AN ABSTRACT OF THE THESIS OF

Sondra Ann Dubowsky for the Master of Science Degree

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Title: Effects of testosterone on oviduct growth in the northern leopard frog (*Rana pipiens*)

Abstract Approved: Katherine N. Smalley

Testosterone has been shown to cause oviduct hypertrophy in some amphibians. To understand the role of testosterone, ovariectomized and immature frogs were injected with estradiol and testosterone and compared to oil-treated animals. Since testosterone can be converted to estradiol, dihydrotestosterone (DHT), which cannot be converted, was also used. Animals treated with testosterone or estradiol plus testosterone had the largest oviducts, whereas oviducts from mature animals were slightly smaller in groups treated with estradiol alone. DHT had no effect in mature animals, and only a very slight effect in immature animals. These results suggest that oviduct growth in testosterone-treated animals was due to the aromatization of testosterone to estradiol. To test this hypothesis oviduct tissue was incubated with testosterone. The medium showed high estradiol levels, supporting the presence of aromatization in the oviduct. Protein and total polysaccharide content of oviducts were examined across groups with little significant difference. Hexose content of the total polysaccharides was not different between groups. Total plasma proteins from groups injected with estrogen plus androgen were smaller than those injected with estrogen alone. This

suggests that androgen effects on plasma proteins are not due to conversion to estradiol.

EFFECTS OF TESTOSTERONE ON OVIDUCT GROWTH IN THE NORTHERN LEOPARD FROG (*RANA PIPIENS*)

A Thesis

Submitted to

the Division of Biological Sciences

Emporia State University

In Partial Fulfillment

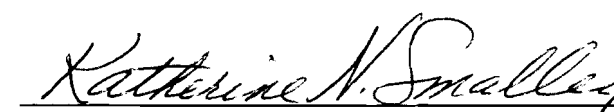
of the Requirements for the Degree

Master of Science

by

Sondra Ann Dubowsky

December, 1991



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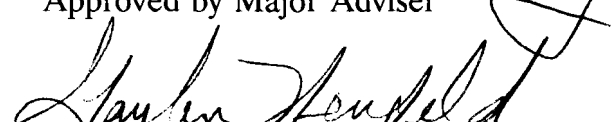

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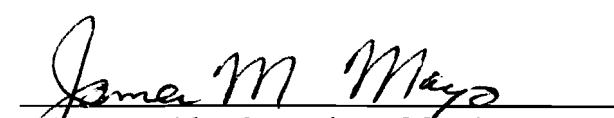

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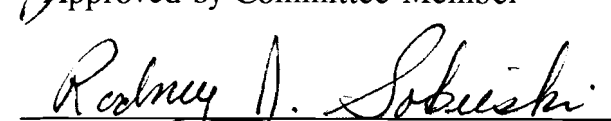

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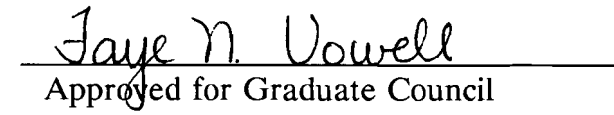

Approved for Graduate Council

ACKNOWLEDGMENTS

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Very special thanks go to Dr. Katherine Smalley for showing me what graduate school is really all about. She allowed me to fall down and pick myself back up when others would have given up. Her patience was extraordinary and her time and understanding is greatly appreciated.

I would also like to express how important my parents Lee and Juanita Dubowsky were in helping me through all the rough times. They not only helped me financially, but also supported me emotionally every step of the way.

I cannot forget the role played by my closest friends. I would not have made it to this point had it not been for their support and encouragement.

PREFACE

This thesis is being submitted for publication in General and Comparative Endocrinology so I followed their publication style.

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INTRODUCTION

Growth of the frog oviduct is stimulated by estrogens (Lofts, 1974). Growth effects of testosterone (T) on the amphibian oviduct have also been examined. Testosterone causes oviduct hypertrophy in urodeles (Moore and Norris, 1973; Guillette *et al.*, 1985; Foote, 1941; Kikuyama *et al.*, 1986; Adams *et al.*, 1941; Kambara, 1964) and in some anurans (Greenberg, 1942; Tôjio and Iwasawa, 1977). However it has little or no effect in other anurans such as *Rana esculenta* (Delrio *el al.*, 1979), *R. nigromaculata* (Iwasawa and Kobayashi, 1974), and *Xenopus laevis* (Redshaw *el al.*, 1969). All of these studies vary in species, size of dosage, length of treatment, age of the animal, and whether or not the ovari ·tact.

The ıı. ·f action of T's paradoxical growth effect has not been investigated. Testoste. ' ` have a direct effect on the oviduct, or in intact animals it could be converted ı. .iol (E) in the ovary (Lofts, 1974), in which case oviduct hypertrophy could be an estrogen effect. The present study was undertaken to investigate these possibilities.

To understand how T elicits growth, one must first determine if the growth is due to an androgen or estrogen effect. In this study possible interference from ovarian steroids and enzymes was avoided by using ovariectomized and immature *R. pipiens*. Dihydrotestosterone (DHT) was also administered to show any androgen effect without the possibility of conversion. The possibility of the oviduct containing an aromatase, so T could be converted to E locally in the tissue, was also considered.

