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The effects of estrogen on egg maintenance were tested in gravid female frogs by injecting them with 0.5 ug estradiol-17B per gram of body weight. Upon autopsy, the animals were found to have greater degeneration of the oocytes, as indexed by absence of the germinal vesicle and pigment mottling, than did the control animals. Experimental animals autopsied 6 days after treatment had more degeneration than those autopsied at 2 days. To determine if endogenous estrogens could also cause degeneration of mature oocytes, an antiestrogen, nafoxidine, was administered in a dosage of 5 ug per gram body weight to gravid female frogs. This was done with spring animals coming out of hibernation and fall animals going into hibernation. Half the experimental and controls were fed and the other half were not fed during the treatment period. Hormone treatment was repeated twice weekly, and the animals were autopsied at the end of 3 weeks time. Nafoxidine treated animals were found to have less degeneration of mature oocytes than were control animals in both spring and fall samples. Additionally, animals who were not fed appeared to have healthier eggs than those animals who were fed. However, the feeding effect was not statistically significant in this sample. Blood studies

on the nafoxidine and control animals from the spring sample show no significant differences in hematocrit, total blood proteins, or blood plasma estradiol-17B levels.

EFFECTS OF ESTROGEN, ANTIESTROGEN, AND FEEDING ON EGG MAINTENANCE IN THE LEOPARD FROG

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INTRODUCTION

The reproductive cycle of Rana pipiens, the North American leopard frog, has been studied primarily in wild-caught animals. Little is known about the mechanisms responsible for normal egg development and maintenance. The purpose of the present work was to investigate some of the factors involved in egg maintenance in this species.

In the wild, R. pipiens females spawn in the spring and then begin a new round of oocyte growth and development. The animals feed throughout the late spring, summer, and early fall, storing resources for the winter hibernation period (Mizell 1965). During this feeding period the oocytes take up yolk-forming proteins from the blood in a process known as vitellogenesis. As fall and winter approach the entry of new oocytes into this cycle stops, but those eggs already in the cycle continue to grow to a mature size of approximately 1.5 mm. When the animal goes into hibernation, she has a large number of mature oocytes in the ovaries.

During the fall, the animal also builds up a supply of metabolic resources in the liver and in the fat bodies that are attached to the tip of the ovaries (Mizell 1965). These resources are to maintain the animal and her eggs in good condition through hibernation. When the animal emerges from hibernation in the spring, she travels to her spawning grounds, mates, and lays her eggs. It has been shown that the female of a related species Rana esculenta (Itamies et al. 1970), does not feed until after her eggs are laid. The metabolic resources built up in the fall must, therefore, not only carry the animal through hibernation, but also be sufficient to sustain it through a period of

high activity and stress in the succeeding breeding season. Since stress itself may have deleterious effects on the reproductive capacity of frogs (Licht et al. 1983b) an attempt was made in the present study to minimize stress effects by feeding some of the frogs as they were taken out of hibernation. Surprisingly, feeding under these conditions seemed to promote egg degeneration rather than egg maintenance.

Wild-caught frogs can be ovulated in the laboratory, but after they have been maintained at room temperature for a period of time vitellogenesis becomes erratic and eggs do not mature normally (Smalley and Nace 1983). Smalley and Nace discovered that treatment with cold improved the frog's reproductive capabilities, although the animals were still not normal. They proposed that different temperature regimes may affect the secretion of the hormones which are necessary for normal vitellogenesis and maintenance.

Estrogens are steroid hormones that are secreted by the follicle and induce the liver to produce the vitellogenic proteins (Wallace and Bergink 1974). Estrogens also inhibit meiotic maturation of mature oocytes *in vitro* (Lin and Schuetz 1983). These results suggest that estrogens may play a role in egg maintainence *in vivo*. However, when Smalley (1985) injected females with estradiol-17B, the estrogen paradoxically caused gross degeneration of the eggs. A similar effect has been reported in the toad *Bufo bufo bufo* (Jorgensen 1974). Two interpretations of this effect are possible:

1. degeneration is a response which may occassionally occur at physiological estrogen levels,

   or,

2. it is due to unphysiologically high doses of estrogen.

