THE MORPHOLOGY, HISTOLOGY AND FUNCTION OF THE SEMINAL VESICLES IN THE ADULT MALE FROG, RANA PIPIENS

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
PAMELA KAY McALLISTER
1973

This is to certify that the

thesis entitled

The Morphology, Histology and Function of the Seminal Vesicles in the Adult Male Frog, Rana pipiens.

presented by

Pamela Kay McAllister

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Zoology

John R. Shaver
Major professor

Date June 13, 1977

O-7639

ABSTRACT

THE MORPHOLOGY, HISTOLOGY AND FUNCTION OF THE SEMINAL VESICLES IN THE ADULT MALE FROG, *RANA PIPIENS*

By

Pamela Kay McAllister

The seminal vesicles in *Rana pipiens* are multiple and branched evaginations of the Wolffian ducts consisting of a layer of pseudostratified columnar epithelium surrounded by connective tissue. The secretory products of the seminal vesicles were seen to contain neutral mucopolysaccharides whereas acid mucopolysaccharides were absent.

Several experiments revealed that materials from the seminal vesicles are able to prolong the fertilizable life of spermatozoa which are stored at refrigerator temperatures. Low levels of fertility were seen if sperm which have been incubated with seminal vesicle homogenate were washed off the eggs after short periods of time. If sperm remained on the eggs for longer periods of time, high levels of fertility were seen. High levels of fertilizability were obtained even if sperm were washed off shortly after insemination if the sperm were exposed to diffusible factors obtained from freshly ovulated eggs before their use in fertilization. Results suggest that the seminal vesicles may be the source of a factor or factors which may bind to the sperm thereby rendering them incapable of fertilizing and that one role of the egg jellies may be to capacitate the sperm. This may

be of significance in natural fertilization in that sperm may thereby be maintained in a fertilizable state during the time they are stored in the seminal vesicles.

THE MORPHOLOGY, HISTOLOGY AND FUNCTION OF THE SEMINAL VESICLES IN THE ADULT MALE FROG, *RANA PIPIENS*

By

Walsworth

Pamela Kay^McAllister

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Zoology

1973

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to my major professor, Dr. John R. Shaver, for his guidance and constructive criticism during my graduate work and his help in the preparation of this manuscript.

I also acknowledge and thank the members of my guidance committee, Drs. W. R. Dukelow, H. Ozaki, and S. Aggarwal, for their helpful suggestions for this thesis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF PLATES

LIST OF TEXT FIGURES

LIST OF APPENDIX TABLES

INTRODUCTION

The Morphology and Histology of the Seminal Vesicles in Anuran Amphibians

Although considerable attention has been directed towards understanding the conditions necessary for fertilization in anuran amphibians, including the role of the oviductal secretions and egg jellies, little information is available on the role that the male accessory glands, the seminal vesicles, may play. The seminal vesicles are generally described as outgrowths of the *vas deferens* or Wolffian duct and have been described as the site of sperm storage prior to ejaculation.

Seminal vesicles have been described in a number of anuran species including *Discoglossus pictus* (Mann, Lutwak-Mann and Hay, 1963), *Rana esculenta, Rana temporaria* (Aron, 1926), *Hyla crucifer* and *Rana pipiens* (Rugh, 1934 and 1939). In histological sections transverse to the long axis the seminal vesicles of *D. pictus* were seen to consist of a thick epithelial layer supported by a thin layer of connective tissue. The epithelium consists of three types of cells: basal cells, long columnar cells containing PAS-positive granules, and small cells surrounding the lumen. The fluid within the seminal vesicles is hypotonic and contains a high content of glycoprotein.

The seminal vesicles of *R. esculenta* and *R. temporaria* are described as enlargements of the distal one-third of the Wolffian duct. Histological sections show them lined with a simple epithelium of mucous cells which invaginate into the connective tissue forming pseudo-glands. During the breeding season the lumina within the seminal vesicles are filled with sperm. At this time the glandular evaginations are larger and more complex. The seminal vesicles of *H. crucifer* and *R. pipiens* are described only as enlargements of the posterior portions of the Wolffian ducts.

Functions of the Seminal Vesicles in Anuran Amphibians

Although the functional significance of the seminal vesicles in anuran amphibians is unknown, several suggestions have been made.

In *R. pipiens* spermatozoa are stored in the seminal vesicles and are expelled during amplexus. Spermatozoa removed from various portions of the male reproductive tract have been found to be functional in regards to fertilizing capacity (Rugh, 1939). In *H. crucifer* the seminal vesicles are not only a storage place for spermatozoa but also may be copulatory accessories, since in *Hyla* as in toads each egg is inseminated singly and the seminal vesicles together with the muscular cloaca supply each egg with a small amount of spermatozoa (Rugh, 1941).

In addition to their storage function the seminal vesicles may be the source of seminal fluids which serve as a vehicle for the passage of sperm to the outside (Mann, 1964; Mann, Lutwik-Mann and Hay, 1963).

Effects of Hormones on the Seminal Vesicles of Anuran Amphibians

The endocrine control of Wolffian duct development has been best studied in *R. temporaria*. The duct is formed independently of the presence or absence of male or female hormones. Later, towards the end of the larval period, frogs treated with androgen display hypertrophy of the Wolffian ducts and develop a seminal vesicle. The sensitivity to testosterone follows a decreasing caudo-cephalic gradient (Gallien, 1955).

In adult *R. pipiens* and *Bufo americanus* the Wolffian ducts including the seminal vesicles hypertrophy following treatment with testosterone propionate (Puckett, 1939). Sperm release from the testes and enlargement of the Wolffian ducts and seminal vesicles has been reported in *R. pipiens* and *H. crucifer* following treatment with pituitary gonadotropins (Rugh, 1939, 1941). Regression of the Wolffian ducts, including a decrease in diameter of the ducts and a decrease in the height of the epithelium, has been observed in *R. esculenta* following hypophysectomy (Sluter, van Oordt and Mighorst, 1950). Transplantation of testicular tissue into castrated male frogs has been reported to induce development of nuptial pads and seminal vesicles (Parkes, 1960).

The Seminal Vesicles in Other Non-Mammalian Vertebrates

Seminal vesicles have also been described in a number of other non-mammalian vertebrates. In teleost fish seminal vesicles have been described as fan-shaped glandular structures lined with typical secretory epithelium. They are believed to produce a fluid resembling mammalian prostatic and vesicular secretions, which may have the function of providing nutrients for the spermatozoa or may secrete a substance which maintains the sperm in an immobilized state (Mann, 1964). Histochemical and biochemical studies on the secretions of the seminal vesicles of the catfish, *Heteropneustes*, indicate the presence of mucoproteins, mucopolysaccharides, proteoses, phospholipids and native proteins (Nayyar and Sundararaj, 1970). Seminal vesicle weights were found to decrease following treatment with an anti-androgen, cyproterone acetate (Sundararaj and Nayyar, 1969). Eggs were successfully fertilized using testis squash with or without seminal vesicles indicating that the seminal vesicles are not essential to fertilization. Thus, while the seminal vesicles are not essential, the seminal fluids probably help in natural fertilization in that they maintain the sperm in an inactive but viable condition (Sundararaj, 1958).

Although seminal vesicles have not been described in reptiles, hypertrophy and secretory activity has been demonstrated in a variety of male lizards and snakes to include the preterminal and terminal segments of the urinary tubules, the collecting canal, and the ureter. The femoral glands, epididymides and *vasa deferentia* are all affected by castration. The *vas deferens* in castrated males of *Anolis carolinensis* were reported to undergo a 63% reduction in diameter when compared to sexually active males and a 37% reduction when compared to sexually quiescent males. Reduction in the height of the epithelial cells lining the *vas deferens* was also reported (Parkes, 1960).

Seminal vesicles have also been reported in passerine birds as swollen portions of the *vas deferens* and are considered to be sites of sperm storage. The seminal vesicles are small during periods of sexual inactivity but enlarge and become highly convoluted during the breeding season (Mann, 1964). During the height of the breeding season the sperm-laden seminal vesicles descend into cloacal protuberances where the temperature is lower than body temperature. Thus the major function of the seminal vesicles in birds as well as in fish may be to maintain the sperm in a viable state until intromission (Parkes, 1960).

The Seminal Vesicles in Mammals

The seminal vesicles in mammals, as in non-mammalian vertebrates with the exception of fishes, are glandular developments of the Wolffian ducts and are dependent on androgens for the maintenance of the epithelium and secretory activity. In some species the secretions of the seminal vesicle contribute a substantial portion to the whole ejaculate, although the secretory output of the seminal vesicles varies considerably and in some species may contribute relatively little volume of the total ejaculate. Seminal vesicles are totally absent in some species such as the dog and cat.

In addition to providing the fluid medium suspending the sperm, the male accessory glands in mammals may be the source of sperm-coating antigens which may have significance in fertilization. The need for a uterine incubation period for spermatozoa (capacitation) was first demonstrated in 1951 (Austin, 1951; Chang, 1951). It was subsequently demonstrated that capacitation could be functionally reversed (decapacitation) by a substance from the seminal plasma referred to as decapacitation factor (Chang, 1957).

In one study utilizing immunofluorescence a single sperm-coating antigen was demonstrated in the human and in the rabbit which was firmly attached to ejaculated sperm. The origin of this antigen was the seminal vesicle (Weil and Rodenberg, 1962). Subsequent studies demonstrated the presence of several sperm-coating antigens of accessory gland origin. Ejaculated sperm did not fluoresce when exposed to antibodies against testicular sperm, indicating that at ejaculation the sperm are coated with seminal plasma antigens which block the sites with which antibodies against testicular sperm would interact (Hunter and Nornes, 1969; Johnson and Hunter, 1972). Two of the sperm-coating antigens were demonstrated to be glycoproteins and to have activity similar to that of decapacitation factor since they were able to block fertilization when sperm were incubated with them (Hunter, 1969).

The present investigation was begun for the purpose of elucidating the functional significance of the seminal vesicles in male *Rana pipiens* since in amphibians, as in mammals, the seminal vesicles may be the source of sperm-coating antigens which may alter the fertilizing capacity of the sperm. For this purpose a series of experiments were performed in which sperm were incubated with a variety of substances, including materials from the seminal vesicles, to determine whether they could influence the fertilizing capacity of the sperm when they were used to fertilize normal jellied eggs. Additionally, seminal vesicles from castrated animals were compared to those from intact animals as to their ability to alter the fertilizing capacity of testicular sperm. The histology and cytochemistry of the seminal vesicles from intact and castrated animals were also compared.

METHODS AND MATERIALS

Animals Used

Adult male and female *Rana pipiens* were obtained from a commercial dealer (Bay Biologicals, Ltd., Port Credit, Canada). The animals were kept in hibernation until use (4-6°C.).

Preparation of Sperm Suspensions

Male frogs were pithed and the testes and seminal vesicles were removed. In some experiments the brains and male rudimentary oviducts were also removed. The tissues were placed in 10% Holtfreter's solution over ice until all dissections were complete. The testes were crushed with forceps, filtered through glass wool, and the resulting suspension diluted with 10% Holtfreter's solution to give a concentration of about 2000 sperm/mm^3. A hemocytometer was used in making the sperm counts. Aliquots of the sperm suspension were then mixed with equal volumes of filtered tissue homogenate or 10% Holtfreter's solution giving a final concentration of 1000 sperm/mm^3. The suspensions were stored for varying times in the refrigerator until use in fertilization experiments. Tissue homogenates were prepared by homogenizing in a glass homogenizer with 10% Holtfreter's solution (1gm. tissue/10ml. Holtfreter's).

In one set of experiments the sperm suspensions were mixed with an equal volume of egg water prior to use. In these experiments the sperm suspension was initially diluted to a concentration of 4000 sperm/mm^3.

Aliquots were mixed with a 20% tissue homogenate. Egg water was prepared by extruding freshly ovulated eggs into a dish and covering them with 10% Holtfreter's solution. One hour later the Holtfreter's solution was pipetted off and was mixed with the sperm suspensions which had been removed from the refrigerator and allowed to come to room temperature. This gave a final sperm concentration of 1000 sperm/mm^3 as in the other experiments. The sperm concentration was again checked for each suspension just prior to use in fertilization experiments.