It has been shown in immature salamanders (Norris and Moore, 1975; Guillette *et al.*, 1985) and ovariectomized newts (Kato *et al.*, 1986) that oviduct growth is greater when E and T are administered together. This suggests that priming by E may be necessary before T can have an effect. Intact frogs have both T and E circulating throughout the blood so the possibility that androgens have an effect in female frogs in combination with estrogen was examined.

In addition to measuring the oviduct weight, the effects of androgens and E on the chemical composition of the oviduct were examined. The oviduct is made up lay cells which secrete jelly to cover ovulated eggs before they exit the body. The is made of large glycoproteins that are fifty percent carbohydrate (Shivers and 1970). These glycoproteins vary in carbohydrate content in *R. esculenta* treated (Menghi *et al.*, 1987). If androgens have a different effect on the jelly be duced, the carbohydrate content of the E and T-treated frogs might be expected to differ.

MATERIALS AND METHODS

Freshly caught female frogs were obtained in the fall of 1989 and 1990 from a Wisconsin supplier (Kons Scientific). Frogs were maintained at room temperature, 21 ± 3 °C, in large tanks containing a wet and dry environment on a 14L:10D photoperiod. All frogs were fed crickets ad lib once a week.

Mature frogs were ovariectomized after being anesthetized with MS 222 (3-aminobenzoic acid ethyl ester). Frogs rested for three weeks following surgery to eliminate the effects of endogenous hormone.

Immature frogs were left intact. Upon autopsy, the ovaries of these frogs were examined to determine if the follicles were transparent. E is produced in the ovary by mature follicles and transparent follicles have probably not reached an E secreting stage in their development (Smalley, 1989). Having no previous exposure to E, immature frogs given E+T serve as a good test for priming.

Frogs were divided into two main groups, mature ovariectomized and immature. These two groups were further divided into 6 treatment groups which were injected as follows:

(1) 10 μg 17β-estradiol 3-benzoate (E)

(2) 100 μg testosterone propionate (T)

(3) 10 μg of E and 100 μg of T

(4) 100 μg of 5α-dihydrotestosterone (DHT)

(5) 10 μg of E and 100 μg of DHT

(6) 0.05 cc Mazola corn oil

The androgen dosages are ten times those of E because *R. pipiens* follicles are known to secrete approximately ten times as much androgen as estrogen (Smalley, 1989). The combinations of E and T in Group 3 and E and DHT in group 5 should test for any possible E priming. DHT can not be aromatized to E so any growth of the oviduct in Group 4 will indicate an androgen effect. Group 6 was injected with the vehicle, Mazola corn oil, to serve as a control.

The indicated dosages in oil were injected into the dorsal lymph sacs once a week for two weeks following the first injection and twice a week for two additional weeks. After five weeks frogs were pithed and the oviducts were removed. Oviducts were lyophilized to obtain dry weights.

The ability that the oviduct can aromatize T to E was tested by incubating oviduct tissue with T and measuring E production. Oviducts from 2-3 E, T and oil injected frogs (groups 1, 2 and 6) were incubated in wells containing 1.5 ml of amphibian saline (Petrino and Schuetz, 1986). Either 5 μl propylene glycol:ethanol (1:1) or 500 ng of T in this vehicle were added to each well as substrate. The tissue was incubated for 13 hours at 21.5 ± 1 °C. The medium was then removed and frozen for later analysis.

E in the medium was measured by radioimmunoassay (RIA) using antibody obtained from Wien Laboratories. According to the manufacturer, cross reactivity of this antibody with other steroids was less than 1% except for estradiol-16-one (8%), estrone (15%), and estrone-1-methyl (3%). The inter-assay coefficient of variation was 5.79% where n = 10. The intra-assay coefficient of variation was

2.82% where n = 4. To validate the antibody for this system, parallelism between the E standard and serial dilutions of medium was tested using a t-test.

Each dried oviduct was minced into a powder which was then divided for the assays. Oviduct protein concentration was measured from a homogenized portion of the dry oviduct using Bio-Rad protein assay (Bradford, 1976). Part of the oviduct was digested to remove protein. Total polysaccharides were precipitated and weighed (Menghi *et al.*, 1987). A biochemical assay for hexoses (Carroll *et al.*, 1956) was used to compare the hexose concentration of the total 'saccharides in different treatment groups. When tested, this assay indicated erage cross reactivity of 30% for fucose. Jelly obtained from ovulated eggs w ·d to validate the carbohydrate technique. Freshly secreted jelly should co vpical carbohydrate concentrations (Minganti, 1961).

lood sample was collected from each frog at the time of death. The ventra ce was opened and one side of the body cavity swabbed out. Frogs were tilt .nen the dorsal aorta was cut, so that blood pooled onto the body cavity wall. The blood was collected with a heparinized syringe and emptied into a heparinized tube in an ice bath. The tubes were centrifuged and the plasma collected and frozen. Blood plasma protein concentration was determined (Bradford, 1976).

All of these data were analyzed using a one-way analysis of variance (ANOVA). The Student-Newman-Keuls Multiple Range test was used to determine significance between groups.

RESULTS

Oviduct weights

Steroid hormone treatment in mature ovariectomized frogs caused significant oviduct growth (Fig. 1). The groups treated with T and T plus E were significantly larger than all other groups. Oviduct weights were only slightly smaller in the group treated with E alone.

