were collected weekly between June and mid-October 1993 (except the weeks of June 6, September 26 and October 3 due to unfavorable river conditions) and monthly during November and December 1993. Hellbenders were caught during the day by turning large rocks in riffles and pools at depths up to 1.5 m. Uncovered hellbenders were grasped by hand and placed in nylon mesh bags and brought ashore. Hellbenders were then anesthetized in a weak solution of tricaine (ethyl-m-aminobenzoate, Sigma Chemical Co.) for easier handling. A standard fish board was used to measure snout-vent length (SVL) to the nearest mm. Gender was determined.either by external examination (by swollen cloacal glands or distended abdomens; Smith, 1907) from mid-July through November, or by internal examination at all other times. When gender was determined by internal examination, an incision approximately 1 cm in length was made on the lower left quadrant of the abdomen. An otoscope was inserted into the body cavity to examine for the presence of yellow follicles or testes. Sutures were made using silk thread. For all hellbenders, 1 ml of blood was collected by cardiac puncture using a 1 ml insulin syringe. Blood was then placed on ice until returning to the laboratory. Hellbenders were branded on the abdomen using the heated wire technique described by Clark (1971). The hellbenders were then placed in a shallow tub of fresh, cold water, allowed to recover from the anesthesia, then released at the site of capture.

Immediately upon returning to the laboratory, blood was centrifuged for 5 minutes at 11,500 RPM. The serum was separated, then stored at -20°C. An

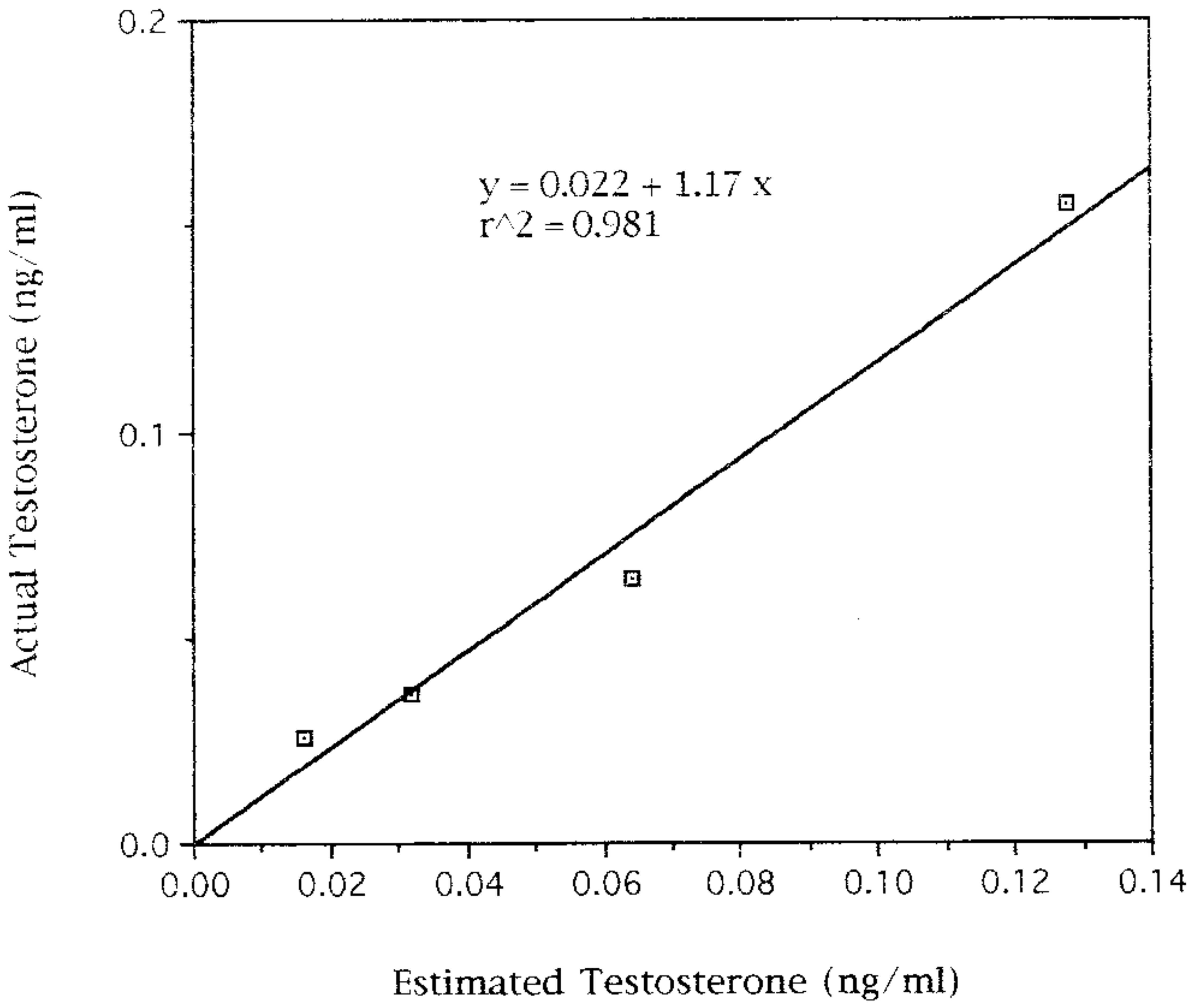

Fig. 5. A regression analysis of Ozark hellbender serum pool (0.064 ng/ml tesosterone) at dilutions of 25%, 50%, 100% and 200% was performed for assay validation.

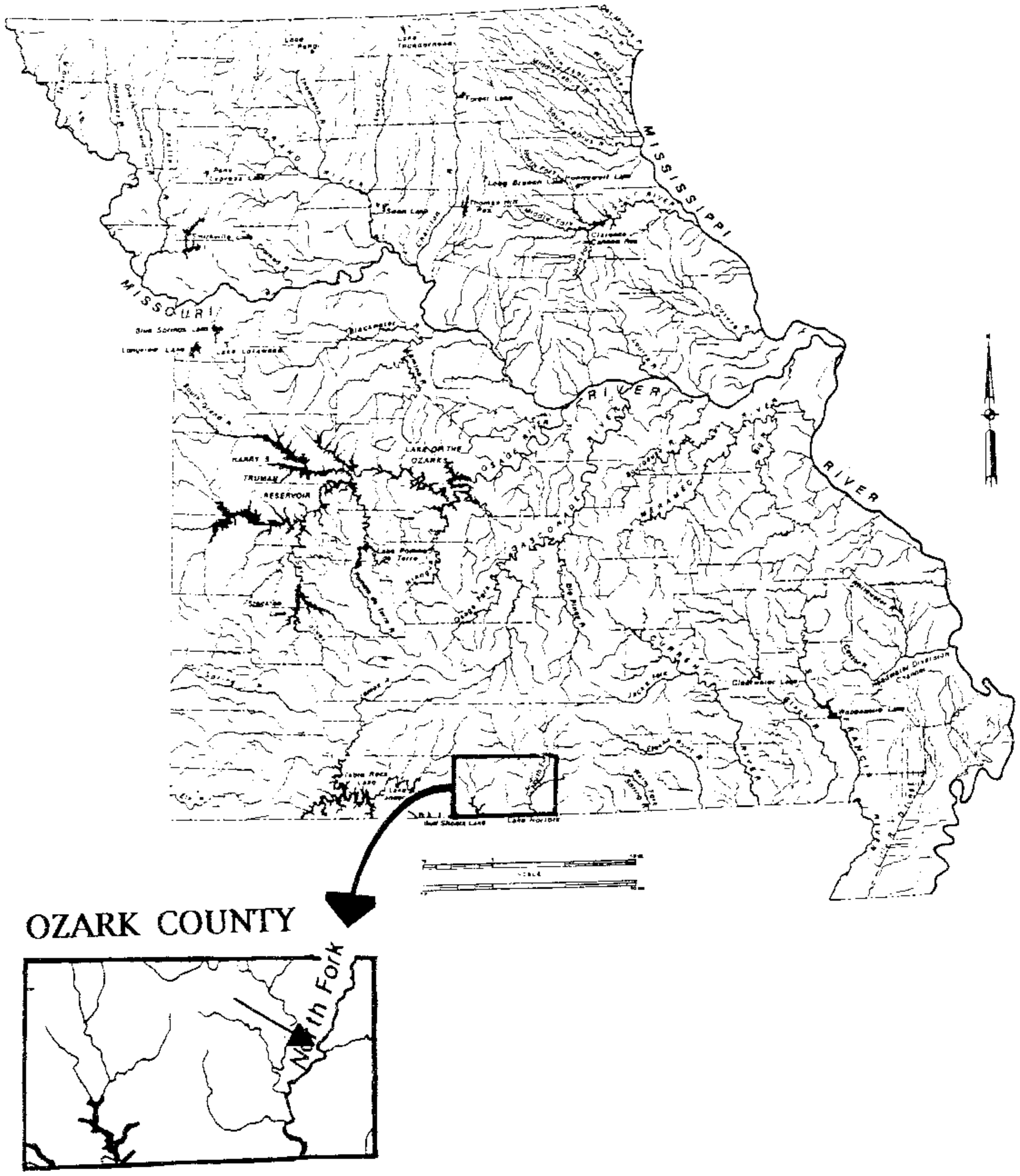

Figure 1. State map showing North Fork River, the river in which collections were made, in south central Missouri. The general area of collection is indicated by the smaller arrow.

extraction procedure was performed (following instructions from Diagnostic Products Corporation, DPC) in which dichloromethane was used to isolate steroids from potentially interfering substances. A 0.5 mL serum sample was added to 5.0 mL of dichloromethane in a screw top tube and mixed by gentle inversion for one hour. Organic and inorganic layers were separated by centrifugation at 1500 x g for 5 minutes. Steroids contained in 2.0 mL of the lower, organic layer were dried under a stream of nitrogen gas and then resuspended in 0.2 mL human serum that had been stripped free of steroids.

The concentrations of estradiol were measured by radioimmunoassay (RIA) via a commercially available kit (DPC; Estradiol Coat-A-Count, TKE22). The standards used for the standard curve were modified to increase sensitivity at low concentrations and ranged from 0.02 to 1.80 ng/mL. The radioactive estradiol was diluted to 50% using distilled water before running the assay. The serum estradiol RIA was validated for the Ozark hellbender by exhibiting assay linearity when serum pool was diluted to 25% and 50% and doubled to 200% (a combination of various extracted Ozark hellbender sera diluted to 50% with estradiol-stripped human serum was utilized as a pool; the pool's estradiol concentration was 0.277 ng/mL). These samples were assayed with results of 0.058, 0.124 and 0.632 ng/mL respectively (Fig. 2). Linearity was also displayed by analysis of 50:50 mixtures of Ozark hellbender pool with estradiol standards (0.05, 0.15 and 0.50 ng/mL). These mixtures were estimated to have estradiol concentrations of 0.165, 0.215 and 0.390 ng/mL respectively, and yielded results of 0.160, 0.211 and 0.352 ng/mL, respectively (Fig. 3).

