AN ABSTRACT OF THE THESIS OF

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Title: Effects of estrogen on selected reproductive parameters of the male plains leopard frog, *Rana blairi*

Abstract approved: Katherine N. Smalley

It has been suggested that the reproductive success of amphibians might be depressed by increasing amounts of estrogenic chemicals released into the environment. In order to explore this possibility, thirty male plains leopard frogs were injected with 0.1 μg 17β-estradiol/g body weight, 0.5 μg 17β-estradiol/g body weight, or a control solution of corn oil twice weekly for a period of 6 weeks. These treatments simulated the effects of long-term, low-level exposure to estrogenic chemicals on the reproductive physiology and endocrinology of the frogs. The yolk protein vitellogenin, used by others as an indicator of estrogen influence, comprised nearly half of the plasma proteins in the estrogen-treated frogs and significantly increased the total plasma protein concentration in the 0.1 μg E2 group ($P<0.05$). Vitellogenin was completely absent in the controls. Estrogen treatment dramatically depressed the plasma androgen levels, decreasing androgen levels by 53% in the 0.1 μg E2 group ($P<0.001$) and 73% in the 0.5 μg E2 group ($P<0.001$). The depressed androgen levels were probably

related to a significant decrease in testis weights ($P<0.05$) at the highest estrogen dose. Sperm motility and spermatogenesis were not different among groups. A significant decrease in liver weights and a 30% mortality rate of frogs in the 0.5 μg E2 group suggested that continuous, low-level exposure to estrogen might be harmful to the frogs. This study showed that vitellogenin synthesis stimulated by continuous, low levels of estrogen could be linked to depressed androgen levels and other possibly detrimental effects on the reproductive system of the plains leopard frog.

EFFECTS OF ESTROGEN ON SELECTED REPRODUCTIVE PARAMETERS OF THE MALE PLAINS LEOPARD FROG, *RANA BLAIRI*

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I thank my parents, Wayne and Sandra Howdeshell, for teaching me the value of education and implanting in me the desire for knowledge. I am indebted to my family and friends for their encouragement and support through my Masters training.

PREFACE

This thesis was prepared following the publication style of *General and Comparative Endocrinology*.

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INTRODUCTION

Amphibian populations are experiencing worldwide declines (Baringa, 1990; Blaustein and Wake, 1990; Wake, 1991). One frog that has been severely affected in the United States is the northern leopard frog, *Rana pipiens*. Hine *et al*. (1981) reported the northern leopard frog absent from 95% of its suitable breeding habitat in Wisconsin. It is estimated the northern leopard frog population has experienced a 50% decline in the past decade (Pechmann and Wilbur, 1994).

Several factors may contribute to the amphibian decline. The introduction of exotic fishes into native frog habitat and the presence of non-indigenous bullfrogs, *Rana catesbeiana*, could be impacting the number of tadpoles that survive to become mature frogs (Hayes and Jennings, 1986). Drought conditions might have had a hand in the disappearance of the golden toad, *Bufo periglenes*, and the harlequin frog, *Atelopus varius*, in Costa Rica (Pounds and Crump, 1994). Ultraviolet radiation is known to cause severe damage to the embryonic development of the Cascades frog, *Rana cascadae*, in Oregon (Blaustein *et al*., 1994). Habitat alteration by humans is also thought to play an important role in the decline of amphibians (Hayes and Jennings, 1986).

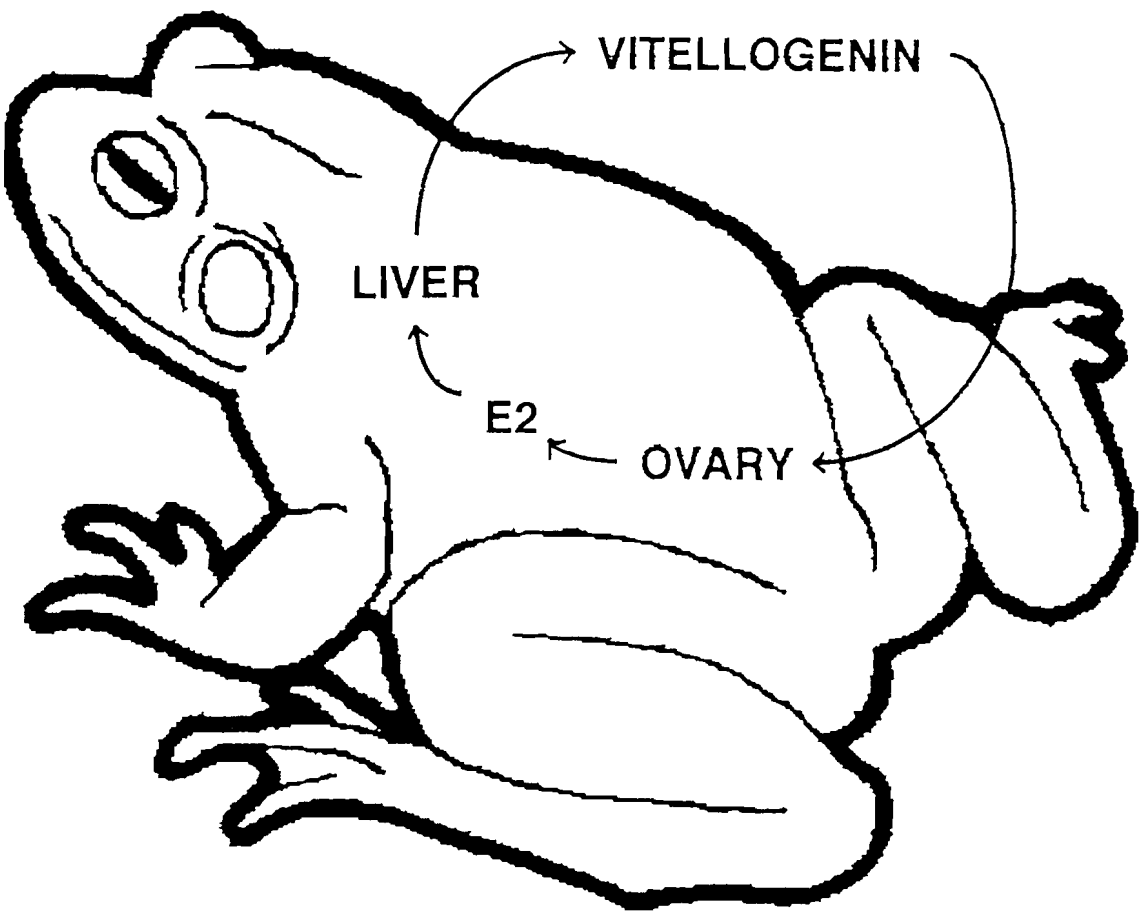
Recent research has explored the possibility that endocrine-disruptive chemicals, such as pesticides and

industrial detergents, may be compromising the reproductive success of wildlife (Colborn *et al.*, 1993; Guillette *et al.*, 1994). These chemicals can bind to estrogen receptors and trigger hormone-like responses. The problem that arises is that the body cannot effectively regulate the stimulus provided by the synthetic hormone as it would with natural hormones.