If degeneration can occur in response to physiological levels of estrogen, then there must be circumstances under which the animal's own estrogens can cause degeneration. This hypothesis can be tested by taking animals which are prone to egg degeneration, such as those emerging from hibernation with inadequate energy reserves, and treating them with a compound which can block the effects of endogenous estrogens. Nafoxidine is known to be an effective anti-estrogen in mammals (Katzenellenbogen et al. 1979), and Smalley (1985) has shown that it can reverse some estrogen-induced effects in male frogs.

Therefore, in this study, female frogs emerging from hibernation in the spring were treated with nafoxidine to see if it would block egg degeneration. Half the nafoxidine treated animals were fed in order to control for the effects of food deprivation during hibernation. The control animals were also divided into fed and unfed groups. The experiment was repeated in the fall with a group of animals preparing for hibernation.

MATERIALS AND METHODS

Gravid female frogs were obtained from commercial suppliers in Wisconsin in December 1985 and October 1986. Upon arrival, the December frogs were gradually cooled to 4 C and then maintained at this temperature with a light/dark cycles of 9 hours light/15 hours dark. When the animals were removed from hibernation in April 1986, they were weighed, measured from snout to vent, and their color and spots recorded for identification purposes. The October frogs were placed at room temperature after arrival and used within three days of shipment. The frogs were divided into four treatment groups: nafoxidine/fed, oil/fed, nafoxidine/not fed, oil/not fed. Initially, each group contained 10 animals. An attempt was made to distribute the larger and smaller frogs evenly among the groups, so all the groups would have similar average weights. The nafoxidine treated frogs received injections of 5.0 ug hormone per gram body weight in corn oil emulsion (0.002 ml). The control animals received 0.002 ml oil per gram of body weight. Injections were made into the dorsal lymph sac. During the treatment period the animals were injected twice weekly. The dosage was the same as the initial treatment.

During treatment the frogs were maintained at room temperature, between 22 and 24 C, in tanks providing both wet and dry surfaces. The two fed groups were given an excess of crickets 2-3 times per week (Lehman 1978). The light/dark cycle was approximately 13 hours light/ 11 hours dark for the remainder of the experiment.

Spring and fall animals were autopsied in the same manner, the spring animals after 2 weeks of treatment and the fall animals after 3

weeks of treatment. Upon autopsy the body cavity was blotted to remove fluids and blood was collected by bleeding the aorta into the body cavity. A sample for a hematocrit was taken first, then plasma was collected in a heparinized syringe and serum in a non-heparinized syringe. The liver, ovaries, oviducts, fat bodies, and spleen were removed and weighed. A piece of the ovary was boiled and 30 eggs were checked for degeneration by breaking them open under a dissecting microscope and noting the presence or absence of the germinal vesicle. A small piece of ovary was weighed and the eggs were categorized according to stage of development and they were looked at for signs of degeneration. Radioimmunoassays (RIA) for estradiol-17B were run on blood plasma (Wein laboratories kit and procedure), and total blood protein analyses were made on blood serum using the micro method of Lowry (Sigma technical procedure no. 690). The sensitivity of the RIA was 25 pg hormone per tube. Assays were conducted with 100-250 ul of plasma in each assay tube. The antisera was extremely specific. It had a cross reactivity of approximately 10 % with estrone, and cross reactivities much less for all other hormones tested (including testosterone, progesterone, estriol, and hydrocortisone). A third experiment was performed in the spring of 1987 in which frogs were given one injection of estradiol-17B (0.5 ug in .002 ml oil per gram of body weight) and autopsied after different lengths of time (2, 4, or 6 days). None of these animals were fed. Control animals were given oil only (0.002 ml/g body weight). Those animals were autopsied similarly to the procedure described above.

RESULTS

Effects of estrogen

The ability of a single injection of estradiol-17B to cause egg degeneration is illustrated in Table 1. Due to the small sample sizes data are not significant, nevertheless a distinct trend toward loss of the germinal vesicle of the oocytes can be shown as early as 2 days after injection. This effect continues over time, resulting in an accumulation of degenerating oocytes in the ovaries. These atretic oocytes exhibit a mottling of the pigments on the surface, and separate animal and vegetal hemispheres cannot be seen in the interior of the egg when it is broken open. As the oocytes progress through degeneration, yolk is reabsorbed by the body and the oocytes shrink in size from about 1.5 mm to about 1.3 or 1.4 mm in just 6 days. Diameter measurements were made with an ocular micrometer. This shrinkage also caused a significant decrease in the overall weight of the ovaries as shown in Table 2. It appears that once the process of degeneration is begun by the estrogen it will continue without additional treatment for at least 6 days.