The concentrations of progesterone were measured by RIA via a commercially available kit (DPC, Progesterone Coat-A-Count, TKPG2). The standards used ranged from 0.025 to 20.0 ng/mL and the radioactive

Statistics were performed using Minitab. One-way analyses of variance (ANOVA) were performed to determine significant gender-specific hormone or SVL differences ($p < 0.05$) over time (Freund, 1988). Fisher's tests (Freund, 1988) were performed on data sets with significant differences to determine which dates were significantly different from one another.

RESULTS

Animals

Fifty females and 75 males were captured between June 2 and December 4, 1993. Of these, 16 females and 15 males were recaptured once or twice. Four juveniles were captured, although sera from these individuals was either not drawn or was used only for the serum pool. Sexually mature females ranged between 250 and 372 mm SVL and sexually mature males ranged between 229 and 341 mm SVL.

Snout-vent length varied little among females and males over time (Tables 1 and 2; Figs. 6 and 7). ANOVA p values for female and male weekly SVL data were 0.014 and 0.078 respectively. A Fisher's test confirmed that females in weeks 13 and 14 (August 26 and September 4, 1993) were smaller than those in most other weeks (Table 3).

Hellbenders became more difficult to capture after the breeding season. A total of only 6 individuals were captured in November and December. Recaptured individuals were captured too few times (2 or 3) to examine individual patterns, but data from recaptured animals are consistent with trends of the entire population as follow.

No hellbenders died during surgery or during recovery from the anesthesis. Two live animals were recaptured with their sutures missing and

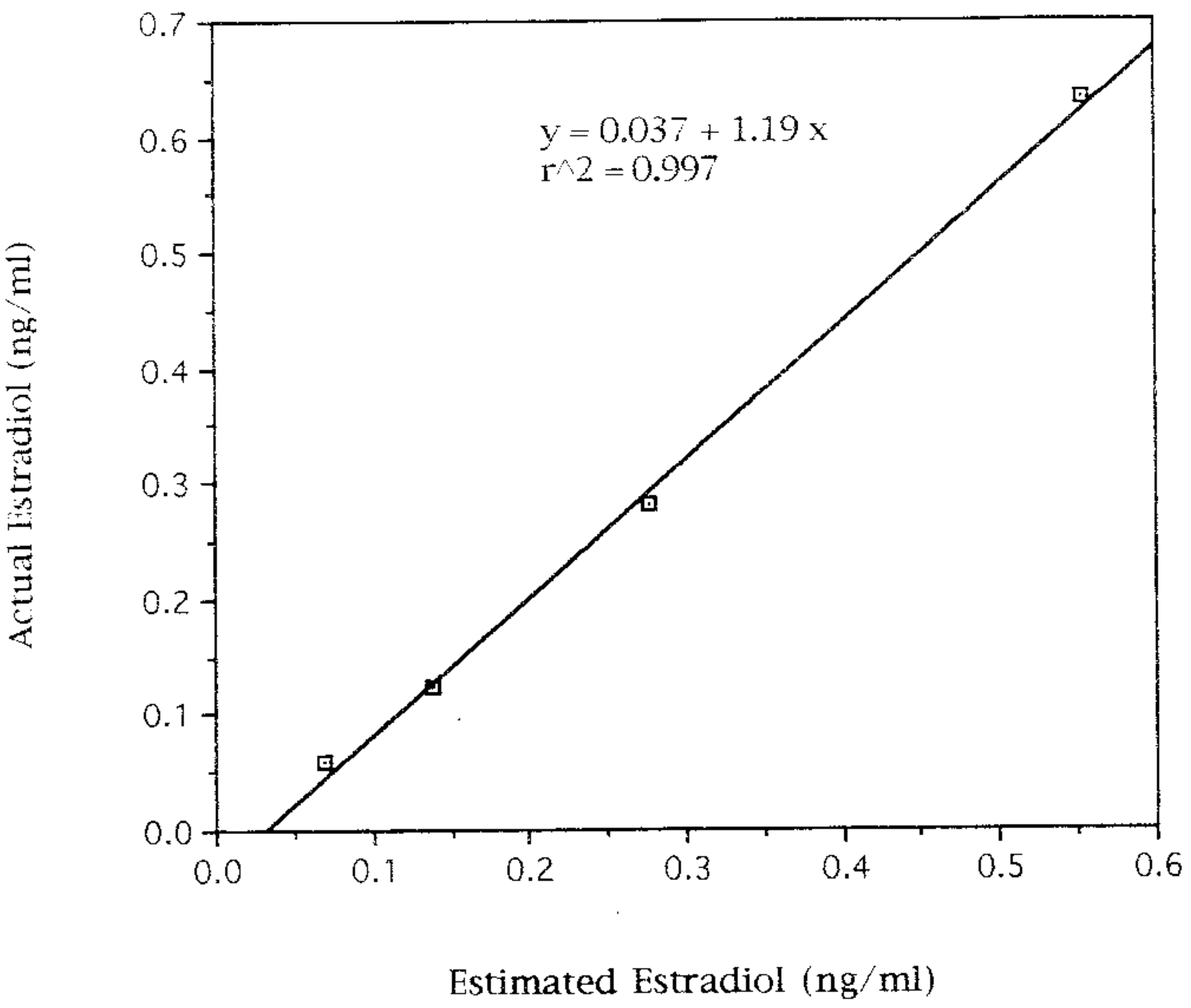

Fig. 2. A regression analysis of Ozark hellbender serum pool (0.277 ng/ml estradiol) at dilutions of 25%, 50%, 100% and 200% was performed for assay validation.

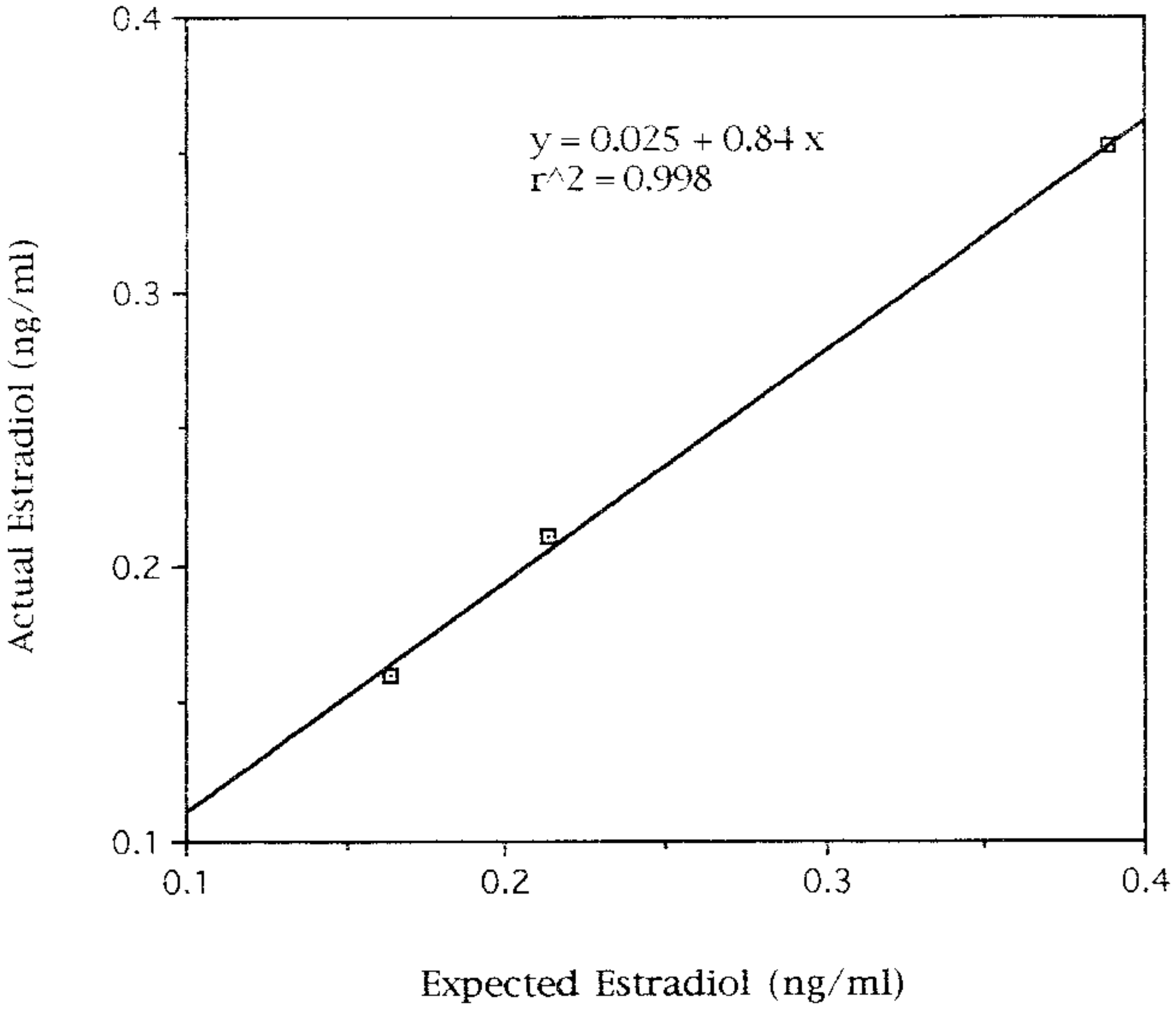

Fig. 3. A regression analysis of 50:50 mixtures of Ozark hellbender serum pool (0.277 ng/ml estradiol) with estradiol standards (0.05, 0.15 and 0.50 ng/ml) was performed for assay validation. Expected estradiol was estimated by averaging the pool and known estradiol values.