The production of vitellogenin in oviparous vertebrates has been used as a bioassay to detect the presence of estrogen-like substances in the environment (Palmer and Palmer, 1995; Sumpter and Jobling, 1995). Vitellogenin is a yolk-protein produced by the liver upon stimulation by estrogen. In females, vitellogenin travels from the liver *via* the blood to the ovaries where it is deposited into the developing eggs (Fig. 1). Male oviparous vertebrates can also produce vitellogenin. Normally, male levels of estrogen are too low to promote the synthesis of the yolk-protein. If males are exposed to exogenous estrogen *via* injection or environmental contamination, they produce detectable levels of vitellogenin in their blood plasma (Palmer and Palmer, 1995; Purdom *et al.*, 1994).

The present study examined the effects of long-term exposure of the male plains leopard frog, *Rana blairi*, to low doses of 17β-estradiol. The low levels of estrogen were intended to simulate the low concentrations of estrogenic

Fig. 1. Hormonal control of vitellogenin production. Estrogen (E2) secreted by the ovaries stimulates hepatocytes in the liver to synthesize vitellogenin.


VITELLOGENIN
LIVER
E2
OVARY

chemicals that are increasingly present in the frogs natural habitat. The plains leopard frog, *Rana blairi*, was selected as the subject of the study because of its relative abundance in Kansas (Collins, 1993), and its close familial relationship to the declining northern leopard frog, *Rana pipiens* (Pace, 1974). The objective of the study was to relate vitellogenin synthesis in response to continuous, low levels of exogenous estrogen to possible adverse effects on the reproductive physiology and endocrinology of the frogs. These effects were assessed by measuring: (1) blood plasma vitellogenin and androgen levels, (2) weights of the liver, fat bodies, and testes; (3) sperm motility, and (4) abundance of intermediate sperm stages within the testes.

MATERIALS AND METHODS

Animals, treatments, and necropsy procedures. Adult male plains leopard frogs were captured by hand and dipnet 29 April 1995 through 14 May 1995 in southeastern Shawnee County, Kansas. The frogs were maintained at room temperature (23 ± 2° C) and housed in stock tanks providing both wet and dry habitat. Frogs were fed crickets *ad libitum* twice a week, and tanks were cleaned once a week. The initial body weights (31.7 ± 5.7 g), snout-vent lengths (64.2 ± 4.0 mm), and spot patterns of the frogs were catalogued for identification purposes. The frogs were placed in one of three treatment groups: control (corn oil), 0.1 μg 17β-estradiol/g body weight (0.1 μg E2), or 0.5 μg 17β-estradiol/g body weight (0.5 μg E2). Animals were distributed between groups by size so that the average weight of the frogs in each group was approximately the same. Injections were administered into the dorsal lymph sac twice weekly for six weeks.

Before necropsy, the frogs were anaesthetized with 0.02 ml/g body weight of 1% MS 222 (ethyl-aminobenzoate methanesulfonate). Blood was collected *via* cardiac puncture using heparin-coated syringes and placed into small, heparin-coated glass tubes. Blood was mixed with two protease inhibitor solutions: 10 μl/ml blood of a solution containing 5 mg soybean trypsin inhibitor and 1 mg leupeptin, and 5 μl/ml blood of a 2 mg/ml solution of aprotinin. Plasma was separated by centrifugation for

5 minutes in a clinical centrifuge, then centrifuged twice at 16,000 g for 10 minutes at 4°C. The resulting plasma was stored at -20°C until used for vitellogenin and hormone analyses. The frogs were sacrificed following cardiac puncture; the liver, fat bodies, and testes were removed and their weights were recorded to the nearest 0.1 mg.

Vitellogenin assay. Vitellogenin was isolated from female *Rana pipiens* blood plasma using $MgCl_2$ precipitation methods modified from Herberner *et al.* (1983). Further purification techniques and attempts to create an antibody against this vitellogenin were unsucessful. See Appendix for details on the purification procedures and antibody preparation methods used.

Plasma proteins were electrophoresed on stacking gels of 4% polyacrylamide, and running gels of 5% and 7.5% polyacrylamide (Laemmli, 1970). Blood plasma was first diluted 1:20 with distilled water, and total plasma protein concentrations were determined by the BioRad protein assay (Bradford, 1976). Each plasma sample was diluted by 4:1 (v/v) in sample buffer (20.2% w/v 2-mercaptoethanol, 7.8% SDS, 0.08% bromophenol blue, and 40.7% glycerol), and heated in boiling water for 3 minutes. Equal amounts of each protein sample (10 μg/lane) were applied to the gel and run at 30 mA until the dye front reached the bottom 1 cm of the gel. Gels were then stained with Coomassie blue. Gel images were analyzed using a Hitachi video camera

(horizontal resolution capability of ≥430 lines) connected to a computer containing NIH Image 1.59 public domain software. Gel images were captured at ≥510 nm using Kodak Wratten gelatin filter #15 and neutral density filters to achieve optimal contrast. Each gel was scanned using the gel plotting macro, and an empty lane of the gel was used as the background lane. The integrated area under each protein peak was then measured. The area under the protein peaks representing vitellogenin (175-kDa), 165-kDa protein, and albumin (64-kDa) was divided by the total area under all peaks in each gel lane to determine the relative percentage of each of these three proteins in each plasma sample. The calculated value of the three proteins (mg/ml) was derived by multiplying the total plasma protein concentration by the relative percentage value of the protein sample.