Effects of nafoxidine

A two way analysis of variance (ANOVA) was made on data from the spring animals (Table 3). The oil-fed group had significantly higher liver weights than the unfed oil animals, as might be expected. The nafoxidine-fed group also had higher liver weights than the unfed nafoxidine animals. There were no significant differences found in fat body, oviduct, or ovary weights. However, there seems to be trend in ovary weights. In both the control and experimental groups the unfed

Table 1. Effects of a single injection of estrogen on percent of germinal vesicles present as an index of oocyte degeneration.

	Percent Germinal Vesicles	
	Oil	Estradiol
2 days	95.5 ± 2.2(3)[a]	85.5 ± 8.8(4)
4 days	78.4 ± 12.6(3)	47.1 ± 12.1(4)
6 days	81.3 ± 4.3(4)	65.5 ± 5.0(4)

[a] Number in parenthesis is the number of animals in that treatment group.

Table 2. Effects of a single injection of estrogen on ovary size over time.

	Ovary Weight (g/100 g body weight)		
	2 day	4 day	6 day
Estrogen	16.07 ± .91(4)[a]	15.8 ± 1.54(4)	12.19 ± 1.66(4)[b]
Oil	15.01 ± .52(3)	17.81 ± 1.99(3)	16.32 ± 2.24(4)

[a] in grams/100 grams body weight ± SEM. Number in parenthesis is the sample size for that group.

[b] significantly different from oil control for that time period at the $p < 0.05$ level.

Table 3. Effects of nafoxidine and feeding on organ weights, spring 1986 animals.

Treatment group	Fat bodies	Liver	Oviducts	Ovaries
Oil fed (8)[a]	.04 ± .01[b]	2.21 ± .17[c]	5.55 ± .21	16.48 ± .92
Oil not fed (10)	.07 ± .01	1.61 ± .09	5.93 ± .24	17.44 ± .73
Nafoxidine fed (8)	.08 ± .01	2.17 ± .13[c]	4.98 ± .28	16.24 ± .67
Nafoxidine not fed (10)	.06 ± .01	1.74 ± .12	5.44 ± .58	17.22 ± .48

[a] number in parenthesis is the number of animals in the treatment group

[b] organ weight in grams/100 grams body weight ± SEM

[c] differ significantly from unfed animals at $p < 0.05$.

animals have heavier ovaries. Table 4 contains data from fall animals. Due to the death of many of the animals, there were only three treatment groups and a two-way ANOVA was not possible. However, a Student T-test showed that fed animals had significantly higher fat body weights and liver weights than unfed animals. Nafoxidine did not appear to affect those weights greatly. There were no significant differences in oviduct weights.

The average percent of germinal vesicles (% GV) present in boiled oocytes was used as an index of egg degeneration and the data have been summarized in Figure 1. The data show that nafoxidine fed animals had significantly higher percentages of germinal vesicles present than their respective control groups for both spring and fall animals. This would indicate a larger percentage of healthy oocytes in the ovaries and less degeneration of the follicles. The two-way ANOVA on spring data also showed a significant interaction term between feeding and nafoxidine treatment. It appears that fed animals respond differently to nafoxidine treatment than do animals that are not fed. If feeding influences the way the animal responds to antiestrogen with regard to egg maintenance, then it seems reasonable that feeding might influence the response to endogenous estrogens with regard to egg maintenance. Although the ANOVA showed no significant difference in % GV between fed and unfed animals, the unfed animals at autopsy were observed to have oocytes in which the animal and vegetal hemispheres were more concisely defined, exhibited a much reduced incidence of pigment mottling, and coloring in each hemisphere was more even. These changes are consistent with the higher % GV in unfed animals and suggest that a

Table 4. Effects of nafoxidine and feeding on organ weights, fall 1986 animals.

