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THE CYTOPLASMIC DROPLET ON MINK (*Mustela vison*) SPERMATOZOA

by

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ABSTRACT

THE CYTOPLASMIC DROPLET
ON MINK (*Mustela vison*)
SPERMATOZOA

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The effects of spermatozoa with cytoplasmic droplets, in normal ejaculations of mink, on the fertilizing capacity of the male were examined. Results show that ejaculates with droplet concentrations up to 9% had no discernible effect on fertilizing capacity.

Concentrations of the cytoplasmic droplet on mink spermatozoa remain constant throughout the epididymis and vas deferens. Active motility of spermatozoa aid in casting of the droplet from the posterior area of the middle-piece.

Scanning electron microscope photographs show that the size of the droplet decreases slightly as it descends down the reproductive tract. The droplet on mink spermatozoa, in transmission electron microscope photographs, have U-shaped tubules and medium to large vacuoles dispersed throughout the membrane-bound matrix.

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INTRODUCTION

The existence of the cytoplasmic droplet (kinoplasmic or protoplasmic droplet) on the tail of mammalian spermatozoan has been known since Ritzius described it in 1909 (Bloom and Nicander, 1961). The majority of the data on the cytoplasmic droplet has been obtained from farm animals and rodents, due to the increasing importance of artificial insemination since the early 1900's. A detailed study on the ultrastructure of mink spermatozoa was not conducted until 1978 (Kim et al., 1968); however, the cytoplasmic droplet was only mentioned briefly. Scanning electron microscope and transmission electron microscope studies were done to obtain knowledge of the surface structure and ultrastructure of the droplet.

Mink ranchers occasionally observed spermatozoa with droplets in semen samples obtained in routine sperm checks during the breeding season. The possibility that male mink displaying spermatozoa with attached droplets in their semen samples have decreased fertilizing capacity was naturally a great concern to mink ranchers. The reproductive trial of this study, which was funded by the Mink Ranchers Research Foundation, was run using standard ranch breeding practices to determine whether or not mink displaying spermatozoa with droplets should be eliminated from breeding programs. Concentrations of spermatozoa with cytoplasmic droplets were calculated, from the caput (head), corpus (middle), and cauda (tail) of the epididymis and the proximal and distal areas of

loss of the droplet on mink spermatozoa.

REVIEW OF LITERATURE

GENERAL DESCRIPTION OF REPRODUCTION IN MINK

Female mink will come into estrus on the average of three times during the breeding season which begins at the end of February and extends through the month of March. Female mink will come into estrus at intervals of 7-10 days and remain in estrus for three days. If the females are checked by a trial breeding every third day, the chances are good for breeding in one of their heat periods.

There is a correlation between litter size and the number of times a female is mated during the breeding season. Age of the females also affects production with first year females being the lowest producers and third year females the highest producers. Mating females more frequently also decreases the number of females that do not whelp. Best results are obtained with a three mating system where the second mating is 7-10 days after the first mating and the third mating is within 24 hours of the second mating (Venge, 1956, 1973). In the latter portion of the breeding season, females that are mated for the first time are rebred the next day as it is not practical to ascertain whether a second mating can be obtained in a latter heat.

The stimulus of copulation is required for ovulation to take place in mink. The time of ovulation is from 36-52 hours after copulation. The length of copulation normally lasts about 1 hour, but ranges from 10-12 minutes to 3-4 hours or more (Adams and Rietveld, 1981). Duration of

copulation is important for sperm transport. When copulation is restricted to 6 minutes or less, fertility is decreased compared to 12 minutes or more (Venge, 1956). This decrease in fertility is not due to lack of sufficient sperm numbers but rather lack of sufficient stimulus. Adams and Rietveld (1981), using vasectomized males to prolong stimulation, increased fertility when normal males were used for only five minutes. The use of the vasectomized male could either precede or be subsequent to the natural mating period. General practice on mink ranches allows copulation to take place for 12-24 minutes. Mating is interrupted after this time so the males can be used more frequently thereby reducing the number of males needed to breed the females on the ranch.

When a male and female mink are placed together for breeding, brutal fighting can occur when the female mink is not in estrus and refuses the advances of the male. If the female is in estrus, there is generally little fighting, and copulation takes place. There are times when a particular male shows no interest in a certain female and vice versa. If the uninterested animal is placed with a new partner, copulation may take place. There is no explanation as to why this occurs (Venge, 1973). Enders (1952) described the anatomy of the mating act. The S-shaped position is assumed by the male following intromission.

GENERAL DESCRIPTION OF MALE REPRODUCTIVE TRACT

The organs which make up the male reproductive tract of mink are the testes, epididymides, vas deferens, penis, and the accessory sex glands. The accessory sex glands consist of a well-developed pair of ampullae of the vas deferens and a prostrate. Male mink lack seminal vesicles and bulbourethral glands (Basrur and Ramos, 1972).

The testes are composed of a pair of ovoid shaped organs that produce spermatozoa and steroid hormones. The testes have an inelastic fibrous capsule, the tunica albuginea. This capsule thickens at the posterior end, forming the mediastinum testis which surround the rete testis. The seminiferous tubules make up 80% of the testicular mass and are connected by the tubule recti to the rete testes which connects to the epididymis. A watery fluid is secreted by the Sertoli cells of the seminiferous tubules and transports the non-motile spermatozoa into the epididymis. The epididymis reabsorbs 99% of this fluid and excretes its own fluids which nourish the spermatozoa.

Continuous with the rete testes is the ductuli efferentes, which connects to the head of the ductus epididymis. The ductules emerging from the mediastinum testes are in the form of strait cords and become slightly coiled and folded as they approach the head region of the epididymis (Basrur and Ramos, 1972). The epididymis is the site where the spermatozoa mature and it is also the primary storage area for spermatozoa. Normally, there is a division

of the epididymis into three regions consisting of the caput (head), corpus (middle), and cauda (tail).

The vas deferens are the tubes that are continuous with the cauda epididymis and ascend, by way of the inguinal canal. Union of the vas deferens with the urethra occurs around the prostrate gland. The ampullae are at the distal end of the vas deferens and are the thickened glandular areas of this structure (Basrur and Ramos, 1972). Circular and longitudinal muscles in the vas deferens contract involuntarily during ejaculation.

The prostrate, being the only other accessory sex gland, besides the ampullae, is responsible for the fluid portion of semen. A well-developed disseminate prostrate gland completely surrounds and is attached to the entire pelvic urethra (Basrur and Ramos, 1972). Secretions in the alveoli of the prostrate are homogenous, eosinophilic and highly PAS-positive (Basrur and Ramos, 1972).

SEASONAL VARIATION IN THE MALE REPRODUCTIVE TRACT

The mink is a seasonal breeder which undergoes changes in testicular development throughout the year. Kits differ from adults in the development of the seminiferous tubules, epididymis and interstitial tissue until December (Bostrum et al., 1968).

Testicular weights of kits increase by 25-fold from March to June, then change very little until mid-November when a progressive increase in testicular weight begins. By March maximum size is attained. When compared to body

weights, the testes decrease slightly from June to mid-November. Adults have a slight but constant decrease in testicular weight from April to November. This trend is also seen when testicular weight is compared to body weight (Bostrum et al., 1968).

