AN ABSTRACT OF THE THESIS OF

Joseph L. Vacca for the Master of Science Degree

in Biology presented on December 16, 1988

Title: Ovarian Steroid Secretion and Effects of Testosterone in Female Rana pipiens

Abstract Approved: Katherine N. Smalley

Gravid leopard frogs (Rana pipiens) were injected with testosterone or oil, and half of each group received food while the other half did not. Among the fed frogs, those injected with oil gained twice as much weight as those injected with testosterone. Among the frogs that were not fed, testosterone injected animals lost almost three times as much weight as oil injected controls. Fed frogs had heavier spleens, fat bodies, livers, ovaries, and left gastrocnemius' than unfed frogs. In addition, in fed frogs fat bodies were 1.5 times heavier in oil injected than in androgen injected animals. These data suggest a higher metabolic rate, increased lipid utilization and/or decreased appetite in frogs that received exogenous testosterone. Not fed, androgen injected R. pipiens had much heavier oviducts than any other group. It is hypothesized that testosterone acted as a precursor for estradiol-17B in producing this effect.

Rana pipiens follicles taken early in hibernation and late in hibernation showed much higher testosterone secretion than estradiol-17B secretion in vitro. Frog pituitary homogenate (FPH) greatly enhanced the androgen secretion while not affecting estrogen secretion. It is hypothesized that high testosterone secretion during hibernation plays a specific, yet uncharacterized, role in successful egg production by Rana pipiens.

OVARIAN STEROID SECRETION AND EFFECTS OF TESTOSTERONE IN FEMALE *RANA PIPIENS*

A Thesis

Submitted to

the Division of Biological Sciences

Emporia State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Joseph Lyle Vacca

December, 1988

Katherine N. Smalley
Approved for Major Department

James F. Wolfe
Approved for Graduate Council

ACKNOWLEDGMENTS

A number of people have given me valuable assistance and reinforcement in completing this study.

The financial support of my parents and the emotional backing offered by family and relatives was extremely helpful to me while working on this project.

I am indebted to Dr. Dwight Moore for his statistical analysis of my data and for his time so unselfishly given. I also want to thank Dr. Carl Prophet for willingly being on my thesis committee during a busy time of the year.

A special thanks to Dr. Ed Rowe for being on my thesis committee and for providing thoughtful, practical advice and support throughout my two years of study at ESU.

My deepest appreciation goes to my major supervisor Dr. Kay Smalley. If not for her teaching and her guidance, this study would have been impossible. More importantly for me, she demanded high work standards while, at the same time, being patient for the outcome. Her encouragement through my difficult times, as well as her proofreading and technical advice, allowed me to complete this thesis.

Finally, I want to thank Dr. John Parrish, Dr. Gaylen Neufeld, Alisa Bridge, Floy Schwilling and all other faculty, staff, and students of Emporia State University who provided much needed friendship and encouragement.

TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION

It has been known for many years that there are high levels of androgens in female anurans. For example, D'Istria et al. (1974) measured plasma testosterone and estradiol-17B levels in Rana esculenta throughout one full year. Their data showed a much higher peak concentration of testosterone (14 ng/ml) than of estradiol-17B (one ng/ml). Fortune (1983) studied the in vitro production of sex steroids by ovarian tissue in the African clawed toad, Xenopus laevis. In this study, follicles at different developmental stages were incubated in amphibian Ringer's solution with or without the addition of frog pituitary homogenate (FPH). The data showed that Xenopus ovarian follicles produced and secreted testosterone, estradiol-17B, and progesterone. Furthermore, the follicles changed their steroidogenic abilities as they matured. The younger follicles produced more estradiol-17B than did older follicles. Older follicles, however, produced more testosterone than did the younger ones. Peak testosterone levels were almost seven times those of the peak estradiol-17B levels. FPH treatment generally enhanced the production of each steroid at all stages of development.

The function of these high androgen levels is not known. One possibility is that testosterone is a precursor for estradiol-17B in amphibians as it is in mammals (Gorbman et al. 1983). Mulner et al. (1978) investigated this possibility in studies of Xenopus laevis follicles in vitro. Their experiments established that the follicles can convert androgens to estrogens, although this activity is much

greater in young follicles than in mature ones. Delrio et al. (1979) also published data showing that amphibian follicles can convert androgens to estrogens. In vitro studies of Rana esculenta steroid metabolism showed that this conversion was seasonal, occurring during ovarian recrudescence, but not later in the year. In vivo work on these frogs showed that testosterone injections increased plasma estrogens at six hours but not at 24 hours.

High levels of androgen in female anurans could have other functions as well. Delrio and Brachet (1980) established the presence of androgen receptors in the skin of mature R. esculenta of both sexes, as well as in tadpoles. The receptors seem to exist throughout the frog life cycle. Testosterone also affects the liver. Injection of testosterone during vitellogenesis increased liver synthesis of a serum protein in the common leopard frog, Rana pipiens (Mohanty-Hejmadi et al. 1978). This particular protein is thought to be incorporated into the maturing eggs. This raises the possibility of a direct physiological role for testosterone in egg maturation.