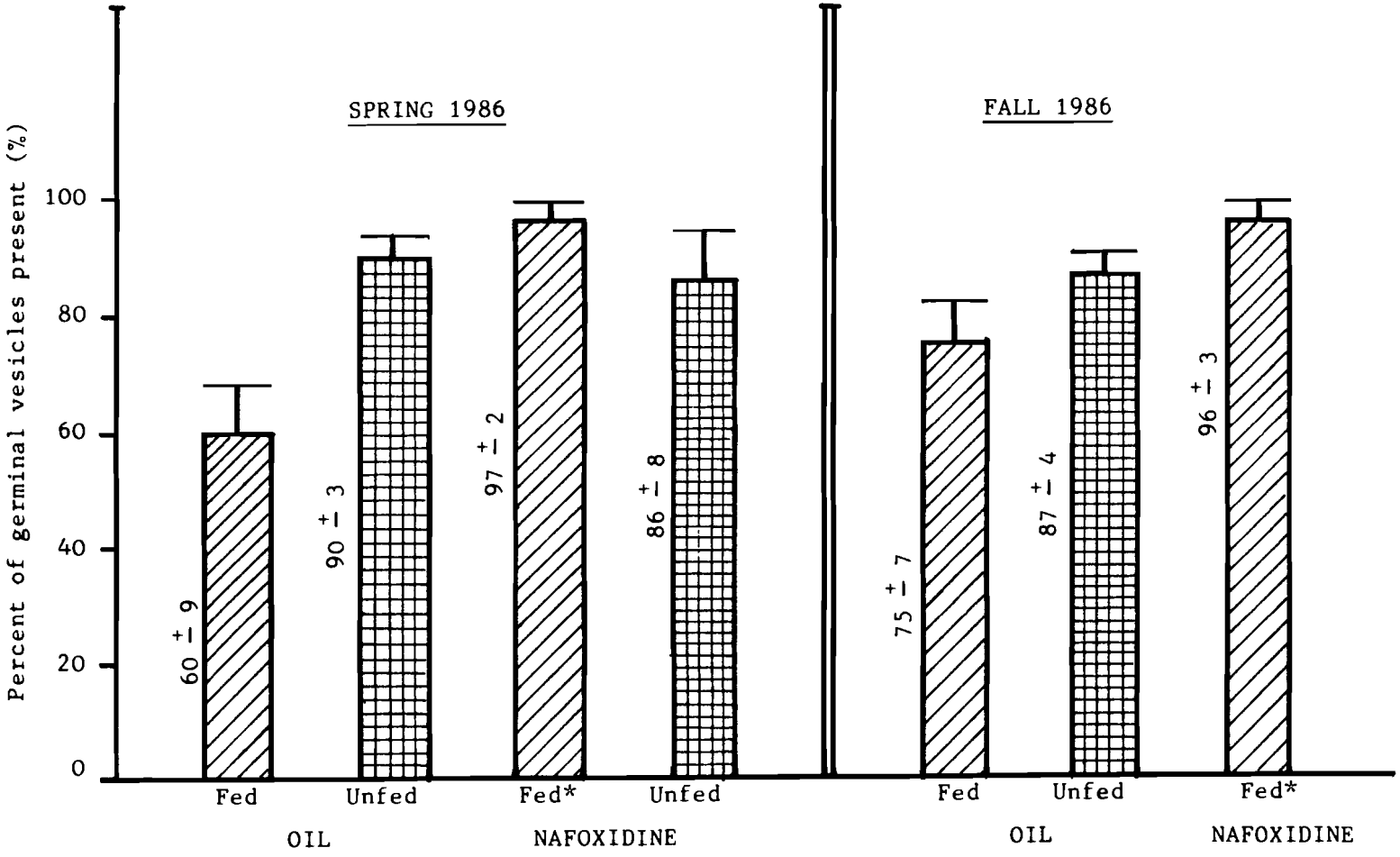
Treatment group	Fat bodies	Liver	Oviduct	Ovaries
Oil fed (9)[a]	0.26 ± .04[b]	5.46 ± .26[c]	4.73 ± .2	11.07 ± 1.34
Oil not fed (6)	0.06 ± .01	3.68 ± .24	4.36 ± .4	8.1 ± 1.54
Nafoxidine fed (6)	0.24 ± .09	5.06 ± .25	4.31 ± .24	10.72 ± .98

[a] number in parenthesis is the number of animals in that treatment group.

[b] organ weights in grams/100 grams body weight ± SEM.

[c] differ significantly from unfed animals at $p < 0.05$.

Figure 1. Effects of nafoxidine and feeding on the percent of germinal vesicles present in boiled oocytes.



Values are graphed ± SEM
*Differ significantly from the Oil/fed control group at $p < 0.05$.

feeding effect may occur even though it was not shown statistically in the present sample.

