The Effect of Castration on the Development of the Bidder's Organ, and Its Role in Sex-Reversal in *Bufo americanus*

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Abstract

All male toads of the genus *Bufo* possess a unique reproductive feature: the Bidder's organ. This organ is a rudimentary ovary, composed of undeveloped ovarian tissue. In some *Bufonids*, the Bidder's organ will develop into an ovary upon castration, and literally reverse the sex of the animal. In this experiment, the American Common Toad, *Bufo americanus*, was castrated to see if sex reversal was possible within that particular species. Through histological analysis and comparison of the Bidder's organs of castrated and non-castrated control animals, it was found that the Bidder's organ of *Bufo americanus* will begin to develop into an ovary after castration. Hopefully, these data will be useful to herpetologists interested in *Bufo americanus*, and will add to scientists' knowledge of sex reversal in all toad species.

Introduction

An anatomical feature common to nearly all toad species is the Bidder's organ. First discovered in the middle 1800's, the Bidder's organ has been an unknown phenomena to biologists for many years after its discovery. Today, the Bidder's organ is known to be a rudimentary ovary present in male toads, attached to the testes (Pancak-Roessler, 1991). In experiments first performed in 1922 by K. Ponse of France, it was discovered that in *Bufo bufo* the male will actually change sex after castration due to sudden growth and development of the Bidders' organ (Rostand, 1934). The Bidder's organ consists of undeveloped oocytes, which normally would not yolk or mature. In Ponse's experiment (Rostand, 1934), the testes of an Italian toad, *Bufo bufo*, were removed in January 1923. The toad was routinely reopened to observance the development of the Bidder's organ. By May 1924, the toad's Bidder's organ had enlarged and almost fully developed into an ovary. On February 12, 1925, the castrated toad spawned

120 eggs (of which nine survived to become toads), after being mated with another male toad. From 1925-June 2, 1928, when the toad finally died, Ponse obtained 608 toads (Rostand, 1934). Some toad species such as *Bufo viridis* do not change sex upon castration or are reproductively sterile (Rostand, 1934). As far as the author knows, no such experiment has ever been attempted using *Bufo americanus* as the subject. Only four or five toad species are known to be hermaphroditic to any extent.

As in all vertebrates from amphibians to humans, the sexual behavior and reproductive cycle of toads is under hormonal control. In males, the reproductive behaviors are controlled by both steroid hormones and neuropeptides (Moore, 1983). Very little research has been done on female endocrinology; therefore, only the male side will be discussed (many female toad hormones are similar to those in female humans such as estrogen, progesterone, and luteinizing hormone). Testosterone and dehydrotestosterone (DHT) influence mating calls and amplexus by stimulating the male to give mating calls

during the breeding season (late April thru early May), and stimulate the male to move into the position of amplexus (Moore, 1983). Amplexus is the position a male and female toad take during sexual intercourse. The male toad climbs onto the back of the female toad and clasps her tightly with his front legs. Moore's study pointed the necessity of steroid hormones for maintaining reproductive behaviors such as mating calls and amplexus. However, it is neuropeptides which actually activate the mating behaviors of frogs and toads. AVT and AVP are both neuropeptides which cause amplexus in male newts upon injection (no similar experiments have been done with toads). It is these neuropeptides that may influence toad mating behavior, and their levels could be involved with sex reversal.

The histology of most vertebrate ovaries is similar, when compared with the ovaries of invertebrates. However, the amphibian ovary, anurans in particular, differ greatly from other vertebrates. The vertebrate ovary is derived from three major sources: primordial germ cells, the "germinal" epithelium, and

the mesenchyme cells (Dodd, 1986). Anuran ovaries, unlike the Gymnophionas (a sub-family of amphibians), are very large, paired and lobed. They consist of many small, fluid-filled sacs (lined heavily with blood vessels), which contain developing follicles (oocytes or eggs). Development of the oocytes (vitellogenesis) occurs in four distinct stages. In stage I, oocytes are 50-300 micrometers in diameter and are previtellogenic (Dood, 1986). The oocytes contain a large yolk nucleus, mitochondria, golgi complexes and cisternae. By stage II, the oocytes have increased in thickness and now possess microvilli. During stage III, actual vitellogenesis begins as the pigment and cortical cells increase in number, and the vitelline envelope forms a continuous layer over the oocyte (Dood, 1986). Stage IV brings vitellogenesis to a close as the microvilli disappear.