Little evidence of spermatogenic activity is seen in kits from June through late September. During this time the seminiferous tubules are lined with a single layer of cells which are composed mainly of Sertoli cells with the rest being resting spermatogonia. A second layer of cells develop in the lining of the tubules in October. There is an increase in the number of large resting spermatogonia and the lining of the tubules consists of two to three layers of cells which contain Sertoli cells, spermatogonia and a few primary spermatocytes. The adult testes are aspermatic and in a resting stage resembling the kits, from June to November. After November, there is little difference between kits and adults. Active spermatogenesis is well under way by early December. The testes contain one to two layers of rapidly dividing primary spermatocytes and numerous active spermatogonia along with secondary spermatocytes and spermatids in many of the tubules. Sertoli cells begin to hypertrophy in early December and continue to enlarge until maximum size is reached in March. By April some regression and degenerative changes are taking place in seminiferous tubules. There are few, if any, spermatozoa in the rete testes, ductuli differentia and proximal portion of the

epididymis. Notable regression of the seminiferous tubules is taking place in June. In late June the tubules are lined with a single layer of cells which is composed mainly of Sertoli cells. From June through November the adult testes are in a resting stage closely resembling the kits (Bostrum et al., 1968).

The interstitial tissue shows little activity in the kits from June through October. Islands of interstitial cells surround the rete testes in the mediastinum. The interstitial cells are small and numerous and about equal in mass to the seminiferous tubules. The nuclear to cytoplasmic ratio of these cells is 1:1.25 to 1:1.5 during this time. In mid-November the nuclear to cytoplasmic ratio begins to increase. The cell ratio is approximately 1:2 in December, and there is an increase in vascularity. Maximum size of the interstitial cells is attained in February, when the nuclear to cytoplasmic ratio is 1:2.25 to 1:2.5. The nuclear as well as the cytoplasmic size increases; therefore, the cellular size has more than doubled since July or August. The increase in size of the seminiferous tubules and interstitial cells account for the apparent decrease in the number of interstitial cells during the breeding season. The interstitial cells remain active until April when the nuclear to cytoplasmic ratio decreases to 1:1.5 to 1:1.75. From April to June, there are yellow-brown pigment granules which are prominent in the interstitial cells; this pigment is present in the cells through November. By June, the

interstitial tissue is in a resting stage. For the same date, the adults nuclear to cytoplasmic ratio is slightly greater than that of the kits (Bostrum et al., 1968).

The epididymis in both the kits and adults shows little activity from June through November. The ducts of the epididymis are lined with low pseudo-stratified columnar epithelium with poorly developed stereocilia. There is little secretory activity at this time. In mid-November secretory activity is increased. The height of the epithelial cells of the ducts is slightly increased and the stereocilia are more apparent. After November the kits and adults resemble each others development. There is a progressive increase in weight from December until March, when the maximum size is reached. The weight increase is due to the hypertrophy of the epithelial cells, smooth muscle fibers, and an increase in sperm concentration. As the epididymis is developing from December through March, the luminal diameter becomes larger, especially in the tail region; and there is an increase in epithelial height with maximum development of the stereocilia, and an increase in secretory activity of the epithelial cells, especially in the tail. By mid-April, there are still mature sperm in the tail but few in the upper regions of the epididymis. This is probably the last time a fertile mating can occur. A progressive atrophy is taking place in the epididymis during this period which is accompanied by an increase in the surrounding connective tissue. There are no sperm left in

any portion of the epididymis by early June and by late June the resting stage is reached (Bostrum et al., 1968).

Individual strain and seasonal variation affect the onset of active spermatogenesis which begins sometime during the months of December and January. Pastel mink generally begin active spermatogenesis later than dark mink.

GENERAL DESCRIPTION OF SPERMATOZOA

There are two principal parts of a mammalian spermatozoa, the head and the tail. The head of the spermatozoa is composed of a nucleus which is capped by an acrosome. The tail is further subdivided into four regions consisting of the neck, middle-piece, principal-piece, and end-piece listed in descending order (Fawcett, 1965).

The head of mink spermatozoa is dorsoventrally flat and ovate in outline. The average length of the head is 5.83um with an average diameter of 0.35um at the equatorial segment. The acrosome covers the anterior 2/3 of the nucleus with the posterior 1/3 of the nucleus being covered by the postacrosomal sheath (Kim et al., 1978). The acrosome of mammalian spermatozoa is enclosed completely in a continuous membrane. It has its major region at the anterior edge of the nucleus with a thin layer extending back over the anterior portion of the nucleus. The acrosome forms a cap-like structure over the anterior part of the nucleus. The acrosome of the head of mammalian spermatozoa can therefore be described as a cap-shaped structure which is limited by a membrane (Fawcett, 1965). The acrosome plays a significant

role in the penetration of the sperm through the outer layers of the ovum. Mink have a relatively small acrosome which has its apical end extending a little beyond the nucleus. This is typical of sperm of man, monkey, bull, boar, rabbit, hare, ram, dog, horse, and cat but is different from chinchilla, guinea pig, and ground squirrel which have a much larger acrosome. (Kim et al., 1978). A special feature of mink spermatozoa is the swellings which occur anterior and posterior to the equatorial segment. Similar swellings have been observed in the rabbit and hare, except that these swellings are confined to the anterior borders (Nicander and Bane, 1966).

The neck region of the mammalian sperm tail can best be defined as the region between the nucleus and the first gyre of the mitochondrial helix of the middle-piece. This definition does not always give a sharp line of demarcation between the neck and middle-piece as in some species, such as the guinea pig, where there is a gradual transition of the orientation of the mitochondria from a longitudinal to a circumferential orientation (Fawcett, 1965). In the mink, however, there are generally one or two mitochondria with a longitudinal orientation which mark the starting point of the mitochondrial helix (Kim et al., 1978). The connecting-piece is the major structural component of the neck which functions to attach the tail to the head. Fawcett (1958) suggested that the connecting-piece is a modified centriole. Fawcett based this on the fact that when the flagellum is formed

there are two centrioles at the base of the early spermatid. The centriole which constituted its basal body can no longer be identified but the other one can still be seen in the interior of the connecting-piece. It can be assumed that the centriole-like basal body has been transformed into the connecting-piece. The only evidence that supports this theory comes from the superficial resemblance of the structure of the connecting-piece to other structures which are derived from centrioles and exhibit periodic structure such as rootlets of cilia. The connecting-piece may be the site of the initiation of the beat of the tail, as this function is generally associated with the basal body or kinetosome of simple flagella, and there is no typical basal body connected with the axial filament of the tail (Fawcett, 1965).

The middle-piece of the mink spermatozoa is about 10um long with 53-57 gyres of mitochondria which are of variable size (Kim et al., 1978). The amount of gyres and the number of mitochondria associated with the middle-piece varies with the species. At the end of the mitochondrial sheath is the annulus, formerly known as the Jensin's ring or ring centriole. The annulus marks the posterior end of the middle-piece separating it from the principal-piece. The function of the annulus is not known (Fawcett, 1965).

The principal-piece is characterized by a fibrous sheath which is composed of ribs that are circumferentially oriented and extend half way around the tail and insert into two

longitudinal columns that are continuous and run along either side. The sheath or tail helix surrounds the axonemal complex. In the mink this sheath ends abruptly at 1.5um from the tip of the tail and makes a junction between the principal-piece and the end-piece.

The end-piece is completely enclosed in a flagellar membrane. Its structure is identical to a cilium with only minor differences between species in the way the fibrils terminate.