Another possible function of the high levels of androgens in female anurans is to act as anti-estrogens. Smalley and Nace (1983) suggested that estrogens might play a significant role in egg maintenance in Rana pipiens. Smalley has continued work on the role of estrogens and has found that anti-estrogens improve egg maintenance in R. pipiens (Smalley 1985; Bridge 1987). This suggests that androgens might act as possible anti-estrogens during egg maintenance. Support for an anti-estrogen role also comes from the work of Rastogi and Chieffi (1975). They demonstrated that two derivatives of

dihydrotestosterone (DHT) were powerful anti-estrogens in female R. esculenta. Their work showed that these DHT derivatives caused a significant decline in oviduct weight, while control oviducts were increasing in size under the influence of endogenous estrogens.

Licht et al. (1983) showed that plasma testosterone levels correlate with ovarian size and oviduct size in female R. esculenta and the female bullfrog Rana catesbeiana. They measured increasing plasma testosterone with increasing follicular size in R. catesbeiana and believe plasma testosterone to be a good indicator of ovarian stage in the bullfrog. The testosterone levels were reported to decline just before ovulation. A periovulatory decline in androgen is supported by the work of Pierantoni et al. (1984) who found that preovulatory R. esculenta had higher plasma androstenedione levels than postovulatory females.

The objective of the present work was to clarify the role of testosterone in female R. pipiens reproductive physiology. Two studies were carried out with this goal in mind. The first study used exogenous testosterone and tested the possibility that testosterone might act as a stimulator of the reproductive tract, or as an anti-estrogen.

The second study was an attempt to measure the steroidogenic capabilities of large follicles taken from frogs early in the hibernation period and late in the hibernation period. This was done in order to determine any differences in relative testosterone and estradiol-17B secretion at these two times. Early and late hibernation times were chosen because of differences in the way Rana pipiens

follicles respond to frog pituitary homogenate *in vitro* (Wright, 1945). It was thought that these data would help explain the interaction of steroid hormones that ultimately leads to successful reproduction in *Rana pipiens*.

MATERIALS AND METHODS

STUDY 1. *in vivo* Testosterone Injection

Testosterone injection experiments were begun in late October of 1986 using gravid female *Rana pipiens* obtained from a Wisconsin supplier in early October, 1986. Before treatment, these leopard frogs were divided into four groups according to size so that each group had a similar average body weight. The four groups were: testosterone/fed, testosterone/not fed, oil/fed, and oil/not fed. The members of the two testosterone groups were injected with testosterone propionate (0.5 mg/ml corn oil) with each frog receiving one microgram/gram body weight. The two control groups were injected with an equivalent volume of oil alone. Injections were given twice weekly, subcutaneously in the dorsal lymph sac, for three weeks. The oil/fed and testosterone/fed groups were given live crickets three times a week.

All animals were kept at room temperature in large metal tubs containing tap water. The water was drained and refilled weekly. A light cycle of approximately 14 hours of light and 10 hours of dark was maintained.

At the end of treatment, each animal was weighed and autopsied. The ventral body wall was cut open with scissors, the body cavity was blotted, and the abdominal aorta was located and severed. This allowed a pooling of blood inside the abdominal cavity for blood collection. A capillary tube was filled with fresh blood and used to measure the hematocrit of each frog. The spleen, liver, oviducts, ovaries, left gastrocnemius, and fat bodies were removed, blotted and weighed to the nearest 0.1 mg on a Sartorious analytical balance. The weights of these

various organs were compared for possible testosterone effects and to provide clues about the health of each animal.

The data obtained from this experiment were analyzed on a Zenith computer using a Biostat I Two-Way Analysis of Variance (ANOVA) (Pimentel and Smith 1985) for testosterone vs oil and fed vs not fed.

STUDY 2. *in vitro* Follicle Incubations

INCUBATIONS:

Steroid hormone secretion was studied in mature follicles isolated from gravid *Rana pipiens* obtained from a Wisconsin supplier in December, 1986. After arrival, the frogs were placed in metal tanks containing aerated tap water in a coldroom at 4 C. This environment was intended to approximate the frogs' natural hibernation environment. No food was given to the animals, and the water was not changed during the experiment.

Follicles obtained from eight different frogs were used for the incubations. Follicles from four frogs were taken early in January, near the beginning of hibernation, and follicles from the other four were taken in late March toward the end of the normal hibernation period. Each frog was chosen randomly from the common tank.

The FPH was made in early January and used throughout the complete experiment. Fifteen pituitary glands, collected from gravid female *Rana pipiens*, were homogenized in 15 ml of cold Ringer's solution with a teflon grinder. This homogenate was frozen for 3–4 hours and then thawed and centrifuged at 10,000 rpm for at least 20 minutes to remove debris. The FPH was brought up to 15 ml with amphibian Ringer's for a

final concentration of one pituitary/one ml. FPH aliquots of one ml were frozen individually until just before use (Lin and Schuetz 1985).