The studies by K. Ponse suggest a possibility for sex reversal in other *Bufonids*. Ponse's work demonstrates that major histological changes must take place in the Bidder's organ in order for it to have a

histology like that of an ovary. Finally, all of the histological changes must be directed by steroid hormones and neuropeptides. Therefore, I hypothesized that after castration, male toads (*Bufo americanus*) would begin to develop a functional ovary from their rudimentary Bidder's organ.

Methods

This study was conducted from May 18, 1992 through July 23, 1992 at the University of Notre Dame Environmental Research Center (UNDERC) in the Upper Peninsula, Michigan. At UNDERC, several vernal ponds, lakes, and wooded areas immediately surrounding the center's residences were used as the primary study area, since *Bufo americanus* were believed to reside there. The study was divided into three major areas: field work at UNDERC, laboratory work at UNDERC, and laboratory work at the University of Notre Dame, Notre Dame, IN (this took place from August 28 through October

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28, 1992).

First, 2-3 toad populations were to be located on the UNDERC grounds ranging in size from 15-80 toads. However, due to the cold temperatures at UNDERC during the primary toad breeding season, the toads, as far as I know, never bred. Consequently, only a handful of toads were located on the property, approximately eighteen. Each toad captured was toe-clipped with small scissors and measured to the nearest millimeter, from snout to ischium, with a plastic ruler. After measurement, the toad was weighed to the nearest milligram using a digital balance. The field work was the most demanding due to the difficulty in locating usable male toads.

The laboratory aspect of the experiment involved the development of the Bidder's organ. Originally, ten male toads would be castrated via a surgical procedure performed by myself in conjunction with Dr. Sunny Boyd, University of Notre Dame. Another ten male toads would undergo an operation to simulate the stress of surgery; however, these toads would not be castrated. Once again the poor weather prevented the capture of twenty male

toads. The final numbers used were two male castrates, two male shams and one female sham operated toad (nine out of the eighteen captured toads were females, the remaining males either died at UNDERC or at Notre Dame in late August and early September).

The surgery involved removing both testes via a bilateral removal operation. The animal was first anesthetized using a benzocaine anesthetic consisting of 10g benzocaine in 100ml of 95% ethanol. When the animal was anesthetized it was placed in a plastic bucket filled with approximately two inches of the anesthetic (4-6ml of the stock solution in one gallon of tap water). The volume of anesthetic was varied frequently, since many toads took up to one hour to go asleep and would only remain asleep for ten to twenty minutes. Usually, 5.5ml of the stock solution was used. This amount put the toad out in about forty-five minutes and kept the animal out for about thirty-five minutes. Still, some toads woke up during surgery, and had to be re-anesthetized the next day. Those toads who awoke during surgery generally had to be physically restrained

and placed in a shallow petri dish of anesthetic, while the procedure was finished. No toads died of shock from waking up during surgery; however, two toads did die from being over-anesthetized.

The surgery itself proved to be quite challenging. The incision was made to the left of the animal's ventral artery using tweezers to hold up the skin and muscle layers, and scissors to cut the actual half inch incision. The testes are located on the left and right sides of the toad's kidneys (which are connected together). The most challenging part of the surgery involved locating the kidneys. The toad's stomach, large intestine, small intestine, pancreas, and bladder all had to be temporarily removed to allow access to the kidneys. One toad died from internal bleeding caused by accidently slicing an intestinal artery while searching for the kidneys.