FEMALE

WK#	DATE	N	ESTRADIOL	PROGESTERONE	TESTOSTERONE	SVL
1	6/02/93	4	0.234 ± 0.024	0.141 ± 0.035	0.693 ± 0.069	340.5 ± 9.314
3	6/18/93	2	0.323 ± 0.120	0.145 ± 0.079	0.742 ± 0.375	332.3 ± 6.860
4	6/23/93	4	0.311 ± 0.156	0.109 ± 0.029	0.848 ± 0.166	324.0 ±13.010
5	6/30/93	5	0.815 ± 0.283	0.168 ± 0.017	1.709 ± 0.371	325.2 ± 9.014
6	7/07/93	2	1.672 ± 0.313	0.391 ± 0.254	4.053 ± 1.298	365.0 ±24.000
7	7/14/93	3	2.393 ± 0.637	0.222 ± 0.034	2.264 ± 0.435	332.7 ± 13.170
8	7/23/93	2	1.208 ± 0.091	0.212 ± 0.185	2.572 ± 0.261	351.0
9	7/28/93	5	0.954 ± 0.484	0.157 ± 0.034	2.999 ± 0.641	312.7 ± 7.517
10	8/04/93	4	2.280 ± 0.188	0.068 ± 0.032	5.392 ± 0.839	338.8 ± 7.024
11	8/12/93	4	2.271 ± 0.065	0.098 ± 0.052	3.069 ± 0.849	298.0 ± 15.590
12	8/18/93	6	1.671 ± 0.321	0.036 ± 0.015	5.404 ± 0.520	317.5 ± 6.951
13	8/26/93	1	0.237	0.009	4.263	264.0
14	9/04/93	3	1.717 ± 0.032	0.038 ± 0.012	14.627 ± 3.845	286.3 ± 19.890
15	9/11/93	3	1.600 ± 0.373	0.005 ± 0.002	12.258 ± 0.078	334.0 ± 10.770
16	9/19/93	3	1.032 ± 0.388	0.006 ± 0.002	4.825 ± 0.039	326.5 ± 6.384
19	10/09/93	1	0.164	0.399	7.049	319.0
20	10/15/93	3	0.165 ± 0.067	0.047 ± 0.029	4.344 ± 1.338	301.7 ±26.210
23	11/11/93	2	0.318 ± 0.123	0.005 ± 0.001	0.227 ± 0.127	285.0 ± 15.000
26	12/04/93	1	0.148	0.001	0.124	333.0

Table 1. Sample size, mean hormone concentration (ng/ml) and snout-vent length (mm; ± standard error) for female Ozark hellbenders.

progesterone was diluted to 50% with distilled water before running the assay. The serum progesterone RIA was validated for the Ozark hellbender by exhibiting assay linearity when serum pool (same pool used for estradiol: the progesterone concentration was 0.255 ng/mL) was diluted to 50% and doubled to 200%. These samples were assayed with results of 0.071 and 0.487 ng/mL respectively (Fig. 4).

The concentrations of testosterone were measured by RIA via a commercially available kit (DPC, Total Testosterone Coat-A-Count, TKTT2). The standards used ranged from 0.05 to 8.0 ng/mL and the radioactive testosterone was diluted to 50% with distilled water before running the assay. The serum testosterone RIA was validated for the Ozark hellbender by exhibiting assay linearity when serum pool (same pool used for estradiol and progesterone: the testosterone concentration was 0.064 ng/mL) was diluted to 25% and 50% and doubled to 200%. These samples were assayed with results of 0.026, 0.036, and 0.155 ng/mL respectively (Fig. 5).

To eliminate problems arising with the use of repeated measures, only one serum sample was assayed for any animal captured more than once. All other samples from animals that were captured more than once were collected for serum pool. The Q test (Fritz and Schenk, 1966) was employed to determine if a questionable value, an outlier, could by rejected with a 90% probability level. Fifteen of 390 values were rejected by the Q test and eliminating all but one set of values for each individual, mean values and standard errors were calculated for hormone concentrations. Mean values with standard errors were plotted against time to display trends of increasing and decreasing hormone concentrations. Mean values of SVL with standard errors were plotted to display variances among SVL over time.

FEMALE PROGESTERONE

WK#	1	3	4	5	6	7	8	9	10	11	12	13	14	15	16	19	20	23
3	NS																	
4	NS	NS																
5	NS	NS	NS															
6	*	*	*	*														
7	NS	NS	NS	NS	NS													
8	NS	NS	NS	NS	NS	NS												
9	NS	NS	NS	NS	*	NS	NS											
10	NS	NS	NS	NS	*	*	NS	NS										
11	NS	NS	NS	NS	*	NS	NS	NS	NS									
12	NS	NS	NS	*	*	*	*	*	NS	NS								
13	NS	NS	NS	NS	*	NS	NS	NS	NS	NS	NS							
14	NS	NS	NS	NS	*	*	NS	NS	NS	NS	NS	NS						
15	NS	NS	NS	*	*	*	*	*	NS	NS	NS	NS	NS					
16	NS	NS	NS	*	*	*	*	*	NS	NS	NS	NS	NS	NS				
19	*	*	*	*	NS	NS	NS	*	*	*	*	*	*	*	*			
20	NS	NS	NS		*	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	*		
23	NS	NS	NS	*	*	*	*	*	NS	NS	NS	NS	NS	NS	NS	*	NS	
26	NS	NS	NS	NS	*	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	*	NS	NS

Table 5. Significant (*) and non-significant (NS) differences among weekly female progesterone concentrations (Fisher's test, $\alpha = 0.05$).

MALE

WK #	DATE	N	ESTRADIOL	PROGESTERONE	TESTOSTERONE	SVL
1	6/02/93	2	0.355 ± 0.341	0.249	0.909	261.5 ±21.500
3	6/18/93	4	0.010 ± 0.002	0.366 ± 0.088	3.998 ± 1.052	293.8 ± 9.977
4	6/23/93	1	0.011	1.808	6.982	253
5	6/30/93	3	0.012 ± 0.002	1.101 ± 0.202	9.595 ± 1.067	299.8 ± 7.336
6	7/07/93	5	0.012 ± 0.000	0.288 ± 0.077	14.473 ± 2.363	305.1 ± 8.572
7	7/14/93	4	0.011 ± 0.001	0.456 ± 0.151	5.453 ± 2.096	302.3 ± 8.782
8	7/23/93	5	0.013 ± 0.001	0.526 ± 0.089	16.242 ± 2.436	319.1 ± 7.986
9	7/28/93	3	0.011 ± 0.002	0.364 ± 0.119	13.184 ± 4.345	303.8 ±14.230
10	8/04/93	4	0.009 ± 0.001	0.185 ± 0.058	12.221 ± 2.185	281.1 ±12.070
11	8/12/93	4	0.015 ± 0.003	0.193 ± 0.040	20.983 ± 3.354	302.0 ± 3.362
12	8/18/93	3	0.011 ± 0.003	0.336 ± 0.201	13.864 ± 4.017	292.3 ±16.130
13	8/26/93	1	0.008	0.005	24.874	303.0
14	9/04/93	2	0.010 ± 0.001	0.116 ± 0.014	27.023 ± 1.582	274.0 ± 31.000
15	9/11/93	5	0.011 ± 0.001	0.039 ± 0.019	38.135 ± 3.250	308.5 ± 6.639
16	9/19/93	5	0.011 ± 0.002	0.013 ± 0.007	22.433 ± 2.699	307.0 ±17.910
19	10/09/93	6	0.011 ± 0.001	0.459 ± 0.255	29.623 ± 8.674	297.8 ± 7.946
20	10/15/93	4	0.009 ± 0.000	0.330 ± 0.015	22.135 ± 7.232	288.5 ±18.010
26	12/04/93	3	0.009 ± 0.001	0.002 ± 0.001	0.066 ± 0.023	293.0 ± 16.000

Table 2. Sample size, mean hormone concentration (ng/ml) and snout-vent length (mm; ± standard error) for male Ozark hellbenders.

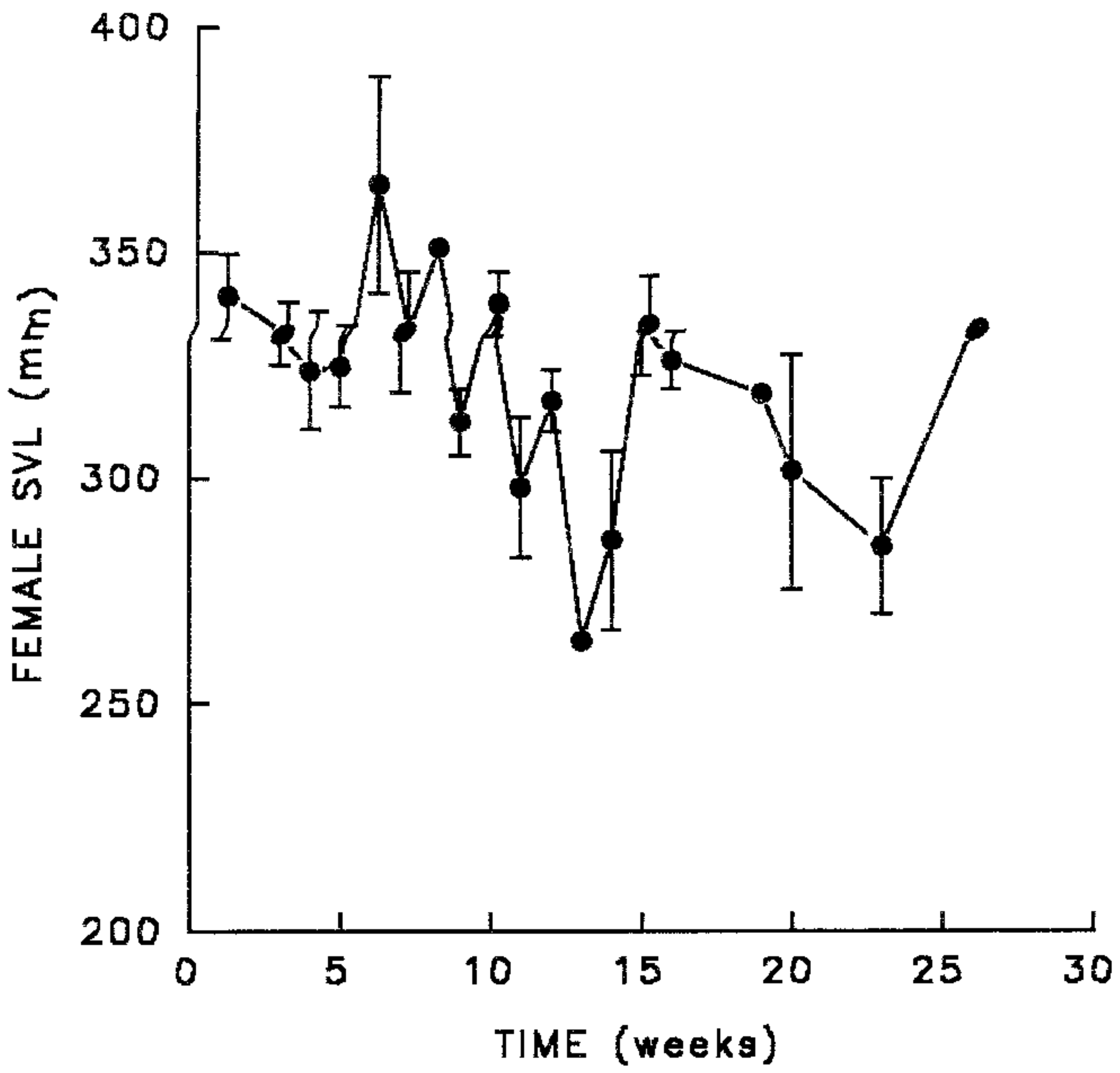

Figure 6. Mean snout-vent lengths (SVL) in female Ozark hellbenders over time, starting June 2, 1993. Vertical lines indicate standard errors. Sample sizes are in Table 1.