The following protocol was employed for obtaining follicles from each animal. The frog was pithed, and the ovaries were removed, rinsed twice in amphibian Ringer's (Lin and Schuetz 1985), and then cut into small pieces. A dissecting microscope and fine-pointed forceps were used to pluck individual follicles from the small pieces of ovary. Twenty such isolated follicles were placed in each of the 24 wells of a Costar cluster dish. Twelve of the wells contained one ml amphibian Ringer's as an incubation medium control. The other twelve wells contained one ml of Ringer's solution plus .05 ml of a frog pituitary homogenate (FPH). The whole Costar dish was incubated on a gentle shaker for a period of eight hours at room temperature (22-25 C).

After eight hours, the Costar dish was removed from the shaker, and the medium from each of the 24 wells was collected and frozen separately without further treatment.

RIA's:

Testosterone and estradiol-17B were measured in the incubation medium by radioimmunoassay (RIA). Frozen media were thawed and extracted with at least 10 ml methylene chloride. The extract was then evaporated to dryness in a water bath at 40 C. The residue was mixed with 50 ul of radioactive estradiol-17B or testosterone, depending on the RIA to be performed. This mixture was dissolved in 1.0 ml of phosphate buffer and the RIA's were completed using RIA kits from Wien Laboratories, Inc. An LKB Wallac 1217 Rackbeta liquid scintillation counter was used to count radioactivity and calculate disintegrations per minute (dpm). Hormone concentrations (expressed as pg/follicle)

were determined from a standard curve which was constructed by running five standards of known hormone concentration with each RIA. The data were analyzed with both One-Way ANOVA's and Two-Way ANOVA's (Pimentel and Smith 1985) for FPH vs controls and January vs March using the Biostat I program and a Zenith computer. Preliminary RIA's showed testosterone and estradiol-17B levels to be quite different; this necessitated dissimilar volumes of medium for the two RIA's.

Testosterone:

Fifty microliters of the collected medium were extracted with 10 ml methylene chloride to provide sufficient testosterone to be measured by the RIA kits. The sensitivity of the testosterone assay was 17 pg/tube. The antibody showed a 65 % cross-reactivity with DHT and a 51 % cross-reactivity with delta-1-testosterone. Other cross-reactivities were negligible.

Estradiol-17B:

Because there were not as high a concentration of estrogen in the samples, 2.4 ml of the medium was extracted with 12 ml methylene chloride to provide adequate estradiol-17B for measurement. The sensitivity of the RIA for estradiol-17B was 36 pg/tube. The antibody showed a 10 % cross-reactivity with estrone and negligible cross-reactivity for other steroids.

RESULTS

STUDY 1. *in vivo* TESTOSTERONE INJECTION

Female *Rana pipiens* were injected with testosterone or oil, and one half of each of these groups was fed while the other half was not fed. The results of this study were analyzed to determine the effects of testosterone vs oil injections and the effects of feeding vs not feeding the animals.

Hematocrit values averaged 29 % and showed no indication of any significant effect ($p > 0.05$) caused by the oil or testosterone injections given the frogs.

There were significant effects ($p < 0.05$) due to both feeding and testosterone injection on body weights of the animals. Table 1 shows that fed frogs gained weight while those not fed lost weight. This table also shows that oil injected fed frogs gained more weight than did the testosterone injected fed group. In addition, testosterone injected not fed frogs lost more weight than did the oil injected not fed group.

Table 1. Mean weight gain or loss by frogs

GROUP	FED (N*)	NOT FED (N)
TEST[a]	4.3 ± 1.2 (7)	-2.6 ± 1.5 (7)
OIL	9.3 ± 2.4 (9)	-0.9 ± 0.8 (6)

weight change given as g/frog ± Standard Error of the Mean
a abbreviation: (TEST) testosterone injected
* number of frogs

Organ weight data are shown in Tables 2 and 3. Analysis of these data showed that fed animals had significantly higher ($p < 0.05$) spleen weights, fat body weights, and liver weights than the unfed animals.

Fat body weights were also significantly higher ($p < 0.05$) in oil injected, fed frogs than in those given testosterone (Table 2). There were no weight differences among the fat bodies of not fed frogs. Oviduct weights showed a significant interaction ($p < 0.05$) between testosterone injections and not feeding, with testosterone injected/not fed frogs having heavier oviducts than any other group (Table 3). Because of the heavy oviducts, the oviduct/ovary ratio was also significantly larger ($p < 0.05$) in the testosterone/not fed frogs than in the other groups.

STUDY 2. *in vitro* FOLLICLE INCUBATIONS

When mature follicles were incubated *in vitro* for eight hours, the incubation medium was found to contain both testosterone and estradiol-17B. This was true whether the follicles came from animals early in hibernation or late in hibernation.