Once the kidneys were located, the testis were easy to identify due their pale yellow color compared to the orange/red color of the kidneys. The testis resembled beans in their shape. The Bidder's organ was also

visible as another white organ attached to the top of the testis. The testis were removed by simply grasping them with the tweezers and snipping them off with scissors. Care had to be taken to avoid damaging the kidney or the Bidder's organ. One toad died after I accidently snipped one of its renal arteries. Once the testis were removed, the incision was stitched using a hooked surgical needle and standard nylon suture. The incision generally required two or three stitches. The stitches were hard to do because the needle and suture were each too thick in diameter for the thinness of the toad's skin and muscle layer. Also, many of the toads began to come out from under the anesthetic by this stage of the surgery and were kicking violently, making the stitching process quite difficult. The entire time for the surgery was usually about twenty minutes and another five minutes for the stitches. Male toads of the control group were merely opened, the testis located to confirm sex, then the incision stitched. Since sex could not be determined without surgery, the females were not identified, until the ovaries were located.

The females were then stitched. Neither the male shams nor the female shams had any organs removed. After surgery, the toads were placed in another bucket containing an angled plastic "bed" to place the toad on, and tepid water to gradually wake up the animal. The animal was kept in the bucket for one full day and observed every few hours.

In order to prevent bacterial infection, the toads were treated with antibiotics for two hours, three times per week for two weeks after surgery. The antibiotic solution consisted of two grams penicillin and two grams streptomycin in two liters tap water. The solution was good for one week if refrigerated. A second form of infection prevention involved the use of "Anti-Ick" fungal medicine. This blue powder was diluted with tap water and used periodically on the toads, two hours at a time, as the condition persisted. Many of the toads did develop fungal infections around their incisions; however, the anti-fungal solution quickly remedied the problem. Several toads did develop fatal bacterial infections which the antibiotics could not cure, even

with more frequent soakings.

When not under the knife, the toads were kept in the basement of the research lab in a large 7'X 4'X 3' wire cage. The cage was lined with cardboard and newspaper. A natural environment was obtained by filling the bottom of the cage with loose soil, leaves, twigs, and rocks. The cage was cleaned and refilled every three weeks. The toads were at first fed a variety of live insects including: dragonflies, damselflies, and grasshoppers. However, five toads died of apparent starvation, so leaf worms were used during the final two weeks at UNDERC with great success. At Notre Dame, the remaining thirteen toads thrived on live crickets, which were unavailable to me at UNDERC. The possible reasons for the toads starvation could be: lack of food (the toads were only fed every two or three days), feeding them the incorrect food, or a complication from surgery which simply rid the toad of its desire to eat. I find the last explanation very attractive, since the surgery involved removing the stomach and intestines before castration and then

replacing them prior to stitching the incision. The internal organs could easily have been damaged in the process or placed back incorrectly.

After returning to the University of Notre Dame in late August, the Bidder's organs of the surviving castrated toads were examined and compared to a normal non-castrated toad's Bidder's organ (*Bufo americanus*). By early September only two male castrates and two male shams were useable for examination. The other nine toads were either female or males who were unusable (two male castrates and one male sham died at Notre Dame between July and August. Their bodies were frozen, but they were too decayed to be examined).

In order to study the histology of the toads, the remaining toads were over-anesthetized in a solution containing twenty grams of benzocaine in 100ml of tap water. The thirteen toads were then placed in a preservative solution of 40% neutrally buffered formalin. Next, the Bidder's organ of the two remaining male castrates and the two remaining male shams (controls) were removed using tweezers and scissors.

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After removing the organs from the toads, the organs were prepared for histological analysis by placing in dry ice and embedding them with O.C.T. embedding agent. The organs were cut into 50 micrometer sections with a microtome at -17 degrees Celsius and placed on snubbed microscope slides. Finally, the sections were stained using the Harris Hematoxylin-Eosin staining process (see sheet at end of paper for detailed staining directions). The eosin stained the cytoplasm orange, while the hematoxylin stained the organelles purple. The stained slides were examined under a microscope at 4X power and 10X power, and drawings of the sections at each power were made.