Eggs were obtained by injecting mature females with progesterone and frog pituitaries (Wright and Flathers, 1961). Eggs were extruded onto microscope slides and inseminated. An average of 25-50 eggs were fertilized from each female for each time period. At varying times after insemination the sperm were poured off the slides and the slides were immersed in a large volume of aerated tap water. Three to five hours later the percentage of cleaving eggs was determined and used as the criterion for fertilization. For statistical analysis, the percentage of cleaving eggs was transformed by the angular transformation (θ = arc sin $\sqrt{p.}$).

Castration of Frogs

Frogs were castrated under ether anesthesia. An incision was made in the lateral body wall midway between the pectoral and pelvic girdles. The testis on that side was pulled through the incision, the mesorchium was sutured to close the testicular blood vessels and the testis removed. The incision was sutured and the testis on the other side was then removed by the same procedure. The frogs were kept at room temperature until use.

Histological Procedures

The seminal vesicles were fixed in Smith's modification of Bouin's fixative (Guyer, 1947), dehydrated, cleared, and embedded in paraffin. One seminal vesicle from each animal was sectioned at 10 microns and stained with hematoxylin and eosin for routine histological examination. The other seminal vesicle from each animal was sectioned at 5 microns and stained for carbohydrates and proteins. The periodic acid-Schiff (PAS) procedure for neutral mucopolysaccharides was used both with and without prior treatments to determine more accurately the nature of the PAS-positive compounds. Schiff's reagent was prepared by the method of de Tomasi (Pearse, 1970). PAS-reactive protein was removed by incubation of sections at 37°C. for 1 hour with trypsin (0.1% in 0.1M sodium phosphate buffer at pH 7.4). Control sections were incubated in buffer only. A 1:1 solution of chloroform and methanol was used to remove PAS-positive lipids. Glycogen was removed from some sections by incubating for 1 hour at 37°C. in a 0.1% malt diastase solution (Nutritional Biochemicals Corp.) in 0.02M sodium phosphate buffer at pH 6.0 containing 0.65% NaCl. The specificity of the reaction was determined by acetylation with a 2:3 mixture of acetic anhydride and pyridine for 2 hours at 60°C. and also by placing slides directly into Schiff's reagent without periodic acid oxidation (Pearse, 1970).

Acid mucopolysaccharides were determined by staining with alcian blue at pH 2.5 (Barka and Anderson, 1963; Mowry, 1963).

The ninhydrin-Schiff procedure was used for the detection of protein. The specificity of the reaction was determined by omitting the ninhydrin oxidation step and also by incubating some sections in a solution of 1% acetic acid in acetic anhydride for 1 hour at 60°C (Umpierre, 1971).

RESULTS

Morphology and Histology

The seminal vesicles in *Rana pipiens* are glandular swellings of the posterior portions of the Wolffian ducts and are enclosed in a connective tissue sheath along with the posterior portions of the male rudimentary oviducts. The urogenital system of an adult male *Rana pipiens* is seen in Plate I, Figure 1, and is illustrated diagrammatically in Text Figure 1. The sperm after leaving the testes pass through the *vasa efferentia* and into the anterior tubules of the kidneys. The sperm then pass into the Wolffian ducts and are stored in the seminal vesicles. During amplexus the sperm pass into the cloaca and are emitted over the eggs (Rugh, 1939).

In transverse sections the seminal vesicles are seen to consist of multiple and highly branched evaginations of the Wolffian duct. Surrounding the lumina of the seminal vesicles and of the Wolffian duct is a pseudo-stratified columnar epithelium. A layer of connective tissue surrounds the epithelium (Plate II, Figure 2).

Five weeks after castration, seminal vesicles were removed from four castrated and four sham-operated controls. Seminal vesicles together with the adjacent portions of the oviducts were removed, weighed, and fixed. One seminal vesicle from each animal was sectioned for routine histological examination and the other was stained for carbohydrates and proteins. Five weeks after castration the height of

Figure 1. The urogenital system of an adult male *Rana pipiens*.

Key to Abbreviations

B - Urinary Bladder

Cl - Cloaca

FB - Fat Body

K - Kidney

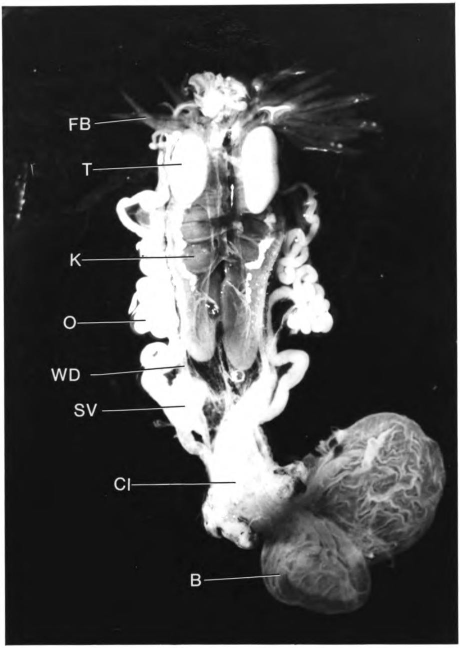
O - Oviduct

SV - Seminal Vesicle

T - Testis

WD - Wolffian Duct

PLATE I



TEXT FIGURE I

Diagrammatic representation of the urogenital system of an adult male *Rana pipiens*.

Key to Abbreviations

B - Urinary Bladder

C - Colon

FB - Fat Body

K - Kidney

O - Oviduct

SV - Seminal Vesicle

T - Testis

VE - *Vasa Efferentia*

WD - Wolffian Duct

TEXT FIGURE I

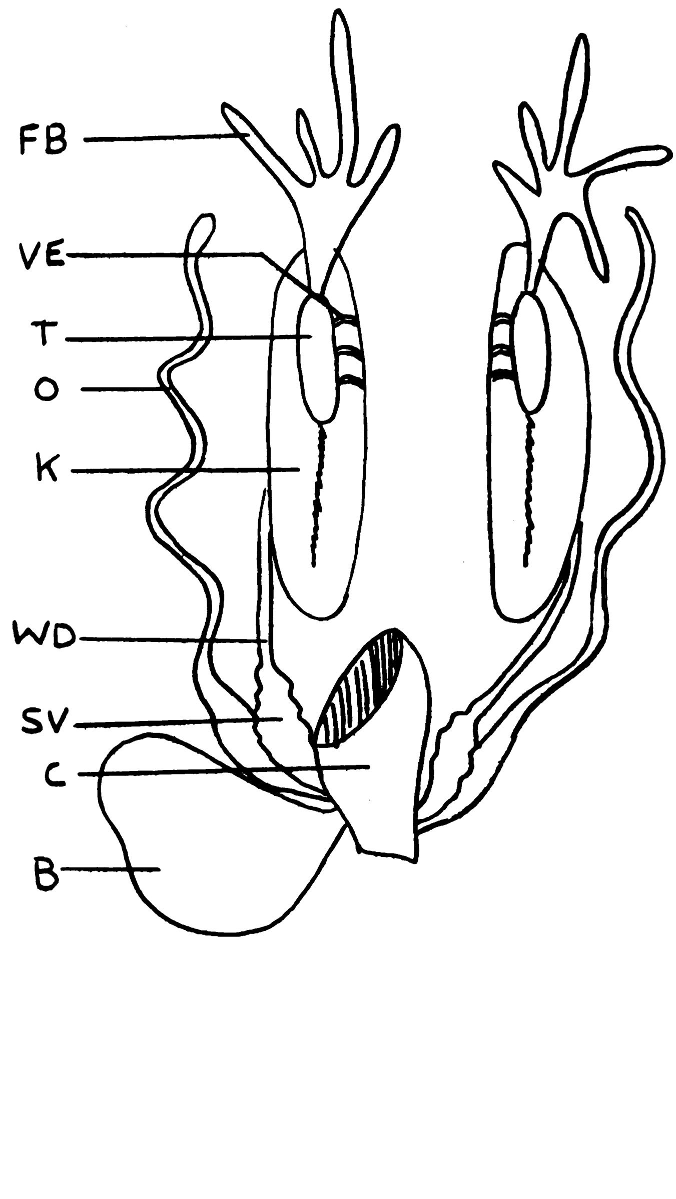


Figure 2. A representative cross section through the seminal vesicle of an intact frog.

Figure 3. A representative cross section through the seminal vesicle of a castrated frog.

PLATE II

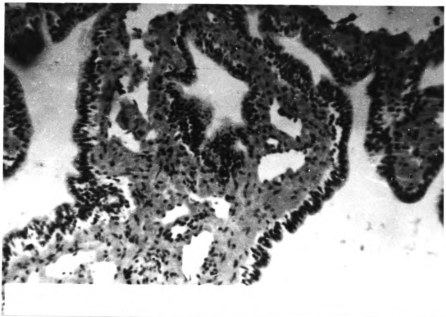


Figure 2. Intact

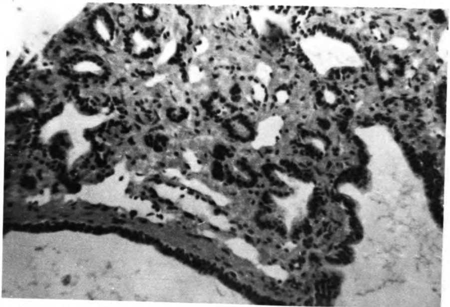


Figure 3. Castrated

the epithelium of the seminal vesicles and the Wolffian duct was reduced (Plate II, Figure 3).

The weights of the seminal vesicles and adjacent portions of the oviducts were reduced following castration (Table 1) although the difference was not significant ($0.1<p<0.2$).

Table 1. Effect of castration on "seminal vesicle weights"*

animals	$\frac{\text{gm. seminal vesicle + oviduct weight}}{\text{gm. body weight}} \times 10{,}000$	S.D.	t
Intact	7.325	1.49	1.36
Castrated	5.955	1.36	

S.D. = standard deviation

*Since the seminal vesicle and the adjacent portion of the oviduct are within the same connective tissue sheath, it was not possible to separate them.

The fact that the differences in weight are not significant is not surprising in view of the fact that the weights include portions of the oviduct which would not be expected to change following castration and which contribute at least half of the total weight.

The cytochemical techniques applied to demonstrate the inclusions in the seminal vesicles are listed in Table 2. Carbohydrates present in the seminal vesicle epithelium and lumen and in the Wolffian duct appear to be in the form of neutral mucopolysaccharides. A strong PAS-positive reaction accompanied by a weak protein reaction was obtained.

Table 2. Cytochemical observations on the secretions of the seminal vesicles and Wolffian ducts of the frog, *Rana pipiens*

Cytochemical test	Sv-ep	Reaction in SV-1	WD-ep	WD-1	Remarks
PAS without oxidation	--	--	--	--	free aldehydes absent
PAS with prior acetylation	--	--	--	--	
PAS	+to+++	--to+++	+	±	1:2 glycol groups present
PAS after trypsin digestion	+to+++	--to+++	+	±	PAS not due to protein
PAS after lipid extraction	+to+++	--to+++	+	±	PAS not due to lipids
PAS after glycogenolysis	+to+++	--to+++	+	±	carbohydrates other than glycogen present
Alcian Blue pH 2.5	--	--	--	--	acid mucopoly-saccharides absent
Ninhydrin-Schiff without oxidation or with acetylation	--	--	--	--	free NH_2 groups absent
Ninhydrin-Schiff	±	±	±	±	reactive NH_2 groups present

-- = no color reaction

± = faint reaction

\+ = moderate reaction

+++ = intense reaction

SV-ep = Seminal vesicle epithelium

SV-1 = Seminal vesicle lumen

WD-ep = Wolffian duct epithelium

WD-1 = Wolffian duct lumen

There was no specific localization of the PAS reaction. Within each seminal vesicle there were some areas which were intensely PAS-positive and other areas showing a weak reaction. The areas with intense PAS-positive reactions were randomly distributed throughout the tissue. The faint positive reaction for proteins was evenly distributed throughout the tissue. No differences were seen between castrated and intact animals in the amounts or distribution of carbohydrates which could be demonstrated by these techniques.