FINE STRUCTURE DESCRIPTION OF THE SPERMATOZOA HEAD

The electron-dense nuclear material of the mink spermatozoa contains numerous vacuoles of varying size dispersed throughout. Vacuoles in the nucleus have an average diameter of about 20nm with a larger portion of the vacuoles in the posterior region of the nucleus. Sagittal sections taken from the sperm head show that the posterior portion of the nucleus is thicker than the anterior part. Covering the anterior 2/3 of the nucleus is the acrosome which is made up of a matrix of material that is less dense than the nuclear material. Division of the acrosome yields three main parts consisting of the apical segment, the main segment, and the equatorial segment. The apical segment is 0.68um long and it occupies the largest area extending a little beyond the tip of the nucleus. The main segment is 1.61um long and is bordered by the apical segment on its anterior end and the equatorial segment borders its posterior end. The equatorial segment occupies the smallest area and

has a length of 1.55um (Kim et al., 1978). Longitudinal sections show the triangular shaped perforatorium is 0.34um long. The postacrosomal sheath has a length of 1.99um and a diameter of 6um. The plasmalemma is firmly anchored only to the tip of the acrosome and the postacrosomal sheath. The remaining area of the acrosome is only loosely associated with the plasmalemma.

Bilateral swellings are formed at the anterior and posterior borders of the equatorial segment where the acrosome is separated from the nucleus. There are 6 of these swellings on the head of the mink spermatozoa. Similar structures have been seen in the rabbit and hare; however, these swellings are limited to the anterior border of the equatorial segment with the caudal part of the postacrosomal sheath being only slightly swollen (Kim et al., 1978).

FINE STRUCTURE DESCRIPTION OF THE SPERMATOZOA NECK

The neck of mink spermatozoa contains such structures as the capitulum, cross-striated longitudinal columns, proximal centriole, a few mitochondria and scrolls. Directly beneath the head is the basal plate, which is positioned within the area of the implantation fossa. This position of the basal plate is common in most species of mammals. The guinea pig is one of the exceptions to the basal plate placement, as its basal plate extends about a half micron or more beyond the implantation fossa (Fawcett, 1965). An electron-dense substance fills the space between the basal plate and the capitulum, which agrees with the findings of Saacke and

Almquist (1964) for the bull. Fawcett and Phillips (1969) observed fine filaments in Chinese hamster spermatozoa, but these structures are not present in mink spermatozoa (Kim et al., 1978).

The capitulum is articulated between the basal plate located above the cross-striated columns of the connecting-piece below. Due to the separation of the capitulum in the lateral aspects, it appears to be composed of a lateral and a ventral plate. The cross-striated columns are composed of alternating dense and light bands, with 10 to 13 striations (Kim et al., 1978). The four cross-striated columns of the proximal area of the neck form two major and five minor columns farther down the neck. Beneath the neck, the major columns split in two, forming nine columns attached to the nine dense fibers.

Fibers of the cross-striated columns are arranged parallel to one another and seem to create a connected ring or cross striations in the anterior region of the neck. In the center of the connected rings, below the inner surface of the capitulum, is the proximal centriole. The centriole has a longitudinal axis of about 70 degrees to the main axis (Kim et al., 1978).

Mink are one of the few species that contain microtubules in the neck region of the spermatozoa. The central fiber and double microtubules may be remains of the centriolar triplets or the extension of the axoneme. Fawcett and Phillips (1969) commented on the existence of the central

pair of microtubules in the connecting-piece of mature spermatozoa of some species (Kim et al., 1978).

The redundant portion of the nuclear envelope moves away from the condensed chromatin and extends into the neck region on both sides of the head. These extra membranes take the form of a pair of scrolls or a number of folds depending on the species (Fawcett, 1965). Mink spermatozoa take up the excess nuclear envelope by forming scrolls, which are similar in structure to those in the bat, dormouse, Russian hamster (Fawcett, 1970) and boar (Nicander and Bane, 1962).

FINE STRUCTURE DESCRIPTION OF THE SPERMATOZOA TAIL

The tail of the mammalian spermatozoa has less species variation than the head. Mink spermatozoa have the usual axial fiber bundle pattern of 9+9+2. These bundles resemble those of other mammals in the region of the middle-piece. Kim et al. (1978) found that dense fibers, numbers 9, 1, 5 and 6 are larger in diameter than the other 5 dense fibers. Saacke and Almquist (1964) have postulated that the comparative size variation between the dense fibers, varies with species and between breeds of the same species, as there have been conflicting reports from researchers working with the same species of mammal.

The mitochondrial sheath of mink spermatozoa appears to be composed of a single helix, and is comparatively longer than in many species. The number of mitochondrial gyres in the mink is greater than in the human, bull, dog, and chinchilla, but it is less than in the dormouse, Russian

hamster, mouse, bat, and rat (Fawcett, 1970).

> The triangular annulus was present in mink spermatozoa as in the bat, dormouse, Chinese hamster (Fawcett, 1970), bull (Saacke and Almquist, 1964b), and human. The annulus prevents caudal displacement of the mitochondria during the tail movement (Fawcett, 1970). On the other hand, the plasmalemma appeared to anchor itself on the annulus, which is similar to the findings of Saacke and Almquist (1964b) in bull spermatozoa.
> (Kim et al., 1978)

GENERAL DESCRIPTION OF THE CYTOPLASMIC DROPLET

Formation of the cytoplasmic droplet is associated with the Sertoli cells. The majority of the cytoplasm from the spermatids moves to the posterior portion of the head to take up a position around the base of the flagellum, as the spermatids develop. During the development of the head, the posterior portion of the spermatids are pushed closer to the lumen, until they finally become free spermatozoa. This process creates slender stalks between the residual cytoplasm which is held in the epithelium and the cytoplasm that is connected to the neck region. When the slender stalks are finally broken, they form the cytoplasmic droplet around the neck of the spermatozoa (Fawcett and Phillips, 1969). The granules and tubules, which are a component of the cytoplasmic droplet, are generally considered to be components from the golgi apparatus and endoplasmic reticulum (Bloom and Nicander, 1961, Greeson and Zlotnik, 1945).

The droplet is located around the posterior region of the head, surrounds the neck, and borders the anterior portion of the middle-piece of early spermatozoa and moves down the mitochondrial sheath during the passage of the

spermatozoa through the epididymis. The movement of the droplet down the middle-piece is the only morphological change which can be observed with a light microscope. Fawcett and Hollenberg (1963), working with guinea pigs, found that the highly characteristic shape of the acrosome, which can be observed in sagittal sections under the transmission electron microscope (TEM), is gradually acquired during the passage of the spermatozoa through the epididymis. With the use of the TEM, the loosening of the plasma membrane that covers the anterior region of the head has been observed in the epididymis in several mammals including the rabbit and the chinchilla (Fawcett, 1965). This evidence, along with the fact that spermatozoa do not attain full maturity until they reach the cauda epididymis, support the theory that the position of the droplet on the middle-piece is an indicator of maturity (Bedford, 1963 and Bloom and Nicander, 1961). Ejaculates which contain a large number of droplets on the anterior portion of the middle-piece are considered to be an indicator of a spermatogenetic disturbancy (Langerlof, 1934 and others, according to Bloom and Nicander, 1961).

Presence of the droplet on the middle-piece of spermatozoa causes a bend in the tail in the area of the droplet, when semen is stored. The active mobility of spermatozoa with the cytoplasmic droplet is inhibited as the average velocity of these spermatozoa was lower than spermatozoa without the droplet (Branham, 1969). However, active mobility helped cast off the droplet when it was on

the posterior position of the middle-piece (Rao and Berry, 1949).