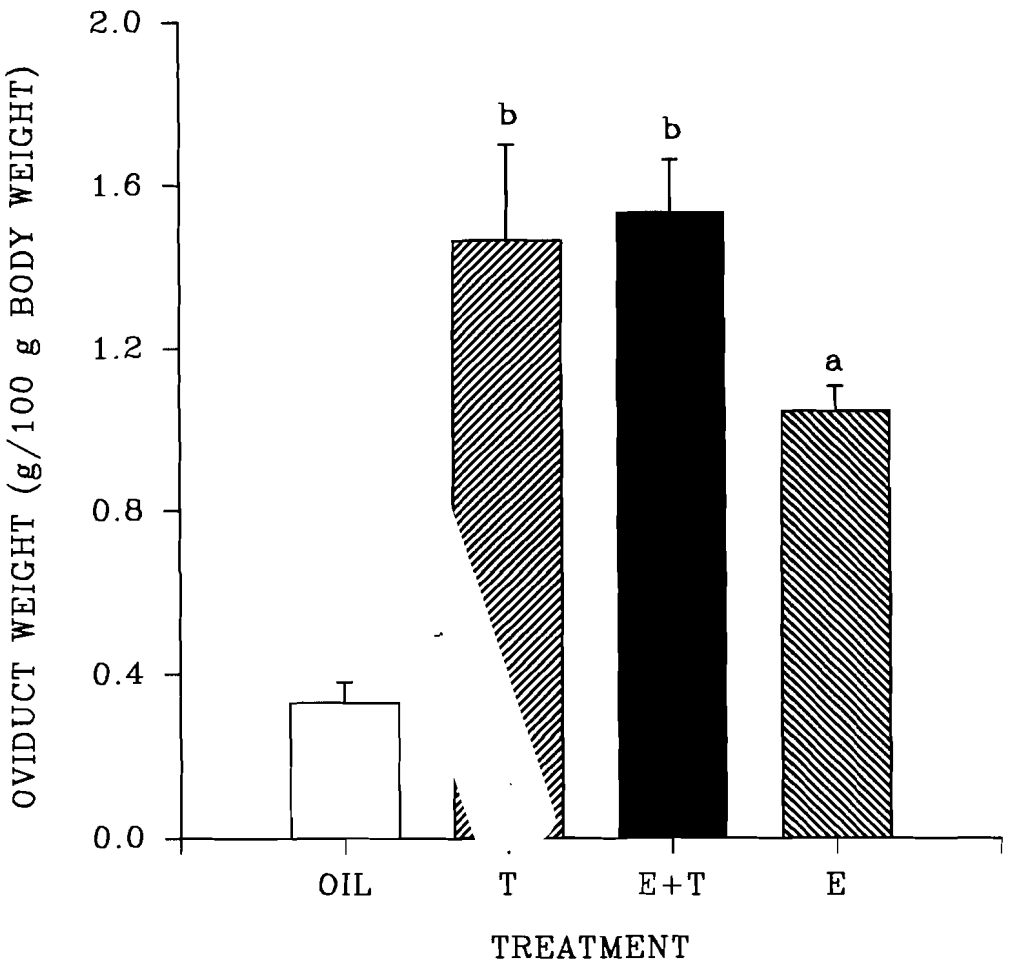
When immature frogs were injected with the same hormones similar results were obtained (Fig. 2). Oviduct weights from the E, T, and E+T groups were significantly r than those in the oil group.

Since ng T did result in oviduct growth it was necessary to determine if this was an en effect due to aromatization of T to E. To test for any androgen effec ire ovariectomized frogs were given DHT which cannot be converted to E. lts of treating frogs with DHT, E+DHT, or E alone are seen in Fig. 3. Th as no significant difference between the DHT group and the oil control. T + DHT group was not different from the E group. These data suggest that the effect seen with T is not an androgen effect but instead may be due to conversion.

Immature frogs were also injected with DHT and E+DHT and compared to E-treated animals (Fig. 4). In this case frogs treated with DHT had slightly larger oviducts than those treated with oil. The group treated with E+DHT was significantly larger than the DHT group.

Since T does cause hypertrophy of frog oviducts in both ovariectomized and immature animals, and since DHT appears to have little or no effect, it is