Results

Ideally, the Bidder's organ would begin to slowly develop into a functional ovary in the castrated male toads, similar to the *Bufo bufo* toads which K. Ponse worked with in 1922. The figures shown below are drawings of the toad sections made with the help of a

microscope's drawing instrument. Figure I is of a male sham's Bidder's organ. The drawing shows the Bidder's organ to contain undeveloped ovarian tissue as expected. The ovarian tissue is in an arrested condition. A female toad would have ovarian tissue resembling this at approximately stage II of development (Figure II). Comparison of Figure II with Figure I reveals a definite similarity of cell types.

Figure III shows the Bidder's organ of a male castrate. Once again, the drawing shows ovarian tissue, but the development is at least stage IV and probably stage V (Figure IV). The Bidder's organ tissue of the male sham shows follicle cells which arch over the oocyte and contain microvilli. The oocyte are approximately 300 to 450 micrometers in diameter (stage II). The Bidder's organ tissue of the male castrate shows less yolk in the follicles and fewer and shorter microvilli. The peripheral cytoplasm also contains irregularly shaped yolk platelets (stage IV or V). Clearly, the Bidder's organ of the male castrate did develop.

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Figure V shows the testis of an uncastrated male sham toad. In the drawing, the flagellum of the sperm are clearly visible. Figure VI shows how an uncastrated male's testis should look. Figures V and VI clearly resemble each other.

During the sectioning process outlined above in the "Methods" section, several mistakes were made. First, one of the male castrates was incorrectly sectioned. Instead of sectioning its Bidder's organ, I sectioned part of a fat body. Secondly, one of the male shams had also been incorrectly sectioned. Instead of Bidder's organ, I sectioned one of its testicles. Therefore, only one sham and one castrate were available for comparison.

Discussion and Conclusions

Since only one castrate and one sham toad were available for comparison, it is difficult to justify any of my forthcoming conclusions. However, the data do provide for some interesting interpretation. According

to my drawings from the stained slides, at least one of the male castrates did begin to show some significant development. Although the Bidder's organ did not completely change into a functional ovary, this does not negate the hypothesis. Time must be taken into consideration. This particular toad was castrated on June 9, 1992 and was over-anesthetized on August 28, 1992. The experiment was only run for two and half months. It is very possible that more development would have taken place, if the experiment had been run for a longer period of time as originally planned. However, the time period was cut short due to the poor health of the toads.

Many other experiments involving sex reversal in amphibians, reptiles, fish, and even mammals have been performed. Many species of amphibians are capable of sex reversal during development. E. Vannini induced sex reversal in *Rana dalmatina* by injecting tadpoles with testosterone in 1975. The tadpoles treated were only two to four weeks along in development. Vannini also used an antibiotic, actinomycin D, to induce sex

reversal. The antibiotic works by inhibiting DNA-dependent RNA-transcription. *Rana sylvatica* shows sex reversal, if tadpoles are subjected to high temperatures (Witschi 1929). The testis are almost totally unaffected by the increase in temperature; however, the ovaries showed dramatic change. After two weeks of increased temperature, new oocyte generation had virtually disappeared. Oocytes already present reverted back to the auxocyte stage, and assumed an abnormal character. Within six weeks, the ovaries became functional testis. Reversal has also been shown in *Rana temporaria* after testosterone injections (Gallien 1937). The process occurs very similarly to the one described above for *Rana dalmatina*. In 1984, an experiment on the salamander *Ambystoma mexicanum* resulted in reversing the sex of males to females using estrogen injections (Bodney 1984).

The above experiments all dealt with complete sex reversal initiated during development. How does sex reversal during adulthood differ? It is already well known that sex reversal in adulthood is not possible in

birds and mammals. Experiments with some fish species such as *Monopterus albus*, *Coris julis*, and *Anthias spuamminpinnis* have been very successful (Norris 1987). What about reptiles and amphibians? Sex in most reptiles is not determined genetically. For most reptile species, sex is determined almost entirely on incubation temperature (Norris 1987). A few lizard species are even unisexual. Due to the difficulties involved in rearing reptiles, little is known about the specifics of their sexual differentiation. Experiments using androgen injections into incubating eggs has caused partial sex reversal. No experiment has been able to cause permanent or total sex reversal by using injections during development or adulthood. Therefore, the sex reversal inducer is unlikely to be a sex steroid. Some postulate that secretions from the pineal gland may be responsible (Norris 1987).