Measurements of testosterone secretion are summarized in Table 4. The results show that the follicles secreted large amounts of testosterone, with average values of 73 pg/follicle in January and 79 pg/follicle in March. FPH caused a significant increase in testosterone secretion from follicles of frogs in both January and March. This increase averaged almost nine times the control secretion level. There was no significant difference ($p > 0.05$) in control or FPH stimulated testosterone secretion between January and March follicles.

Table 2. Average non-reproductive organ weights.

GROUP	SPLEEN	FAT BODY	LIVER	LEFT GASTROCNEMIUS
TEST F(6)[ab]	0.09 ± .008[cd]	0.17 ± .033[d*]	5.74 ± .196[d]	1.73 ± .050
TEST NF(7)	0.06 ± .005	0.06 ± .013	4.33 ± .206	1.53 ± .054
OIL F(9)	0.07 ± .005[d]	0.26 ± .033[d]	5.46 ± .241[d]	1.56 ± .077
OIL NF(6)	0.05 ± .006	0.06 ± .007	3.68 ± .217	1.37 ± .227

[a] abbreviations: (F) fed, (NF) not fed, (TEST) testosterone injected

[b] number in parenthesis is the number of animals in the group

[c] organ weights in grams/100 grams body weight ± standard error of the mean

[d] differ significantly from not fed groups at p less than 0.05

* differ significantly from oil group at p less than 0.05

Table 3. Average reproductive organ weights.

GROUP	OVIDUCT	OVARY	OVIDUCT/OVARY RATIO
TEST F(6)[ab]	4.91 ± .165[c]	8.47 ± 1.36	0.65 ± .084
TEST NF(7)	5.98 ± .217[d]	7.04 ± 0.824	0.92 ± .099[d]
OIL F(9)	4.73 ± .189	11.07 ± 1.27	0.49 ± .427
OIL NF(6)	4.35 ± .360	8.10 ± 1.40	0.60 ± .060

[a] abbreviations: (F) fed, (NF) not fed, (TEST) testosterone injected

[b] number in parenthesis is the number of animals in the group

[c] organ weights in grams/100 grams body weight ± standard error of the mean

[d] differ significantly from oil groups at p less than 0.05

Table 4. Mean testosterone secretion by isolated follicles.

GROUP	JANUARY (N*)	MARCH (N)
CONTROL	73 ± 12 (240)	79 ± 18 (260)
FPH	599 ± 44 (340)	759 ± 70 (380)

testosterone values given as pg/follicle ± standard error of the mean
* number of follicles

Estradiol-17B secretion is summarized in Table 5. The data shows no significant difference ($p > 0.05$) in secretion between FPH stimulated follicles and controls or between January and March follicles. It should be noted that there was much less estradiol-17B secreted into the medium per follicle than testosterone.

Table 5. Mean estradiol-17B secretion by isolated follicles.

GROUP	JANUARY (N*)	MARCH (N)
CONTROL	1.2 ± 0.23 (200)	1.4 ± 0.24 (240)
FPH	2.2 ± 0.46 (240)	1.8 ± 0.35 (220)

estradiol values given as pg/follicle ± standard error of the mean
* number of follicles

DISCUSSION

Two different experiments were designed to help elucidate the role of testosterone in the reproductive physiology of female Rana pipiens. Since the experiments were different, the results will be discussed separately.

STUDY 1: in vivo TESTOSTERONE INJECTION

This study investigated the effects of testosterone injection in fed and not fed frogs. It was undertaken to check for a possible anti-estrogen role or a specific stimulatory role of this steroid. Effects of feeding were also compared to those of not feeding in this study.

Hematocrits showed no significant differences between any of the four groups. This finding is different from that of Redshaw et al. (1969) who reported a slight increase in hematocrit due to testosterone injection in Xenopus laevis. This difference could be due to the different species studied, or to differences in the dosage and timing of injections. Redshaw's frogs got more frequent injections of a lower concentration of testosterone than the frogs in the present study.

Both testosterone injection and feeding showed separate significant effects on body weight change of the frogs (Table 1). It is not surprising that feeding led to weight increases and not feeding led to weight decreases. However, the testosterone/fed group did not gain as much weight as the oil/fed frogs. Also, testosterone/not fed frogs lost more body weight than did the oil/not fed group. It could be that testosterone caused an increased metabolic rate that lessened potential weight gain in fed frogs and increased weight loss in the not

fed animals. Sinha (1981) studied effects of testosterone injection on the lipid content of female Rana esculenta that were fed. Oil injected controls had significantly higher liver and ovarian fat, ovarian cholesterol, and body fat than did the testosterone treated group. This finding suggests that testosterone increased lipolysis. The effect was specific to testosterone because the lipid content of the same organs was significantly higher after estrogen treatment than in the controls. However, water content of Sinha's frogs was significantly higher in steroid injected animals than in oil controls. While lipolysis would lead to a decrease in body weight, and increased water content would lead to an increase in body weight, differences in the amount of steroid injected could explain any discrepancies between Sinha's study and the present one. Increased lipolysis due to testosterone could account for the data in Table 1, and there could still have been higher water content in the steroid injected frogs.