Sperm motility and spermatogenesis study. Sperm motility was assessed by macerating one testis in 5 ml of dechlorinated water and allowing the sperm to capacitate for 15 minutes. The sperm kinetics were visually rated on a three point scale: slightly decreased, normal, or slightly increased. The remaining testis was fixed in 10% buffered formalin, sectioned at 7 μm, and stained with hematoxylin and eosin. Five animals from each treatment group were randomly selected for morphometric analysis. The number of intermediate sperm stages found within the testis sections was captured using a Hitachi video camera connected to a

computer with Adobe Photoshop (Adobe Systems Inc., Mountainview, CA) and NIH Image 1.59 public domain software. The seminiferous tubules selected for quantification passed certain criteria to ensure evaluation of cross-sectioned tubules. The criteria were: (1) an intact basement membrane, (2) a round or slightly oval appearance, the difference between width and length not more than 20% of length, and (3) the presence of a lumen near the center of the tubule. The number of spermatocytes and spermatids were counted, then divided by the area of the tubule to determine the number of intermediate sperm stages present per 1000 μm^2 of seminiferous tubule. One 7 μm section per animal was evaluated. Only four to six seminiferous tubules per section met the criteria for image analysis.

Hormone assays. Plasma sample aliquots of 150 μl were double-extracted with 17 ml of methylene chloride before RIA analysis. Duplicate samples were extracted when a sufficient amount of plasma was available. The extraction efficiency of the assay was 95 ± 5.8% (mean ± SE); RIA readings were not corrected for this value. Androgen plasma levels were measured by testosterone radioimmunoassay (RIA) using an antibody purchased from Wien Laboratories. According to the manufacturer, the cross reactivity of the testosterone antibody was 50% with dihydrotestosterone; thus, the results are expressed as androgen levels instead of testosterone levels. Standard curves were prepared in

buffer with known amounts of radioinert testosterone (0, 25, 50, 100, 250, and 500 pg). The minimum concentration per tube that was distinquishable from 0 was 25 pg. The intra-assay coefficient of variation was 5.1%.

Statistical analysis. The data were analyzed using SigmaStat 1.01 statistical program (Jandel Corporation, San Rafael, CA). The liver, fat bodies, and testes weights were evaluated by one-way analysis of variance (ANOVA) and Student-Newman-Keuls range tests. Plasma protein and androgen data were analyzed by Kruskal-Wallis non-parametric ANOVA and Dunn's method range test. The slide study data were analyzed by nested ANOVA using BIOSTAT I statistical software (Sigma Soft Inc., Placentia, CA).

RESULTS

The mean total plasma protein concentration was significantly higher in the 0.1 μg E2 frogs than in the controls (Table 1). Although the 0.5 μg E2 group showed an increase in mean total plasma proteins, the value was not statistically different from either the control or the 0.1 μg E2 values.

Figure 2 is a representative sample of a stained gel. A protein of approximately 175-kDa was present in the estrogen-treated males, but was absent in the plasma of the controls. The protein was presumed to be vitellogenin because of this distribution as well as its similarity in molecular weight to purified *Rana pipiens* vitellogenin (180-kDa). The estrogen treatments decreased the relative percentages of a 165-kDa protein and a 64-kDa protein in plasma (Table 1). The 64-kDa protein was identified as albumin because it was abundant in the control plasma, was present in all of the plasma samples, and its molecular weight was similar to red-eared turtle (*Trachemys scripta*) and human albumin (66-kDa).

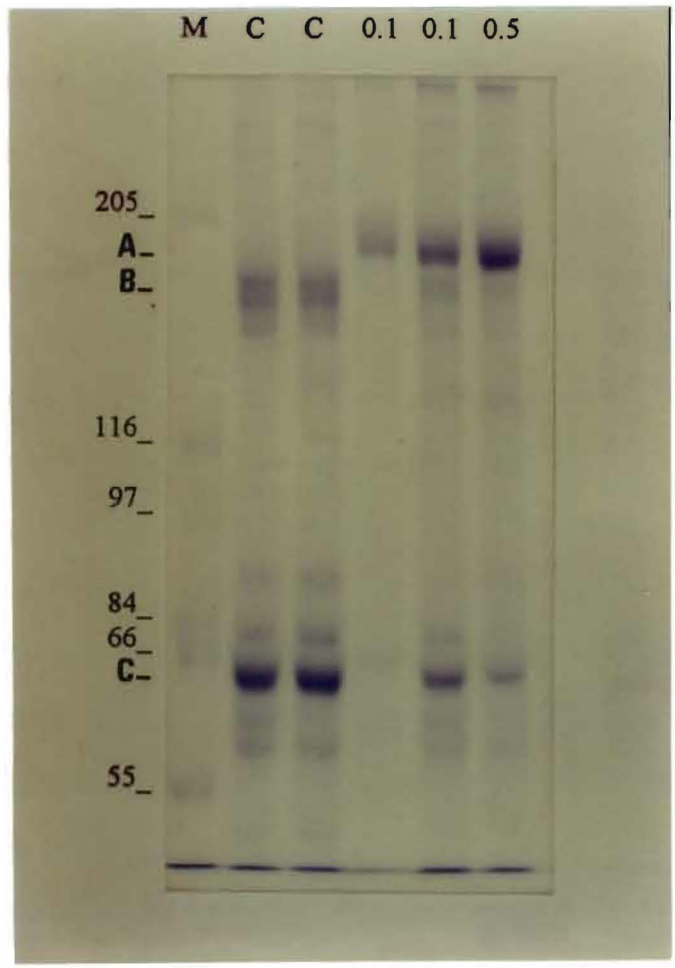
Vitellogenin comprised 45 to 46% of the total plasma proteins on the gels of the estrogen-treated frogs and was totally absent in the controls (Table 1). Although the relative percentage of albumin in plasma significantly decreased in the estrogen-treated animals, the calculated albumin level (mg/ml) did not show any significant difference between treatment groups. Similarly, the

Table 1. Mean (± SE) relative percentages and calculated concentrations of selected plasma proteins of male frogs in control (9), 0.1 µg E2 (9), and 0.5 µg E2 (7) treatment groups. Number of animals is shown in parentheses.