Amphibian differentiation occurs entirely during the post-hatching larval stage. The experiments described above clearly indicated that complete sex reversal can occur during development by using steroid

injections. In nearly all cases, male sex steroids dominated with ovaries being masculinized, but testis not being femininized. Obviously, sex steroids are the gonadal inducers in most cases. But, what about *Bufo*? Sex steroids do not induce sex reversal in Bufo larvae or adults (Norris 1987). The actual inducer remains unknown. Castration of male *Bufonids* does occasionally cause sex reversal. Removal of the testis in *Bufo vulgaris* and *Bufo bufo* both result in sex reversal (Gallien 1974).

In amphibians, the onset of sex reversal causes a few hormonal and structural changes. Without female steroids such as estrogen, the mullerian ducts will not develop. In urodeles, increased levels of female sex hormones, especially estrogen, causes shrinkage in cloacal glands (Norris 1987). If female *Xenopus* are reversed, they develop large nuptial pads and an increased larynx size due to higher levels of testosterone.

Studies of the behavior of sex reversed adults has yielded mixed results. Reversed *Xenopus* females did

begin to clasp normal females; however, estrogen treated amphibians showed no differences in behavior. The vocal mechanism of *Bufo americanus* is controlled by the magnocellular portion of the pretrigeminal nucleus (located in the midbrain). The area is larger in males than in females, and can be completely developed in adult females upon injection of testosterone (Norris 1987). Finally, studies with *Rana pipiens* show that male laryngeal nerves (isolated from their brainstems) possess call-like firing patterns not found in females. However, upon injection of male androgens, the females begin to exhibit the same call-like firing patterns.

These data suggest that some amphibians may be sex reversed in adulthood; therefore, the mechanisms for sexual differentiation of the brain, gonad and behavior must be fundamentally different from those of mammals and birds.

Hematoxylin/Eosin Staining Procedure

Solution	Time
1. xylene	3 minutes
2. 100% EtOH	2-3 minutes
3. 95% EtOH	2-3 minutes
4. 70% EtOH	2-3 minutes
5. distilled water	5 minutes
6. distilled water	5 minutes
7. Harris hematoxylin	2-5 minutes
8. distilled water	2 minutes
9. distilled water	2 minutes
10. Scott solution	3 minutes
11. distilled water	3 minutes
12. Eosin	1 minute +
13. 70% EtOH	5 dips
14. 95% EtOH	5 dips
15. 100% EtOH	3 minutes
16. 100% EtOH	3 minutes
17. xylene	3 minutes
18. xylene	3 minutes

19. Permount and coverslip while sections are still moist from xylene

Figure Legend

Figure 1-Male sham Bidder's organ from *Bufo americanus* (10X)

Figure 2-Female ovarian issue from *Xenopus laevis* at stage II (Dood, 1986)

Figure 3-Male castrate Bidder's organ from *Bufo americanus* (10X)

Figure 4-Female ovarian tissue from *Xenopus laevis* at stage IV (Dood, 1986)

Figure 5-Male sham testicular tissue from *Bufo americanus* (10X)

Figure 6-Male testicular tissue from *Rana nigromaculata* (Nagahama, 1986)