Fig. 1. Mean dry oviduct weights from mature ovariectomized frogs treated with oil (7), T (5), E+T (5), or E (5). Number of animals is shown in parentheses. Error bars represent ± SEM. [a] Significantly larger than oil at $P<0.05$. [b] Significantly larger than oil and E at $P<0.05$.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0.8
0.4
0.0
b
b
a
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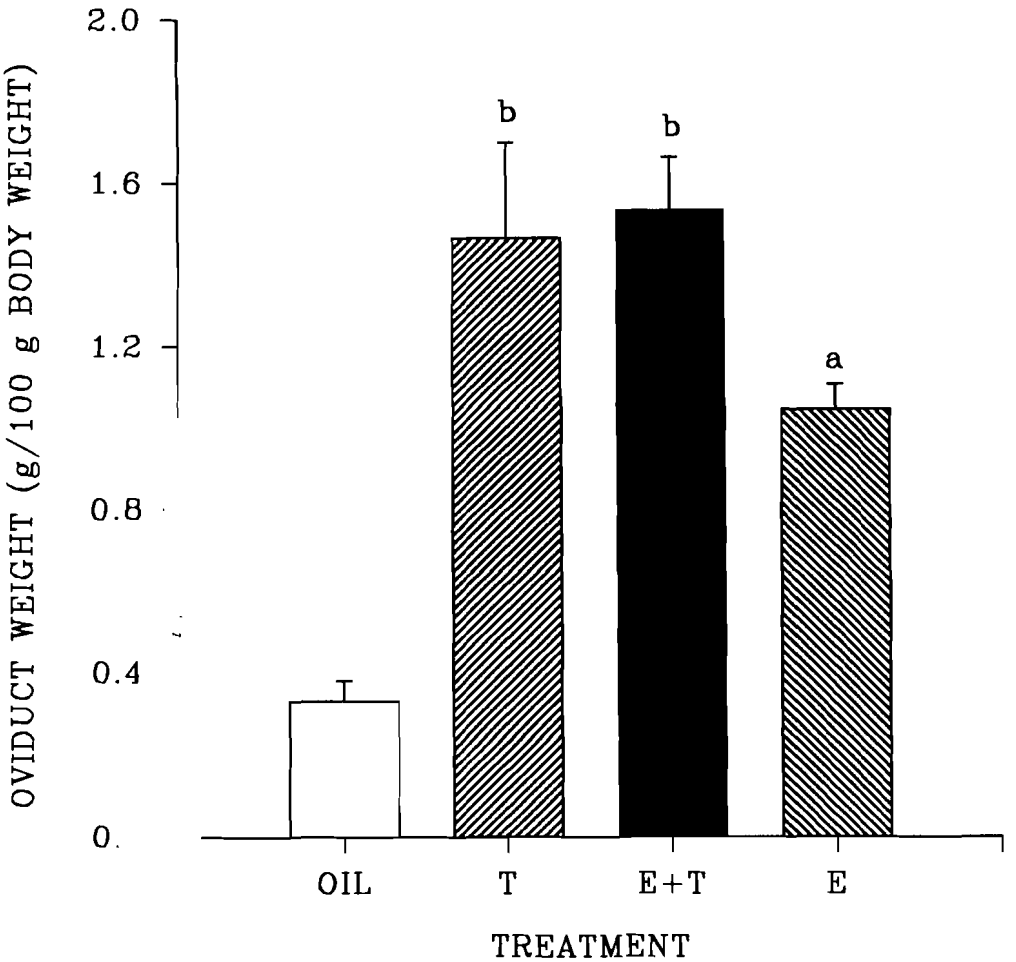
T
E+T
E
TREATMENT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1.2
0.8
0.4
0
b
b
a
OIL
T
E+T
E
TREATMENT

Fig. 2. Mean dry oviduct weights from immature frogs treated with oil (5), T (4), E+T (5), or E (4). Other conventions as in Fig. 1. [a] Significantly larger than oil at $P<0.05$.