	Treatment group		
	Control	0.1 µg E2	0.5 µg E2
Total plasma protein (mg/ml)	15.3 ± 1.2[a]	27.6 ± 6.9[b]	21.8 ± 9.4[a,b]
Vitellogenin as % of total protein	0.0 ± 0.0[a]	46.6 ± 12.7[b]	45.1 ± 13.7[b]
Calculated vitellogenin in plasma (mg/ml)	0.0 ± 0.0[a]	13.5 ± 6.4[b]	9.2 ± 4.5[b]
Albumin as % of total protein	34.8 ± 1.8[a]	14.8 ± 6.5[b]	15.9 ± 8.1[b]
Calculated albumin in plasma (mg/ml)	5.3 ± 0.6[a]	3.8 ± 1.6[a]	3.5 ± 2.3[a]
165-kDa protein as % of total protein	30.0 ± 1.0[a]	11.8 ± 2.7[b]	13.2 ± 2.8[b]
Calculated 165-kDa protein in plasma (mg/ml)	4.6 ± 0.4[a]	3.1 ± 0.4[b]	3.1 ± 1.8[a,b]

[a,b] Treatments with different subscripts are significantly different at $P<0.05$.

Fig. 2. Representative SDS-PAGE gel of plasma samples from control (C), 0.1 μg E2 (0.1), and 0.5 μg E2 (0.5) male frogs. Molecular weight markers (M) are, from the top, 205-, 116-, 97-, 84-, 66-, and 55-kDa. A = 175-kDA protein, identified as vitellogenin. B = 165-kDa protein, identity unknown. C = 64-kDa protein, presumed to be albumin.


M
C
C
0.1
0.1
0.5
205
A
B
116
97
84
66
C
55

relative percentage of 165-kDa protein in plasma fell precipitously under estrogen treatment. However, the calculated amount of 165-kDa protein (mg/ml) was significantly decreased only in the 0.1 μg E2 treatment group (Fig. 3).

Estrogen treatment had a dramatic effect on plasma androgen levels. Androgens were depressed by 52.5 ± 0.1% in the 0.1 μg E2 group and by 73.1 ± 0.1% in the 0.5 μg E2 group (Fig. 4).

The testis weights were significantly decreased in the 0.5 μg E2 group (Fig. 5). Overall, the testes of the estrogen-treated frogs were smaller and more dense than the controls. A few of the testes of the estrogen-treated frogs exhibited abnormal, kidney-bean shapes not observed in the oval-shaped testes of the controls.

The seminiferous tubules of the testes possessed a range of developmental stages including primary and secondary spermatocytes, spermatids, and mature sperm. The observational study of the slides suggested that the estrogen-treated animals possessed fewer intermediate sperm stages (spermatocytes and spermatids) than did the controls. However, the calculation of cells per 1000 μm^2 of seminiferous tubule did not show any significant difference between treatment groups (Table 2). An analysis of intermediate sperm cell data indicated 63% of the total variance was due to differences among individuals in a

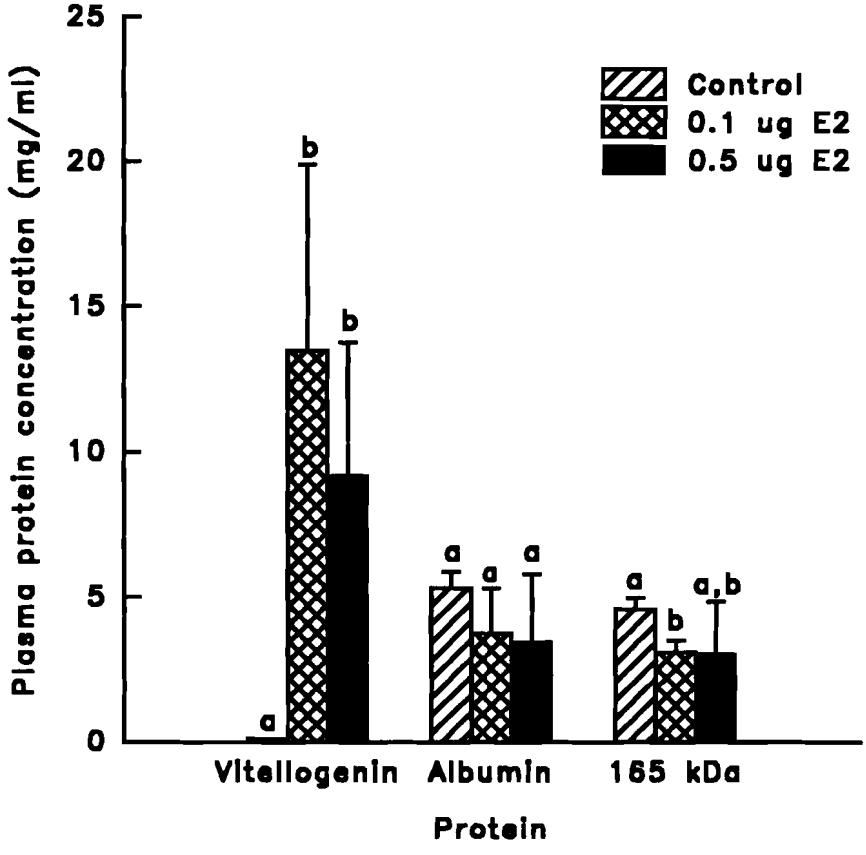

Plasma protein concentration (mg/ml)
25
20
15
10
5
0
Control
0.1 ug E2
0.5 ug E2
a
b
b
a
a
a
a
b
a,b
Vitellogenin
Albumin
165 kDa
Protein

Fig. 4. Blood plasma androgen levels of male frogs in control (10), 0.1 μg E2 (10), and 0.5 μg E2 (7) treatment groups. Number of animals is shown in parentheses. Error bars represent ± SE. Treatment groups with different letter designations are statistically different at $P<0.001$.