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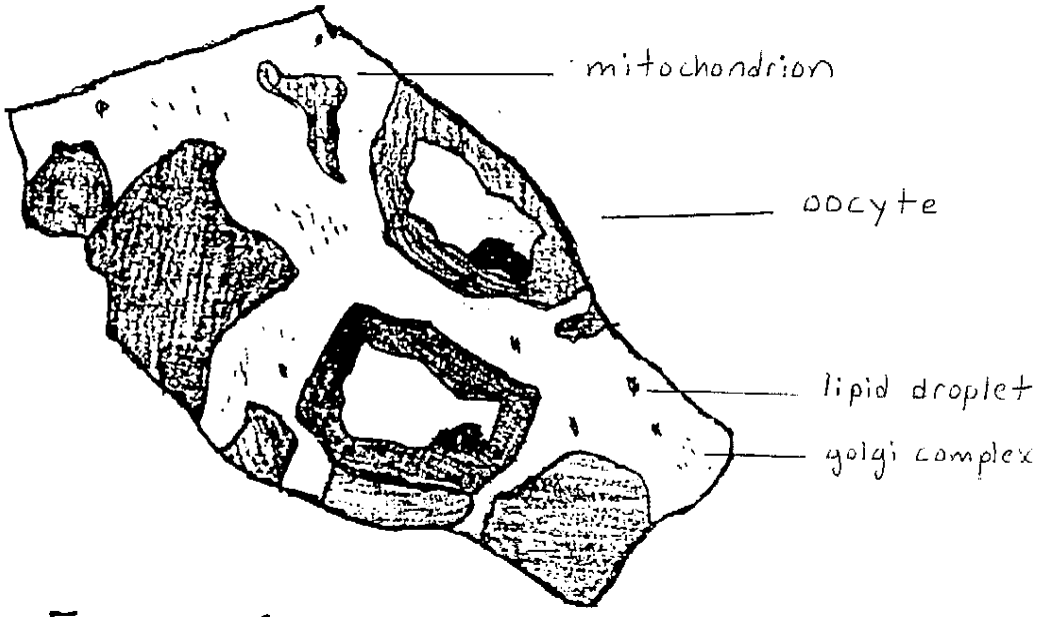
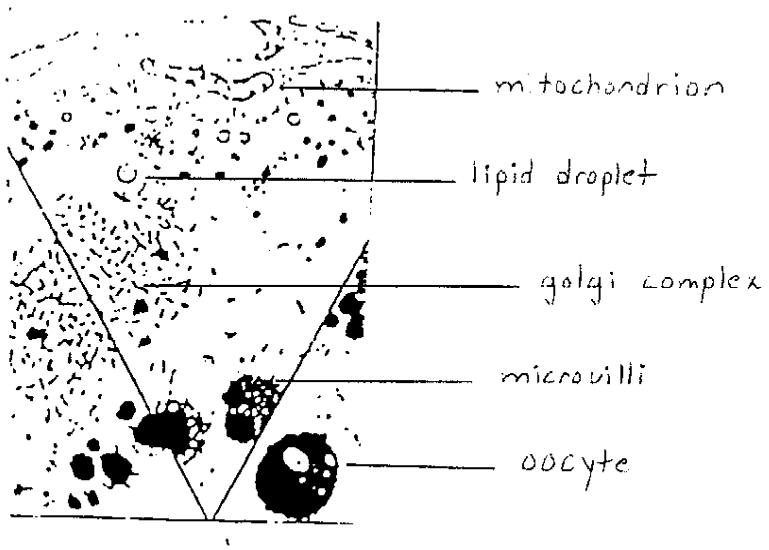


Figure 1



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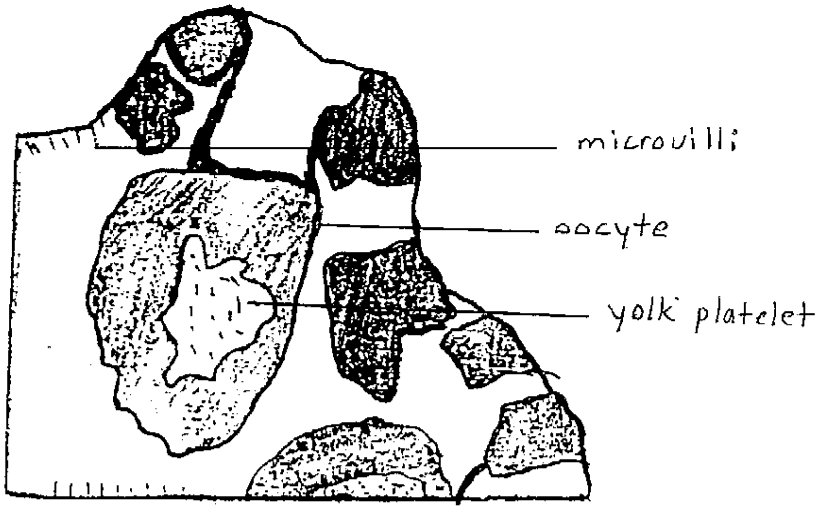