Effects of Seminal Vesicles on Fertilization

Sperm suspensions with or without seminal vesicle homogenates added were incubated for varying times and tested for fertilizing capacity. The experimental procedure is illustrated in Text Figure II.

Sperm incubated for less than 12 hours in the presence of seminal vesicle homogenate did not differ in their fertilizing capacity from those incubated in 10% Holtfreter's solution. In both cases fertilization was higher if 30 minutes was allowed to elapse before washing the sperm off than if only one minute was allowed (Text Figure III based on data in Appendix Table 1).

If sperm were incubated for 24 hours and then used to fertilize eggs, there was a significant difference in fertilizing capacity. Sperm incubated with seminal vesicle homogenates fertilized more eggs than sperm in Holtfreter's solution alone. As in the previous experiment more eggs were fertilized after allowing 30 minutes insemination time regardless of the treatment the sperm had received (Text Figure IV based on data in Appendix Table 2).

When sperm were incubated for 48 hours before fertilization significant differences in fertilizing capacity were again seen between

TEXT FIGURE II. Experimental Procedure

sperm suspension (2000 sperm/mm^3)

diluted with equal volume
Holtfreter's solution
(1000 sperm/mm^3)

diluted with equal volume
seminal vesicle homogenate
(1000 sperm/mm^3)

each
divided into 3
aliquots

Aliquots incubated in refrigerator for less than 12 hours, 24 hours, or
48 hours

One aliquot from each suspension used to fertilize 2 slides of eggs from
each female

1 min.

30 min.

sperm poured off one slide
from each female and slide
immersed in tap water

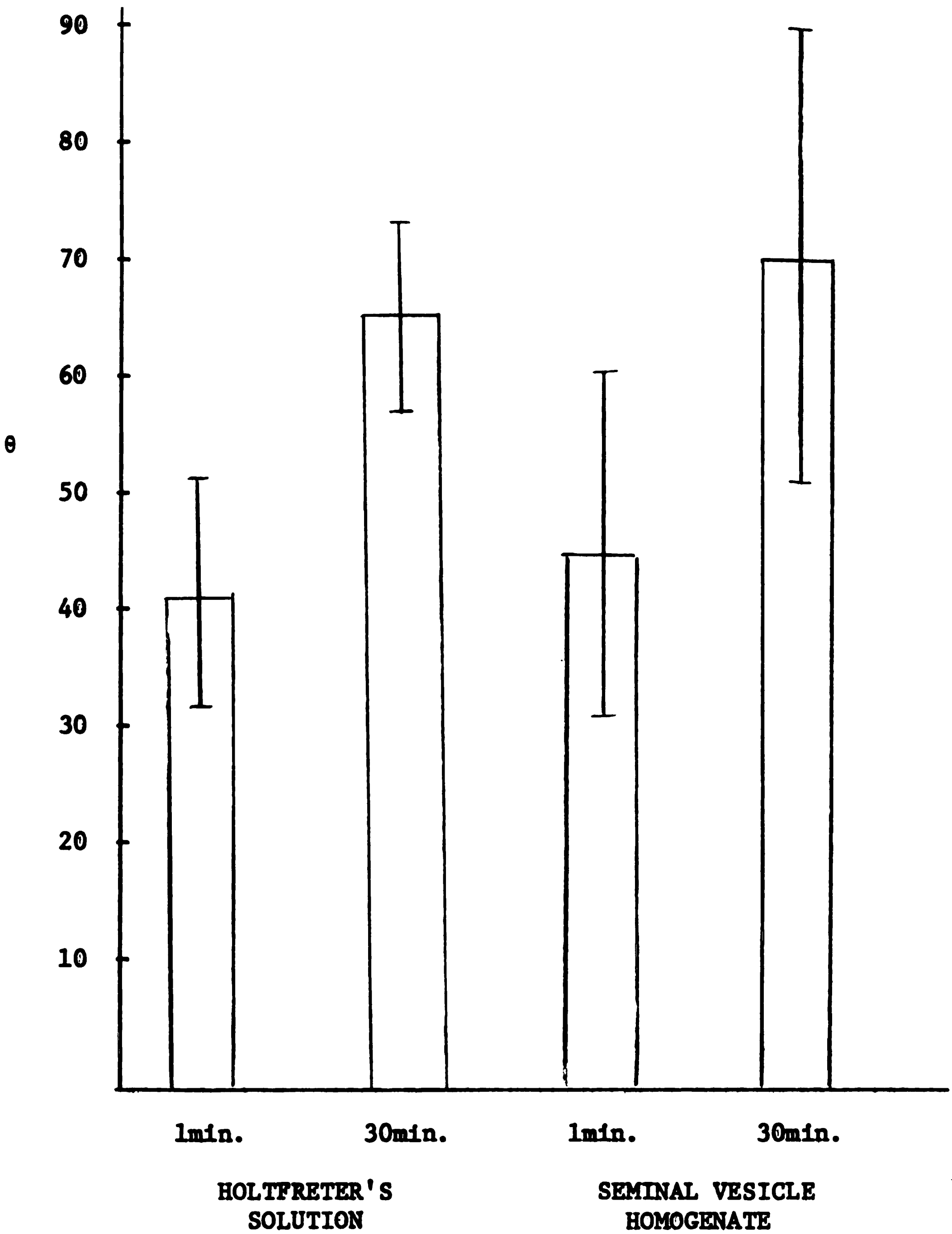
sperm poured off one slide
from each female and slide
immersed in tap water

3-5 hours later

% cleaving eggs counted

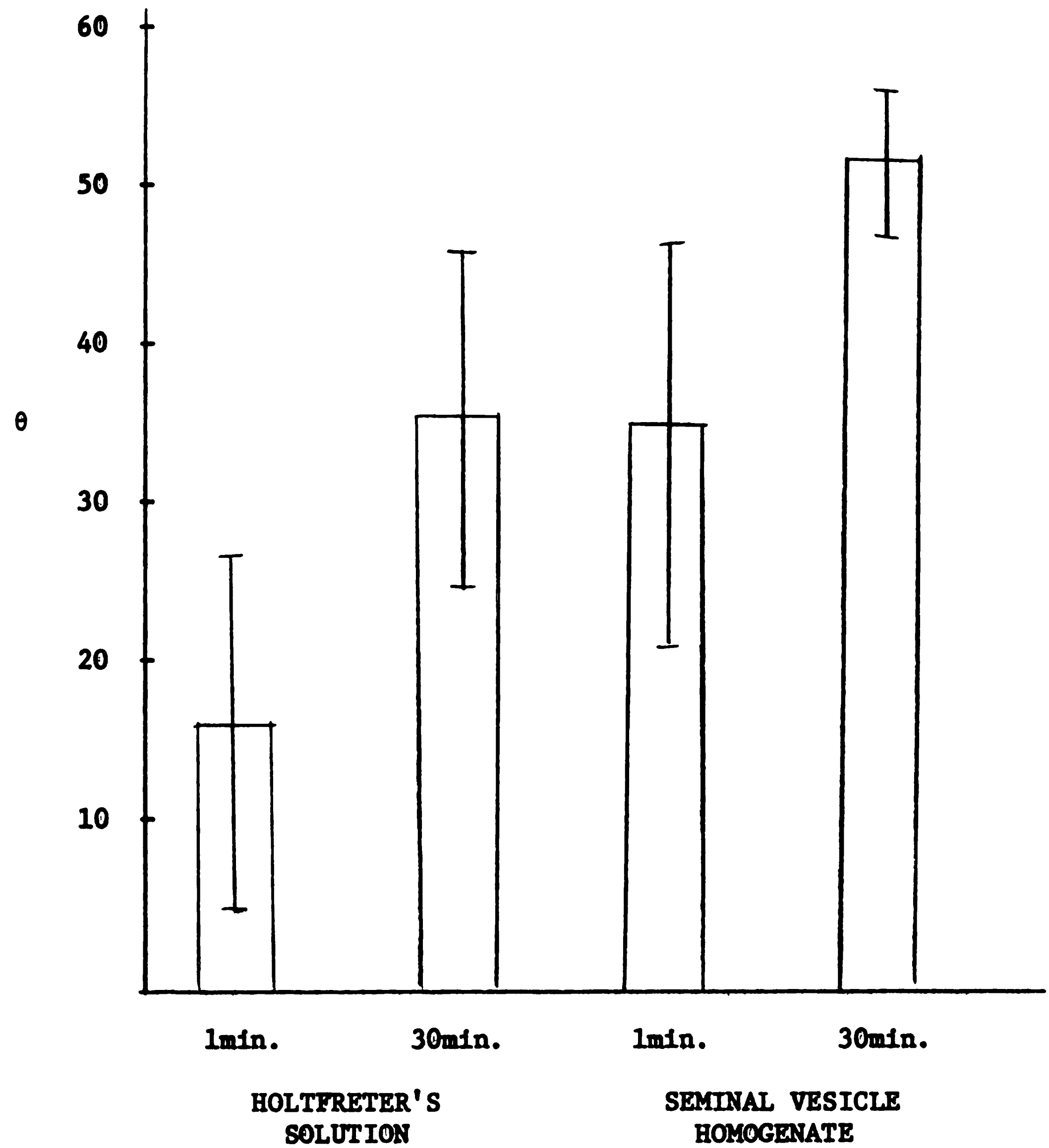
TEXT FIGURE III

Effect of seminal vesicle homogenate on the fertilizing capacity of sperm incubated less than 12 hours.



TEXT FIGURE IV

Effect of seminal vesicle homogenate on the fertilizing capacity of sperm incubated 24 hours.



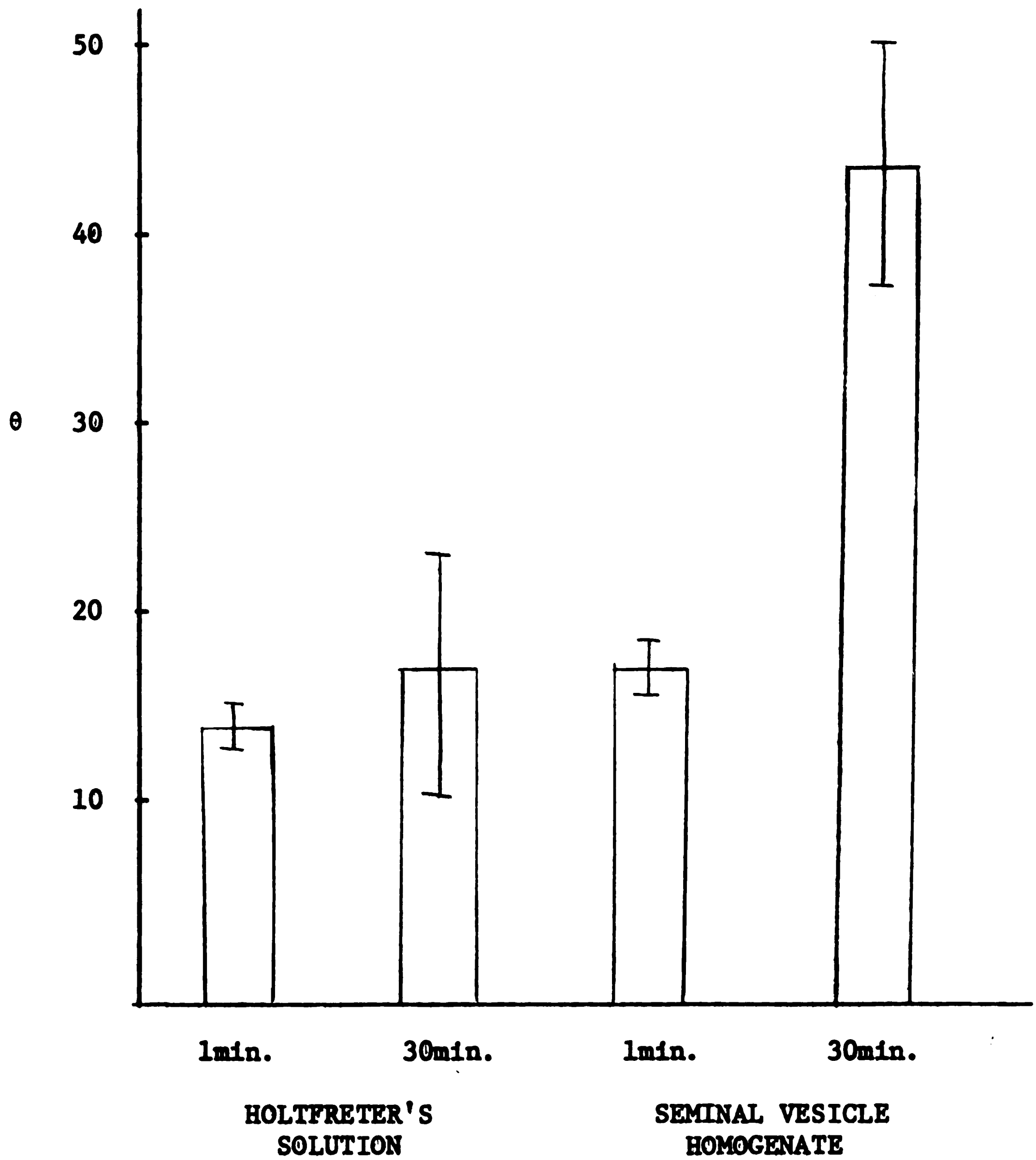
sperm incubated with seminal vesicle homogenate and those incubated in Holtfreter's solution alone. In this instance there was also a significant interaction between the treatments and times indicating that the increase in fertility as a function of time was dependent on the prior treatment the sperm had received. All of the differences in this experiment were due to the increase in fertility seen with seminal vesicle treated sperm after 30 minutes insemination time. The fertilization achieved by sperm in Holtfreter's solution alone did not increase over time and was equal at both time periods to the fertilization which occurred with sperm in seminal vesicle homogenates after 1 minute insemination (Text Figure V based on data in Appendix Table 3).