FEMALE TESTOSTERONE

WK#	1	3	4	5	6	7	8	9	10	11	12	13	14	15	16	19	20	23
3	NS																	
4	NS	NS																
5	NS	NS	NS															
6	NS	NS	NS	NS														
7	NS	NS	NS	NS	NS													
8	NS	NS	NS	NS	NS	NS												
9	NS	NS	NS	NS	NS	NS	NS											
10	*	*	*	*	NS	NS	NS	NS										
11	NS	NS	NS	NS	NS	NS	NS	NS	NS									
12	*	*	*	*	NS	NS	NS	NS	NS	NS								
13	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS							
14	*	*	*	*	*	*	*	*	*	*	*	*						
15	*	*	*	*	*	*	*	*	*	*	*	*	NS					
16	*	NS	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	*	*				
19	*	*	*	*	NS	NS	NS	NS	NS	NS	NS	NS	*	*	NS			
20	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	*	*	NS	NS		
23	NS	NS	NS	NS	NS	NS	NS	NS	*	NS	*	NS	*	*	*	*	NS	
26	NS	NS	NS	NS	NS	NS	NS	NS	*	NS	*	NS	*	*	NS	*	NS	NS

Table 6. Significant (*) and non-significant (NS) differences among weekly female testosterone concentrations (Fisher's test, $\alpha = 0.05$).

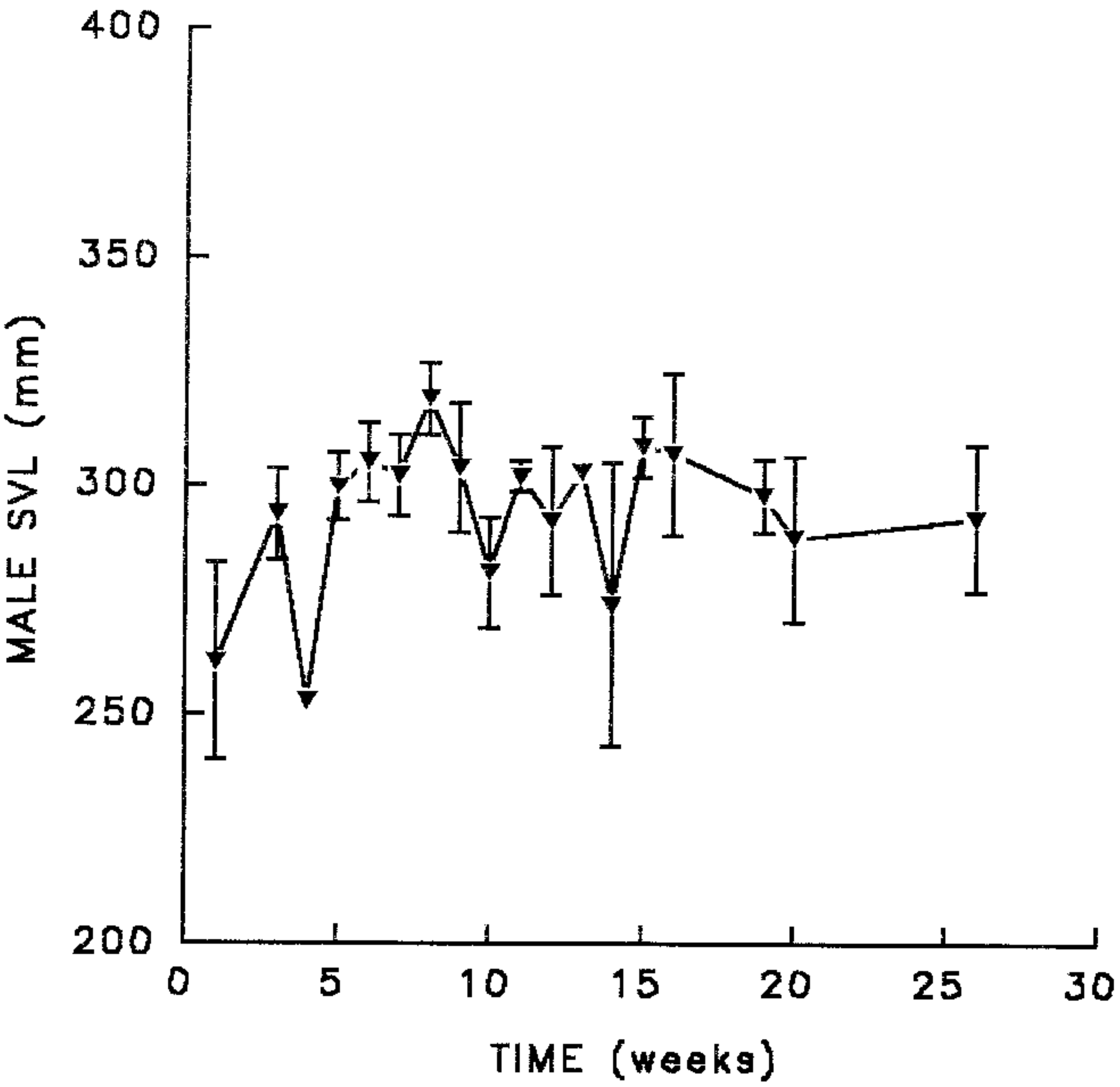

Figure 7. Mean snout-vent lengths (SVL) in male Ozark hellbenders over time, starting June 2, 1993. Vertical lines indicate standard errors. Sample sizes are in Table 2.

FEMALE SVL

WK#	1	3	4	5	6	7	8	9	10	11	12	13	14	15	16	19	20	23
3	NS																	
4	NS	NS																
5	NS	NS	NS															
6	NS	NS	NS	NS														
7	NS	NS	NS	NS	NS													
8	NS	NS	NS	NS	NS	NS												
9	NS	NS	NS	NS	*	NS	NS											
10	NS	NS	NS	NS	NS	NS	NS	NS										
11	*	*	NS	NS	*	NS	NS	NS	*									
12	NS	NS	NS	NS	*	NS	NS	NS	NS	NS								
13	*	*	*	*	*	*	*	NS	*	NS	*							
14	*	*	*	*	*	*	*	NS	*	NS	NS	NS						
15	NS	NS	NS	NS	NS	NS	NS	NS	NS	*	NS	*	*					
16	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	*	*	NS				
19	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS			
20	*	NS	NS	NS	*	NS	NS	NS	*	NS	NS	NS	NS	NS	NS	NS		
23	*	*	NS	NS	*	*	*	NS	*	NS	NS	NS	NS	*	NS	NS	NS	
26	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Table 3. Significant (*) and non-significant (NS) differences among weekly mean values for female snout-vent lengths, SVL (Fisher's test, $\alpha = 0.05$).

MALE ESTRADIOL

WK#	1	3	4	5	6	7	8	9	10	11	12	13	14	15	16	19	23
3	*																
4	*	NS															
5	*	NS	NS														
6	*	NS	NS	NS													
7	*	NS	NS	NS	NS												
8	*	NS	NS	NS	NS	NS											
9	*	NS	NS	NS	NS	NS	NS										
10	*	NS	NS	NS	NS	NS	NS	NS									
11	*	NS	NS	NS	NS	NS	NS	NS	NS								
12	*	NS	NS	NS	NS	NS	NS	NS	NS	NS							
13	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS						
14	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS					
15	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS				
16	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS			
19	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		
20	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
26	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Table 7. Significant (*) and non-significant (NS) differences among weekly male estradiol concentrations (Fisher's test, $\alpha = 0.05$).

their intestines partly hanging out of the body cavity. These animals were resutured and released. Based on the large numbers of apparently healthy, fully recovered recaptures, surgical mortality was judged to be minimal.

Hormone Analyses

One-way analyses of variance were performed on six data sets: female estradiol ($p < 0.001$), progesterone ($p = 0.002$), and testosterone ($p < 0.001$; Table 1); and male estradiol ($p = 0.007$), progesterone ($p < 0.001$), and testosterone ($p < 0.001$; Table 2). The results of the Fisher's tests (Tables 4-9) indicated which weekly means within a given data set were significantly different from one another.

Female estradiol concentrations started increasing on week 5 (June 30, 1993) and dropped to levels seen in early June by week 19 (October 9, 1993; Fig. 8). On weeks 8, 9 and 13 (July 23, 28 and August 26, 1993), estradiol levels were quite low. Significant differences between means were seen scattered throughout (Table 4). Weeks 7, 10, 11, 12, 14 and 15 were the most different from other weeks. Female progesterone concentrations peaked at week 6 (July 7, 1993) and week 19 (October 9, 1993; Fig. 9). Week 6 was significantly higher than most other weeks; week 19 was significantly higher than most other weeks, but the value for week 19 is from only one individual (Table 5). Female testosterone concentrations peaked at weeks 14 and 15 (September 4 and 11, 1993; Fig. 10). These weeks were significantly higher than most other weeks (Table 6).

Male estradiol concentrations showed little variance between June and December 1993, with the exception of 1 male on June 2, 1993 that had a very high estradiol level, making the mean for that week exceptionally high (0.355 ng/ml; Fig. 11) and significantly different from all other weeks (Table 7).

FEMALE ESTRADIOL

WK#	1	3	4	5	6	7	8	9	10	11	12	13	14	15	16	19	20	23
3	NS																	
4	NS	NS																
5	NS	NS	NS															
6	*	*	*	NS														
7	*	*	*	*	NS													
8	NS	NS	NS	NS	NS	NS												
9	NS	NS	NS	NS	NS	*	NS											
10	*	*	*	*	NS	NS	*	*										
11	*	*	*	*	NS	NS	NS	*	NS									
12	*	*	*	NS	NS	NS	NS	NS	NS	NS								
13	NS	NS	NS	NS	NS	*	NS	NS	*	*	NS							
14	*	*	*	NS	NS	NS	NS	NS	NS	NS	NS	*						
15	*	*	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS					
16	NS	NS	NS	NS	NS	*	NS	NS	*	*	NS	NS	NS	NS				
19	NS	NS	NS	NS	*	*	NS	NS	*	*	*	NS	*	*	NS			
20	NS	NS	NS	NS	*	*	NS	NS	*	*	*	NS	*	*	NS	NS		
23	NS	NS	NS	NS	*	*	NS	NS	*	*	*	NS	*	*	NS	NS	NS	
26	NS	NS	NS	NS	*	*	NS	NS	*	*	*	NS	*	*	NS	NS	NS	NS

Table 4. Significant (*) and non-significant (NS) differences among weekly female estradiol concentrations (Fisher's test, $\alpha = 0.05$).