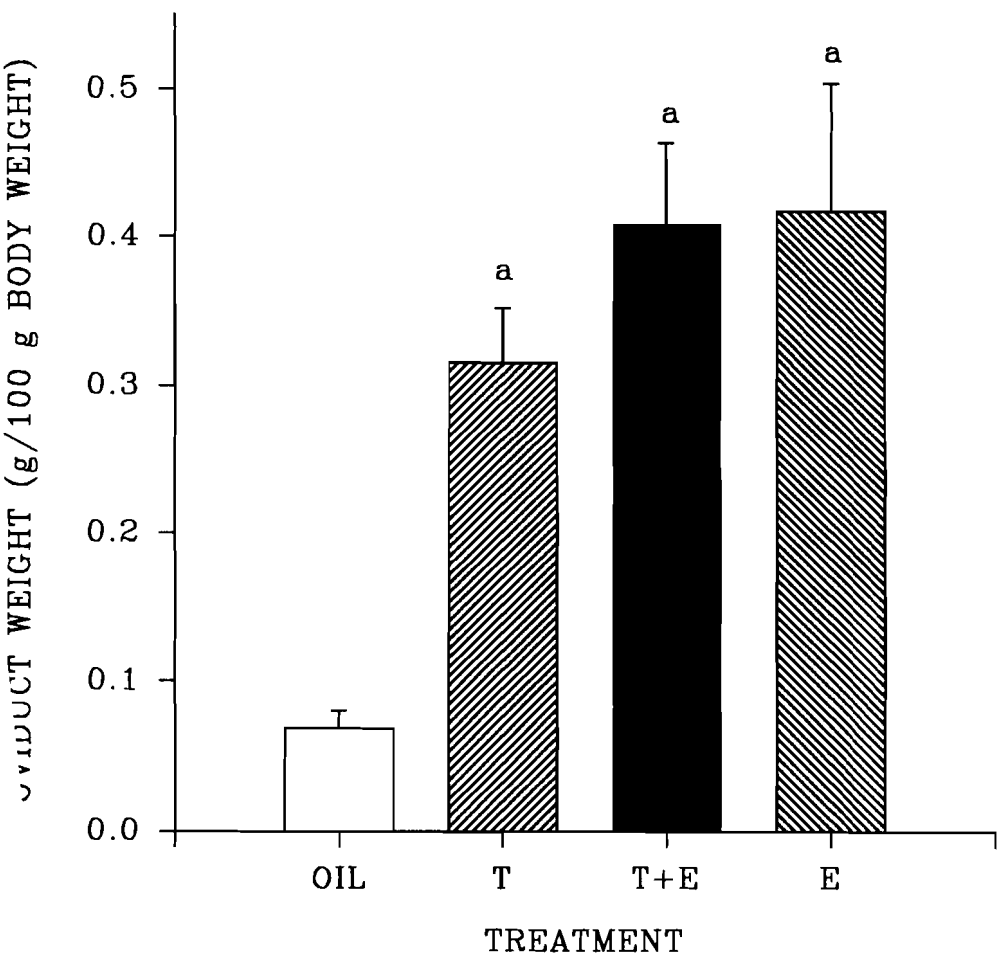
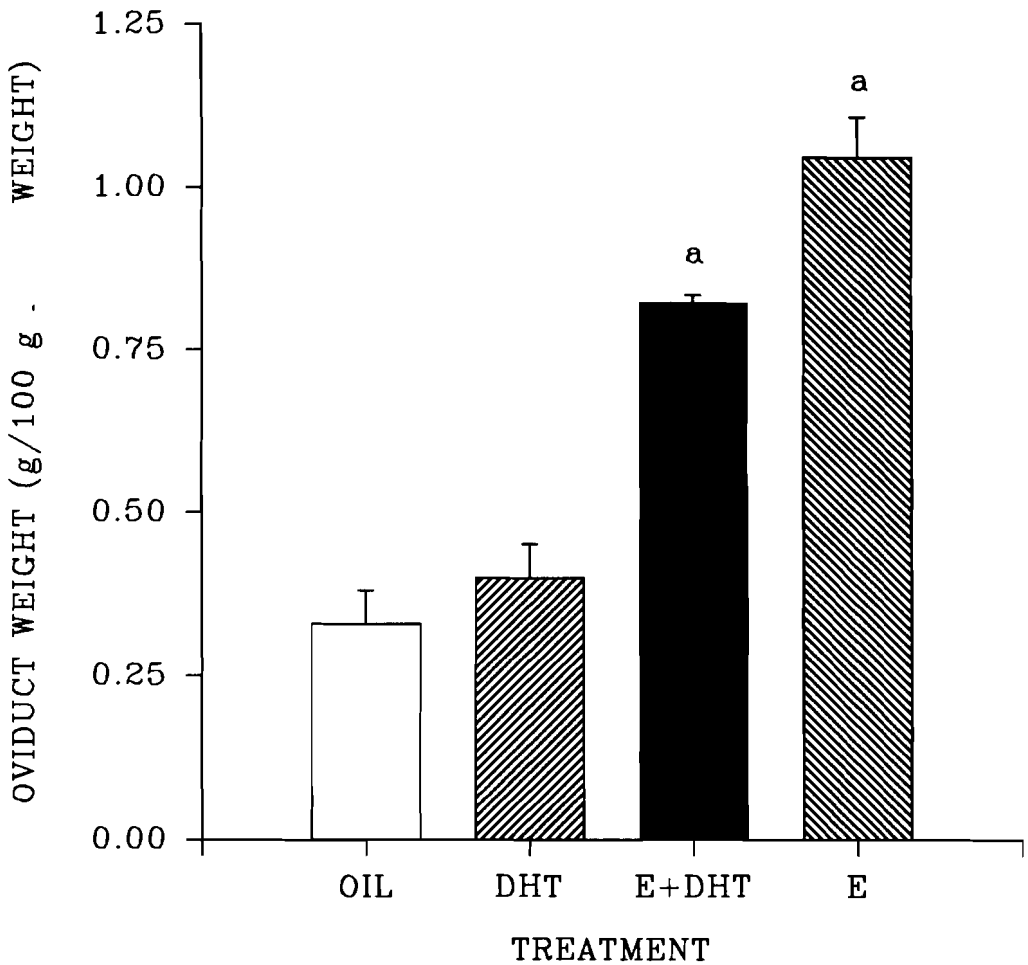