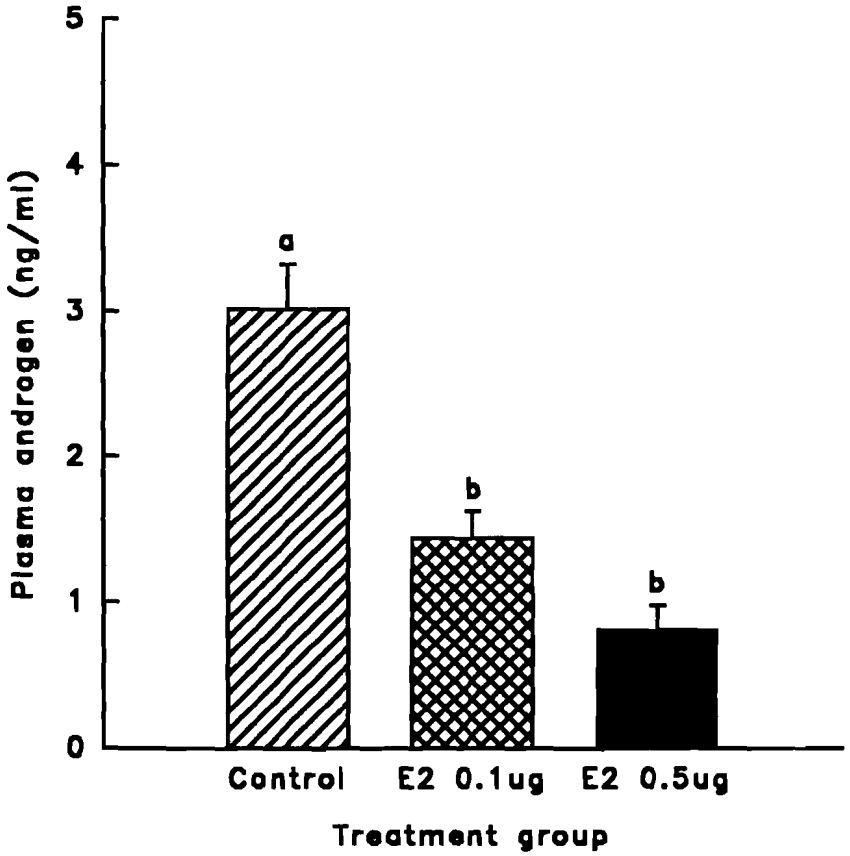

5
4
3
2
1
0
Plasma androgen (ng/ml)
a
b
b
Control
E2 0.1ug
E2 0.5ug
Treatment group

Fig. 5. Testis weights as percentage of total body weight of male frogs in control (10), 0.1 μg E2 (10), and 0.5 μg E2 (7) treatment groups. Number of animals is shown in parentheses. Error bars represent ± SE. Treatment groups with different letter designations are statistically different at $P<0.05$.

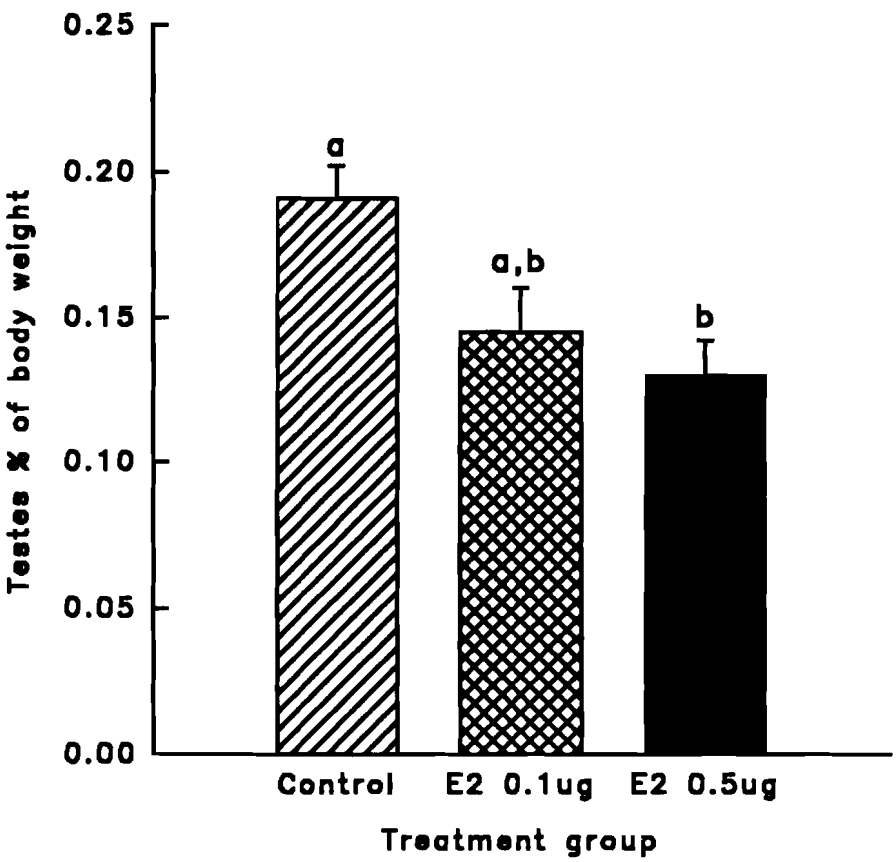

0.25
0.20
0.15
0.10
0.05
0.00
Testes % of body weight
a
a,b
b
Control
E2 0.1ug
E2 0.5ug
Treatment group

Table 2. Mean (± SE) number of intermediate sperm cells per 1000 μm^2 seminiferous tubule of male frogs in control (5), 0.1 μg E2 (5), and 0.5 μg E2 (5) treatment groups. Number of animals is shown in parentheses. No statistical difference was found between treatment groups.

TREATMENT GROUP	NUMBER OF CELLS/1000 μm^2 TUBULE
CONTROL	2.64 ± 0.25
0.1 μg E2	2.11 ± 0.29
0.5 μg E2	2.32 ± 0.23

treatment group, and the remaining 37% variance was due to differences among seminiferous tubules in an individual (Table 3). Sperm motility did not vary significantly between groups (Table 4).

The liver weights were significantly decreased in the 0.5 μg E2 group (Fig. 6). Fat body weights were not significantly affected by estrogen treatment (Fig. 7).

Table 3. One-way nested ANOVA of the number of intermediate sperm cells per 1000 μm^2 seminiferous tubule of male frogs in control (5), 0.1 µg E2 (5), and 0.5 µg E2 (5) treatment groups. Number of animals is shown in parentheses.

	DF	MS	F-value	Significance Probability	Variance[a]
Differences among treatment groups	2	1.54	0.30	0.75	
Differences among individuals in a treatment group	12	5.04	8.36	<0.001	63.05
Differences among seminiferous tubules in an individual	50	0.60			36.95

[a] Percent total variance attributed to each level of the one-way nested ANOVA.

Table 4. Sperm motility of male frogs in control (10), 0.1 μg E2 (10), and 0.5 μg E2 (7) treatment groups. Number of animals is shown in parentheses. Percentages represent number of animals in each catagory per total animals in each treatment group.