MALE PROGESTERONE

WK#	1	3	4	5	6	7	8	9	10	11	12	13	14	15	16	19	23
3	NS																
4	*	*															
5	*	*	*														
6	NS	NS	*	*													
7	NS	NS	*	NS	NS												
8	NS	NS	*	NS	NS	NS											
9	NS	NS	*	*	NS	NS	NS										
10	NS	NS	*	*	NS	NS	NS	NS									
11	NS	NS	*	*	NS	NS	NS	NS	NS								
12	NS	NS	*	*	NS	NS	NS	NS	NS	NS							
13	NS	NS	*	*	NS	NS	NS	NS	NS	NS	NS						
14	NS	NS	*	*	NS	NS	NS	NS	NS	NS	NS	NS					
15	NS	NS	*	*	NS	*	*	NS	NS	NS	NS	NS	NS				
16	NS	NS	*	*	NS	*	*	NS	NS	NS	NS	NS	NS	NS			
19	NS	NS	*	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	*		
20	NS	NS	*	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
26	NS	NS	*	*	NS	*	*	NS	NS	NS	NS	NS	NS	NS	NS	*	NS

Table 8. Significant (*) and non-significant (NS) differences among weekly male progesterone concentrations (Fisher's test, $\alpha = 0.05$).

MALE TESTOSTERONE

WK#	1	3	4	5	6	7	8	9	10	11	12	13	14	15	16	19	23
3	NS																
4	NS	NS															
5	NS	NS	NS														
6	NS	NS	NS	NS													
7	NS	NS	NS	NS	NS												
8	NS	NS	NS	NS	NS	NS											
9	NS	NS	NS	NS	NS	NS	NS										
10	NS	NS	NS	NS	NS	NS	NS	NS									
11	NS	*	NS	NS	NS	*	NS	NS	NS								
12	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS							
13	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS						
14	*	*	NS	*	NS	*	NS	NS	NS	NS	NS	NS					
15	*	*	*	*	*	*	*	*	*	*	*	NS	NS				
16	*	*	NS	NS	NS	*	NS	NS	NS	NS	NS	NS	NS	*			
19	*	*	*	*	*	*	*	*	*	NS	*	NS	NS	NS	NS		
20	*	*	NS	NS	NS	*	NS	NS	NS	NS	NS	NS	NS	*	NS	NS	
26	NS	NS	NS	NS	*	NS	*	NS	NS	*	NS	*	*	*	*	*	*

Table 9. Significant (*) and non-significant (NS) differences among weekly male testosterone concentrations (Fisher's test, $\alpha = 0.05$).

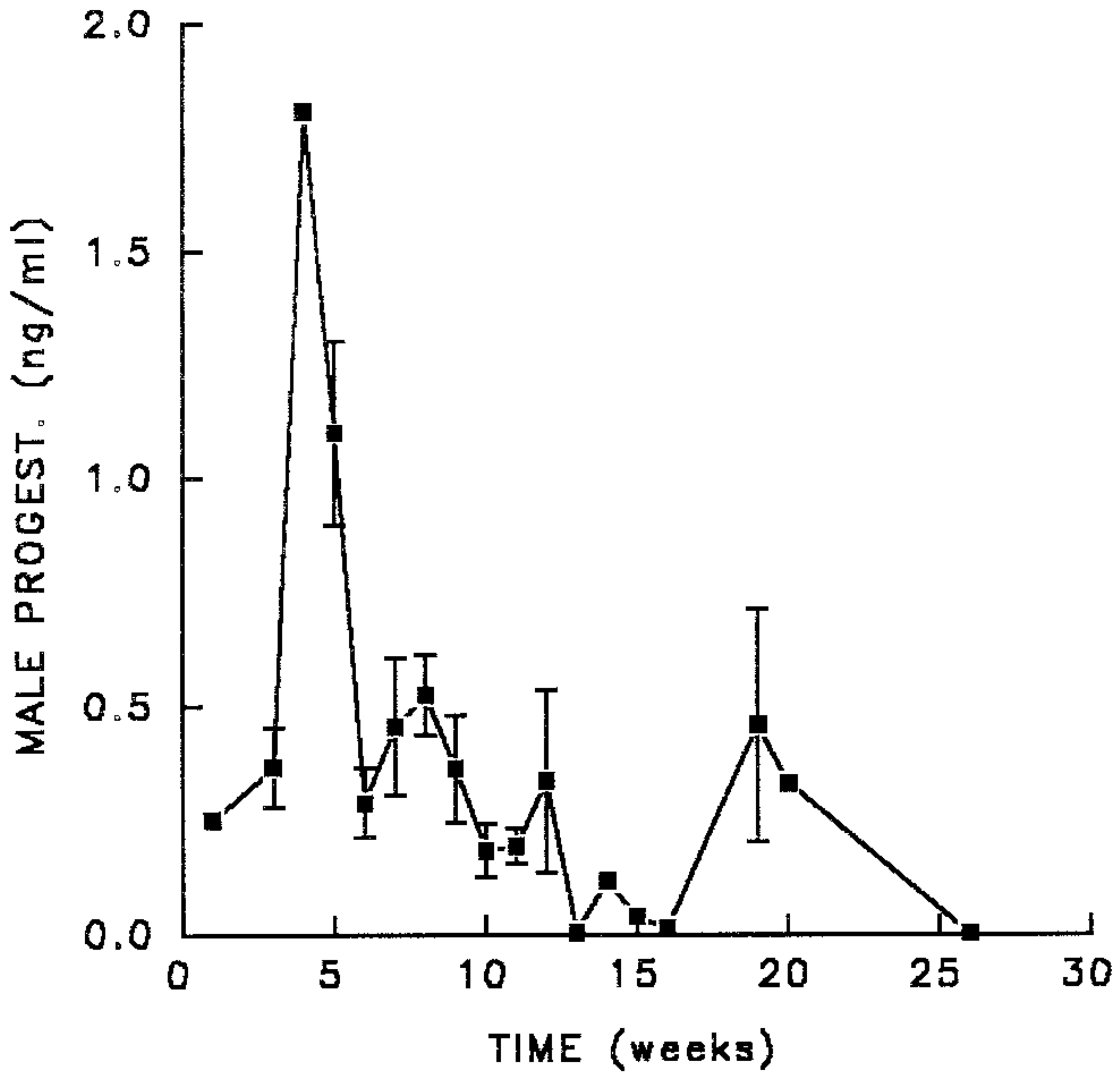

Figure 12. Mean concentrations of serum progesterone (ng/ml) in male Ozark hellbenders over time, starting June 2, 1993. Vertical lines indicate standard errors. Sample sizes are in Table 2.

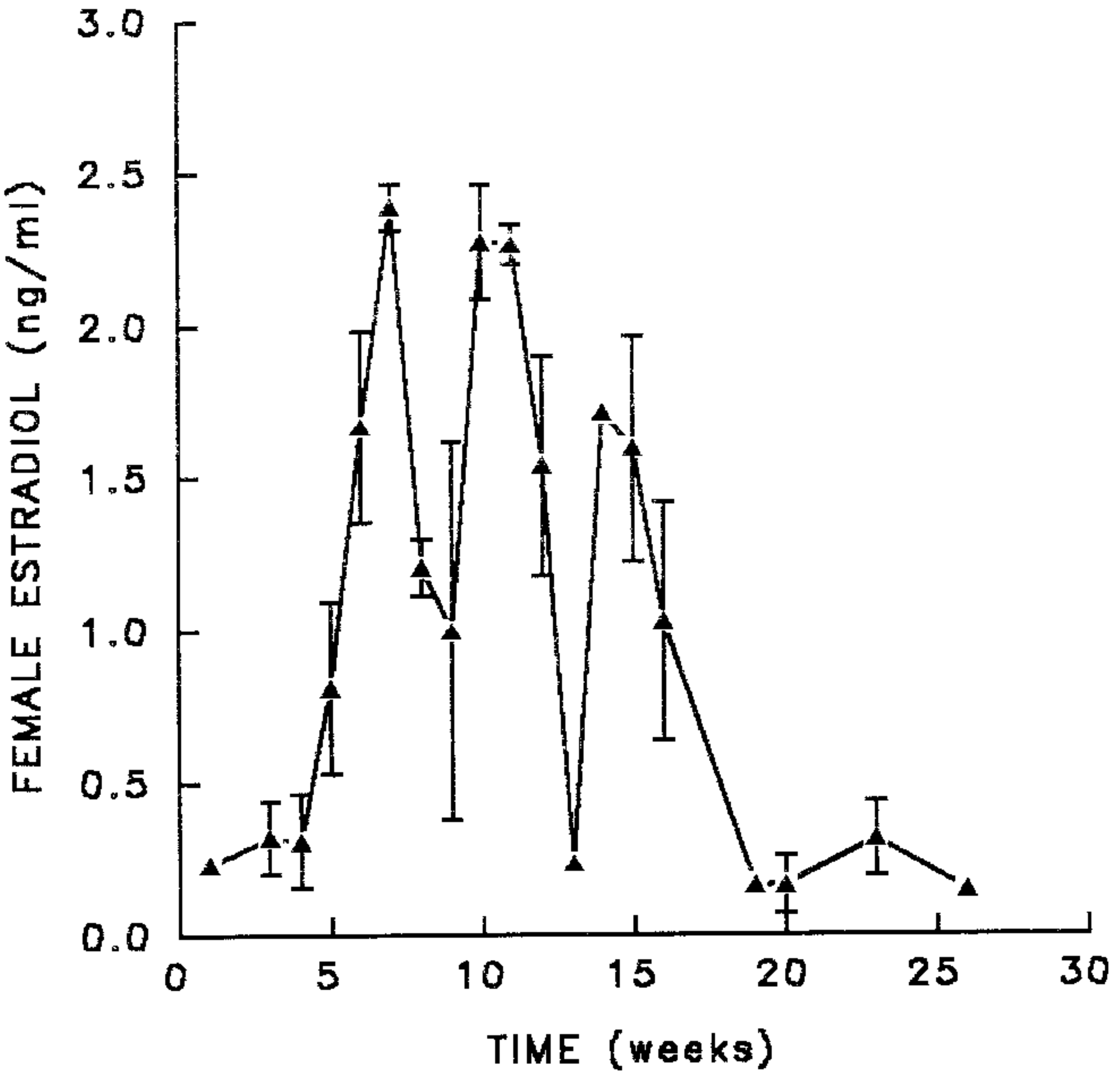

Figure 8. Mean concentrations of serum estradiol (ng/ml) in female Ozark hellbenders over time, starting June 2, 1993. Vertical lines indicate standard errors. Sample sizes are in Table 1.