Organ weight data (Table 2) showed significantly lower fat body weights in testosterone injected frogs. This supports the hypothesis that increased lipolysis might account for the body weight changes in the testosterone injected groups. The high lipid content of the fat bodies would allow these organs to show such a testosterone effect more clearly.

The data in Table 2 show that fed frogs had significantly higher spleen weights, fat body weights, and liver weights than unfed frogs. These data also show that the left gastrocnemius was generally heavier in fed frogs, although this was not significant at $p < 0.05$. Table 3 shows that ovary weights were generally greater in fed frogs, although

not significantly at $p < 0.05$. The only excised organs that were not heavier in fed frogs were the oviducts. Interaction between testosterone injection and not feeding caused this difference, so it will be discussed separately. All other organs that were excised and weighed from the fed group of frogs were heavier. Since organ weights were corrected for differences in body weight, this must mean that these organs were differentially affected by the feeding regime. Two equally likely explanations of these data are possible. Feeding caused maintenance of the weights of these organs while not feeding caused a decrease, or feeding caused an increase in organ weights while not feeding caused either maintenance or a decrease.

The spleen, along with the liver and kidney, is a lymphoid organ in frogs. No published data were found suggesting a reason for increased weight of the spleen due to feeding or decreased weight due to not feeding. Since the hematocrit data showed no significant differences, it is unlikely that the spleen housed more blood cells in fed frogs. However, because the liver weights were heavier in fed frogs and both liver and spleen are lymphoid organs, there may be a connection between the two. Nutritional status of the animals must affect these organs, but the exact effect on the spleen is unknown.

Mizell (1965) showed seasonal differences in liver glycogen content in Rana pipiens that had been obtained from a supplier and acclimated in the laboratory for two weeks. Glycogen is a readily usable form of stored energy, and his work showed that Rana pipiens increased liver glycogen stores to a peak in November followed by a sharp decline in December. The liver glycogen then abruptly increased

to near peak levels in February followed by a decline to the lowest point in March. He hypothesized that the frogs built glycogen stores while feeding in late summer and early fall and that these stores were used for energy during late fall and into the hibernation period. The significantly higher liver weights of the fed frogs in the present study are probably due to a combination of nutrient storage in fed frogs and use of liver glycogen stores by non-feeding frogs.

The data in Table 2 show that fat body weights were significantly lower in unfed frogs than in fed animals. Mizell (1965) also reported seasonal differences in fat body weights of Rana pipiens. He discounted feeding as a controlling factor in these results. He reported that the fat bodies decreased in weight from a peak in late summer through fall, winter, and spring to a low value in June. He hypothesized that this weight decrease was correlated with preparation for hibernation in fall months, hibernation during the winter, and ovulation in the spring. He also suggested that the weight decreases of the fat bodies were due to the breakdown of fat for energy during these seasons.

The fact that the fed frogs in the present study had higher fat body weights suggests that feeding may be more of a controlling factor than Mizell thought. The difference in results may be due to the fact that the frogs in the present study were maintained in the laboratory at room temperature and, therefore, were not exposed to normal seasonal influences. As a result, feeding may have played a larger role in the fat body weight gain than normal. It should also be remembered that the frogs in Mizell's study were held captive without feeding for two

weeks before the fat bodies were excised and weighed. This captive period might have decreased normal fat body weights of his frogs.

It is apparent that nutritional status of the frogs affected the weights of the animals during the three weeks of my study. Autopsies of incoming frogs should be done in future studies of this type to determine whether it is feeding, not feeding, or a combination of the two that causes weight fluctuations. This laboratory protocol does not provide insight to what normally occurs in nature, but it does indicate that the frogs in the fed groups actually ate the crickets that were provided them.

The oviduct weights were significantly higher in testosterone injected frogs, but the ANOVA revealed that this was due to a significant interaction between testosterone injection and not feeding the frogs. The oviducts in the testosterone injected not fed frogs were heavier than in any other group (Table 3). There is evidence that the oviducts of anurans can metabolize testosterone. Delrio et al. (1979) found that the oviduct of R. esculenta could, in vitro, metabolize testosterone into DHT and 11-ketotestosterone (11-KT) but not into estrogens. However, this conversion was only possible during a short period of the year. Data from their biological assays showed that testosterone had no effect on the mass of the oviducts in these frogs. Their ultimate conclusion was that the oviducts of these frogs were not directly controlled by either testosterone or DHT.