TREATMENT GROUP	KINETICS		
	SLIGHTLY DECREASED	NORMAL	SLIGHTLY INCREASED
CONTROL	10%	80%	10%
0.1 μg E2	20%	80%	--
0.5 μg E2	11%	89%	--

Fig. 6. Liver weights as percentage of total body weight of male frogs in control (10), 0.1 μg E2 (10), and 0.5 μg E2 (7) treatment groups. Number of animals is shown in parentheses. Error bars represent ± SE. Treatment groups with different letter designations are statistically different at $P<0.05$.

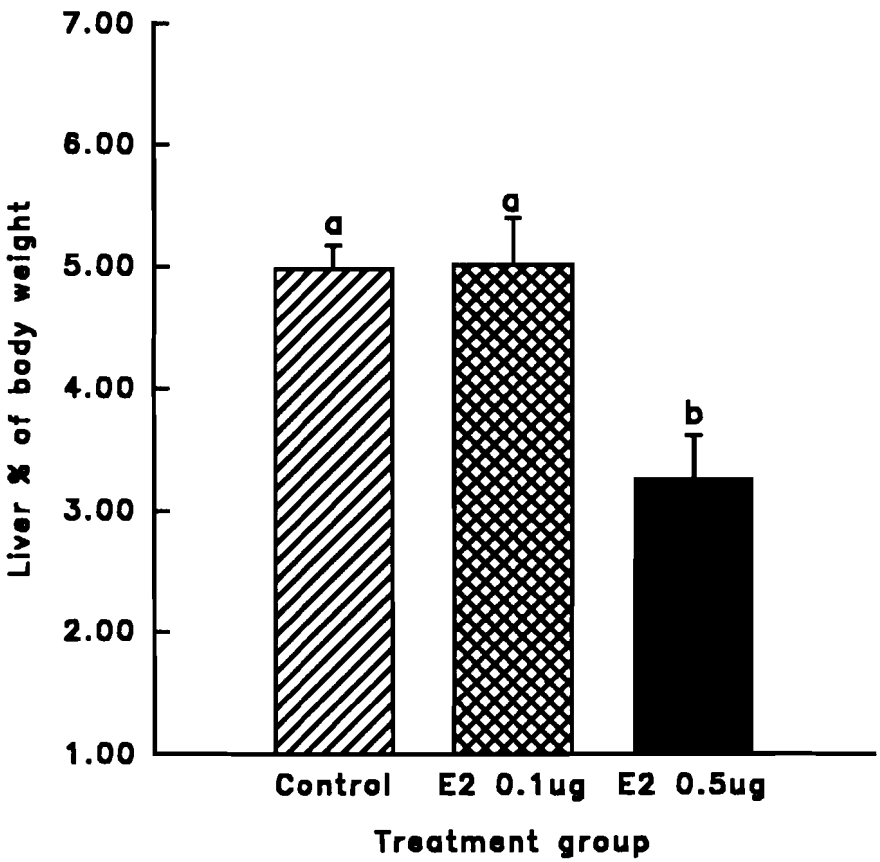
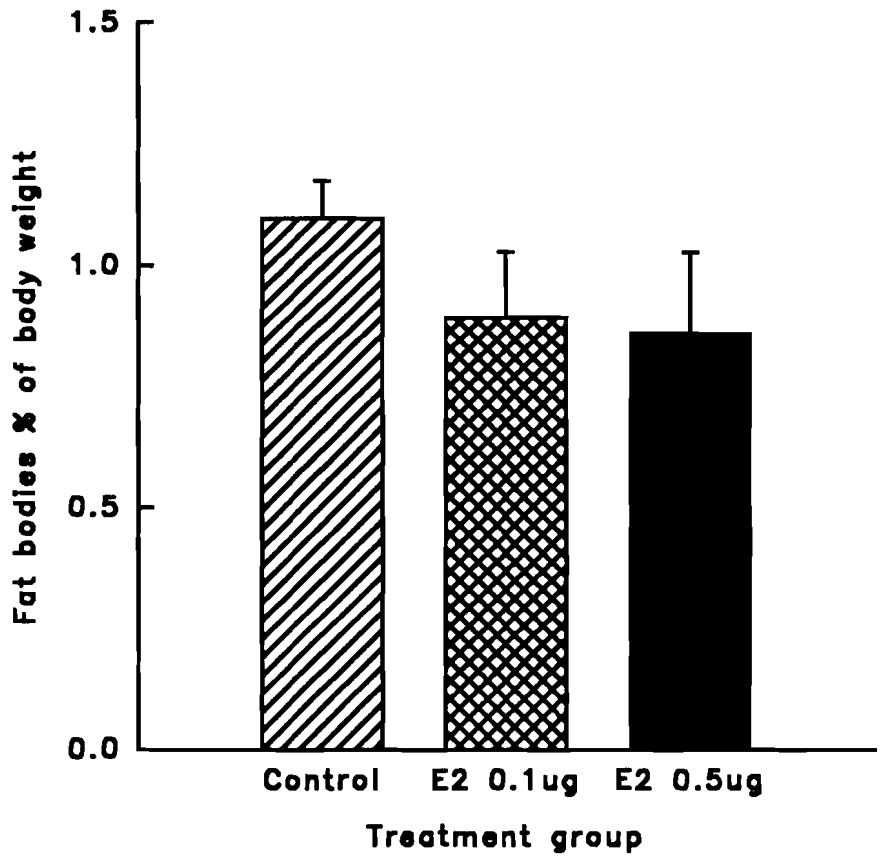

7.00
6.00
5.00
4.00
3.00
2.00
1.00
Liver % of body weight
a
a
b
Control
E2 0.1ug
E2 0.5ug
Treatment group

Fig. 7. Fat body weights as percentage of total body weight of male frogs in control (9), 0.1 μg E2 (10), and 0.5 μg E2 (7) treatment groups. Number of animals is shown in parentheses. Error bars represent ± SE. No statistical difference was found between treatment groups.