0.5
0.4
0.3
0.2
0.1
0.0
a
a
a
OIL
T
T+E
E
TREATMENT

Fig. 3. Mean dry oviduct weights from mature ovariectomized frogs treated with oil (7), DHT (7), E+DHT (5), or E (5). Other conventions as in Fig. 1.
[a] Significantly larger than oil at $P<0.05$.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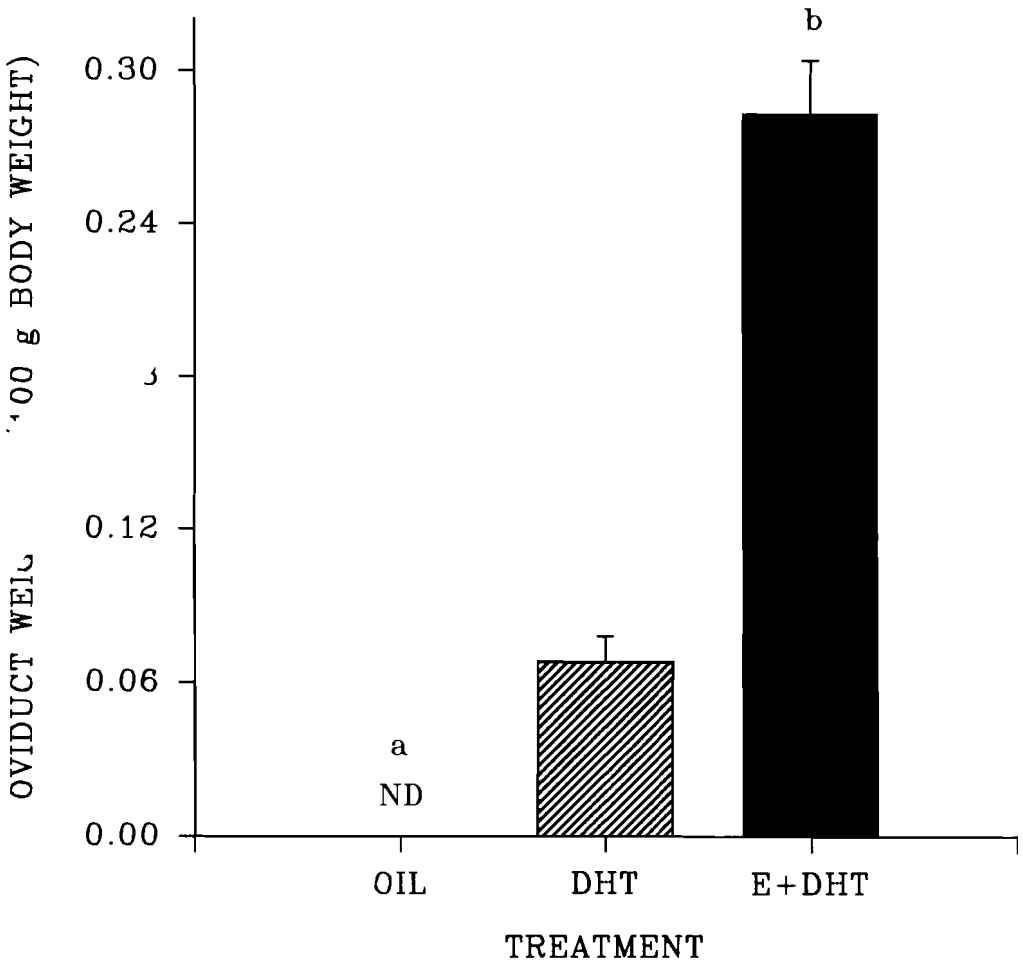
0.75
0.50
0.25
0.00
OVIDUCT WEIGHT (g/100 g . WEIGHT)
a
a
OIL
DHT
E+DHT
E
TREATMENT

Fig. 4. Mean dry oviduct weights from immature frogs treated with oil (9), DHT (4), or E+DHT (9). Other conventions as in Fig. 1. [a] ND, not detectable. [b] Significantly larger than DHT at $P < 0.05$.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0.24
0.12
0.06
0.00
a
ND
OIL
DHT
E+DHT
TREATMENT

possible that the oviduct contains an aromatase. To test this possibility, oviducts from mature ovariectomized frogs injected with either oil, T, or E were incubated with and without T. E levels in the wells containing T were significantly greater than those with tissue alone (Table 1). No difference between tissue from frogs injected with oil, T, or E was seen. Estradiol measured in the wells without T was at the level of detection.

Since it appears that T is converted to E, it is possible that the high dosages of T could account for the greater oviduct weights found in T-treated mature ovariectomized frogs. To test this possibility, two different dosages of E were injecte[illegible]nto ovariectomized mature frogs. One group was given 10 μg per dose as b[illegible] while the other was given 40 μg per dose. The results in Fig. 5 show n[illegible]rence in oviduct weight between these two groups. This suggests that [illegible] achieved its maximum growth effect at 10 μg and that the additional gr[illegible]ı the T group might be due to an androgen difference.

[illegible]ct content

The protein content from the immature oil and DHT groups were significantly greater than all other immature groups (Table 2). The remaining immature groups were not different from each other. Protein content of oviducts from mature frogs did not vary significantly (Table 2).