The combination of testosterone injection and not feeding the frogs must have caused the higher oviduct weights in the present study. Testosterone's role in this effect might have been mediated by

conversion into estrogens. Marsh and Tata (1987) established X. laevis oviduct cell cultures and reported that estrogen receptors were present and that the oviduct was an important target organ for estrogen. Smalley and Nace (1983) reported that estrogens increase oviduct weights in R. pipiens. Delrio et al. (1979) reported that the ovaries of R. esculenta could, in vitro, metabolize testosterone into estradiol-17B during early vitellogenesis. If this ability is present in vivo for R. pipiens, it is possible that the injected testosterone was converted by the ovaries into estradiol-17B which subsequently acted on the oviducts in a growth enhancing manner. Support for in vivo conversion of testosterone into estrogens also comes from Delrio et al. (1979). They showed that October frogs had three times as much estrogen in their plasma six hours after testosterone injection than did controls.

Endogenous estrogen is required for normal oviduct growth and vitellogenesis. If healthy R. pipiens follicles can convert testosterone into estradiol-17B in vivo during the fall, then an explanation of the testosterone/not feeding interaction becomes possible. Bridge (1987) showed that there was greater egg degeneration in R. pipiens ovaries from fed frogs than from frogs that were not fed. This suggests that feeding the frogs in the present study could have led to fewer functional ovarian follicles and, therefore, less ability to convert testosterone to estrogen. The injected testosterone would then have acted as substrate for a greater number of healthy follicles in the not fed frogs, and thus led to more estrogen production and higher oviduct weights. This could explain why testosterone had a

stimulatory effect on the oviduct only in not fed frogs.

Study 1 investigated effects of testosterone *in vivo*. It appears that this hormone stimulated lipolysis in both fed and not fed frogs. The data also point to a possible precursor role of testosterone. Its conversion to estradiol-17B seems to be the most likely explanation for its ability to stimulate oviduct growth.

STUDY 2: *in vitro* FOLLICLE INCUBATIONS

This study tested the steroidogenic capabilities of *R. pipiens* ovarian follicles. Incubations were performed in January and March with or without frog pituitary homogenate (FPH) added to the medium. The results clearly show that these mature follicles produced large amounts of testosterone and much smaller amounts of estradiol-17B (Table 4).

As Table 4 illustrates, FPH caused a nine-fold increase in testosterone secretion over basal levels. There was no significant change between January and March. Despite differences in species and methods, these results are remarkably similar to those Fortune (1983) obtained in mature *Xenopus laevis* follicles. Both experiments showed considerable testosterone secretion by follicles *in vitro*, and FPH enhancement of this secretion.

Rana pipiens and *Rana catesbeiana* ovarian incubations were performed by Hubbard and Licht (1986). They showed that both species secreted significant amounts of testosterone, and this secretion was generally enhanced by addition of pituitary extract. They concluded that luteinizing hormone in the extract was the probable cause of this enhancement. These studies, as well as the present work, establish that

mature amphibian ovarian follicles are able to produce and secrete testosterone *in vitro* and that this secretion can be enhanced by addition of FPH, or a pituitary extract, to the incubation medium.

Estradiol-17B secretion by *R. pipiens* follicles (Table 5) did not seem to be affected by month or FPH treatment. Average values were between 1.2 and 2.2 pg/follicle throughout the four incubation settings. Fortune (1983) showed comparable values for estradiol-17B secretion from *X. laevis* follicles of approximately the same stage. In *Xenopus*, however, FPH treatment caused a two-fold increase in estradiol-17B secretion. Fortune also showed that intrafollicular levels of estradiol-17B were higher than the levels actually secreted into the medium. These tissue levels were also increased two-fold by FPH addition. Initial attempts to measure intrafollicular levels of testosterone and estradiol-17B in the present study gave unreliable results. It is therefore not possible to conclude that *R. pipiens* follicles retain more estradiol-17B than they secrete. However, Fortune's work shows that intrafollicular estrogen levels, while they do not necessarily mimic secretory levels, are subject to the same proportional increase caused by FPH. Therefore, since FPH did not produce a rise in estradiol-17B secreted from the *R. pipiens* follicles of the present study, it is likely that the production was not significantly enhanced inside the follicles either.

It was mentioned in the introduction of this paper that different developmental stages of *Xenopus* follicles secreted much different levels of both estradiol-17B and testosterone (Fortune, 1983). Follicles of early developmental stages showed much higher secretion of

estradiol-17B and much lower testosterone secretion. This finding suggests that as Xenopus follicles mature, they switch from secreting high estrogen quantities and low androgen quantities to just the opposite. If this pattern is also present in Rana pipiens, and if it occurs in vivo, then this steroid secretion switch must be important for normal reproduction. Future studies should measure steroid secretion from less mature R. pipiens follicles in order to determine if they also switch steroid secretion patterns. The present study did show that R. pipiens follicles secrete much testosterone and little estradiol-17B in both January and March. This suggests that the switch in steroid secretion, if it exists in this species, must occur before hibernation begins.