Subsequent experiments were designed to determine what factors may have contributed to the interaction between sperm treatments and insemination times and also to determine why sperm incubated in seminal vesicle homogenates retained fertility longer than those incubated in Holtfreter's solution alone.

Since the retention of fertilizing capacity may have been due to a non-specific tissue effect, sperm were incubated in brain homogenate in addition to seminal vesicle homogenate. It was expected that if brain, being a highly specialized tissue not related to the reproductive system, could have an effect similar to that of the seminal vesicle, then any specific role of the seminal vesicle in fertilization could be ruled out. Also, since the seminal vesicles are in the same connective tissue sheath with the male rudimentary oviducts and the tissues cannot therefore be separated, sperm were incubated in oviducal homogenate to determine whether the results could have been due to oviducal contamination of the seminal vesicle homogenates. In addition to the 1 and 30 minute time periods previously used, sperm were washed off at

TEXT FIGURE V

Effect of seminal vesicle homogenate on the fertilizing capacity of sperm incubated 48 hours.



10, 15, 20, and 25 minute intervals. Sperm in all cases were incubated 48 hours prior to use. Data are plotted from the regression lines calculated by the method of least squares.

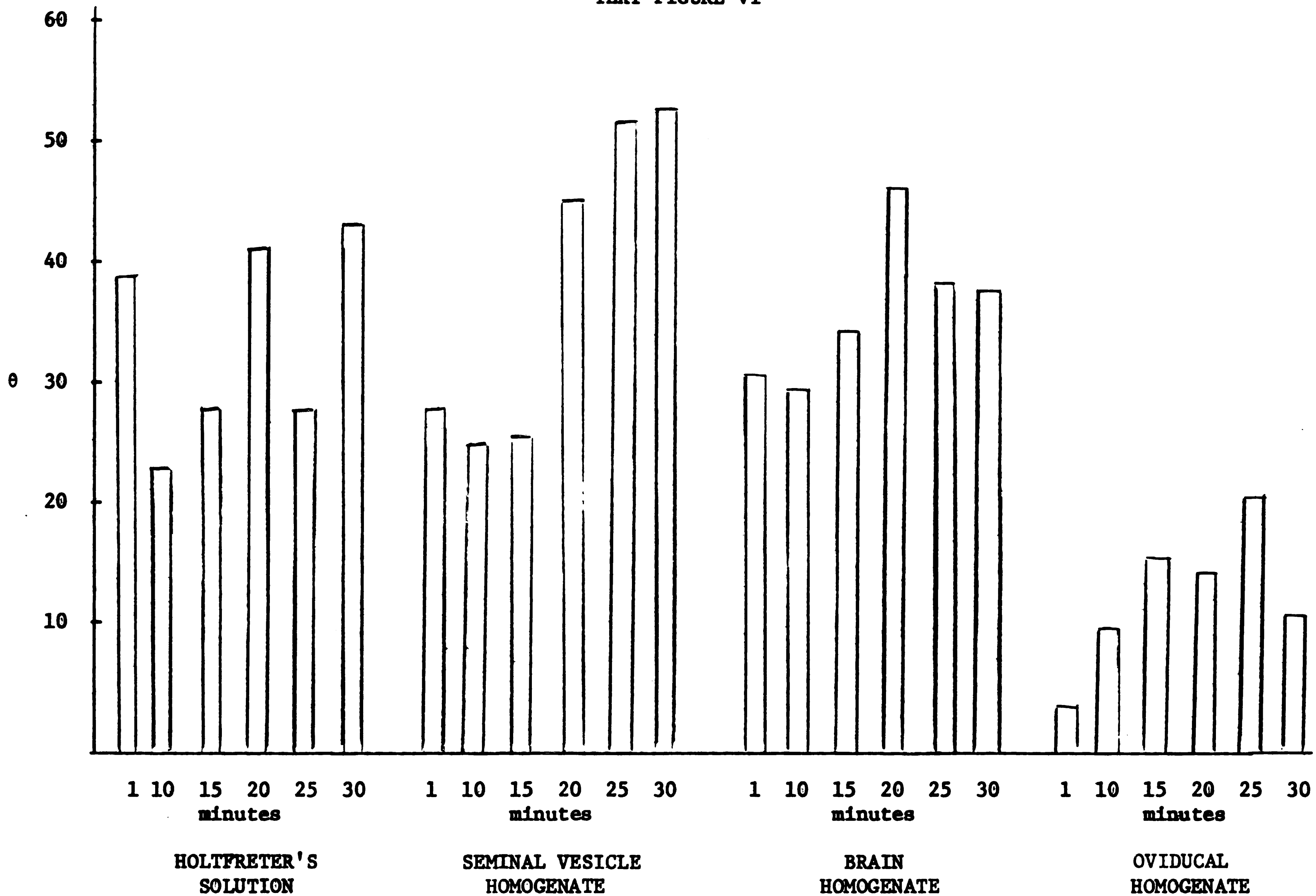
The data show that oviduct-treated sperm retain very little fertilizing capacity and the differences in fertilizing capacity previously observed with seminal vesicle-treated sperm could not therefore have been due to oviducal contamination. In fact, the fertility seen with seminal vesicle-treated sperm would be a minimal estimate since oviductal contamination is invariably present. Sperm incubated with brain homogenate do not differ from those in Holtfreter's solution alone. Thus a non-specific tissue effect could also not account for the differences previously seen. The increase in fertilization as a function of time is not significant when sperm are incubated with oviduct, brain, or Holtfreter's solution alone. Fertilization did significantly increase with time when sperm were incubated with seminal vesicle homogenates thus accounting for the significant interaction in the preliminary ANOVA (analysis of variance). The increase in fertilization as a function of time is not a simple linear relationship. Low levels of fertilization are seen after short insemination times. Following this initial lag, fertilization increased rapidly reaching a maximum when 25 to 30 minutes were allowed to elapse before washing the sperm off (Text Figure VI and Text Figure VII based on data in Appendix Table 4 and Appendix Table 5).

Since the sperm may interact with materials diffusing from the eggs and their enveloping jelly layers, and since this interaction may have been responsible for the increase in fertilization as a function of time after treatment of sperm with seminal vesicle homogenate, in the next experiment sperm were treated with egg water for 25 minutes

TEXT FIGURE VI

Effects of brain, oviduct, and seminal vesicle homogenates on the fertilizing capacity of frog sperm.

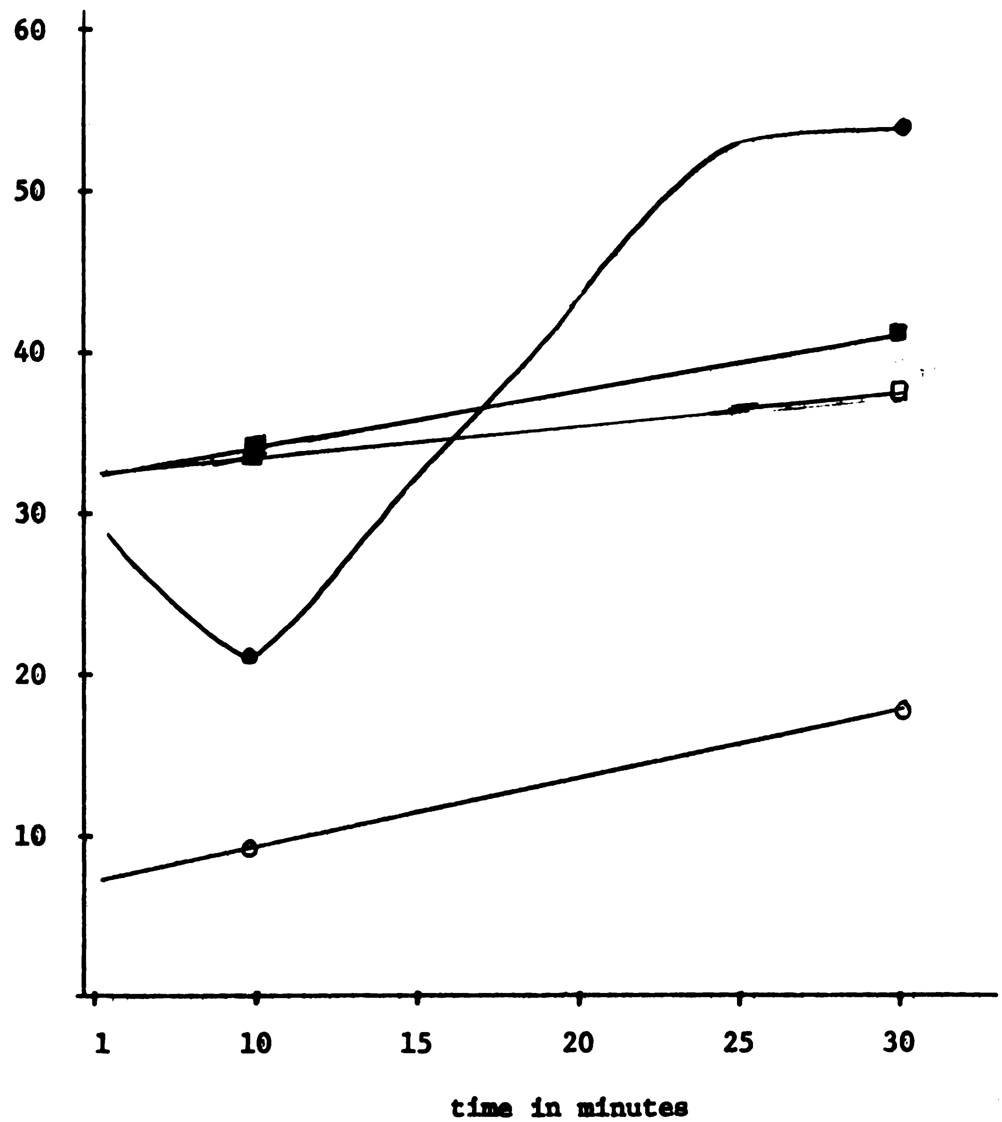
TEXT FIGURE VI



TEXT FIGURE VII

The effect of brain, oviduct, and seminal vesicle homogenates on the fertilizing capacity of frog sperm. Data plotted by the method of least squares.