THE FINE STRUCTURE OF THE CYTOPLASMIC DROPLET

The cytoplasmic droplet is composed of vesicles, vacuoles, and tubules or lamellae randomly oriented and limited by a cell membrane which encloses the entire spermatozoa (Bloom and Nicander, 1961, Dott and Dingle, 1968, Fawcett, 1965, Kim et al., 1978). The interior membranes of the droplet are smooth-surfaced and the surrounding cytoplasmic matrix does not contain ribosomes, glycogen or other granular inclusions (Fawcett, 1965). The same ultrastructure is seen in the droplets of the neck region and those that have moved down the mitochondrial sheath. There is a close similarity in fine structure between the droplets of spermatozoa from the caput epididymis and those from semen and the two types of droplets are believed to be identical (Dott and Dingle, 1968). No change in the middle-piece has been observed that can be associated with the movement of the droplet (Bloom and Nicander, 1961).

No difference has been observed between the cytoplasmic droplet of the bull and the ram (Bloom and Nicander, 1961). The cytoplasmic droplet, which is located around the head, is crowded with vesicles which are generally small but a few large vesicles are usually present. The tubules are fine and orientated randomly throughout the cytoplasmic matrix. The spermatozoa from the posterior region of the epididymis have droplets that are located at the posterior end of the middle-

piece and these droplets have a change of fine structure. The tubules are flattened and some are bent to a U-shaped or nearly circular profile. Two or more flattened profiles are often present, but these complexes are generally absent in the area close to the mitochondrial sheath. The location of the middle-piece is now eccentric to the periphery of the droplet. Spermatozoa from the anterior epididymis of the rabbit have droplets which have similar ultrastructure to the bull and ram but the cytoplasm is denser and strongly curved tubules or lamellae are already present. The cytoplasmic droplets of rats taken from the epididymis are larger than the other species and tubules and vesicles are generally restricted to the peripheral region (Bloom and Nicander, 1961).

FUNCTION OF THE CYTOPLASMIC DROPLET

The cytoplasmic droplet on mammalian spermatozoa could be a remnant of the excess cytoplasm of the spermatid with no function, or it may have a role in the maturation process of the spermatozoa. Research supports the theory of a nutritive function. Lasley and Bogart (1944) have shown that boar sperm from the epididymis survived longer than sperm from the ejaculates of the same boar even though both specimens were stored under similar conditions. The only morphological difference that Lasley and Bogart (1944) detected between the sperm from the epididymis and ejaculates was the presence or absence of the cytoplasmic droplet. The majority of sperm from the epididymis carried the droplet, while the droplet

was absent on the majority of sperm from the ejaculates. Metron (1939), working with albino mice, determined that the droplet was necessary for the survival of spermatozoa in the epididymis, but not for the ejaculated spermatozoa. Gresson and Zlotnik (1945) have suggested a role in the formation of the middle-piece since Golgi material is included in the cytoplasmic droplet and the Golgi material is already associated with proacrosome formation.

Recent research into the enzyme activity in the cytoplasmic droplet shows a high alkaline phosphatase activity in the rabbit and ram, which is restricted to the droplet except in mature spermatozoa (Moniem and Glover, 1972). Moniem and Glover (1972) demonstrated a corresponding decrease in alkaline phosphatase activity of the droplet with the increase in activity of the enzyme in the middle-piece of spermatozoa. Alkaline phosphatase may have a function in the dephosphorylation and transport of phosphate groups between epididymal plasma and the spermatazoon (Moniem and Glover, 1972). Acid and alkaline phosphatase, B-galactosidase, B-glucosidase, aryl sulfatase A and B, acid and alkaline protease, and hyaluronidase activities were shown to decrease in rat droplets as the spermatozoa matured (Roberts et al., 1976). Immature rat droplets were able to synthesize greater amounts of inositol from glucose than mature droplets, which suggests an important role of the droplet in spermatozoon maturation, possibly through inositol synthesis and metabolism (Roberts et al., 1976).

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

The animals used in all experiments were natural dark and pastel mink (*Mustela vison*) raised at the Michigan State University mink ranch. All animals were kept under natural lighting conditions, in open houses with roofs, and were housed in separate cages with individual water cups and nest boxes. The feeding and care of these experimental animals were identical to the feeding and care of the ranch stock not on experiment. Standard commercial mink ranch procedures were used in the care of the mink. Feed and water were provided to the mink *ad libitum*. Composition of the basal diet fed to the mink is listed on Table 1.

The majority of the male mink used in the reproductive trial were selected the previous year (1980) based on their semen samples from vaginal aspirations. Those which contained at least 2-3% sperm with cytoplasmic droplets were selected. One male was selected in the 1981 breeding season because of cytoplasmic droplets on the sperm from vaginal aspirations in the early part of the breeding season. All other males used for obtaining spermatozoa samples were selected randomly from the ranch stock.

A vaginal aspiration was performed by inserting a glass pipette, which had been rinsed in water and then in physiological saline 3 to 4 times, into the vagina of the female within 10 minutes after copulation had occurred. The

Table 1. Composition of basal diet

Ingredient	Percentage
Commercial mink cereal*	13.3
Whole chicken	16.0
Fish/fish products	30.0
Beef tripe	5.3
Beef liver	2.7
Beef lung	2.7
Beef trimmings	2.7
Cooked eggs	2.7
Water	24.7
Total	100.1

* Xk-40 Mink Cereal, Xk Mink Foods, Thiensville, WI.

glass pipette contained 1 to 2 drops of physiological saline at 37 C and the saline was placed into the vagina of the female and then withdrawn with a sample of the vaginal contents. The vaginal aspirations contained a sperm sample if a successful mating had occurred.

The females for the 1981 reproductive trial had been selected in 1980 and were proven breeders that had whelped that year. Proven breeders were used to lower the possibility of sterility problems with the females.

REPRODUCTION TRIAL

During the 1981 breeding season, test crosses were made between March 7th using seven males and 21 proven breeder female mink. Breeding records were kept for each individual animal. Each male was bred to 3 of the females on the trial as well as to other ranch females. The females on the study were randomly mated to the males. The first female that was willing to accept one of the males selected as the breeder on a particular day was assigned to the male's group that bred her. The males were allowed to breed the females for 20 minutes from the time the pair was successfully engaged in mating. After 20 minutes, the female was removed from the male's cage and a vaginal aspiration taken. The females were mated only once during the breeding season so an accurate record of the percent of cytoplasmic droplets found in the vaginal aspiration could be compared with the whelping record. Female mink were checked every day, from the 4th week in April through May, to determine when they whelped and

to record the number of live and dead kits(young), sex of the kits and the weight of the kits at birth and female at whelping.

The vaginal aspiration from each female was divided into two samples, one for light microscopy examination and the other for electron microscopy examination. About 1/3 of the sample (1 drop which is about equal to 0.05ml) was used to make the live-dead smear for use under the light microscope (American Optical light microscope with 400 magnification). The live-dead smear was prepared by placing a small amount of the vaginal wash at one end of a warmed glass slide and adding a drop of warm live-dead stain (eosin-opal blue stain by Lasely and Bogart, 1944) to it. All materials used in the preparation of live-dead stained specimens were pre-warmed to body temperature before being used. The original slide was laid flat while a second slide, held at a 45 degree angle, was used to pull the specimen across the original slide. Another slide was made by lifting the dispersion slide off the original slide and placing it on a new pre-warmed slide. The procedure of dispersing the sample was repeated with the second slide, except that no new sample was added. The second specimen was generally much thinner than the first and generally covered about 1/4 to 1/2 of the second slide.

Live-dead and droplet counts were made with the first slide unless it was unusable, due to the thickness of the specimen. The remainder of the vaginal aspiration was placed in a glass vial which contained 3 to 4 drops of a 5%

glutaraldehyde and was allowed to fix for 2 hours at room temperature. Phosphate buffer was then added to the vials until they were full (approximately 15ml). The specimens were placed in a refrigerator at 4 C for 24 hours and then 2/3 of each vial was removed and replaced with fresh phosphate buffer. The specimen vials were then placed back in the refrigerator for future processing for transmission and scanning electron microscopy studies.