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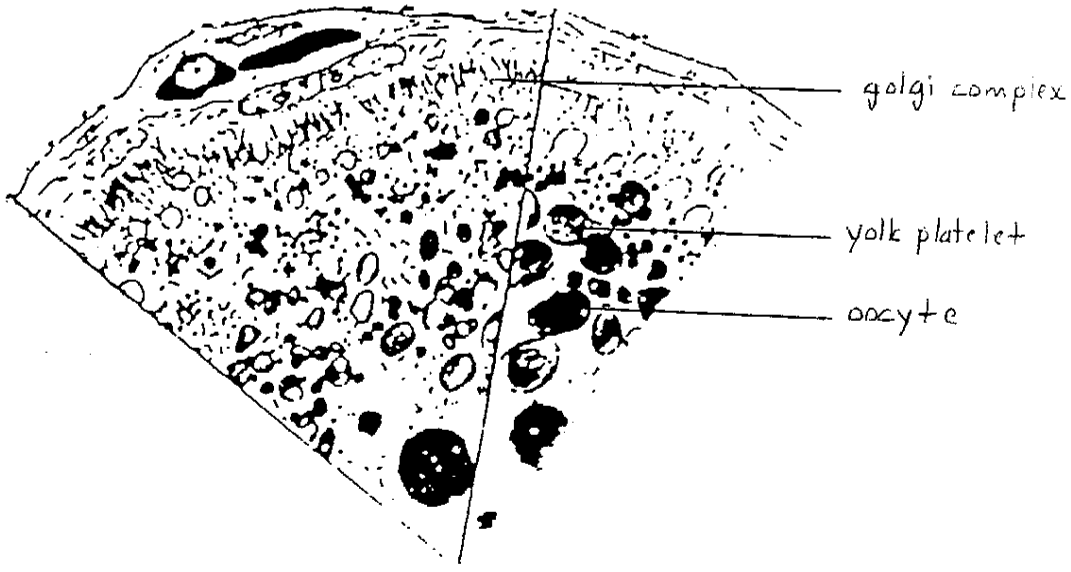

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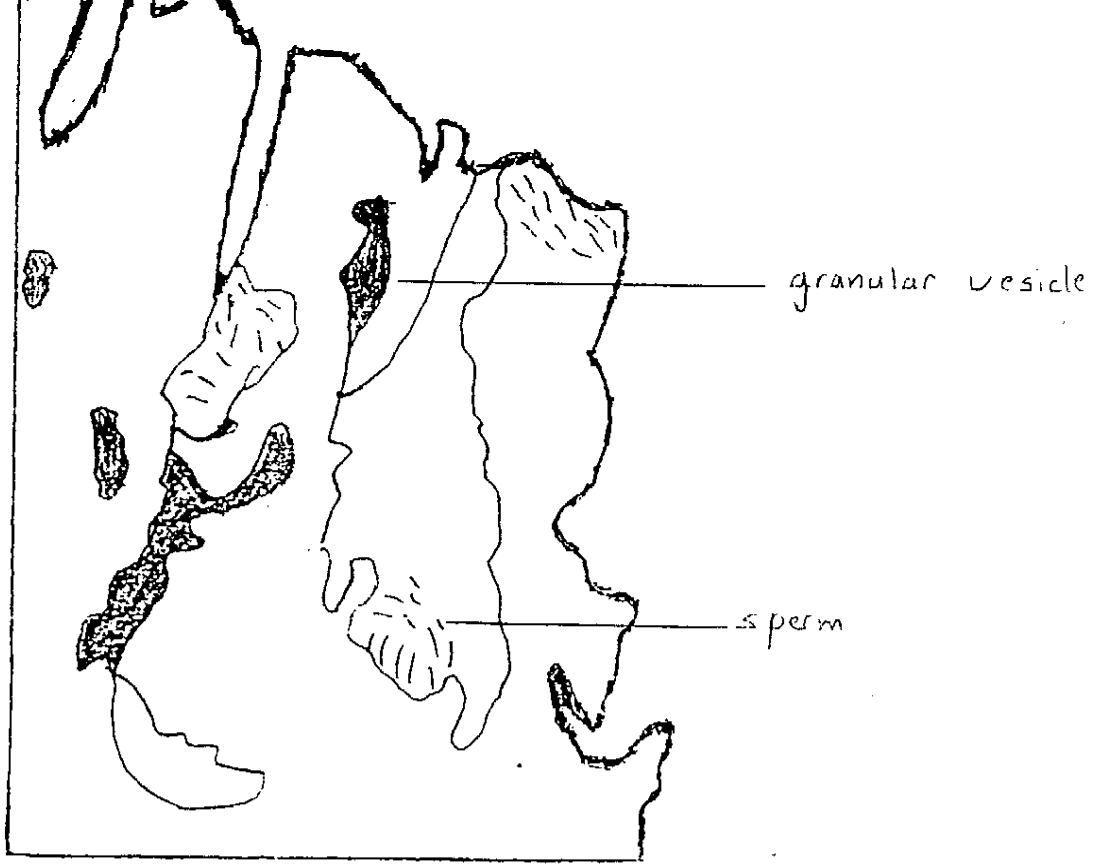