—□— - Holtfreter's Solution
—■— - Brain Homogenate
—○— - Oviducal Homogenate
—●— - Seminal Vesicle Homogenate



before use in fertilization. As in the previous experiment, the sperm were incubated with seminal vesicle homogenate or with Holtfreter's solution alone for 48 hours in the refrigerator. The sperm suspensions were divided into two aliquots and egg water added to one aliquot of each suspension. Ten percent Holtfreter's solution was added to the other aliquot of each suspension. The data were analyzed by three-way analysis of variance. The data are plotted from regression lines calculated by the method of least squares.

Results show that egg water has no effect on sperm incubated in Holtfreter's solution alone. In both cases (sperm in Holtfreter's solution with and without egg water added) there was no significant increase in fertilization as a function of time and the average fertility does not differ. Egg water did have a significant effect on sperm incubated in seminal vesicle homogenate. As in the previous experiment, fertility was low when seminal vesicle treated sperm which were not incubated with egg water were allowed short periods of time to interact with the eggs. Fertility increased when increasing time was allowed for interaction. The overall fertility seen with seminal vesicle-treated sperm without egg water did not differ from that seen with Holtfreter's-treated sperm because of the low fertility of the seminal vesicle-treated sperm at the short time periods. If seminal vesicle-treated sperm were treated with egg water prior to insemination fertility was high at all times and there was no significant increase in fertility over time. Further, the average fertility seen when seminal vesicle-treated sperm were incubated with egg water was significantly higher than the fertility achieved by sperm in Holtfreter's solution with or without egg water or with sperm in seminal vesicle homogenates without egg water (Text Figure VIII and Text Figure IX based on data in Appendix Table 6 and Appendix Table 7).

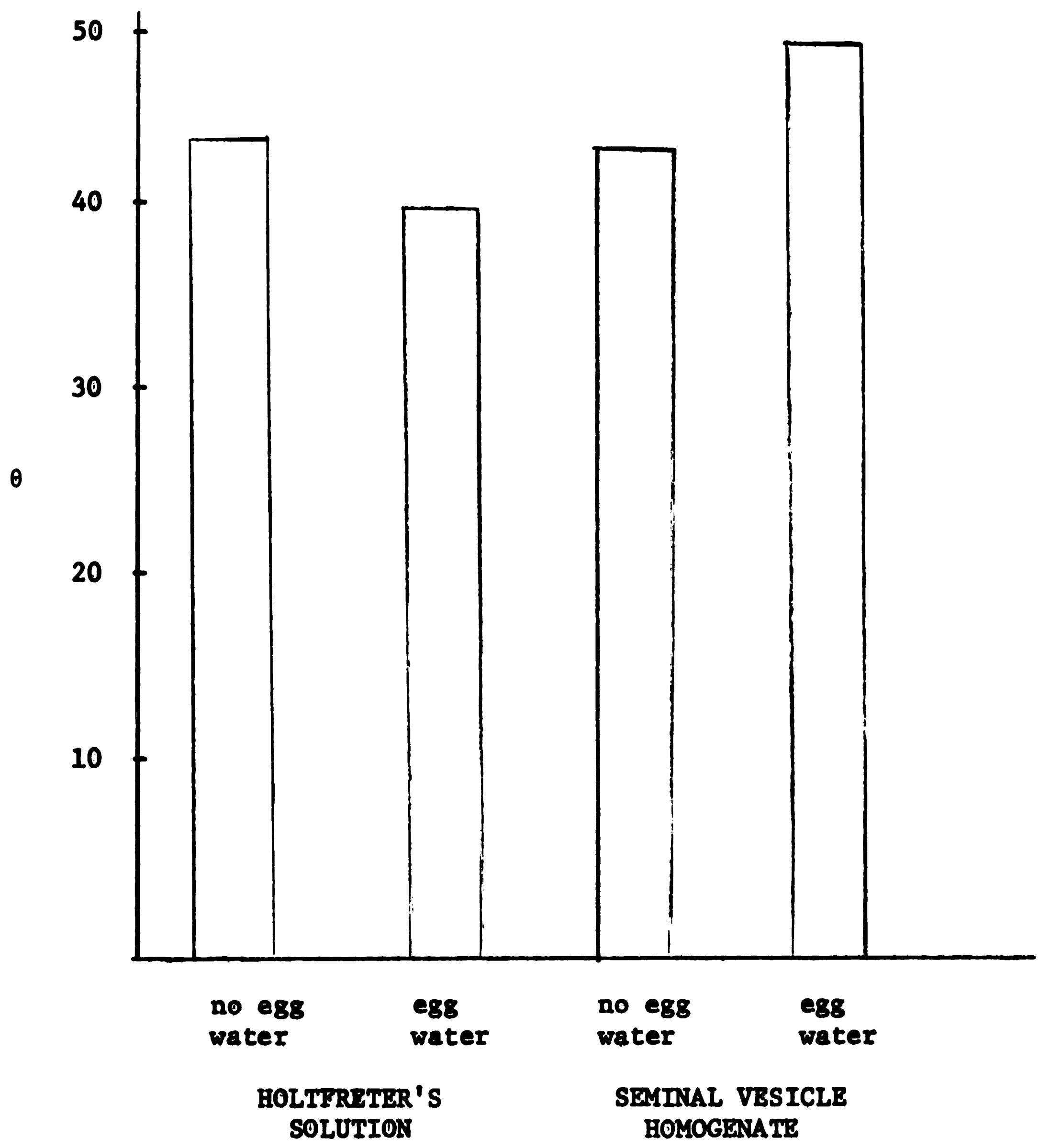
Since the differences in fertility could have been due to differences in activation rather than fertilization embryos were counted in the tailbud stage and the percentage of developing embryos was compared with the percent fertilization. Since many embryos had the external characteristics of the haploid syndrome, embryos were scored as normal or abnormal. Embryos which resulted from fertilization with sperm incubated in Holtfreter's solution alone were frequently retarded in their development as compared to the embryos resulting from fertilization with sperm incubated in seminal vesicle homogenate. Since these embryos were otherwise normal in appearance they were scored as normals. There were no significant differences either in the percent of embryos which developed or in the percent of the developing embryos which were abnormal in appearance (Appendix Table 8). Thus, the differences seen in previous experiments were due to differences in the fertilizing capacity of the sperm.

Since the materials from the seminal vesicle which influence the fertilizing capacity of the sperm may be dependent on androgens or other factors from the testes, male frogs were castrated and after five weeks their seminal vesicles were removed, homogenized, and incubated with sperm. Sperm were incubated in the refrigerator for 48 hours as in the previous experiments. Sperm incubated with Holtfreter's solution alone and with seminal vesicle homogenate from intact frogs were used as controls. Eggs from only one female were used due to the small amount of seminal vesicle homogenate available from castrated animals.

Results show that seminal vesicles from castrated and intact animals do not differ in their ability to maintain the sperm in a

TEXT FIGURE VIII

The effect of egg water on the fertilizability of sperm incubated in seminal vesicle homogenate or Holtfreter's solution alone.



TEXT FIGURE IX

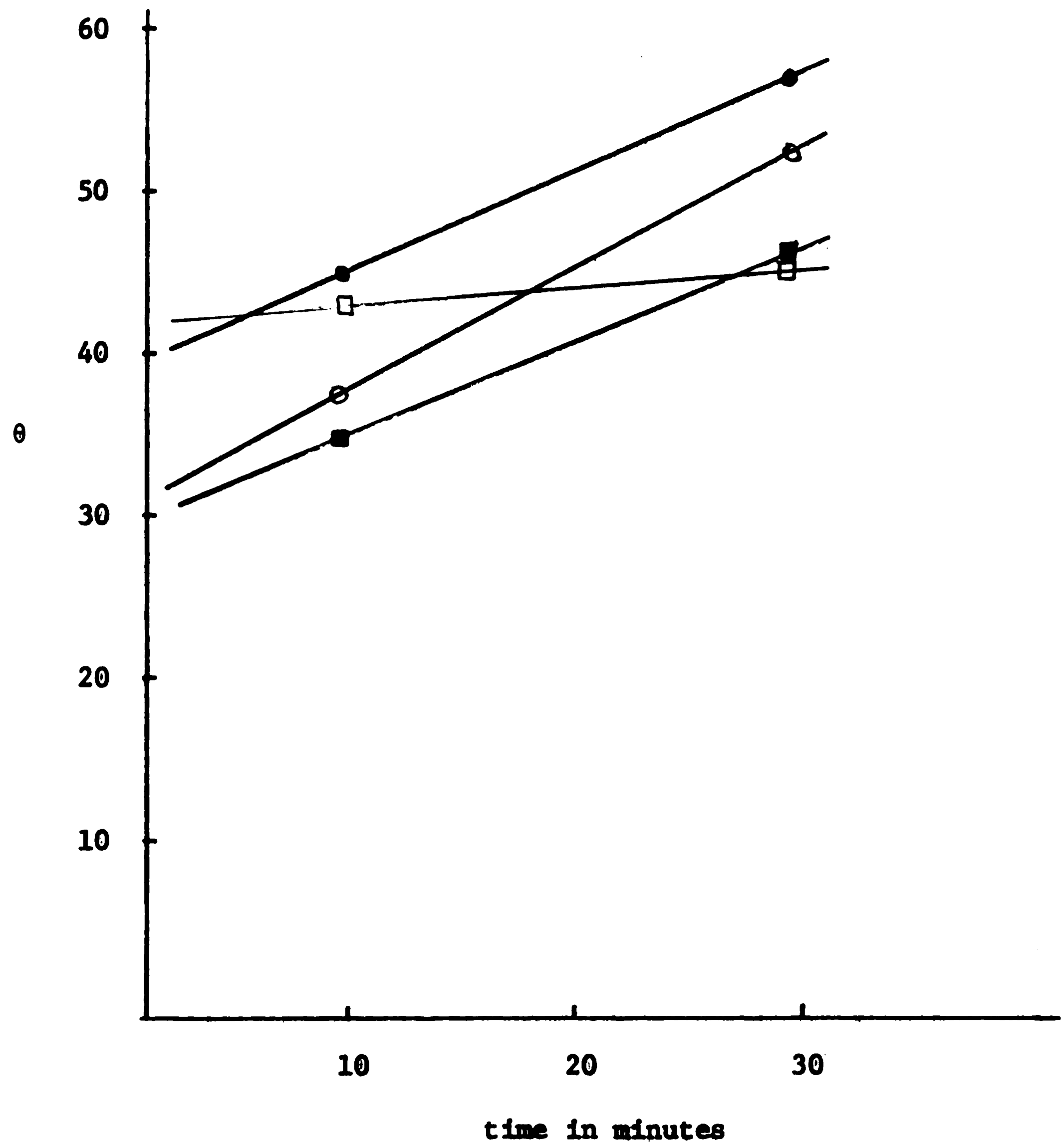
The effect of egg water on the fertilizability of frog sperm incubated in seminal vesicle homogenate or in Holtfreter's solution alone. Changes in fertilization with increasing insemination times.