ELECTRON MICROSCOPY

All specimen preparation for transmission electron microscopy was done by the Center for Electron Optics at Michigan State University. Procedures and materials for specimen preparation can be found in Exercises in Electron Microscopy (Hooper et al., 1979). Any changes in procedures and materials are described later in this section. The microscopes used for electron microscopy were a Philips Transmission Electron Microscope, a JEOL 35C Scanning Electron Microscope and a ISI Super III Scanning Electron Microscope.

For scanning electron microscopy a drop of 1% poly-l-lysine solution was placed on a petri dish and a glass cover slip placed on the top of the drop. The cover slip was removed after 5 minutes and gently washed with several drops of deionized water. Excess water was drained but the cover slip was not allowed to dry. A few drops of the specimen were added to the poly-l-lysine side of the cover slip (enough to cover the entire cover slip). The specimen was

covered for 10 minutes while the sperm settled and adhered to the cover slip. The cover slip was washed gently with several drops of deionized water to remove the sperm that did not adhere to the cover slip, and then the specimen was dehydrated in several increasing concentrations of ETOH for 15 minutes in each concentration. The concentrations of ETOH used were 25%, 50%, 75% and 3 changes in 100% ETOH. After the final dehydration step the specimen was critical point dried and then the cover slip was attached to the specimen stubs with adhesive tape and Tube Koat[1]. The specimen was sputter-coated with about 3.0nm of gold, then stored in a desicator for future viewing. When processing a sperm sample 3 duplicates were made to insure a good specimen was available for study.

ELECTROEJACULATION

The electroejaculation of dark and pastel mink was done on February 27, 1981 and again on March 23, 1981. The technique used was described by Aulerich et al. (1972). A total of 12 attempts were made at electroejaculation but only 3 electroejaculations produced good samples. All animals were anesthetized with 0.5-0.7ml of Tilazol[2]. Specimens for the light microscope were prepared as previously described except that glass test tubes were used in place of the glass vials. The live-dead sample was taken from the glass test

1 - Television Tube Koat. Product of G.C. Electronics, Div. of Hydrometals, Inc., Rockford, IL. U.S.A. 61101.
2 - Trade name for a 1-1 combination of tiletamine hydrochloride and zolaespam(a diazepinione tranquilizer). Product of Parke Davis Co., Ann Arbor, MI.

tube with a glass capillary pipette and placed on a slide which was then diluted with a drop of physiological saline before the live-dead stain was added.

REPRODUCTIVE TRACT

Five sexually mature mink were castrated in 1981 and had one of their testes removed near the junction of the vas deferens and prostrate gland. These samples were taken from freshly killed animals. Six sexually mature mink were castrated in 1984 from animals anesthetized with 0.2-0.3 ml of 100g/ml Vetalar[1] for TEM and SEM work. Samples were taken from the caput epididymis (head), corpus epididymis (middle), cauda epididymis (tail) and vas deferens which were sectioned according to Figure 1.

The sperm from the individual sections were removed by making slashes in the wall of the specimen, then touching the specimen to a pre-warmed slide to obtain a sample. A small drop of warm saline was placed on the slide to dilute the sperm sample before a drop of the live-dead stain was added to the sample. The live-dead slide was prepared for microscopic examination as previously described. The remainder of the tissue was dipped into a glass vial that contained 4-5 drops of 5% glutaraldehyde solution, at room temperature, and agitated gently to suspend the sperm in the fixative. This specimen was processed for electron microscopy as previously described.

1 - Vetalar. Product of Parke Davis Co., Division of Warner-Lambert Co., Morris Plains, N.J. 07950.

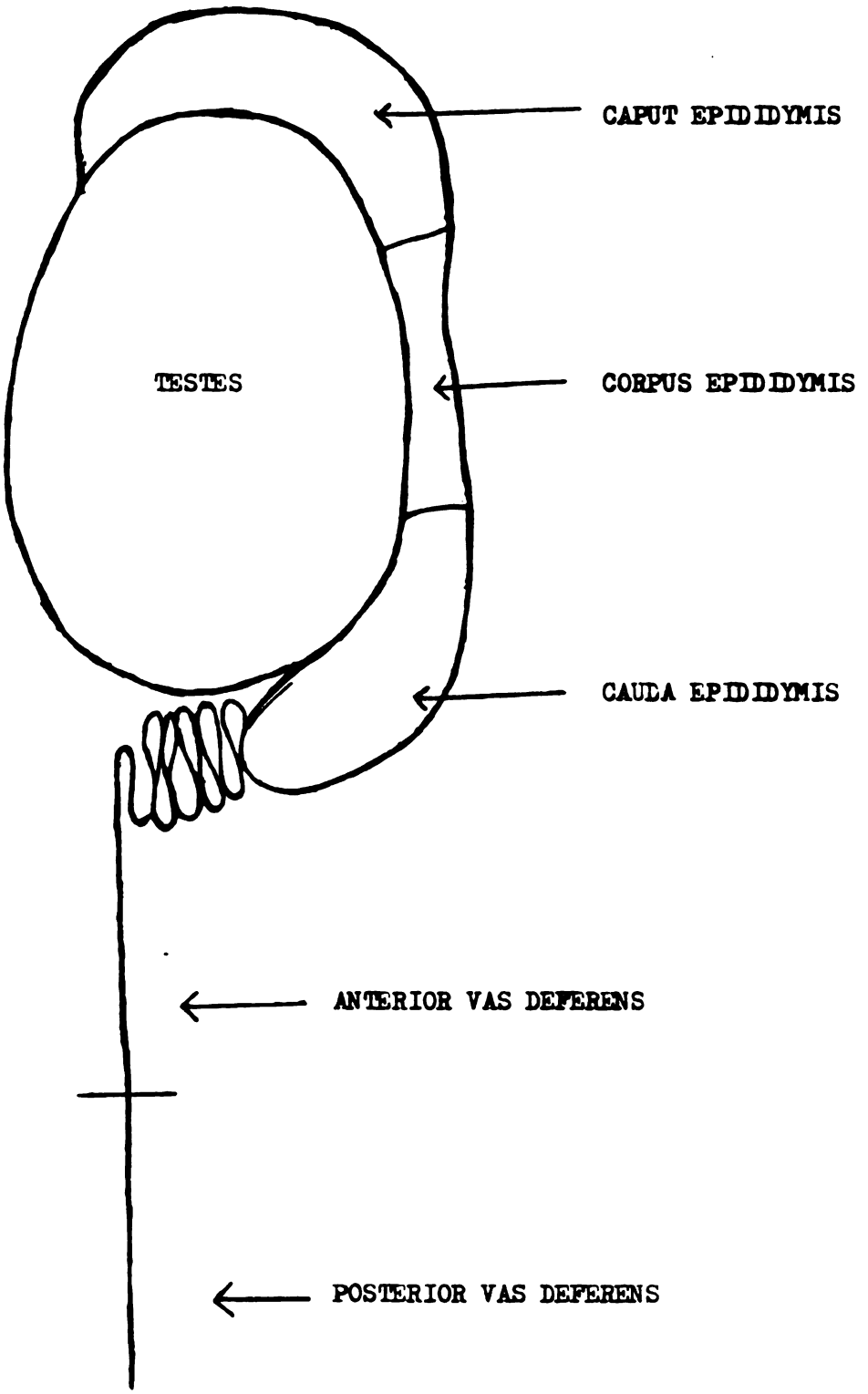

Figure 1: Schematic drawing of the testes, epididymis, and vas deferens of a mink showing the various specimen collection sites.