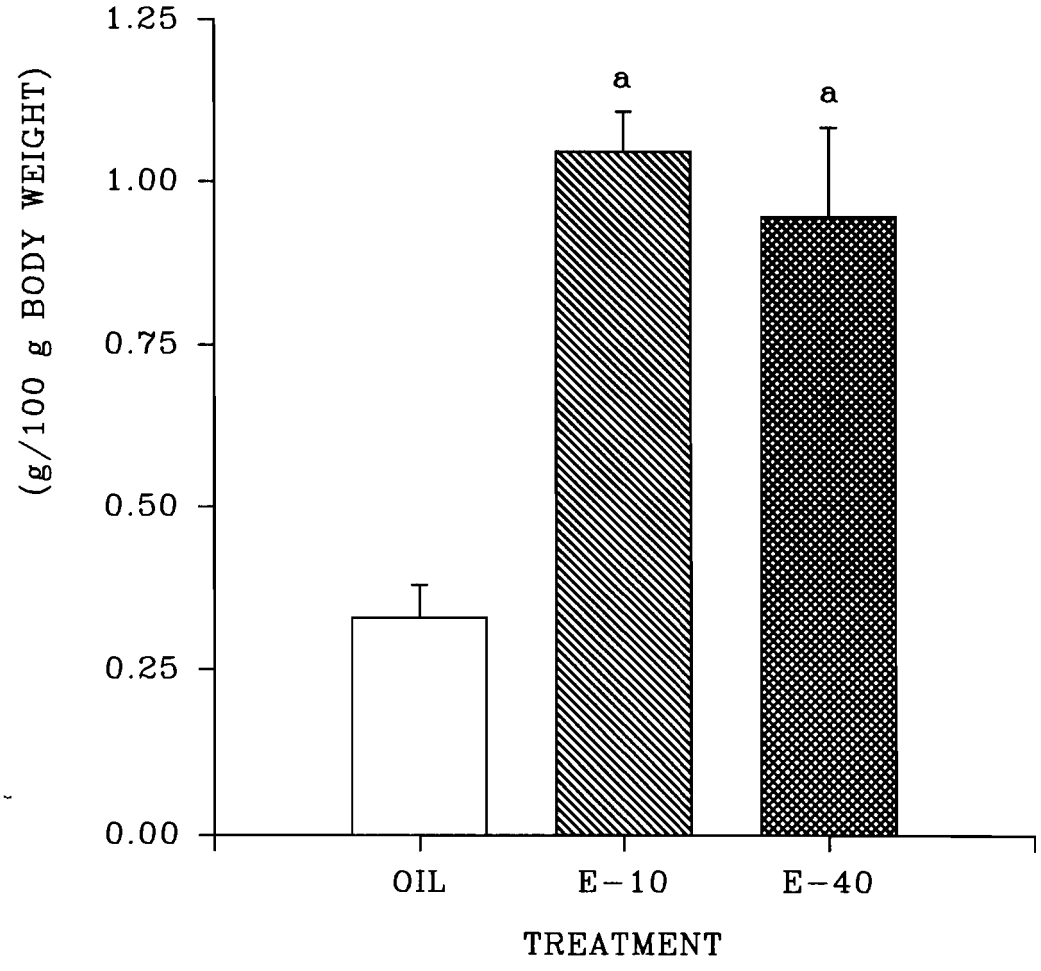
The total polysaccharide concentration from the mature ovariectomized oviducts is shown in Table 3. All of the groups were the same with the exception of the DHT group which contained a significantly smaller amount of total

Table 1. E (pg/mg tissue) levels in the incubation medium of oviducts taken from mature ovariectomized frogs injected with oil, T, or E.

	Substrate	
tion	No T [a,b]	T (500 ng) [a]
3)[c]	2.60 ± 0.47 (11) [d]	59.62 ± 5.57 (12) [e]
(3)	10.65 ± 2.48 (11)	65.80 ± 2.60 (10) [e]
(2)	5.00 ± 0.72 (12)	54.28 ± 7.55 (10) [e]

Mean ± SEM
5 μl of 1:1 propylene glycol:ethanol (vehicle)
number of animals in group
number of incubation wells
significantly different from vehicle at $p<0.05$

5. Mean dry oviduct weights from mature ovariectomized frogs treated with
7), 10 μg E (7), or 40 μg E (5). Other conventions as in Fig. 1.
gnificantly larger than oil at $P < 0.05$.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1.00
0.75
0.50
0.25
0.00
(g/100 g BODY WEIGHT)
a
a
OIL
E-10
E-40
TREATMENT

Table 2. Percent protein of dry oviducts from immature and mature ovariectomized frogs in each treatment group.

Treatment Group	Immature [a]	Mature [a]
Oil	31.39 ± 2.57 (5) [b,c]	10.89 ± 1.39 (5)
DHT	31.04 ± 4.21 (4) [c]	15.71 ± 0.58 (6)
E+DHT	17.74 ± 1.07 (9)	13.68 ± 1.15 (5)
E	13.62 ± 2.07 (4)	12.60 ± 1.50 (7)
E+T	15.86 ± 2.12 (5)	11.12 ± 0.49 (5)
T	16.16 ± 2.70 (4)	12.44 ± 1.86 (5)

[a] Mean ± SEM
[b] Number of animals
[c] Significant difference from other immature groups at $p<0.05$

Table 3. Total polysaccharide (Pol.) and hexose content of oviducts from mature ovariectomized frogs in each treatment group.

Treatment Group	N [a]	% Pol. of dry weight [b]	% Hexose of Pol. [b]
Oil	5	42.92 ± 2.47	16.76 ± 0.82
DHT	6	35.92 ± 1.87 [c]	16.43 ± 0.38
E+DHT	5	45.92 ± 2.39	16.74 ± 0.39
E	7	47.90 ± 2.65	16.96 ± 0.30
E+T	5	50.36 ± 2.20	16.96 ± 0.85
T	5	47.25 ± 3.02	17.08 ± 0.17

[a] N, number of animals
[b] Mean ± SEM
[c] Significant difference from other groups at $p<0.05$

polysaccharide than the others. Hexose content of the total polysaccharides was not significantly different in any group (Table 3). Protein, polysaccharide, and hexose content from frogs injected with 10 μg and 40 μg of E did not differ.

Blood proteins

Plasma was collected from all of the frogs injected. When plasma proteins in these frogs were examined no significant difference between mature and immature animals was found so the data were pooled (Fig. 6). Groups treated with T and DHT were not different from the controls. The E+DHT group was significantly larger than the oil and DHT groups, but was smaller than E alone. The E and E+T groups had the highest levels of plasma proteins.