Blood data

Mean values for hematocrit, total blood protein, and blood estradiol levels are summarized in Table 5 for spring animals. There were no significant differences in hematocrit or blood protein levels among the four groups. Although the blood estradiol level appears to be lower for the nafoxidine-fed group the difference is not significant due to the extreme variability within each group of animals. The values in each group ranged from 50 pg/ml to over 2000 pg/ml. One animal in the fall nafoxidine/fed group had undetectable levels of estradiol. Estradiol levels appear to be very individualized for these animals.

Table 5. Effects of nafoxidine and feeding on blood parameters.

Spring 1986 Animals			
Treatment group	Hematocrit (%)	Blood protein (g/dl)	Blood estradiol (pg/ml)[a]
Oil fed (8)[b]	29 ± 3	3.45 ± .38	823 ± 275
Oil not fed (10)	31 ± 2	3.61 ± .19	887 ± 180
Nafoxidine fed (8)	32 ± 2	3.55 ± .31	651 ± 146
Nafoxidine not fed (10)	30 ± 1	2.97 ± .34	908 ± 238

[a] Values are standardized at a recovery rate of 90 %. The interassay coefficient of variation is 14.37 %.

[b] number in parenthesis is the number of animals in that treatment group.

DISCUSSION

Effects of estrogen

The role of estrogens in the reproductive cycle of frogs has been the subject of several studies. Estrogen has been shown to induce the production of vitellogenic proteins by the liver in the toad Bufo bufo bufo (Emmerson and Kjaer 1974), as well as in the leopard frog Rana pipiens (Mohanty--Hejmadi et al. 1978). Estrogen has been shown by Lin and Schuetz (1983, 1985) to inhibit meiotic maturation of Rana pipiens oocytes in vitro. They also have shown (1985) that it blocks the secretion of progesterone by the follicle cells. Estrogen blocks pregnenolone-induced meiotic maturation in vitro, possibly because it blocks the conversion of pregnenolone to progesterone (Speigel et al. 1978). Estrogen also causes increases in oviduct weight in vivo as shown by Madgiri and Saidapur (1985) with the tropical frog, Rana cyanophlyctis.

The present work suggests that estrogen is also capable of causing atresia in mature oocytes. Gravid females treated with a single injection of estradiol-17B showed evidence of atresia within two days of treatment and further degeneration of the oocytes was observed after four and six days. This type of effect has also been obtained by Smalley (1985). In the toad Bufo bufo bufo estrogen has also been shown to have degenerative effects on oocytes. Jorgensen (1974) found that 5 ug of injected estrogens reduced the size and number of second growth phase oocytes and caused atresia among these oocytes. Madghiri and Saidapur (1985) treated hypophysectomized Rana cyanophlyctis with estrogens and found that they could partially maintain second growth

phase oocytes, but only for 15 days, after which the follicles underwent atresia. They do not believe that estrogen is responsible for long term egg maintainence.

Effects of nafoxidine

Nafoxidine has been shown to block the effects of estrogen on residual oviduct growth and vitellogenin production in intact male Rana pipiens (Smalley 1985). A related antiestrogen, tamoxifen, has been shown to suppress the induction of vitellogenin by estrogen in Xenopus laevis (Riegel et al. 1986). The results of the present nafoxidine study strongly suggest that endogenous estrogens may play a role in the degeneration of mature oocytes. In mammals the action of nafoxidine is to bind competitively to the estrogen receptor (Katzenellenbogen et al. 1979), thus blocking estrogen action. Therefore, any estrogen circulating in the blood of nafoxidine-treated frogs can have little, or no, effect on the oocytes. As the blood study shows, estrogen is present in the blood of animals of all groups in similar amounts. Thus, the improved maintainence of oocytes can be attributed to a reduction in the effects of endogenous estrogens in these animals.

The results of this study indicate that nafoxidine treatment has very little, if any, effect on the blood estrogen levels. Although there is a large variation in blood estrogen levels within each treatment group, the variation is similar among all groups. It is not uncommon to have this type of variation in gravid female frogs. Smalley (observations unpublished) has also found this type of variation in the leopard frog, and Licht (personal communication) has seen this type of variation in the bullfrog, Rana catesbiana. Such

variability suggests that changes in the estrogen levels may be more important to the frog's reproductive physiology than a specific set level of estrogen.