The discussion of Study 2 has thus far centered around in vitro results and has not fully addressed the situation in vivo. Plasma steroid levels are routinely used to study testosterone and estradiol-17B production in vivo. D'Istria et al. (1974) showed that female Rana esculenta had peak plasma testosterone between January and late April and that estradiol-17B levels during this time period averaged just seven percent those of testosterone. The work of McCreery and Licht (1983) on R. catesbeiana showed that plasma testosterone was about 50 times greater than plasma estradiol-17B at all sample time collections. They used a continuous infusion of mammalian gonadotropin releasing hormone as part of their study. They also noted that preovulatory females had high testosterone levels, though steadily declining, whereas postovulatory females had low levels of this steroid. These studies provide direct evidence of higher plasma testosterone levels

than those of estradiol-17B in these anurans. They also show that testosterone levels were greater than those of estradiol-17B during winter months or when mature oocytes were present in the ovary. This latter observation is in good agreement with the *in vitro* results of Study 2 in the present work.

POSSIBLE FUNCTIONS OF TESTOSTERONE

The initial objective of the present research was to clarify the role of testosterone in the reproductive physiology of female *Rana pipiens*. High levels of circulating testosterone are present throughout the Ranidae, but the specific role of this steroid is not yet apparent. Licht et al. (1983) noted a high correlation between plasma testosterone levels and ovarian as well as oviduct weight in *R. catesbeiana* and *R. esculenta*. On the other hand, Delrio et al. (1979) discounted testosterone control of oviduct mass in *R. esculenta*. Study 1 of this paper showed that testosterone effects on oviduct weight were greater in *R. pipiens* when the animals were not fed.

It is apparent that testosterone effects are complex, and there is much more work to be done before the specific actions of testosterone at different times of year can be elucidated. Species differences need to be more fully studied. A repeat of Study 1 with higher numbers of frogs and data on the initial condition of the animals would also help define specific target organs of testosterone. A study on the conversion of testosterone to DHT or estradiol-17B in specific tissues during different seasons would be very helpful. The testosterone RIA kits used in Study 2 showed a 65 % cross-reactivity with DHT. McCreery

and Licht (1984) implicated DHT as an important hormone in female Rana catesbeiana. They felt that testosterone had to be converted to DHT in order to act at the pituitary level.

Although the exact functions of testosterone are unknown, some possibilities can be suggested based on Study 2 results showing high testosterone secretion by follicles from hibernating frogs. I propose that testosterone is being used as a precursor to estradiol-17B or DHT during early fall. If this conversion to estrogen occurs, it must halt just before hibernation so that the oocytes can remain healthy. If such a switch takes place in vivo, then testosterone must play a different role during the hibernation period. This role could be to help create a microenvironment necessary for successful ovulation.

Several studies have addressed the hormonal changes and effects that influence ovulation. Lin and Schuetz (1985) have shown that progesterone is needed for ovulation. They also reported that estradiol-17B inhibits follicle production of progesterone. McCreery and Licht (1983) noted that R. catesbeiana estradiol-17B plasma levels tended to decline just before ovulation, when progesterone levels were rising. In addition to this, Kostellow et al. (1982) reported that progesterone and testosterone both induce resumption of meiosis in amphibian oocytes but that estradiol-17B is a noninducer. In fact, estradiol inhibits ovulation (Lin and Schuetz 1985) and may be deleterious to the mature follicles. Smalley (1985) reported that estrogens decrease the stability of mature follicles. With this in mind, it makes functional sense to have the ovaries quit converting testosterone to estradiol-17B during hibernation. It is therefore

possible that the low levels of estradiol-17B secreted by the R. pipiens follicles in the present work are a natural phenomenon associated with egg maintenance and preparation for progesterone production.

The high levels of testosterone during hibernation may then play a modulating role in preparing for ovulation. It could be that the endogenous testosterone is acting as an anti-estrogen during hibernation or that it works with progesterone to reinduce meiosis. It is also possible that high testosterone levels could be influencing another, yet unreported, reproductive process.

In conclusion, the present study has shown that testosterone exerts complex effects on body weight and on the weights of fat bodies and oviducts in mature female Rana pipiens. It has also been shown that ovarian follicles from hibernating females secrete large amounts of this hormone. Several hypotheses as to the function of this hormone are discussed, but further studies are required to elucidate its role in the reproduction of female leopard frogs.

LITERATURE CITED

LITERATURE CITED

Bridge, A. 1987. Effects of estrogen, antiestrogen, and feeding on egg maintenance in the leopard frog. M.S. Thesis, Emporia State Univ., Emporia. 23pp.

Delrio, G. and J. Brachet. 1980. Steroids and Their Mechanism of Action in Nonmammalian Vertebrates. Raven Press, New York. p.189-199.

Delrio, G., M. D'Istria., L. Iela and G. Chieffi. 1979. The possible significance of testosterone in the female green frog Rana esculenta. Boll. Zool., 46:1-9.

D'Istria, M., G. Delrio., V. Botte and G. Chieffi. 1974. Radioimmunoassay of testosterone, 17B-oestradiol and oestrone in the male and female plasma of Rana esculenta during sexual cycle. Steroids Lipids Res., 5:42-48.