STATISTICS

A one way analysis of variance was conducted on the data from the samples taken from the reproductive tract. A student's t-test was used to compare the size of the cytoplasmic droplet from the caput and cauda epididymis (Gill, 1978).

RESULTS AND DISCUSSION

REPRODUCTION TRIAL

Semen samples from 58 male mink were collected and screened for the presence of the cytoplasmic droplet on their sperm, during the 1981 breeding season. Only 12 males (20.7%), out of the 58 males screened, contained 2 to 3% droplets on their spermatozoa. This value may be complicated by the fact that repeated samples were taken from some of the mink, but only one sample from about half of the males screened. It is further complicated by the fact that individual animals may not consistently have droplets or the same concentration of droplets present on their sperm throughout the breeding season (Table 2 and 3). Table 2 lists two males that had 2 to 3% droplets in two of their semen samples but none in their third sample. Therefore, the value of 20.7% represents those male mink that did show the droplets in at least one of their semen samples obtained during the period of observation.

The fertility evaluations of the males showing the cytoplasmic droplets were carried out using seven males. The reproductive performance of these males is shown in Table 2. The percentage of live sperm and sperm with droplets were made by counting 100 sperm from live-dead smears under a light microscope. Standard errors were calculated for 5 samples with 3 individual counts of 100 sperm made for each sample (Table 4). Calculation of the standard error was done to test the variability of the individual samples and the

Table 2. Reproductive performance of female mink mated[A] to males whose sperm previously showed the presence of cytoplasmic droplets in vaginal washes.

Male	Female	No. kits (young) whelped Live	Dead	Live kits/ litter	Cyto-plasmic[B] droplet (%)	Avg.[B] % droplets	Live[B] sperma-tozoa (%)
G 185	1	7	0		3		55
	2	4	0	4.7	3	2.0	55
	3	3	0		0		58
G 41	1	0	0		6		53
	2	9	0	3.0	9	6.3	53
	3	0	0		4		53
E 525	1	5	1		3		66
	2	5	0	5.3	C	4.0	16
	3	7	0		5		41
D 1247	1	5	1		0		54
	2	7	0	6.0	1	0.7	62
	3	7	0		1		43
H 711	1	2	0		0		94
	2	0	1	0.7	0	0.0	83
	3	0	0		0		41
FP 73	1	3	0		3		D
	2	0	0	2.3	0	1.7	69
	3	4	0		2		42
CP 711	1	0	0		4		80
	2	1	0	1.7	7	4.3	55
	3	4	0		2		50

A Single 20 minute matings.

B Percentage determined by live-dead staining of vaginal aspirations from counts of 100 spermatozoa.

C Blood obscured tails so the droplets could not be counted.

D The stain was not taken up by any of the sperm (could be due to the pH of the sample being off enough to inhibit the uptake of the stain.

Table 3. Concentration and occurrence of cytoplasmic droplets on mink spermatozoa from Table 2.

Cytoplasmic droplets (%)	Incidence
0	6
1-2	4
3-4	6
5-6	2
7-8	1
9-10	1

Table 4. The variability of 3 separate sperm counts taken from the same sample.

Sample	Average[A] % live	Average[A] % droplets
1	70.7 +/- 0.63	3.0 +/- 0.58
2	15.3 +/- 1.30	4.3 +/- 0.92
3	60.0 +/- 2.87	6.3 +/- 0.46
4	69.3 +/- 2.07	4.3 +/- 0.92
5	48.0 +/- 3.21	2.0 +/- 1.15

[A] Mean +/- standard error

accuracy of the counts. The standard errors for the droplet percents and live percents showed that the counts of 100 sperm are accurate representatives of the samples.

The presence of the cytoplasmic droplets in the semen samples was not constant for the males with a low percent of droplets (average < or = 2%) but the males with the higher percentages (average >2%) consistently showed the droplet in their ejaculates. Male H711 had droplets on his sperm in the early part of the 1981 breeding season but when he mated for the study, on the 13th, 16th, and 17th of March, the droplets were no longer present on his sperm.

The reproduction of the female mink from 1980 was 5.4 live kits/litter with the females being mated twice during the breeding season. Reproduction for the same females in 1981 was 4.5 live kits/litter with single matings. The whelping percent of the females on the trial in 1981 was 76.2%. The whelping percent of 76.2 and 4.5 live kits/litter compares very well to what Venge(1973) reported as the difference between a single mating system and a double mating system (where the second mating was seven days after the 1st mating). Venge found that with the double mating system, the whelping percent was increased by 16.79% and increased the litter size by 1.27 kits/litter over the single mating system. In comparing females that were double mated in 1980 and single mated in 1981, the whelping percent decreased by 23.8% in 1981, which is only 7.1% lower than what Venge reported. The number of kits whelped between 1980 was 0.9

kits/litter lower than in 1981, which is 0.37 kits/litter greater than what Venge reported. Based on the limited amount of data obtained from these matings, fertility was not affected by the presence of the droplets even at the highest concentration (9%) observed.

ELECTROEJACULATION

The two attempts at electroejaculation produced only 3 good semen samples which contained enough sperm for an accurate count of cytoplasmic droplets (all samples were from the collection on February 28, 1981). The electroejaculation results are compared in Table 5 with vaginal aspirations from females mated to the same males. An average of 22.7% was observed for the electroejaculates from the 3 males. This is greater than twice the highest concentration of 9% seen in the vaginal aspirations listed on Table 2. The droplets appear to be released from the spermatozoa after ejaculation in the vagina of the female. Active motility allows the droplet to be cast off by the spermatozoon when the droplet is located on the posterior portion of the middle-piece (Roa and Hart, 1948). Sample counts of 100 sperm were taken so they would be comparable to those counts for the vaginal aspirations.

REPRODUCTIVE TRACT

The average percentage of sperm with cytoplasmic droplets from the five areas of the male reproductive tract is listed on Table 6. Samples from five different males were used for this study. There was more variation between the

Table 5. Percentage of cytoplasmic droplets in samples from vaginal washes and electroejaculates.

Mink #	Cytoplasmic droplets (%) in sperm samples from Vaginal aspirations	Electro-ejaculation
1	6	5
2	8	43
3	0	20
Average	4.7	32.7

Table 6. Variability of the cytoplasmic droplets on the spermatozoa from the 5 areas of the reproductive tract of mink.

VAS DEFERENS	VARIABILITY[A]
posterior	33.6 +/- 10.9
anterior	38.4 +/- 8.21
EPIDIDYMIS	
cauda	44.2 +/- 9.7
corpus	40.2 +/- 7.55
caput	36.4 +/- 4.18

[A] Mean +/- standard error

individual animals than there was between the areas of the reproductive tract which showed the percentage with droplets to remain fairly constant. This data is in contrast to a study by Rao and Berry (1949) who found a decrease in the percentage of sperm with droplets along the reproductive tract of boars. Kim et al. (1978) reported that only some of the spermatozoa from the mink cauda epididymis had droplets which is in contrast to this study, where nearly half the spermatozoa had droplets in the cauda epididymis. A greater number of male reproductive tracts taken at different times during the breeding season should be analyzed to ascertain what is taking place with the droplet concentrations on spermatozoa from different segments of the reproductive tract. The time that the samples are obtained may be more important with mink as they are seasonal breeders with a short breeding season.