1.5
1.0
0.5
0.0
Fat bodies % of body weight
Control
E2 0.1ug
E2 0.5ug
Treatment group

DISCUSSION

Vitellogenin synthesis has been used in determining the estrogenic influence of certain chemicals in the environment (Palmer and Palmer, 1995; Sumpter and Jobling, 1995). In England, the production of vitellogenin by male fish downstream of several sewage treatment plants confirmed the presence of estrogen-like chemicals in the effluent (Purdom *et al.*, 1994). The week-long exposure of the African clawed frog, *Xenopus laevis*, and the red-eared turtle, *Trachemys scripta*, to low levels of estrogen or known estrogen-like chemicals (DES and DDT) stimulated substantial vitellogenin production (Palmer and Palmer, 1995). Vitellogenin was not produced in the control animals (Palmer and Palmer, 1995; Purdom *et al.*, 1994). In the present study, multiple low doses of estrogen elicited considerable vitellogenin production in male *Rana blairi*. These results were striking because the total estrogen administered was 78.6% (0.5 μg E2) to 17.1% (0.1 μg E2) of the total estrogen dose used by Palmer and Palmer (1995).

The similar relative percentages of vitellogenin in the plasma of the two estrogen treatment groups indicates that both doses were higher than the threshold necessary to elicit vitellogenin production. Thus, it is possible that even lower estrogenic stimuli, such as those found in the environment, may be capable of eliciting vitellogenin synthesis. Further investigations are necessary to

determine the minimum estrogen levels necessary to stimulate vitellogenin production.

Whereas estrogen elicits the production of vitellogenin, it has been found to downregulate other plasma proteins (Herbener *et al.*, 1983; Selcer and Palmer, 1995). Calculated plasma albumin levels dropped from 14.8 ± 4.4 to 8.7 ± 3.1 mg/ml following estrogen exposure in male *Rana pipiens* (Herbener *et al.*, 1983). Estrogen treatment also elicited substantial downregulation of a 170-kDa protein in the plasma of ovariectomized *Trachemys scripta* (Selcer and Palmer, 1995). In the present study, the calculated albumin levels were not significantly downregulated by estrogen treatment. However, a protein of 165-kDa molecular weight was significantly depressed in the 0.1 μg E2 group. This 165-kDa protein may be comparable to the downregulated 170-kDa protein reported by Selcer and Palmer (1995).

The liver is the principal site of production of vitellogenin. A number of changes occur within this organ to facilitate increased protein synthesis. Synchronous increases in rough endoplasmic reticulum and liver RNA occur in hepatocytes of male *Rana pipiens* approximately two weeks following treatment with a single large dose of estrogen (Herbener *et al.*, 1983). Spolski *et al.* (1985) reported an increase in DNA synthesis and proliferation of the liver parenchymal cell population in male *Xenopus laevis* two weeks

after a single estrogen dose similar to that of the Herberner *et al.* study (1983). The liver also responds to an estrogen stimulus by increasing the number of estrogen receptors in a dose-dependent manner (Wright *et al.*, 1983).

Reported effects of estrogen treatment on liver weight are conflicting. Some studies observed hypertrophy of the anuran liver under the influence of estrogen (Follett and Redshaw, 1974; Vacca, 1988); other researchers did not observe any change in liver mass (Herbener *et al.*, 1983). The proliferation of *Xenopus laevis* liver parenchymal cells under the influence of estrogen produced cells that were as much as 50% smaller than the average parenchymal cell; the decreased size and close packing of the cells prevented an increase in liver mass (Spolski *et al.*, 1985).

The constancy of liver weights between the control and 0.1 μg E2 treatment groups was similar to that of the estrogen-treated male frogs reported by Herberner *et al.* (1983) and Spolski *et al.* (1985). The significant decrease in liver weight of the 0.5 μg E2 group was unexpected. The plasma of the 0.5 μg E2 group showed a substantial increase in vitellogenin production, yet the total plasma protein concentration was not significantly different from the control value. These results suggest that the higher estrogen dose might have been deleterious to normal liver function. In this regard, it is also worth noting that

there were three deaths in the 0.5 μg E2 group during the 4th and 5th weeks of treatment. Dubowsky and Smalley (1993) also reported a high mortality rate in female *Rana pipiens* treated for 4 weeks with low doses of 17β-estradiol 3-benzoate. These results, therefore, suggest that long-term exposure to low levels of estrogen, such as might occur in the wild, could be more harmful to the frogs than a single dose would suggest. This is a cause for concern as increasing numbers of chemicals are being released into the environment that have been found to elicit estrogenic responses (Soto *et al.*, 1995; Sumpter and Jobling, 1995).

There is increasing evidence that estrogenic chemicals in the environment may be compromising the reproductive success of wildlife (Colborn *et al.*, 1993; Fry, 1995; Guillette *et al.*, 1995). One of the most direct effects of exogenous estrogen exposure is a disruption of the normal hormone balance, which is essential for proper reproductive functioning.

Plasma androgen levels were dramatically suppressed by estrogen treatment in the frogs of this study. There are several factors that can affect androgen levels. Guillette *et al.* (1994) reported that juvenille alligators, *Alligator mississippiensis*, from a heavily polluted lake in Florida, exhibited severely decreased plasma testosterone levels. Under normal conditions, plasma androgen levels are known to

increase during spermatogenesis in the toad, *Bufo japonicus* (Itoh *et al.*, 1990). Also, the stress of capture depresses plasma androgen levels in *Rana catesbeiana* (Licht *et al.*, 1983). The stress of capture might have depressed overall androgen levels in the present study, but could not account for the significant difference in androgen levels between the estrogen-treated and control animals.

The dramatic decrease in androgen levels might have directly accounted for the decreased testis size in the 0.5 μg E2 treatment group. The smaller, denser appearance of the testes in the estrogen-treated frogs was similar to results reported by Burgos in *Bufo arenarum* when treated with a high dose of estrogen for 2 to 3 months (1953 reviewed in Dodds, 1960). Sharpe *et al.* (1995) found that prenatal and lactational exposure of rats to estrogenic chemicals causes a significant decrease in testis weights and reduced sperm production. Exogenous estrogen may suppress the proliferation of Sertoli cells during development in the rat resulting in smaller testes and lower sperm counts in adults (Sharpe *et al.*, 1995). The decreased size of the testes in the 0.5 μg E2 group might have been caused by a decrease in Sertoli cell size or atrophy of interstitial tissue (Burgos, 1953 reviewed by Dodds, 1960).

There was no apparent effect of estrogen on spermatogenesis or sperm motility of *Rana blairi* in this

study. The males were collected after their primary breeding season, and the assumption was made that they had emptied their testes. Because *Rana blairi* are continuous breeders (Collins, 1993), it is possible some of the frogs had not participated in the spring reproductive effort. Thus, these frogs would have been in a more quiescent stage of spermatogenesis during estrogen treatment than those frogs undergoing active spermatogenesis during the treatment period.