Fortune, J.E. 1983. Steroid production by Xenopus ovarian follicles at different developmental stages. Dev. Biol., 99:502-509.

Gorbman, A., W.W. Dickhoff., S. Vigna., N.B. Clark and C.L. Ralph. 1983. Comparative Endocrinology. John Wiley & Sons, New York. 572p.

Hubbard, G.M. and P. Licht. 1986. In vitro ovarian responses to pulsatile and continuous gonadotropin administration on steroid secretion and oocyte maturation in the frogs, Rana pipiens and Rana catesbeiana. Gen. Comp. Endocrinol., 61:417-423.

Kostellow, A.B., S.P. Weinstein and G.A. Morrill. 1982. Specific binding of progesterone to the cell surface and its role in the meiotic divisions in Rana oocytes. Biochimica et Biophysica Acta, 720:356-363

Licht, P., B.R. McCreery., R. Barnes and R. Pang. 1983. Seasonal and stress related changes in plasma gonadotropins, sex steroids, and corticosterone in the bullfrog, Rana catesbeiana. Gen. Comp. Endocrinol., 50:124-145.

Lin, Y.P. and A.W. Schuetz. 1985. Intrafollicular action of estrogen in regulating pituitary-induced ovarian progesterone synthesis and oocyte maturation in Rana pipiens: temporal relationship and locus of action. Gen. Comp. Endocrinol., 58:421-435.

Marsh, J. and J.R. Tata. 1987. Establishment and characterization of Xenopus oviduct cells in primary culture. Exp. Cell Res., 173:117-128.

McCreery, B.R. and P. Licht. 1983. Induced ovulation and changes in pituitary responsiveness to continuous infusion of gonadotropin-releasing hormone during the ovarian cycle in the bullfrog, Rana catesbeiana. Biol. Reprod., 29:863-871.

McCreery, B.R. and P. Licht. 1984. The role of androgen in the development of sexual differences in pituitary responsiveness to gonadotropin releasing hormone (GnRH) agonist in the bullfrog, Rana catesbeiana. Gen. Comp. Endocrinol., 54:350-359.

Mizell, S. 1965. Seasonal changes in energy reserves in the common frog, Rana pipiens. J. Cell. Comp. Physiol., 66:251-258.

Mohanty-Hejmadi, P., O.J. Safriel and G.W. Nace. 1978. Serum proteins & vitellogenesis in the frog Rana pipiens. Indian J. Exp. Biol., 16:531-537.

Mulner, O., C. Thibier and R. Ozon. 1978. Steroid biosynthesis by ovarian follicles of Xenopus laevis in vitro during oogenesis. Gen. Comp. Endocrinol., 34:287-295.

Pierantoni, R., L. Iela., G. Delrio and R.K. Rastogi. 1984. Seasonal plasma sex steroid levels in the female Rana esculenta. Gen. Comp. Endocrinol., 53:126-134.

Pimentel, R.A. and J.D. Smith. 1985. Biostat I. Sigma Soft, Placentia, CA.

Rastogi, R.K. and G. Chieffi. 1975. The effects of antiandrogens and antiestrogens in nonmammalian vertebrates. Gen. Comp. Endocrinol., 26:79-91.

Redshaw, M.R., B.K. Follett and T.J. Nicholls. 1969. Comparative effects of the oestrogens and other steroid hormones on serum lipids and proteins in Xenopus laevis (Daudin). J. Endocrinol., 43:47-53.

Sinha, R.C. 1982. Effects of gonadal hormones on the lipid contents of the frog Rana esculenta. Experientia, 38:1066-1067.

Smalley, K.N. 1985. Effects of an estrogen and anti-estrogen in the northern leopard frog. Kansas Academy of Science Abstracts, 4:53.

Smalley, K.N. and G.W. Nace. 1983. Vitellogenic cycles in laboratory-maintained females of the leopard frog, Rana pipiens. J. Exp. Zool., 226:211-219.

Wright, P.A. 1945. Factors affecting in vitro ovulation in the frog. J. Exp. Zool., 100:565-575.

Joseph L. Vacca
Signature of Graduate Student

Katherine N. Bradley
Signature of Major Advisor

I, Joseph L. Vacca, hereby submit this thesis/report to Emporia State University as partial fulfillment of the requirements of an advanced degree. I agree that the Library of the University may make it available for use in accordance with its regulations governing materials of this type. I further agree that quoting, photocopying, or other reproduction of this document is allowed for private study, scholarship (including teaching), and research purposes of a nonprofit nature. No copying which involves potential financial gain will be allowed without written permission of the author.

Joseph L. Vacca
Signature of Author

December 1, 1988
Date

Ovarian Steroid Secretion and Effects of Testosterone in Female Rana pipiens
Title of Thesis/Research Report

Jackie LaClair
Signature of Graduate Office Staff Member

December 5, 1988
Date Received