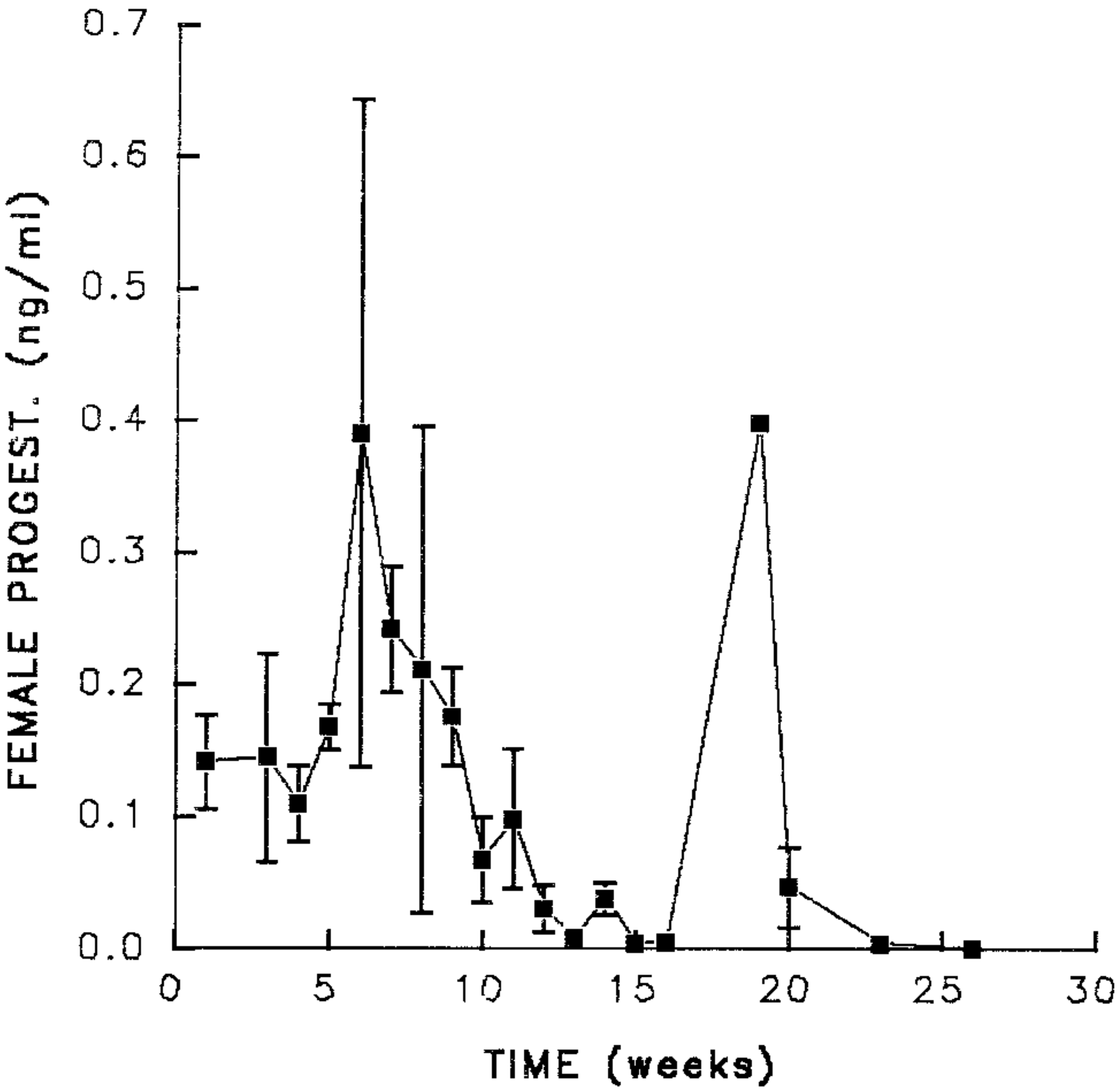

Figure 9. Mean concentrations of serum progesterone (ng/ml) in female Ozark hellbenders over time, starting June 2, 1993. Vertical lines indicate standard errors. Sample sizes are in Table 1.

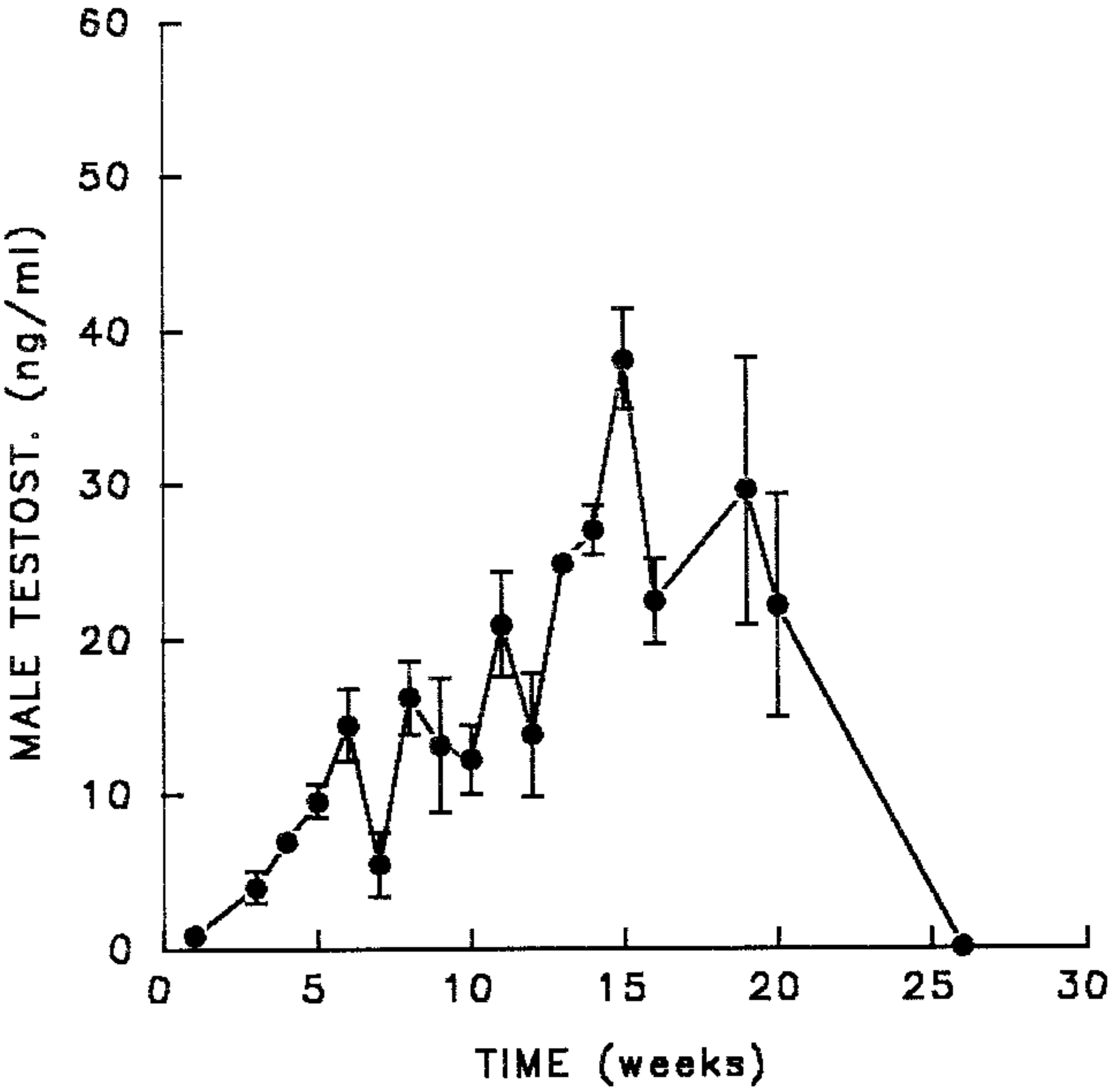

Figure 13. Mean concentrations of serum testosterone (ng/ml) in male Ozark hellbenders over time, starting June 2, 1993. Vertical lines indicate standard errors. Sample sizes are in Table 2.