The droplet counts included the droplets located at the anterior and posterior portion of the middle-piece for all experiments. No distinction was made because early screening consistently found the droplets located on the anterior portion of the middle-piece almost exclusively in the caput and corpus epididymis. The vas deferens and the cauda epididymis had less than 1% of the droplets located in the anterior position. Even the corpus epididymis generally had less than 10% anterior droplets in the sperm counts. There was a slightly greater chance of finding an anterior droplet in the vaginal aspirations than in the vas deferens or cauda

epididymis because the active motility of the spermatozoa would eliminate the majority of the posterior droplets from the tail of the spermatozoa.

ELECTRON MICROSCOPY

Spermatozoa with (Fig. 3-9) and without (Fig. 2, 4) the cytoplasmic droplet are located throughout the epididymis and vas deferens of mink. Scanning electron microscope (SEM) pictures show that the spermatozoa from the caput epididymis exhibit the cytoplasmic droplet surrounding the base of the head, the connecting-piece, and part of the middle-piece (Fig. 3, 5, and 6). The descent of the droplet down the middle-piece has begun for some of the spermatozoa from the caput epididymis (Fig. 3 and 7) but most of the spermatozoa have the droplet on the anterior portion of the middle-piece (Fig. 3, 5, 7). Once the spermatozoa have reached the cauda epididymis, the droplets are found almost exclusively on the posterior portion of the middle-piece (Fig. 4, 8, 9).

The size of the cytoplasmic droplets vary between the caput epididymis and the cauda epididymis. SEM pictures were used to estimate the maximum length and width of the droplets. The average length for five droplets from the caput epididymis was 2.26u with the average width being 1.42u. The range for the length of these droplets was 2.0 to 2.8u and the range for the width was 1.3 to 1.7u. The same measurements were taken for five droplets on spermatozoa from the cauda epididymis and the average length was 1.98u with a range of 1.7 to 2.1u and the average width was 1.20u with a

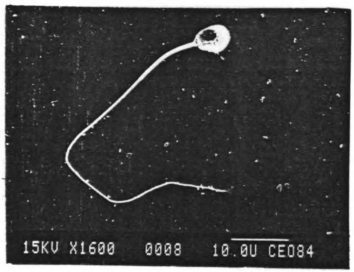

Figure 2: Normal spermatozoon without the cytoplasmic droplet (SEM, 2,000X).

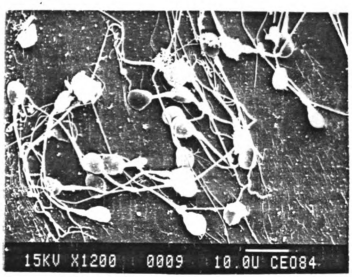

Figure 3: Spermatozoa from the caput epididymis displaying the cytoplasmic droplet in the anterior position on the middle-piece (SEM, 600X).

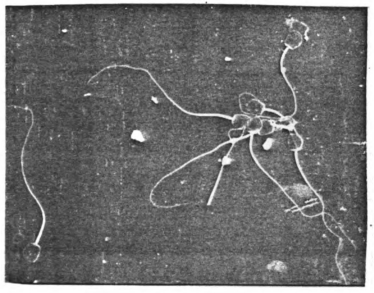

Figure 4: Spermatozoa from the anterior vas deferens displaying the cytoplasmic droplets in the posterior position on the middle-piece (SEM, 728X).

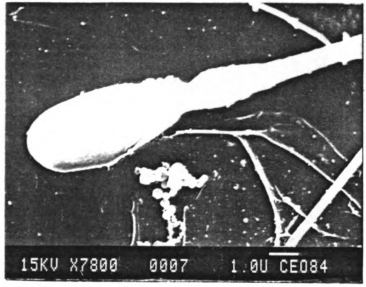

Figure 5: Spermatozoon from the caput epididymis with the cytoplasmic droplet in the anterior position on the middle-piece. The attachment to the head is becoming weaker as the droplet starts its descent down the middle-piece. This droplet has an oval shape on this spermatozoon (SEM, 7,800X).

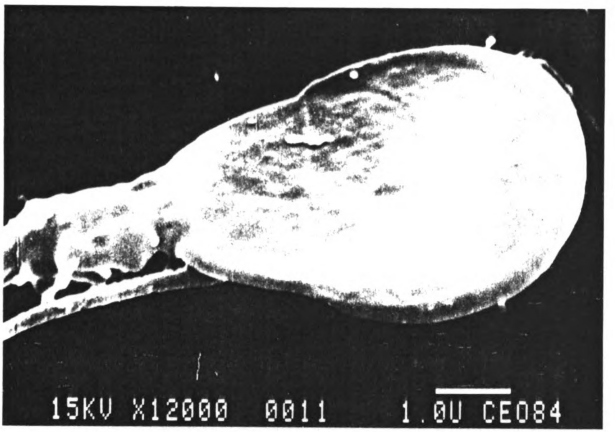

Figure 6: Spermatozoa from the caput with the cytoplasmic droplet surrounding the connecting-piece and part of the middle-piece. Droplets in this position are still partially attached to the spermatozoon. The surface texture of this droplet is very uneven with many protrusions on the surface (SEM, 19,500X).

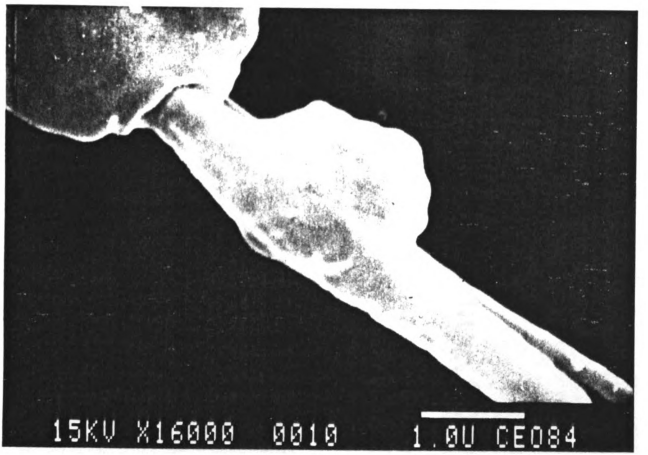

Figure 7: Spermatozoon from the caput epididymis that no longer has the droplet attached to the base of the head. The droplet on this spermatozoon has a globular appearance (SEM, 28,800X).

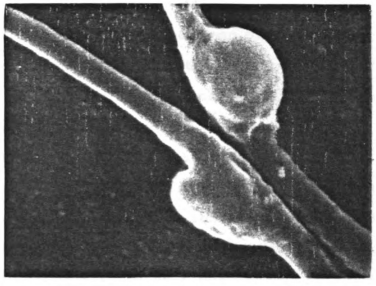

Figure 8: Spermatozoa from the posterior vas deferens displaying the cytoplasmic droplets in the posterior position on the middle-piece. These droplets are eccentrically located on the middle-piece (SEM, 20,000X).

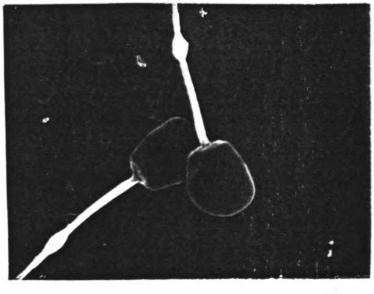

Figure 9: Spermatozoa from the posterior vas deferens displaying the cytoplasmic droplet in the posterior position on the middle-piece. These droplets surround the entire middle-piece (SEM, 3,100X).

range of 1.1 to 1.3u. A comparison of the sizes of the droplets was made using the student's t-test and the length and width of droplets from the caput epididymis were found to be larger at a significance of $P<0.01$.