Fig. 6. Mean plasma protein concentrations from frogs in each treatment group: oil (21), DHT (11), E+DHT (17), E (11), E+T (10), and T (9). Ovariectomized and immature frogs were pooled. Other conventions as in Fig. 1.
[a,b,c] Treatments with different superscripts are significantly different at $P<0.05$.

DISCUSSION

Although many investigators have noted a paradoxical growth effect in the amphibian oviduct due to T (reviewed by Norris, 1987), the exact mechanism of action has not been explained. This study examined the action of T on the oviduct in immature and ovariectomized *R. pipiens*. These frogs did not have functional ovaries present which could interfere with the interpretation of the effects of injected estrogen and androgens.

After confirming that T causes hypertrophy of the oviduct (Figs. 1 and 2), DHT was administered as an androgen which could not be converted to E. When DHT did not have the same effect as T (Figs. 3 and 4), oviduct tissue was incubated to look for an aromatase. These incubations indicated that the oviduct was capable of converting T to E (Table 1).

The presence of an aromatase in the oviduct would allow for localized conversion and oviduct growth when E levels were low but T levels were high. These conditions have been observed in *R. esculenta* during the breeding season (D'Istria *et al.*, 1974). In addition, Licht *et al.* (1983) observed a correlation between oviduct weight and blood T, but not E, levels in *R. catesbeiana*. Furthermore, Smalley (1989) in *R. pipiens* and Fortune (1983) in *Xenopus laevis* have reported that follicles in the later stages of development secrete high levels of T and very little E.

These observations suggest that high blood androgen levels help maintain the oviduct by providing a substrate for the aromatase. However this may not be true in all species, because Delrio *et al.* (1979) found no T effect on the oviduct in

R. esculenta. In addition they found no conversion of T to E in this tissue. Also, Iwasawa and Kobayashi (1974) found minimal effects of T in immature *R. nigromaculata.*

Another explanation of T's effect on the oviduct is that T and E both act on the same receptor (Norris, 1987). This is supported by the fact that Kato *et al.* (1986) were unable to distinguish between T and E binding sites in a newt oviduct.

In addition to working through an aromatase system, androgens may also have a direct effect on the oviduct. Evidence for this in the present study is that there was a slight increase in growth in the immature animals treated with DHT (Fig. 4). This is similar to the effects seen by Norris *et al.* (1991) in immature salamanders. Delrio *et al.* (1979) also observed a small DHT effect in oviducts of ovariectomized *R. esculenta.* Since DHT cannot be converted to E these could be androgen effects.

Evidence for an androgen effect in the mature ovariectomized animals in the present study is questionable. No differences were found between the effects of T and E in the oviduct, except for their weights. Testosterone and E+T frogs had larger oviducts than those treated with E alone (Fig. 1). This could be due to the ten-fold higher dose of T. If T was converted to E, the animals could be receiving E dosages higher than 10 μg. However, when a higher dose of E (40 μg) was given, additional hypertrophy was not seen (Fig. 5). This may imply that the oviduct has reached a maximum E growth effect at 10 μg. If that is the case

the additional growth by T and E+T could possibly be an androgen effect.

When total polysaccharides and hexoses (Table 3) were examined, there were no differences between E and T. This supports the contention that T does not have an independent effect. The DHT data also suggest no androgen effect since DHT-treated animals did not differ from oil controls except for a slight decrease in total polysaccharides (Table 3).

The only difference in total protein content was seen in the immature animals (Table 2). Immature animals treated with oil and DHT had significantly more protein than other groups. The oviducts in these frogs, at death, were very small and appeared to contain very little or no jelly. Groups given any T or E had larger oviducts filled with large quantities of jelly. When present, the large carbohydrate concentration of jelly would reduce the total protein content in an oviduct.

These protein and hexose data differ from those found in *R. esculenta* (Menghi, *et al.*, 1987). They found that both protein and hexose content in E-treated frogs were significantly different from their non-treated animals. One explanation for the difference in this present study is sample size. My groups were considerably smaller than those used by Menghi, *et al* (1987). This means that there was considerably less tissue available for testing, leading to greater variation in the data.

It would have been interesting to compare DNA and protein content of each oviduct, but it was not possible to measure the DNA with any precision.

Apparently the monosaccharides of the jelly glycoproteins were detected by the DNA tests. Using the diphenylamine method (Bradshaw, 1966), jelly alone appeared to have between five and six percent DNA content. When using UV absorbance (Bradshaw, 1966), a similar problem occurred. Part of the problem is that jelly precipitates out with DNA, so it was not possible to separate the two.

The effect of T on blood proteins appears to be quite different from its effects on the oviduct (Fig. 6). Groups injected with T or DHT have significantly lower total plasma proteins than groups injected with E. When E is combined with DHT the plasma protein levels are lower than with E alone. Although T+E was not significantly lower than E alone, there was a trend toward a lower protein content. These data suggest that androgens inhibit the production of one or more plasma proteins when combined with E. This inhibition, unlike T effects seen in the oviduct, appears to be clear evidence of an androgen effect.

Several studies have noted the effects of T on oviducts in various amphibians. These effects have differed from species to species. In *R. pipiens*, hypertrophy of the oviduct by T is most likely due to conversion of T to E within its tissue. Even though a conversion occurs, this does not explain all of the results. These differences imply that direct comparisons between species do not always work. This especially applies to the role of E and T in the oviduct.

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LITERATURE CITED

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