—□— - sperm in Holtfreter's solution alone

—■— - sperm in Holtfreter's solution, egg water added

—○— - sperm in seminal vesicle homogenate

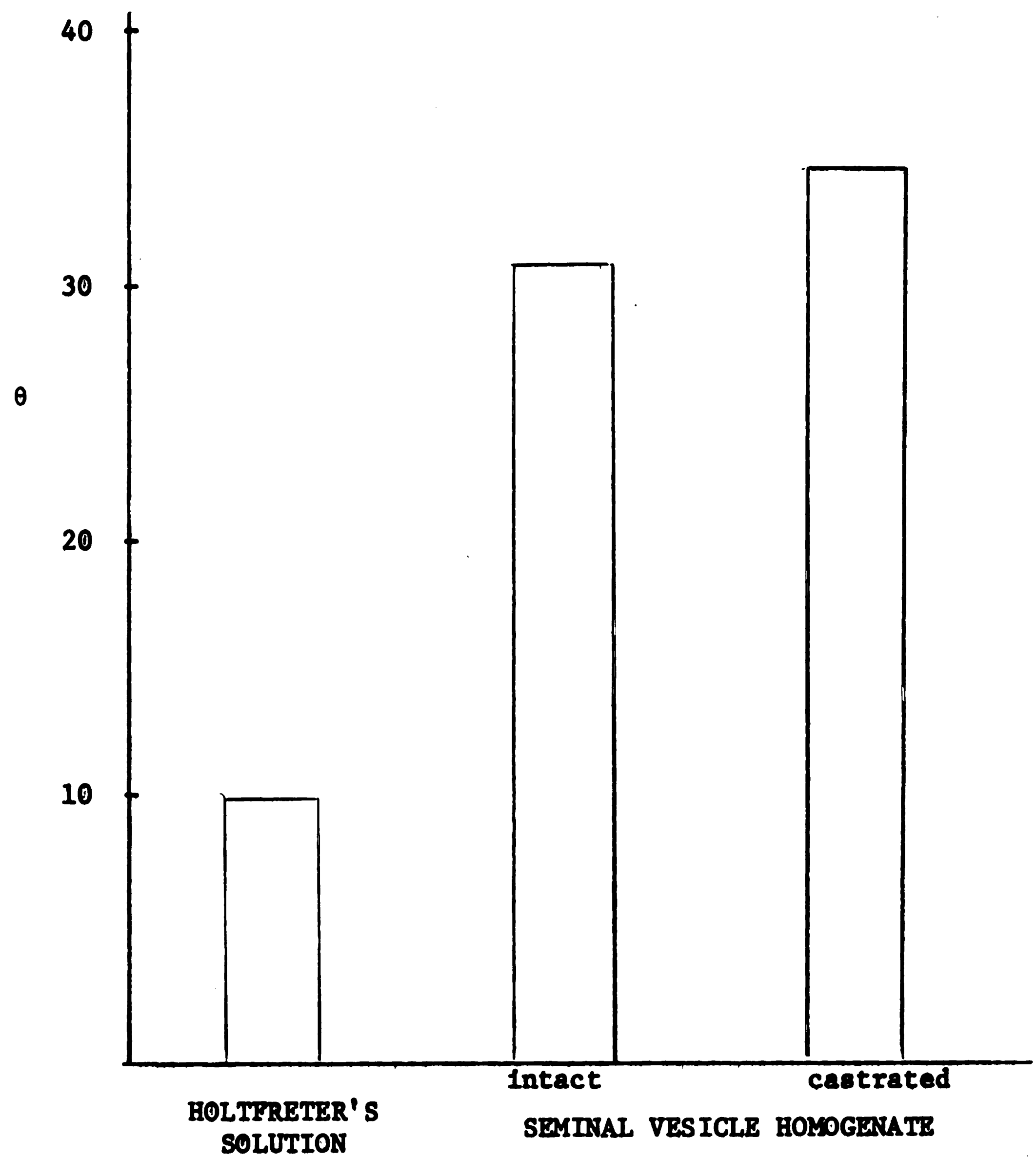
—●— - sperm in seminal vesicle homogenate, egg water added



fertilizable state during 48 hours of sperm storage. Sperm incubated with seminal vesicle homogenate from both intact and castrated animals fertilized significantly more eggs than sperm incubated with Holtfreter's solution alone (Text Figure X and Text Figure XI based on data in Appendix Table 9).

TEXT FIGURE X

A comparison of the effect of seminal vesicle homogenates from intact and castrated animals on the fertilizing capacity of frog sperm.



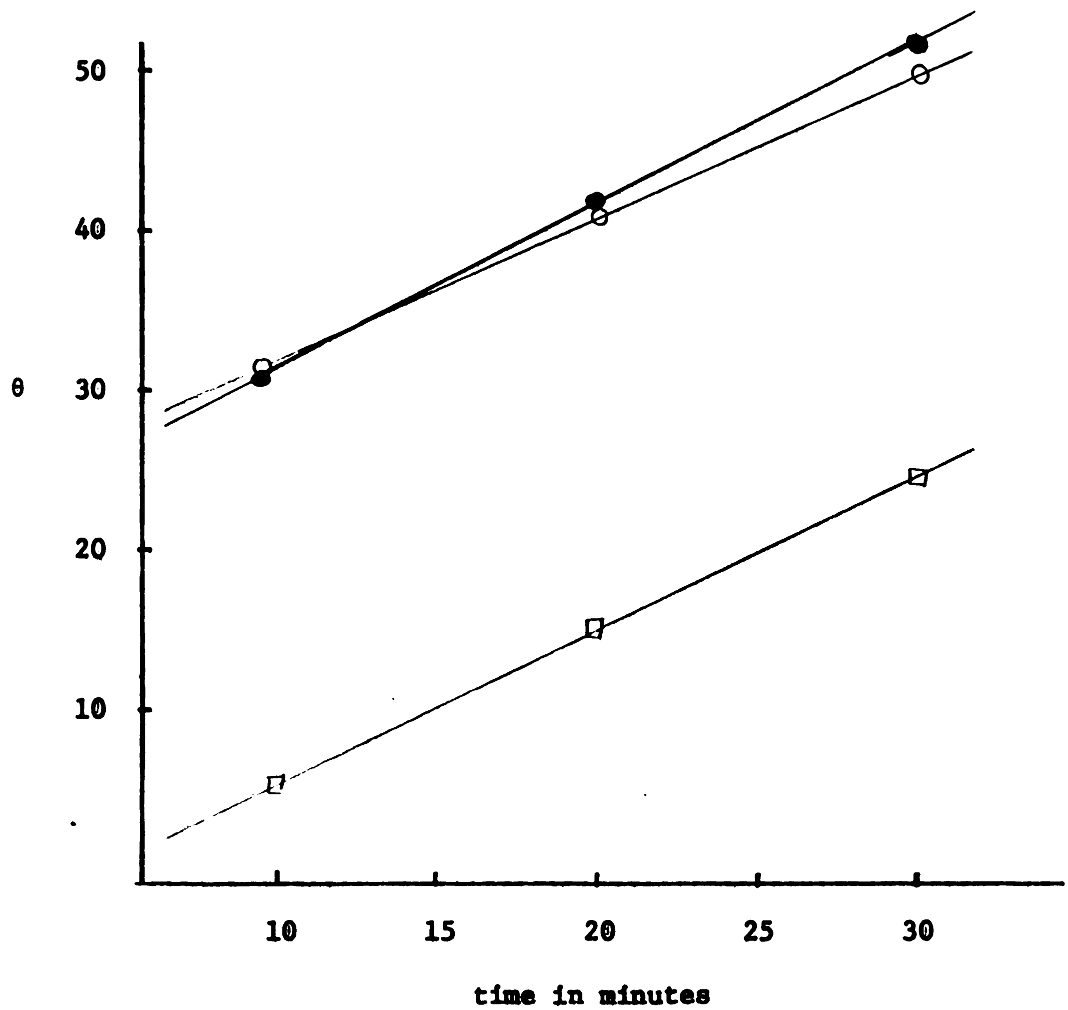
TEXT FIGURE XI

A comparison of the effect of seminal vesicle homogenates from intact and castrated animals on the fertilizing capacity of frog sperm. Changes in fertilization with increasing insemination times.

—□— - Holtfreter's Solution

——— - Seminal Vesicle Homogenate - Intact

——— - Seminal Vesicle Homogenate - Castrated



DISCUSSION

The seminal vesicles of the male frog, *Rana pipiens*, were demonstrated to be similar histologically to those described in other species of *Rana* (Aron, 1926). They consist of multiple evaginations of the Wolffian ducts lined by pseudo-stratified columnar epithelium which is surrounded by connective tissue.

Histochemical techniques which can identify general classes of carbohydrates were used to demonstrate the nature of the materials which are secreted by the seminal vesicles. Although the techniques employed can demonstrate only general classes of carbohydrates, by use of the appropriate controls differences in the chemical nature of their constituents can be detected. The PAS reaction is used to demonstrate carbohydrates rich in neighboring hydroxyl groups or equivalent amino substitutions (Mowry, 1963). Greater accuracy in identification of the reactive compounds can be obtained with controls such as protein digestion, lipid extraction, and diastase digestion (Barka and Anderson, 1963). Alcian blue at pH 2.5 stains carbohydrates rich in free acidic groups although sulfate groups may also contribute to the staining (Mowry, 1963). The complex carbohydrates in the epithelium and lumina of the seminal vesicles appear to be in the form of neutral mucopolysaccharides since they are PAS-positive and diastase-resistant and stain weakly with a protein stain. Acidic mucopolysaccharides are absent.

Results clearly indicate that materials from the seminal vesicles can indeed influence the fertilizing capacity of sperm. The fertilizable life of the sperm was prolonged when they were exposed to materials from the seminal vesicles. In fact, sperm which are incubated in seminal vesicle homogenate still retain some fertility after three days at refrigerator temperatures whereas sperm in Holtfreter's solution alone are totally inviable at this time (data not presented).

Interestingly, after two days at refrigerator temperatures, sperm incubated in seminal vesicle homogenate were capable of fertilizing greater numbers of eggs than sperm incubated with brain, oviduct, or Holtfreter's solution alone but only if allowed considerable time (25-30 minutes) to interact with materials from the eggs or their enveloping jelly coats. That the lag in fertilization was due to an interaction between the sperm and some material which diffuses from the egg or its jelly coat was demonstrated since pre-incubation of seminal vesicle-treated sperm with egg water resulted in elimination of the lag. This interaction can be called capacitation by analogy with the phenomenon in mammals where sperm must interact with materials from the female reproductive tract before gaining fertilizing capacity (Austin, 1951; Chang, 1951).

Incubation of sperm with male rudimentary oviduct results in an almost complete loss of fertilizability. This raises the possibility that the materials from the male oviduct may contain the same materials as the female oviduct and in this instance the sperm may have been capacitated during the two day incubation period and that once capacitated the sperm have a short fertilizable life. Most of the sperm incubated with male oviduct were immotile and were probably dead. That the male rudimentary oviduct does contain some materials similar to those found in female oviducts has been demonstrated (Umpierre, 1971). If indeed the loss of fertilizability of sperm incubated in oviduct homogenate was due to premature capacitation, it would be fruitful to utilize male oviducts rather than female ones in an attempt to isolate the capacitating materials since the secretions of the male oviduct are less complex than those from the female (Umpierre, 1971).

In anuran amphibians it has been established that the egg jellies are essential in fertilization since coelomic eggs or eggs from which the jellies have been removed are not fertilizable (Shaver and Barch, 1960; Shaver, 1966; Katagiri, 1966a and 1966b). Coelomic eggs, however, can be fertilized by sperm which have been exposed to jellied eggs (Shaver, 1966). The nature of the dependency on the jelly coat for fertilization is unknown although experimental evidence indicates that changes in the sperm are involved. The egg jellies are heterogeneous and may contain more than one factor which is necessary for fertilization. Hydrated anuran eggs are not normally fertilizable but fertility can be restored by treating hydrated eggs with materials which diffuse from them. The "diffusible factor" which is produced by the oviducts and retained by the jelly before hydration would under physiological conditions presumably activate the sperm before they penetrate the jelly coats (Barbieri and Raisman, 1969). The authors also report that de-jellied eggs could be fertilized either in the presence of solubilized jelly or the diffusible factor.

Sperm which have been exposed to diffusible materials from egg jellies are also capable of fertilizing eggs whose jelly coats have been blocked by antibodies. Eggs which have been exposed to antibodies prepared against egg jellies are normally not fertilizable. In this case the alteration of the sperm induced by diffusible materials from uterine eggs has been called capacitation (Shivers and James, 1970; Roberts, 1970).