The shape of the cytoplasmic droplet, on SEM pictures, ranged from globular to oval form with the surface having an uneven texture. The transmission electron microscope (TEM) pictures from the cauda epididymis (Fig. 10-17) depict the cytoplasmic droplet as being a membrane-bound sack filled with tubules and vacuoles in a granular, amorphous matrix. These structures inside the membrane give the surface its uneven and somewhat lumpy appearance.

The tubules that are dispersed throughout the droplet have U-shaped, S-shaped, slightly curved and almost circular shapes. The internal structure of the droplet is random with no set pattern to the orientation of its components. This is different than what Bloom and Nicander (1961) reported for the ram. The ram's tubules and vacuoles are generally restricted to the outer region of the droplet. Droplets from the cauda epididymis of the ram appear to have smaller vacuoles and a less granular matrix than the mink. This difference between the ram and the mink may also hold true for the bull, as Bloom and Nicander, in the same paper, state that there was no important difference between the droplets of the bull and ram.

A definite membrane encloses the droplets and encircles the entire middle-piece (Fig. 13-16) but is in a state of

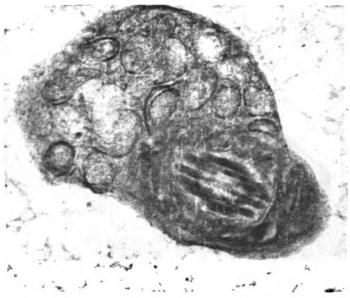

Figure 10: The membrane on the droplet has begun to degenerate but the components of the droplet are only in contact with part of the middle-piece (TEM, uranyl acetate stain, 45,000X).

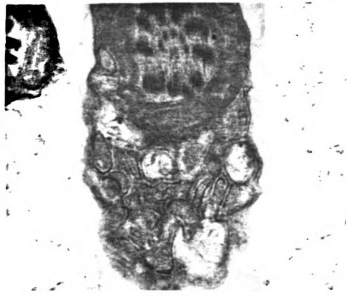

Figure 11: This droplet has many medium to large vacuoles displaced throughout the matrix. The membrane on this droplet has begun to degenerate but the components of the droplet are still intact (TEM, uranyl acetate stain, 45,000X).

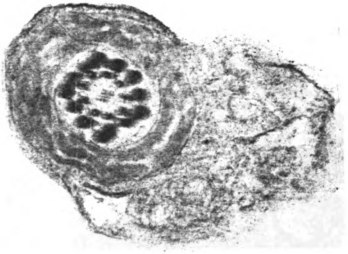

Figure 12: This entire droplet is in a state of degeneration. The droplet no longer surrounds the entire middle-piece (TEM, uranyl acetate stain, 70,000X).

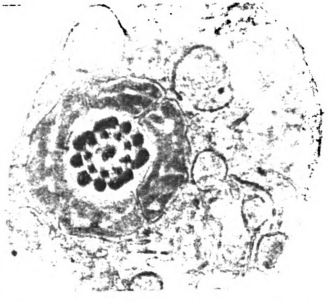

Figure 13: The droplet encircles the entire middle-piece and there is a definite membrane surrounding the droplet (TEM, uranyl acetate and lead citrate stain, 70,000X).

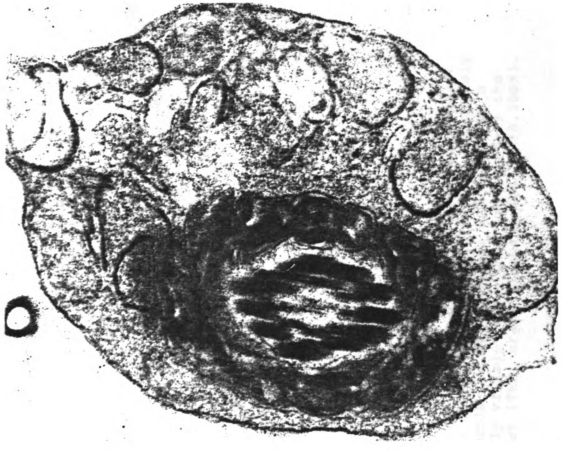

Figure 14: A definite membrane surrounds this droplet which encompasses the entire middle-piece. There are vacuoles on the periphery of the droplet which are causing the surface of the droplet in that area to protrude (TEM, uranyl acetate and lead citrate stain, 73,846X).

Figure 15: A lateral section of the cytoplasmic droplet on the middle-piece. This droplet completely surrounds the middle-piece and still has a clearly visible membrane around most of the droplet (TEM, uranyl acetate stain, 10,500X).

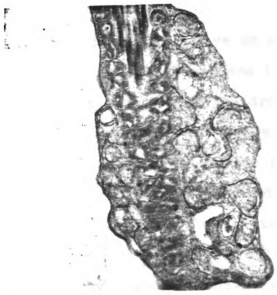

Figure 16: Higher magnification of Figure 15. There is a large V-shaped vacuole present in this area (30,000X).

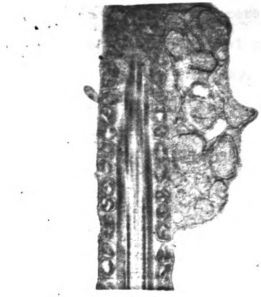

Figure 17: A lateral section of a cytoplasmic droplet on the middle-piece. This droplet is eccentrically located to one side of the middle-piece and its outer membrane is less distinct than the membrane from the droplet in Figure 15, which was taken from the same sample, which also had the droplets surrounding the entire middle-piece (TEM, uranyl acetate stain, 15,000X).

degeneration on the droplets that have an extreme eccentric location to one side of the middle-piece (Fig. 10-12). The degeneration of the membrane around the droplet would weaken the attachment of the droplet to the spermatozoa. The degeneration of the membrane could be the final stage before the droplet becomes detached from the middle-piece.

The granular nature of the cytoplasmic droplet in mink may be due to enzyme activity in the droplet. The research that was done with identifying the enzyme activity of acid and alkaline phosphatase, B-galactosidase, B-glucosidase, aryl sulfatase A and B, DNAase, and alkaline protease, and hyaluronidase in the rat droplet (Roberts et al., 1976), and alkaline phosphatase in rabbits, hamsters, and rats (Moniem and Glover, 1972) showed that the level of activity decreased in the droplet once it reached maturity.

SUMMARY AND CONCLUSIONS

1. The fertility of the female mink was not affected by the presence of the cytoplasmic droplet on the spermatozoa at the concentrations observed. The highest incidence of the droplet observed in the vaginal aspirations was 9%.
2. The cytoplasmic droplets are cast off after ejaculation as the vas deferens and electroejaculates have droplet concentrations greater than twice the highest concentration seen in the vaginal aspirations. Active motility of the spermatozoa aids the spermatozoa in casting off the droplet.
3. Concentration of the droplets on mink spermatozoa remained constant, on the average, throughout the reproductive tract.
4. The size of the droplet decreases slightly in width and length as the droplet descends down the reproductive tract.
5. The irregular surface area of the droplet in the SEM photographs is due to the tubules and vacuoles of the droplet. The matrix is more granular than the matrix in the droplets of the ram and the mink droplet has a more homogenous dispersion of its structures in the droplet than the rat. The vacuoles are also larger in the mink droplets. The same general structures are present in the droplet of the mink as in other mammals with the exception of vesicles.
6. A degeneration of the membrane of the droplet was observed in the droplets which have an extreme eccentric location on the middle-piece. This could be the final stage in the preparation of the loss of the droplet from the middle-piece or the degeneration of the droplet on a dead spermatozoa.

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