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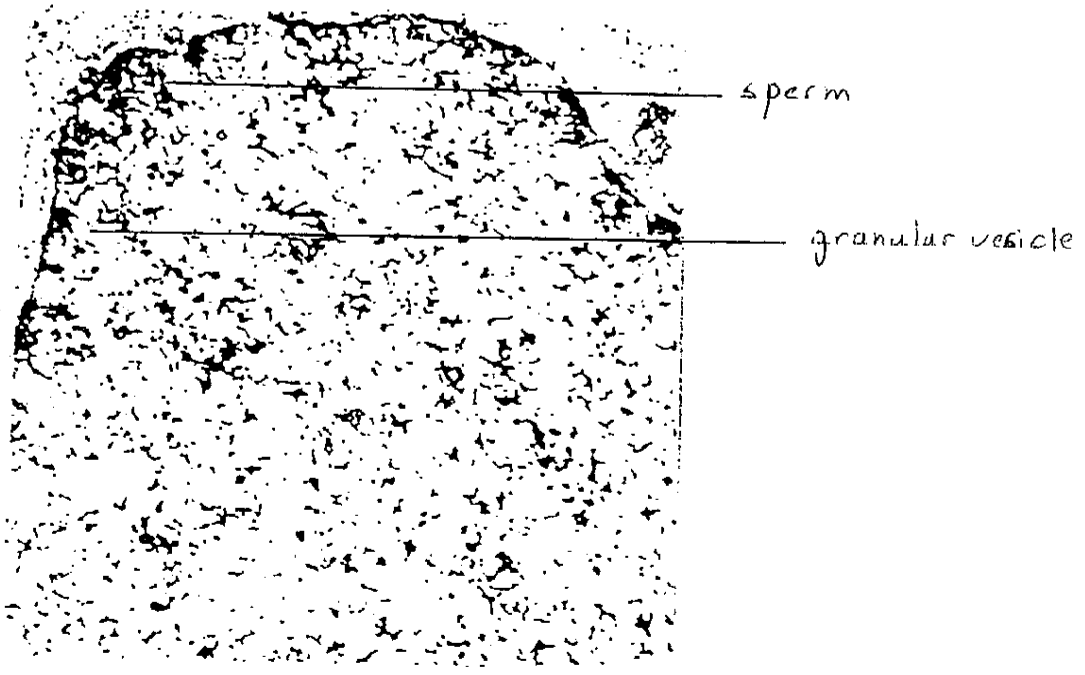

Figure 6