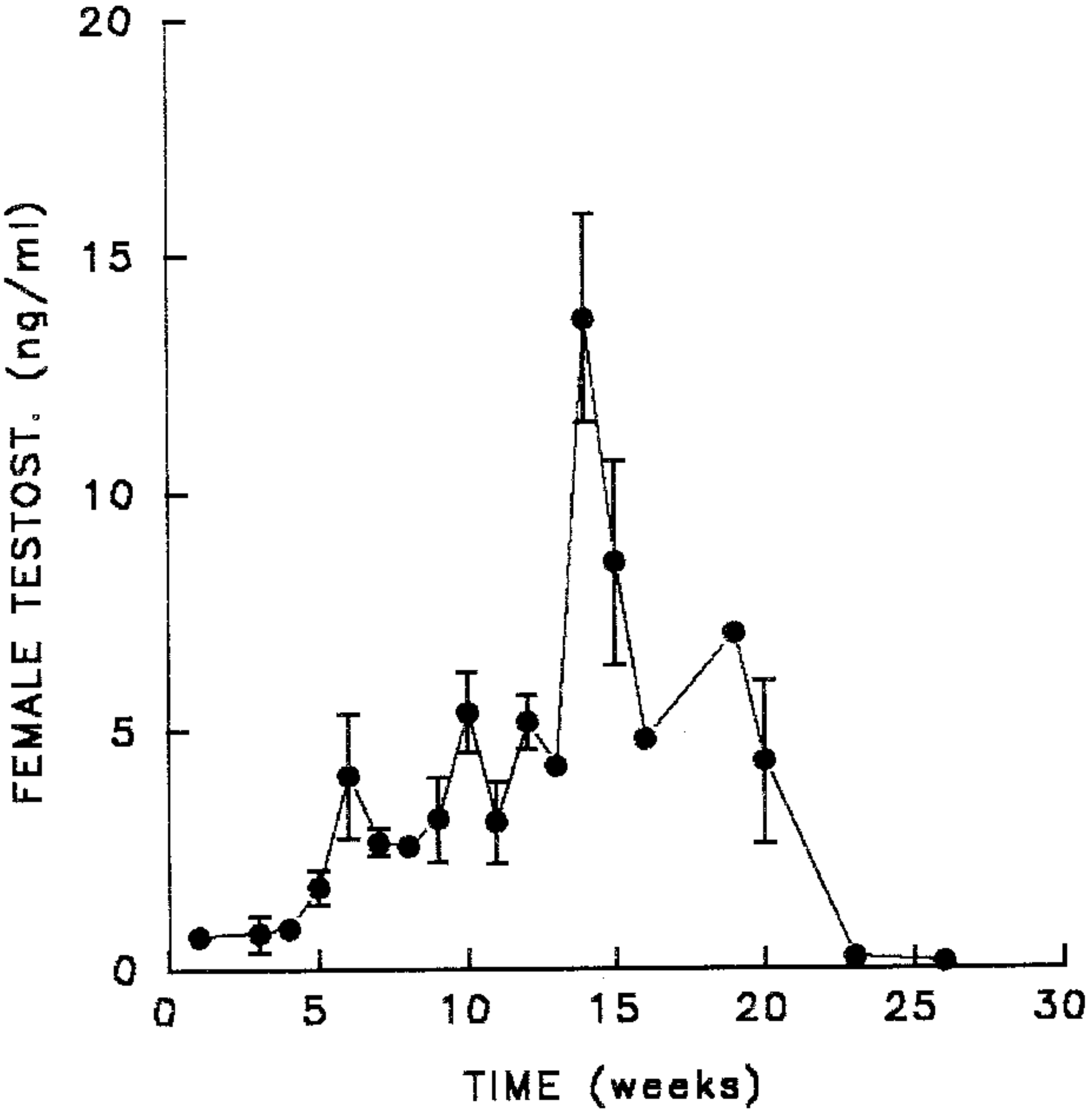

Figure 10. Mean concentrations of serum testosterone (ng/ml) in female Ozark hellbenders over time, starting June 2, 1993. Vertical lines indicate standard errors. Sample sizes are in Table 1.

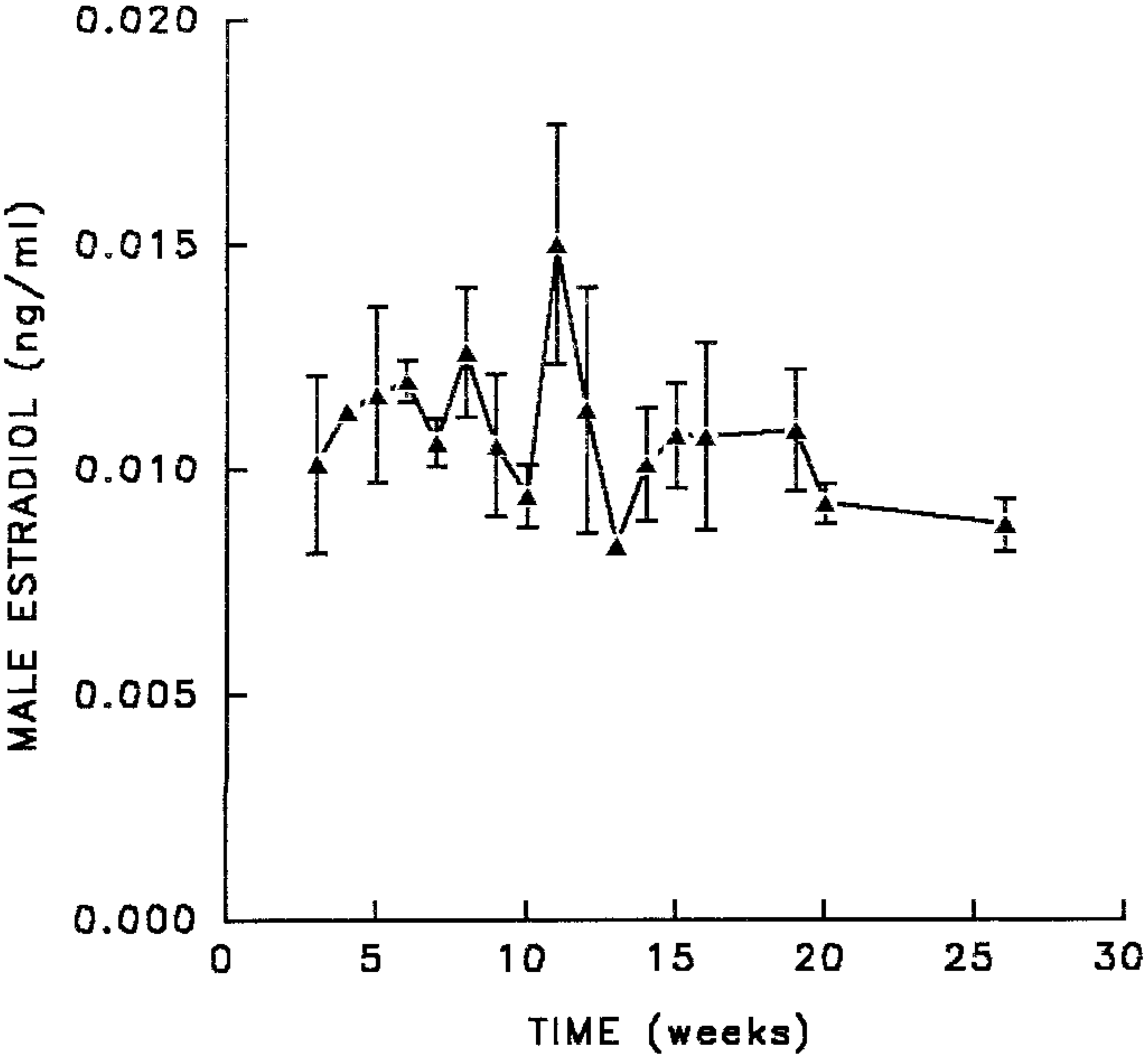

Figure 11. Mean concentrations of serum estradiol (ng/ml) in male Ozark hellbenders over time, starting June 2,1993. Vertical lines indicate standard errors. Sample sizes are in Table 2.

a. bishopi individuals for sexual maturity. Therefore, there may be males within the population smaller than reported that are sexually mature. Further, a study by Wikramanayake and Dryden (1985) described a male *C. a. alleganiensis* (200 mm SVL) with testicular sperm and *C. a. alleganiensis* is larger than *C. a. bishopi* (Nickerson and Mays, 1973b). Another possibility for the differences may be due to population differences. Dundee and Dundee (1965) captured most of their animals from the Eleven Point and Spring Rivers, and only one animal was collected from the North Fork of the White River.

By October, 1993 hellbenders became difficult to capture. There could be many explanations for this occurrence. Males guard nests containing eggs for four to six weeks after breeding and may feed on the eggs within their nests during this time to avoid leaving the nest to find food (Smith, 1907). Also, hellbenders may move to deeper pools of water or into deeper crevices to avoid cooler winter temperatures.

By early July, the cloacal glands of males began enlarging and the abdomens of females began to appear distended. The enlargement of the cloacal glands and distention of abdomens continued until mid-September (Fig. 14). On one occasion in mid-September, two males were observed under the same rock which was within 1 m of a female under another rock. Both males emitted copious amounts of milt upon contact. These observations, along with noticable changes in reproductive hormone concentrations led to the conclusion that breeding took place the last two weeks of Septmeber, 1993 in the North Fork of the White River. Hellbenders are the only salamander species in Missouri to fertilize externally. Thus, oviposition takes place concurrently with courtship and mating (Fig. 14).

Male progesterone concentrations peaked during weeks 4 and 5 (June 23 and 30, 1993) and week 19 (October 9, 1993; Fig. 12). The dates at which progesterone levels peaked are approximately the same dates that progesterone levels peaked in the female (Fig. 9). Weeks 4 and 5 (June 23 and 30, 1993) were significantly higher than most other weeks (Table 8). Male testosterone levels were high from week 6 (July 7, 1993) through week 20 (October 15, 1993; Fig. 13). Weeks 15 and 19 (September 11 and October 9, 1993) were significantly higher than most other weeks, while week 26 (December 4, 1993) was significantly lower than most other weeks (Table 9).

DISCUSSION

Dundee and Dundee (1965) described sexually mature Ozark hellbender females as having SVLs greater than 238 mm, which is in agreement with the current study in which the sizes of sexually mature females ranged from 250 to 372 mm SVL. The weekly female SVL values remained nearly constant with the exception of the last week of August and the first week of September (Weeks 13 and 14; Fig. 6), which were significantly shorter than other weekly values (ANOVA, $p = 0.014$; Table 3). These results are considered an artifact and to represent nothing biological.

The weekly male SVL values varied little between June and December (ANOVA, $p = 0.078$; Fig. 7). Sexually mature males captured in this study, identified by a swollen cloaca, emission of cloacal secretions, and high testosterone levels, had SVLs ranging from 229 to 341 mm. Dundee and Dundee (1965) described the lower limit as 244 mm SVL. This discrepancy may be due to the fact that the previous study only analyzed a total of 14 male and female *C.*