In all of these experiments the authors have been primarily interested in elucidating the role of the egg jellies in the fertilization process and have employed fertilization of eggs which are abnormal (either lacking in jelly or with jellies blocked with antibodies) in an attempt to demonstrate jelly-induced sperm alterations. The authors have also employed sperm obtained from the testes which may not be the same as sperm which are emitted during amplexus, since in normal fertilization sperm must be exposed to materials from the seminal vesicles.

The present experiments suggest that the seminal vesicles may be the source of materials which decapacitate the sperm inasmuch as sperm which have had contact with seminal vesicle materials require a long period of interaction with eggs or diffusible materials from the eggs if high levels of fertilization are to be achieved.

The sperm may be coated by materials from the seminal vesicles and this coating material may then be removed during exposure of the sperm to diffusible materials from the egg jellies. Alternatively, the materials from the seminal vesicles may simply alter the sperm surface without actually binding to it. The role of the seminal vesicle materials may thus be to bind to or alter the sperm surface such that the sperm is maintained in a fertilizable state during storage of the sperm in the seminal vesicle.

The seminal vesicles may not be the only source of factors which render the sperm in a non-capacitated state (decapacitating factors) since immunological studies in our laboratory have failed to demonstrate any antigenically unique material in the seminal vesicles when compared to other portions of the male reproductive tract. The decapacitating factor(s) may also be present in the testis but insufficient in amount to maintain the fertilizability of a dilute sperm suspension for a prolonged period of time. In all previous experiments in which sperm capacitation in anuran amphibians has been described testicular sperm were utilized. From these studies it appears that capacitation is a two step phenomenon involving a small diffusible molecule which interacts with the sperm before contact with the egg jelly and a non-diffusible molecule with which the sperm interacts as it passes through the jelly (Barbieri and Raisman, 1969; Wolf and Hedrick, 197; Katagiri, 1966a and 1966b). In all of these cases capacitation occurred very quickly. In the present experiments capacitation required 25 to 30 minutes. The difference appears to be only a matter of the time required since in this case, as in the previous experiments, capacitation could be induced by the same materials (diffusible factors from the egg jellies). This does not imply that in natural fertilization capacitation requires such a long period of time, since under the artificial conditions of these experiments the sperm may have been exposed to a much greater concentration of decapacitating factors than would be the case in natural fertilization. Exposure of sperm in 10% Holtfreter's solution to egg jelly materials prior to use in fertilization of normal jellied eggs has been reported to reduce the percent of eggs fertilized by about 17% (Shivers and James, 1971). The authors suggest that the decrease in fertility is due to premature capacitation possibly involving an acrosomal reaction. Similarly, in the experiments reported here, sperm which have been incubated in Holtfreter's solution and then exposed to egg water showed a slight decrease in fertilizing capacity although the difference was not significant.

This suggests that sperm from the testis are capacitated by diffusible materials from the eggs and that capacitation in this case occurs rapidly. Thus the difference in capacitation between the experiments described herein and those of other authors may be due simply to the amount of time required and not to differences in the nature of the changes which are occurring in the sperm. Sperm exposed to the seminal vesicles may have a greater amount of material bound to their surface and thus a longer period of time may be required for its removal.

The fact that seminal vesicles from castrated and intact animals do not differ in their ability to alter the fertilizing capacity of the sperm may reflect the fact that they are not dependent upon continuous stimulation by materials from the testes for their function or may have been due to the fact that insufficient time had elapsed after castration for the level of hormones to decline sufficiently for the loss of function to occur.

In conclusion, the present experiments suggest that the role of the seminal vesicles in *Rana pipiens* may be to maintain the sperm in a fertilizable state during the time sperm are stored in it. Upon release and exposure to materials from the egg jellies the sperm then acquire fertilizing capacity. The seminal vesicles may be the source of materials which coat or alter the sperm surface. The role of the egg jellies would then be to remove the coating substance and/or further alter the sperm so that fertilizing capacity is attained.

LITERATURE CITED

LITERATURE CITED

Aron, M. 1926. Recherches morphologiques et experimentales sur le determinisme des caracteres sexuales males chez les Anoures. Arch. de Biol., Paris, 36:3.

Austin, C. R. 1951. Observations on the penetration of the sperm into the mammalian egg. Aust. J. Scient. Res. B., 4:581.

Barbierri, F. D. and Raisman, J. S. 1969. Non-gametic factors involved in the fertilization of *Bufo arenarum* oocytes. Embryologia, 10:363-372.

Barka, T. and Anderson, P. J. 1963. *Histochemistry, Theory, Practice and Bibliography*. Harper and Row, N.Y., pp. 65-95.

Chang, M. C. 1951. Fertilizing capacity of spermatozoa deposited into the fallopian tubes. Nature, Lond., 168:697.

_______. 1957. A detrimental effect of seminal plasma on the fertilizing capacity of sperm. Nature, Lond., 179:258.

Gallien, L. 1955. The action of sex hormones on the development of sex in Amphibia. In: *Memoire of the Society for Endocrinology of Vertebrates. Part I. The Comparative Physiology of Reproduction and the Effects of Sex Hormones in Vertebrates.* Cambridge University Press, pp. 188-204.

Guyer, M. F. 1947. *Animal Micrology*. University of Chicago Press, Chicago, p. 242.

Hunter, A. G. 1969. Differentiation of rabbit sperm antigens from those of seminal plasma. J. Reprod. Fert., 20:413-418.

Hunter, A. G. and Nornes, H. O. 1969. Characterization and isolation of a sperm coating antigen from rabbit seminal plasma with capacity to block fertilization. J. Reprod. Fert., 20:419-427.

Johnson, W. L. and Hunter, A. G. 1972. Immunofluorescent evaluation of male rabbit reproductive tract for sites of secretion and absorption of seminal antigens. Biol. of Reprod., 6:13-22.

Katagiri, C. 1966a. Fertilization of dejellied uterine toad eggs in various experimental conditions. Embryologia, 9:159-169.

_______. 1966b. The fertilizing capacity of frog sperm in the homologous and heterologous egg-jellies and polyvinyl-pyrolidone (PVP). J. Fac. Sci. Hokkaido Univ. (Zool.), 16:77-84.

Mann, T. 1964. *The Biochemistry of Semen and of the Male Reproductive Tract*. Methuen and Co., London.

_______ and Lutwak-Mann, C. and Hay, M. F. 1963. A note on the so-called seminal vesicles of the frog, *Discoglossus pictus*. Acta Embryologia et Morph. Exptl., 6:21-25.

Mowry, R. W. 1963. The special value of methods that color both acidic and vicinyl hydroxyl groups in the histochemical study of mucins. With revised directions for colloidal iron stain, the use of alcian blue G8X and their combination with the periodic acid-Schiff reaction. Ann. N.Y. Acad. Sci., 106:402-423.

Nayyar, S. K. and Sundararaj, B. I. 1970. Seasonal reproductive activity in the testes and seminal vesicles of the catfish, *Heteropneustes fossilis* (Bloch). J. Morph., 130:207-226.

Parkes, A. S. 1960. *Marshall's Physiology of Reproduction*. Vol. I, Part 2. Longmans, Green and Co., Ltd., London.

Pearse, A. G. E. 1970. *Histochemistry, Theoretical and Applied*. 3rd edition. J. and A. Churchill, London.

Puckett, W. O. 1939. Some effects of crystalline sex hormones on the reproductive structures of several anurans. Anat. Rec., 75:127.

Roberts, C. R. 1971. Sperm-egg interactions in *Rana pipiens*. Master's Thesis, Michigan State University.

Rugh, R. 1934. Induced ovulation and artificial fertilization in the frog. Biol. Bull., 66:22.

_______. 1939. The reproductive processes of the male frog, *Rana pipiens*. J. Exp. Zool., 80:81-105.

_______. 1941. Experimental studies on the reproductive physiology of the male spring peeper, *Hyla crucifer*. Proc. Amer. Philosoph. Soc., 84:617-632.

Shaver, J.R. 1966. Immunological studies of the jelly-coats of anuran eggs. Am. Zool., 6:75-87.

_______ and Barch, S. H. 1960. Experimental studies on the role of jelly coat material in fertilization in the frog. Acta Embryol. Morphol. Exp., 3:180-189.

Shivers, C. A. and James, J. M. 1971. Fertilization of antiserum-inhibited frog eggs with "capacitated" sperm. Biol. of Reprod., 5:229-235.

Sluter, J. W., Oordt, G. J. van and Mighorst, J. C. A. 1950. A study of the testis tubules, interstitial tissue and sex characters (thump pads and Wolffian ducts) of normal and hypophysectomized frogs (*Rana esculenta*). Quart. J. Micr. Sci., 19:131.

Sundararaj, B. I. 1958. The seminal vesicles and their seasonal changes in the Indian catfish, *Heteropneustes*. Copeia, 4:289-297.

_______ and Nayyar, S. K. 1969. Effects of estrogen, SU-9055, and cypterone acetate on the hypersecretory activity in the seminal vesicles of the castrate catfish, *Heteropneustes fossilis* (Bloch). J. Exp. Zool., 172:399-408.

Umpierre, C. C. 1971. Studies on the distribution of mucopolysaccharides in adult *Rana pipiens* male rudimentary oviducts after hormonal stimulation. Doctoral Thesis, Michigan State University.

Weil, A. J. and Rodenberg, J. M. 1962. The seminal vesicles as a source of the spermatozoa-coating antigen of seminal plasma. Proc. Soc. Exp. Biol. Med., 109:567-570.

Wolf, D. P. and Hedrick, J. L. 1971. A molecular approach to fertilization. III. Development of a bioassay for sperm capacitation. Develop. Biol., 25:360-376.

Wright, P. A. and Flathers, N. R. 1961. Facilitation of pituitary-induced frog ovulation by progesterone in early fall. Proc. Soc. Exp. Biol. Med., 106:346-347.

APPENDIX

Appendix Table 1. Sperm incubated for less than 12 hours in Holtfreter's solution and seminal vesicle homogenate

Insemination time	Holtfreter's Solution # cleaved / total #	% Fert.	θ	Seminal Vesicle Homogenate # cleaved / total #	% Fert.	θ
	9/28	32.1	34.51	9/23	39.1	38.70
1 minute	13/35	37.1	37.52	10/30	33.3	35.24
	20/32	62.5	52.24	20/26	76.9	61.27
	28/32	87.5	69.30	38/42	90.5	72.05
30 minutes	24/26	66.7	54.76	15/29	51.7	45.97
	23/26	88.5	70.18	28/28	100	90.00

ANOVA Table

source of variation	df	SS	MS	F
Treatments	1	50.9232	50.9232	.2382
Times	1	1698.844	1698.844	7.9478*
Interaction	1	.6721	.6721	.0031
Error	8	1710.0004	213.75	
Total	11	3460.4397		

LSD $t_{.01}$ = 34.57 Holtfreter's $\overline{X}_1-\overline{X}_{30}$ = 23.32*

$t_{.05}$ = 22.2 Seminal Vesicle $\overline{X}_1-\overline{X}_{30}$ = 24.27*

Test for Homogeneity of Variances $s^2max./s^2min.$ 6.4527

*Significant at the 5% level.

Appendix Table 2. Sperm incubated for 24 hours in Holtfreter's solution and seminal vesicle homogenate

Insemination time	Holtfreter's Solution # cleaved / total #	Holtfreter's Solution % Fert.	Holtfreter's Solution θ	Seminal Vesicle Homogenate # cleaved / total #	Seminal Vesicle Homogenate % Fert.	Seminal Vesicle Homogenate θ
1 minute	2/25	8.0	16.43	7/29	24.1	29.4
	3/33	9.0	17.56	23/31	74.2	59.47
	0/36	0.0	0.00	4/25	16.0	23.58
	1/30	3.3	10.47	5/26	19.2	25.99
	4/29	13.8	21.81	9/26	34.6	36.03
	8/27	29.6	32.96	7/24	29.2	32.71
30 minutes	21/53	39.6	39.00	20/36	55.6	48.22
	11/45	24.4	29.60	25/38	65.8	54.21
	5/37	13.5	21.56	26/41	63.4	52.77
	6/28	21.4	27.56	14/27	51.9	46.09
	14/26	53.8	47.18	17/28	60.7	51.18
	16/29	55.2	47.98	24/35	68.6	55.92

ANOVA Table

source of variation	df	SS	MS	F
Treatments	1	1724.8322	1724.8322	16.2663*

Appendix Table 2 (cont'd.)

source of variation	df	SS	MS	F
Times	1	1923.5342	1923.5342	18.1402*
Interaction	1	6.448	6.448	.0608
Error	20	2120.7322	106.0366	
Total	23	5775.5476		

LSD $t_{.01} = 16.9$ Holtfreter's $\bar{X}_1 - \bar{X}_{30} = 18.9$*

$t_{.05} = 13.6$ Seminal Vesicle $\bar{X}_1 - \bar{X}_{30} = 16.9$*

Test for Homogeneity of Variances $s^2 max./s^2 min. = 12.37$

*Significant at the 1% level.