The suppression of plasma androgen in the estrogen-treated frogs may be occurring at the level of the brain and pituitary (Pavgi and Licht, 1993). Pavgi and Licht (1993) suggested low levels of circulating plasma estrogen inhibit pituitary FSH and LH secretion in male *Rana pipiens*. The inhibition of gonadotropins by exogenous estrogen could result in depressed plasma androgen levels and further reproductive effects caused by the decreased androgen levels.

Fat bodies are areas of adipose tissue in amphibians that are thought to provide nutrients to the gonads (Rugh, 1951). Fat bodies could also be a source of lipids necessary for the production of the phospholipoprotein vitellogenin (Follett and Redshaw, 1974; Pierantoni *et al.*, 1984). Fat body size in estrogen-treated frogs of the present study did not differ from controls. This could be a result of the constant food supply, which served to restore

any fat body lipids used in vitellogenin synthesis.

This study indicated that an increase in vitellogenin synthesis could be correlated with the suppression of plasma androgen levels and other possibly negative effects on reproductive functioning, such as decreased testes weight. These results reinforce the use of vitellogen as a biomarker for the deleterious consequences of environmental contamination.

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APPENDIX

This appendix is a description of the methods used for vitellogenin purification and antibody production in this study. As antibody production was not successful, this section serves to notify future researchers of the methods that were not effective in creating *Rana pipiens* vitellogenin antibody.

Animals, injections, and vitellogenin isolation. Northern leopard frog females (*Rana pipiens*) were obtained from Kons Scientific Company (Germantown, WI). Animals were maintained at room temperature (23 ± 2°C) and housed in stock tanks providing both wet and dry habitat. Each frog was injected with 1 mg 17β-estradiol in 1 ml of corn oil into the dorsal lymph sac. Animals were anaesthetized with 0.015 ml/g bw of 1% MS 222 (ethyl-aminobenzoate methanesulfonate) on the 8th day following the estrogen injection. Plasma was collected and prepared as previously indicated. Vitellogenin was isolated from the resulting plasma solution by $MgCl_2$ precipitation and dialysis following Herbener *et al.* (1983). The protein solution was stored at -20°C until used in antibody production. Herbener *et al.* (1983) found that this procedure yielded a 95% pure vitellogenin preparation.

Vitellogenin purification and antibody preparation. Further purification of the vitellogenin was attempted by several methods. A sample of the vitellogenin preparation

was run on a Sephacryl S-200 (Pharmacia, Piscataway, NJ) column (1.5 X 100 cm) in 0.5 M Tris-HCl (pH 7.0 containing 0.1 M NaCl) to separate the 180-kDa *Rana pipiens* vitellogenin from lower molecular weight components. However, gel electrophoresis and subsequent silver-staining of the vitellogenin peak revealed several lower molecular weight bands, presumed to be the breakdown products of vitellogenin.

Further purification of the Sephacryl column fractions was attempted using lecithin affinity chromatography on a one-millimeter packed Concanavalin A-linked Sepharose column (Con A, Pharmacia, Picsataway, NJ). The column was initially washed with 25 mM Tris-HCl (pH 7.0) containing 0.154 M NaCl. One ml of a fraction containing vitellogenin was added to the column. The column was then rinsed with 25 mM Tris-HCl (pH 7.0) without NaCl to remove salt from the column before eluting off vitellogenin. Finally, 50 mM α-methyl mannoside was added to the column to elute off the vitellogenin. In theory, vitellogenin should bind to the Con-A by its carbohydrate portion, but this method proved unsuccessful as the protein did not appear when the column was eluted with 50 mM α-methyl mannoside. Two ml fractions were collected from all the rinses and the elution step, yet no protein was detected in any of the Con-A fractions.

Because of the failure to obtain a pure vitellogenin

preparation by the above procedures, the 95% pure vitellogenin preparation was subjected to SDS-PAGE electrophoresis on 7.5% polyacrylamide (Laemmli, 1970) and antibody production was attempted using the vitellogenin from the gel. Each plasma sample was diluted by 4:1 (v/v) in sample buffer and boiled for 3 minutes. Seven-hundred fifty μg of the vitellogenin preparation was applied to 1 lane of a 2-well gel and run at 30 mA until the dye front reached the bottom 1 cm of the gel. A side strip of the gel containing the molecular weight standards and a portion of the vitellogenin preparation was removed and stained with Coomassie blue. The strip was then lined up with the remainder of the gel and the band containing vitellogenin was cut out. The gel band containing vitellogenin was homogenized, mixed with equal parts of complete Freund's adjuvant (Sigma), and injected into a rabbit. Two gel preparations were injected per rabbit. The total amount of 100% vitellogenin injected was approximately 750 μg per rabbit. A booster shot containing approximately 750 μg of 100% vitellogenin was administered 4 weeks later.

Rabbit serum was collected 6 weeks after the initial injection by bleeding the ear vein. The serum tested negative for the vitellogenin antibody by a precipitin ring test. The animals were given a second booster of antigen preparation.

After 2 weeks, rabbit serum was again collected and

antibody detection was performed by Western Blot against the 95% pure vitellogenin preparation. The vitellogenin preparation was electrophoresed as above, then transferred to a nitrocellulose membrane. The transfer was performed in 25 mM Tris, 192 mM glycine (20% methanol) with 0.075% SDS (pH 8.3) using a BioRad Trans-Blot apparatus (Richmond, CA) at 70 V overnight at 4°C. Following transfer, the membranes were blocked with 5% nonfat dry milk for 1 hr at 37°C. This solution was then replaced with 3% nonfat dry milk containing either control rabbit serum, *Rana catesbeiana* vitellogenin antibody (graciously supplied by Dr. Rick Feldoff), or the *Rana pipiens* anti-serum and incubated for 16 hours at 37°C. The membrane was secondarily probed with anti-rabbit IgG linked to horseradish peroxidase and an avidin-biotin complex following BioStain Super ABC Kit instructions (Biømeda, Foster City, CA). Antibody binding was visualized with diaminobenzidene (DAB Kit, Vector Laboratories, Burlingame, CA). The *Rana catesbeiana* antibody exhibited strong binding to vitellogenin, but the control serum also showed some non-specific binding. The *Rana pipiens* anti-serum response was non-specific.

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