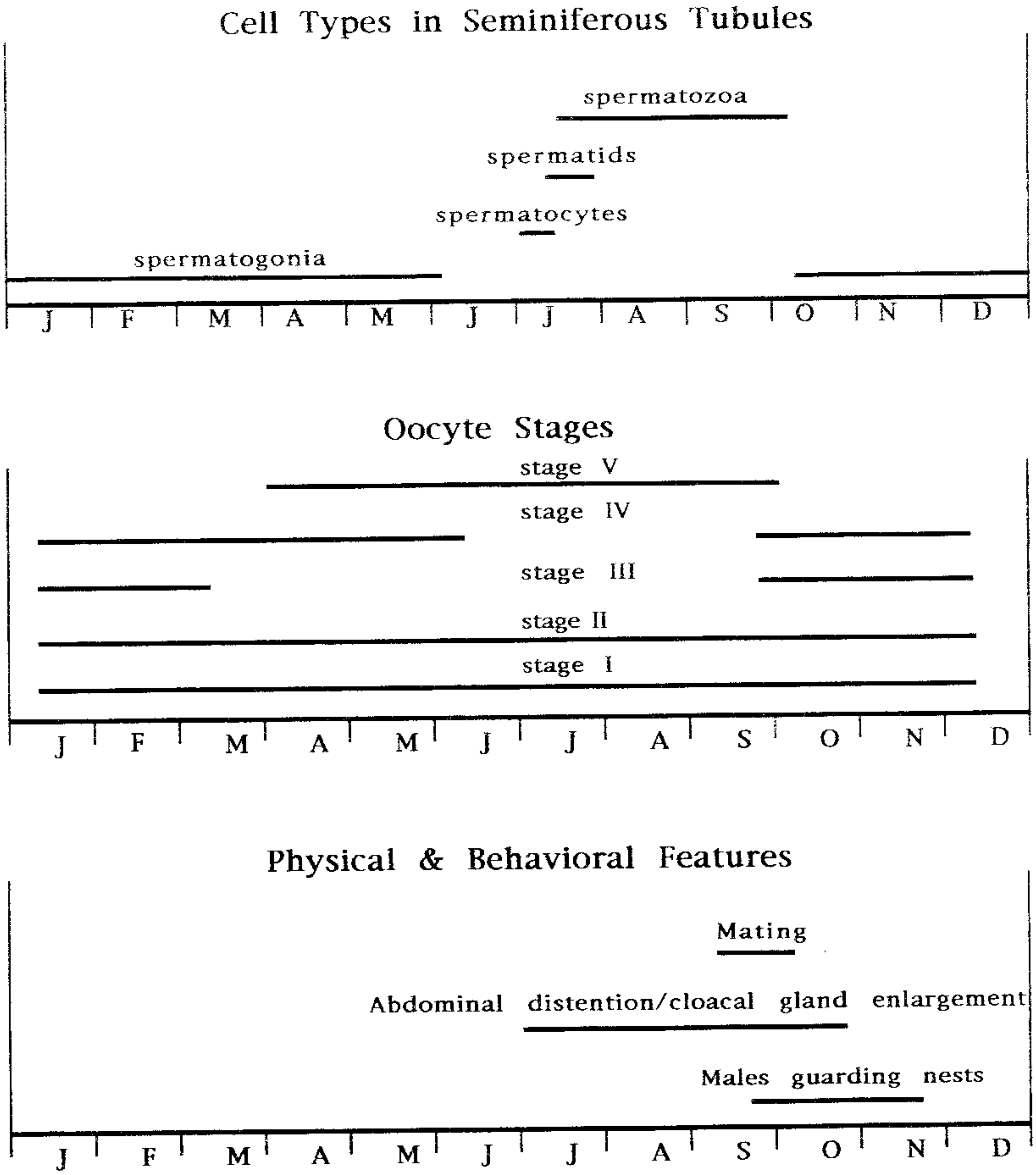

Figure 14. Developmental stages of gametes (Ingersol, 1982) and physical and behavioral features (Smith, 1907) of Ozark hellbenders. Oocyte stages indicate the placement and deposition of yolk in the oocyte.

Levels of female estradiol started increasing in late June and remained high until early October, with the exception of August 26 on which only one female was captured (Fig. 8). This female may be an outlier, having a hormone concentration very different than that of most other female hellbenders at that time. This may be because her size was smaller than the mean size of other sexually mature females (Table 1 and Fig. 6). Alternatively, she may have been sexually mature, but non-breeding (Ingersol et al., 1991). During the time at which estradiol levels were high, progesterone levels were low; when estradiol levels were low, progesterone levels were high. It is possible that these both occur as more or less progesterone is converted to estradiol. Estradiol levels were high at a time when others report oocytes undergoing maturation (Ingersol, 1982; Fig. 14) and breeding (Nickerson and Mays, 1973b). These levels support the belief that estradiol plays an important role in oocyte maturation (Jameson, 1988). A second peak in estradiol, reported for *Triturus carnifex* at the end of the reproductive cycle (Gobbetti et al., 1991; Gobbetti and Zerani, 1992a), was not seen in the Ozark hellbender. The highest levels seen in female Ozark hellbenders (2.3 ng/ml) did not approximate the highest levels seen in *T. carnifex* (12 ng/ml). By early October, levels approximated those seen before the beginning of the maturation of oocytes in late June, and remained low through November and December, 1993. These near-zero levels suggest that estradiol is not playing a physiological role at times other than the period of oocyte maturation and the breeding season.

Female progesterone levels peaked in early July and early October (Fig. 9). At all other times, levels were low, often less than 0.1 ng/ml. The two peaks did not coincide with the hormone's proposed role in ovulation (Lofts, 1974), nor did they coincide with a peak at the onset of the breeding season, (which occurs in September in Ozark hellbenders; Nickerson and Mays, 1973b) as was reported in *Taricha granulosa* (Moore et al., 1979). The highest levels of progesterone in female hellbenders (0.39 ng/ml) were only half the concentrations in *T. granulosa* (0.75 ng/ml). Further, the highest concentrations in *T. granulosa* were reported at the end of the reproductive cycle at which time progesterone levels in female hellbenders were low (0.006 ng/ml) . The progesterone peaks observed were not expected. It is interesting that the progesterone peaks were found just before the beginning of the final maturation of oocytes, stage V (Fig. 14; Ingersol, 1982) and just after the end of the breeding season. Also, these peaks correspond with the rise and fall of testosterone and estradiol levels. Since progesterone is biochemically converted to testosterone and then estradiol, it seems reasonable that decreasing levels of progesterone would accompany increasing levels of testosterone and/or estradiol. It may also be that in Ozark hellbenders, progesterone plays a role similar to that of estradiol in *Triturus carnifex* (Gobbetti et al., 1991; Gobbetti and Zerani, 1992a). Progesterone may trigger the initiation of oocyte maturation and termination of breeding processes or may trigger the development of the next year's eggs. More research in this area is needed to confirm this suggestion and to propose a possible mechanism of action.

Female testosterone levels peaked in early to mid-September (Fig. 10). These weeks correlate with the weeks during which breeding presumably took place. While the role of androgens in female amphibians is unclear, these peaks suggest that testosterone might play a role in mating behavior, egg-laying behavior or oviposition, although egg-laying behavior has previously been suggested to be under the control of estradiol (Moore et al., 1992). A study by

Moore, F.L. (1987). Reproductive endocrinology of amphibians. In "Fundamentals of Comparative Vertebrate Endocrinology" (Chester-Jones, I., Ingleton, P.M., and Phillips. J.G., eds.), pp. 207-222. Plenum Press, New York.

Moore, F.L., McCormack, C., and Swanson, L. (1979). Induced ovulation: Effects of sexual behavior and insemination on ovulation and progesterone levels in *Taricha granulosa*. *Gen. Comp. Endocrinol.* **39**, 262-269.

Moore, F.L., Wood, R.E., and Boyd, S.K. (1992). Sex steroids and vasotocin interact in a female amphibian (*Taricha granulosa*) to elicit female-like egg-laying behavior or male-like courtship. *Horm. Behav.* **26**, 156-166.

Nickerson, M.A., and Mays, C.E. (1973a). A study of the Ozark hellbender *Cryptobranchus alleganiensis bishopi*. *Ecology* **54**, 1164-1165.

Nickerson, M.A., and Mays, C.E. (1973b). The hellbenders: North American giant salamanders. *Milwaukee Public Mus. Pub. Bio. Geol.* **1**, 1-106.

Peterson, C.L., Ingersol, C.A., and Wilkinson, R.F. (1989). Winter breeding of *Cryptobranchus alleganiensis bishopi* in Arkansas. *Copeia* **1989**, 1031-1035.

Smith, B.G. (1907). The life history and habits of *Cryptobranchus alleganiensis*. *Biol. Bull.* **13**, 5-39.

Specker, J.L., and Moore, F.L. (1980). Annual cycle of plasma androgens and testicular composition in the rough-skinned newt, *Taricha granulosa*. *Gen. Comp. Endocrinol.* **42**, 297-303.

Topping, M.S., and Ingersol, C.A. (1981). Fecundity in the hellbender, *Cryptobranchus alleganiensis*. *Copeia* **1981**, 873-876.

Gobbetti et al. (1991) on female *Triturus carnifex* correlated high androgen levels during the reproductive period with temperature and with ovarian and oviductal weights and they suggested that androgens may play a role in eliciting sexual behavior. Injection of testosterone was shown to initiate male mating behaviors in female *Taricha granulosa* but not female mating behaviors (Moore et al., 1992). Although the present and previous studies show the same testosterone peak, more research is needed in this area to determine testosterone's physiological role in female salamanders.

Estradiol levels in male Ozark hellbenders were one to two orders of magnitude less than females, and remained unchanged from June through December, 1993, with the exception of June 2 in which 1 male with a very high level of estradiol skewed the weekly mean (Fig. 11). Estradiol has no known role in male amphibians with the possible exception that the hormone plays a role in terminating breeding processes in *T. carnifex* (Gobbetti et al., 1991; Gobbetti and Zerani, 1992a). No peak was seen at the end of the breeding season to suggest such a role in Ozark hellbenders. If estradiol does play a role in male salamanders, the critical factor may be the increase or decrease in the number of receptors for the hormone, not the increase or decrease in estradiol levels.

Male progesterone levels peaked during late June and again in mid-October (Fig. 12). These dates are approximately the same dates at which progesterone peaked in females (Fig. 9) but the values are generally higher than seen in the females. Levels of progesterone were high when levels of testosterone were low, and were low when levels of testosterone were high. As in females, progesterone levels may decrease as the hormone is being enzymatically converted to testosterone. It has been suggested that progesterone may act as a mating pheromone or stimulate the release of phermones in *Triturus carnifex* (Gobbetti and Zerani, 1992b), but if this were the case in male Ozark hellbenders, a peak during September would be expected. This was not seen. As in the female, progesterone may play a role in triggering the initiation of gamete maturation and the termination of the breeding processes, since peaks are seen just before the beginning of the final maturation of spermatogonia (Fig. 14) and just after the end of the breeding season.

Male testosterone levels were high from early July through late October (Fig. 13). Although values were higher (approximately 3-fold), this pattern is similar to that seen in females. Elevated levels coincide with the period of development of spermatozoa and the breeding season (Fig. 14). At times when spermatozoa were not developing and breeding was not taking place, testosterone levels were near zero. These observations support the idea that testosterone plays a role in the development of secondary sexual characteristics, development of the reproductive tract and spermatozoa, and mating behavior (Moore, 1987). Andreoletti et al. (1983) showed that in male *T. carnifex* sex behavior is depressed by castration and partially reinstated by replacement therapy with testosterone.

Much more research is needed in the area of reproductive endocrinology of not only hellbenders, but urodeles in general. Results of the present study leave many questions unanswered and raise several new questions, especially concerning the role of progesterone in Ozark hellbenders. Laboratory studies involving manipulations may be the easiest way to answer some of these questions.

Trauth, S.E., Wilhide, J.D., and Daniel, P. (1993). The Ozark hellbender, *Cryptobranchus bishopi*, in Arkansas: Distributional survey for 1992. *Bull. Chicago Herp. Soc.* **28**, 81-85.

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