Appendix Table 3. Sperm incubated for 48 hours in Holtfreter's solution and seminal vesicle homogenate

Insemination time	Holtfreter's Solution: # cleaved / total #	% Fert.	θ	Seminal Vesicle Homogenate: # cleaved / total #	% Fert.	θ
1 minute	$\frac{3}{42}$	7.1	15.34	$\frac{2}{25}$	8.0	16.43
	$\frac{3}{55}$	5.4	13.44	$\frac{3}{28}$	10.7	19.09
30 minutes	$\frac{2}{45}$	4.4	12.11	$\frac{20}{36}$	55.6	48.22
	$\frac{8}{56}$	14.3	22.22	$\frac{17}{45}$	37.8	37.94

ANOVA Table

source of variation	df	SS	MS	F
Treatments	1	428.8056	428.8056	15.69*
Times	1	394.6645	394.6645	14.44*
Interaction	1	254.1385	254.1385	9.30*
Error	4	109.2881	27.322	
Total	7	1186.8967		

LSD $t_{.01} = 24.07$ Seminal Vesicle $\bar{X}_1 - \bar{X}_{30} = 25.32^{**}$

$t_{.05} = 14.51$

Test for Homogeneity of Variances $s^2 max./s^2 min. = 29.2737$

*Significant at the 5% level.
**Significant at the 1% level.

Appendix Table 4. Sperm incubated for 48 hours in Holtfreter's solution, seminal vesicle homogenate, brain homogenate and oviducal homogenate

Time	Holtfreter's Solution* θ	Seminal Vesicle Homogenate θ	Brain Homogenate θ	Oviducal Homogenate θ
1 min.	39.03±10.4	28.03±8.26	20.78±18.63	2.86±7.0
10 min.	23.43±19.1	25.07±14.9	29.65±15.5	9.30±11.0
15 min.	28.46±17.3	26.10±11.2	33.60±10.6	15.50±18.8
20 min.	40.47±22.2	45.12±13.7	45.67±16.6	13.37±20.9
25 min.	28.36±7.7	50.67±6.1	37.56±15.1	20.25±7.9
30 min.	42.85±6.8	51.88±8.4	37.14±11.6	10.37±12.1

ANOVA Table

source of variation	df	SS	MS	F
Treatments	3	15526.8665	5175.6221	26.5591**
Times	5	4677.7593	935.5518	4.8008**
Interaction	15	4306.1269	287.0751	1.4731***
Error	120	23384.5467	194.8712	
Total	143	47895.2994		

*Each value is an average of six replicates and represents the average of the arc sin equivalents of the percentages of eggs fertilized for each replicate ± the standard deviation.

**Significant at the 1% level.

***Significant at the 5% level.

Appendix Table 5. Basic statistics, regression statistics, and calculation of higher order regression coefficients for seminal vesicle data

	sperm incubated for 48 hours in:			
	Holtfreter's Solution	Seminal Vesicle Homogenate	Brain Homogenate	Oviducal Homogenate
Basic statistics				
ΣY	1215.51	1362.14	1286.36	429.93
ΣY^2	49836.0099	59957.5872	53680.3662	12045.0748
$\Sigma Y^2/n$	42957.3237	56389.5142	46985.0748	6189.4626
n	36	36	36	36
a	6	6	6	6
ΣnX	606	606	606	606
ΣnX^2	13506	13506	13506	13506
$\Sigma X(\Sigma Y)$	21023.65	26373.35	22787.1	8479.5
Regression statistics				
Σx^2	3305.0	3305.0	3305.0	3305.0
Σxy	562.565	3443.9934	1133.3734	1242.345
SS grps	1916.6415	4849.9204**	1020.5735	1055.0236
SS lin. regress.	95.7577	3588.8322*	388.6642	466.9957
SS dev. regress.	1820.8838	315.272*	631.9093	588.0279
SS within	6878.6862	118.9357	6695.2914	5855.8635
$b_{y.x}$	0.1702	1.042	0.3429	0.3758
$\bar{\bar{Y}}$	33.76	37.84	35.73	11.94
$\bar{\bar{X}}$	16.83	16.83	16.83	16.83
Y inter.	30.8991	20.297	29.9601	5.6166

Appendix Table 5 (cont'd.)

	sperm incubated for 48 hours in:			
	Holtfreter's Solution	Seminal Vesicle Homogenate	Brain Homogenate	Oviducal Homogenate

Calculation of higher order regression coefficients for seminal vesicle data. Data were coded for analysis. Regression coefficients only have been decoded.

x^2 = 5.5084	x^2y = 196.3861	SS dev. regress. = 180.4264
$(x^2)^2$ = 57.6751	x^3y = 585.3866	SS within = 3568.073
$(x^3)^2$ = 545.3462	SS grps = 4849.9204*	Y inter. = 32.8387
xx^2 = 17.1092	SS lin. regress. = 3931.9776**	$b_{y.x}$ = -4.2469
xx^3 = 49.5401	SS quad. regress. = 113.0699	$b_{y.x^2}$ = 0.3857
x^2x^3 = 174.9045	SS cubic regress. = 624.4465*	$b_{y.x^3}$ = -0.0074
xy = 57.411		

*Significant at the 5% level.
**Significant at the 1% level.

Appendix Table 6. Effect of egg water on the fertilizability of sperm incubated in seminal vesicle homogenate or Holtfreter's solution

Time	Holtfreter's Solution θ	Holtfreter's Solution + Egg Water θ	Seminal Vesicle Homogenate θ	Seminal Vesicle Homogenate + Egg Water θ
1 min.	44.28±13.9	31.09±15.1	33.14±6.7	40.48±12.0
10 min.	40.68±12.9	34.61±18.8	34.71±10.5	43.71±7.6
15 min.	43.04±6.6	42.97±8.4	35.05±13.0	48.55±4.6
20 min.	42.28±14.2	39.62±11.4	51.00±7.2	52.03±6.3
25 min.	49.79±9.6	37.73±16.8	46.24±12.5	52.01±10.0
30 min.	40.68±9.8	49.56±14.8	53.83±9.3	55.05±10.9

ANOVA Table

source of variation	df	SS	MS	F
Treatments (Holtfreter's or seminal vesicles)	1	688.4939	688.4939	5.3126*
Times	5	3150.3374	630.0674	4.8618**
Egg Water (present or absent)	1	23.4498	23.4498	0.1809
Times vs Treatments	5	701.6723	140.3344	1.0828
Treatments vs Egg Water	1	1090.2653	1090.2653	8.4128***

Appendix Table 6 (cont'd.)

source of variation	df	SS	MS	F
Times vs Egg Water	5	629.1357	125.8271	0.9709
Times vs Treatments vs Egg Water	5	946.974	189.3948	1.4614
Error	121	15681.0315	129.5953	
Total	143			

Bartlett's Test for Homogeneity of Variances $\chi^2 = 23.6297$ $0.5<p<0.9$

*Significant at the 5% level.

**Significant at the 1% level.

***Significant at the 0.1% level.

Appendix Table 7. Regression statistics based on data in Appendix Table 5

	Holtfreter's Solution	Holtfreter's Solution + Egg Water	Seminal Vesicle Homogenate	Seminal Vesicle Homogenate + Egg Water
Basic statistics				
ΣY	1564.48	1395.42	1523.8	1750.97
ΣY^2	72319.2338	63185.8874	68908.1043	88489.7261
$\Sigma Y^2/n$	68335.345	56757.2002	67010.0593	86092.4877
n	36	36	36	36
a	6	6	6	6
ΣnX	606	606	606	606
ΣnX^2	13506	13506	13506	13506
$\Sigma X(\Sigma Y)$	26443.92	25463.93	28181.66	31188.58
Regression statistics				
Σx^2	3305	3305	3305	3305
Σxy	108.5067	1974.36	25.31.0738	1713.952
SS grps	346.5209	2668.3953	2510.9916**	928.7116
SS lin. regress.	3.5623	1179.4545	1938.3765*	888.8446
SS dev. regress.	342.9586	1488.9408	572.6151	39.867
SS within	3983.888	6428.6872	1898.045	2397.2384
$b_{y.x}$	0.0328	0.5973	0.7658	0.5185
$\bar{\bar{Y}}$	43.4577	38.7616	42.3277	48.638
Y inter.	42.9056	28.7071	29.4368	39.9152

*Significant at the 1% level.

**Significant at the 5% level.

Appendix Table 8. Effect of sperm treatments on subsequent embryonic development

Sperm incubated in	Frog 1* θ	Frog 2 θ	Frog 3 θ
Holtfreter's solution	71.21	79.04	80.55
Seminal vesicle homogenate	56.51	67.75	69.45
Holtfreter's solution + egg water	64.04	60.88	68.95
Seminal vesicle homogenate + egg water	69.59	65.45	80.42

ANOVA Table

source of variation	df	SS	MS	F
Frogs	2	898.3799	449.1899	0.7989
Treatments	3	1652.0536	550.6845	0.9794
Interaction	5	498.7693	99.7538	0.1744
Error	48	26987.7707	562.2452	

Sperm incubated in	Frog 1** θ	Frog 2 θ	Frog 3 θ
Holtfreter's solution	60.8	60.0	60.6
Seminal vesicle homogenate	73.57	58.69	62.1
Holtfreter's solution + egg water	54.76	58.69	62.1
Seminal vesicle homogenate + egg water	59.21	60.53	60.53

Appendix Table 8 (cont'd.)

ANOVA Table

source of variation	df	SS	MS	F
Treatments	3	64.8403	21.6131	0.62
Error	8	279.7062	34.9632	

*Data are expressed as arc sin equivalents of % developing embryos/% fertilized eggs. Each value given is an average of six.

**Data are expressed as arc sin equivalents of no. of normal embryos/ total no. embryos.

Appendix Table 9. Effect of castration on the ability of seminal vesicle homogenates to alter fertilizing capacity of sperm

Insemination Time	Holtfreter's Solution θ	Seminal Vesicle Homogenate Intact θ	Seminal Vesicle Homogenate Castrated θ
	sperm incubated for 48 hours in:		
1 min.	0.0	26.57	14.77
10 min.	0.0	29.60	39.00
15 min.	17.36	32.33	43.11
20 min.	19.82	35.79	33.09
25 min.	12.92	33.09	42.92

ANOVA Table

source of variation	df	SS	MS	F
Treatments	2	1470.6931	735.3465	11.468
Holtfreter's vs seminal vesicles	1	1470.5947		22.93
Times	5	2230.1654	446.033	6.96
Error	10	641.2153	64.1215	
Total	17	4362.0738		

Regression Statistics	Holtfreter's Solution	Seminal Vesicles Intact	Seminal Vesicles Castrated
Σx^2	342.8	342.8	342.8
Σxy	268.38	281.04	331.398

Appendix Table 9 (cont'd.)

Regression Statistics	Holtfreter's Solution	Seminal Vesicles Intact	Seminal Vesicles Castrated
SS regress.	210.16	230.364*	320.375
SS error	149.01	55.83	236.47
$b_{y.x}$	0.783	0.820	0.967
Y intercept	0.0	22.892	20.851

*Significant at the 5% level.