The annual cycle of estrogen in Rana esculenta has been charted (D'Istria et al. 1974). The researchers show that estradiol-17B levels increase sharply from September to October, then fall abruptly toward the end of November as the animals are entering hibernation. The levels of estradiol-17B were too low to be measured by radioimmunological methods the rest of the year. Blood levels of estrone peak in March, when the animals are coming out of hibernation and preparing to lay their eggs. The estrone levels fall very sharply toward May and are very close to zero the remainder of the year. The yearly changes shown by these researchers in levels of both hormones are very dramatic. The hormones never increase gradually, nor do they taper off. This would support the idea that changes in the blood levels of estrogens are important in inducing the seasonal changes in the reproductive cycle.

Effects of feeding

Previous work with the effects of feeding on the amphibian reproductive cycle have indicated that feeding is beneficial to oocyte growth and development. Jorgensen (1982), working with Bufo bufo bufo, found that induction of a new vitellogenic cycle was dependent on a proper nutritional state in the animal. Another study has shown that starvation during the vitellogenic period of the cycle stops endocytosis of yolk proteins by the oocytes of Xenopus laevis (Holland and Dumont 1975). Redshaw (1972) reports that amphibians in captivity

that are underfed exhibit ovarian regression. It is important to note that this previous work has all dealt with frogs in the vitellogenic phase of the reproductive cycle, not with gravid females.

The present study shows some trends toward better egg maintainence by gravid females who were not fed. Although the data are not conclusive, the unfed animals have overall healthier appearing ovaries as described in the experimental results. The frogs in this study were either late fall or early spring frogs. Thus, they were either in hibernation or going into hibernation when the experiments were begun. It has been shown (Itamies and Koskella 1970) that Rana temporaria does not eat during hibernation. Jorgenson (1982) has shown that reactivation of the gonadotropic system, necessary for the reestablishment of the oocyte growth phase of the cycle, is dependent on the resumption of feeding. It is possible that feeding gravid frogs may initiate the start of a new growth phase, through the hypothalamic-hypophysial gonadotropic system. Although estrogen is responsible for the production of vitellogenin proteins by the liver, it is the gonadotropins that are necessary for their incorporation into the oocyte (Emmerson and Kjaer 1974). The renewed secretion of gonadotropins necessary for vitellogenesis could involve a different gonadotropin, or a different proportion of gonadotropins than what is necessary for egg maintenance, thus upsetting maintenance and precipitating degeneration.

Feeding might also cause its deleterious effect by stimulating endocytosis of yolk proteins by the oocytes. This could cause yolk to be deposited in already mature eggs, overstuffing them and resulting in their degeneration. Alternatively, endocytosis might stimulate the

entry of new oocytes into the vitellogenic phase. With more oocytes in the vitellogenic phase of the cycle more estrogen will be produced. Thus, feeding could also cause degeneration of the mature oocytes by indirectly increasing estrogen levels. More studies on the effect of feeding on maintainence of mature oocytes will be needed before firm conclusions can be made.

SUMMARY

Estrogen treated animals were found to have greater degeneration of the oocytes than oil control animals. This degeneration was indexed by absence of the germinal vesicle and pigment mottling. Estrogen treated animals autopsied six days after treatment had a higher degree of degeneration than did animals autopsied two days after treatment. To determine if endogenous estrogens could precipitate oocyte degeneration, an antiestrogen, nafoxidine, was used to treat spring and fall gravid females. Half of the animals were fed and the other half were unfed. Hormone treatment was repeated twice weekly for two weeks before the animals were autopsied. Nafoxidine treated animals were found to have less degeneration of mature oocytes than were control animals in both spring and fall animals. Additionally, unfed animals appeared to have healthier eggs than those animals who were fed. However, the feeding effect is not statiscally significant in this sample. The blood studies run on the spring animals show no significant differences in hematocrit, total blood protein, or blood plasma estradiol-17B levels.

The data on estrogen treated animals indicate that estrogen did cause degeneration of mature oocytes in those animals. Further, the nafoxidine data indicate that endogenous estrogens also were capable of causing oocyte degeneration. The two-way ANOVA indicated that feeding can affect the way the animals responded to antiestrogen treatment, with regard to egg maintenance. It's possible that feeding could also affect the animal's response to endogenous estrogens, thus influencing egg maintenance and degeneration through the estrogens.

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