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The effect of different honey concentrations on the ultrastructure profile of spermatozoa in Dewa Mahseer (*Neolissochilus soro*)

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Abstract. *Abinawanto A, Lestari S, Bowolaksono A, Dwiranti A, Lestari R, Gustiano R, Kristanto AH. 2023. Title The effect of different honey concentrations on the ultrastructure profile of spermatozoa in Dewa Mahseer (*Neolissochilus soro*). Biodiversitas 24: 1025-1031.* Dewa Mahseer (*Neolissochilus soro*) is one of Indonesia's endemic freshwater fish at risk of population decline due to water pollution and asynchronous maturation of gonads. One of the efforts to conserve this species is preserving their spermatozoa using honey as a protector at certain temperatures. Therefore, this study aims to evaluate the effect of multiflora honey concentrations on ultrastructure spermatozoa profile of Dewa Mahseer. The temperature was maintained at 4°C and stored for 48 hours, followed by the cement dilution (1:10) in a stock solution containing fish ringer and honey. Transmission electron microscopy (TEM) examined the spermatozoa profiles at a magnification of ×1,500. Furthermore, ANOVA one-way analysis of variance was conducted to test the effect of head length, width, and area of spermatozoa on honey. The results showed a statistically significant difference in head width ($F=13.929$, $p=0.001$). Also, there is a difference in area between honey spermatozoa, fresh spermatozoa, and without honey at a cold of 4°C. The fresh spermatozoa stored at cold temperatures without honey observed considerable membrane damage. The novel aspect is an investigation of the effect of honey as a natural protector in the storage of *Neolissochilus* spermatozoa at 4°C for 48 hours. This study can be applied as a guide for the 48 hours of spermatozoa in a cold temperature in fish farming.

Keywords: Aquaculture, cold storage, conservation, honey protector, Indonesia

INTRODUCTION

Cold storage of fish sperm is a common technique used for short-term gamete conservation in aquaculture (Shaliutina et al. 2013). It is kept at a low temperature, usually -196-4ºC, for short periods, such as hours up to weeks, to maintain motility, fertility, and metabolic activity (Shaliutina et al. 2013). Furthermore, the technique improves the reproductive process in fertilization using post-preservation sperm because it can maintain the condition of the sperm in the preservation process (Contreras et al. 2019). The process should be performed with low light intensity, temperatures between 0 to 4°C, and a saturated oxygen atmosphere (Shaliutina et al. 2013). Cold storage is necessary for several reasons, such as (i) maintaining control and continuous supply of gametes to have a constant stock of oocytes, (ii) lowering the cost of permanently storing broodstock for artificial fertilization, (iii) maintaining sperm quality during transport from the culture site to the fertilization site and incubation at different positions, (iv) keeping the stock of available sperm once sexual maturation between males and females is out of sync, and (v) conducting studies for conservation of genetic inheritance useful for hybridization and genetic selection programs, as well as sperm cryopreservation (Contreras et al. 2019). Therefore, procedures for storage have been developed with modified methods for specific optimization (Dietrich et al. 2021). However, an optimal storage procedure without decreasing sperm quality is yet to be developed.

The study by Cheng et al. (2022) reported cellular and molecular damage that affects mitochondrial function and spermatozoa quality after cold storage in common carp (*Cyprinus carpio*). Changes during storage result in decreased sperm motility, plasma membrane function, acrosome integrity, DNA fragmentation (Amidi et al. 2016), and decreased fertility (Park et al. 2022). That is due to oxidative stress, which causes an imbalance between free radicals and antioxidant protective activity. Therefore, methodological approaches are continuously being developed to evaluate the integrity of spermatozoa membranes. Those approaches start from the biochemical environment and physical conditions that need to be changed sequentially to improve spermatozoa preservation (Contreras et al. 2019). One methodological approach

being developed is the addition of protective compounds containing antioxidants. Honey is a mixture of 25 sugars with other bioactive substances, such as organic acids, enzymes, antioxidants, and vitamins (Cheepa et al. 2022). Therefore, it can potentially protect the extracellular environment during preservation because it contains large amounts of different sugars and supports to increase in the flow of intracellular fluid, thereby protecting the cytoplasmic regions of sperm (Fakhrildin et al. 2014). In addition, several antioxidant compounds are also contained in honey, including flavonoids, galagin, pinobaxin, and vitamin C (El-Sheshtawy et al. 2014). The antioxidant properties of honey play an important role in protecting cells from thermal damage by reducing reactive oxygen species (ROS) (Cheepa et al. 2022).

The relevant parameter for the evaluation of conventional semen is spermatozoa morphology which can be used as a valid biomarker of functional deficiency, as well as providing information about the possibility of fertilization (Ilieva et al. 2012). Information from spermatozoa morphology as part of a complete semen analysis is becoming increasingly significant from a clinical point of view for infertility (Oehninger and Krugel 2021). Ultrastructural studies using electron microscopy specifically identify spermatozoa morphological defects and degenerative changes that could decrease fertilizing capacity (Ilieva et al. 2012). Uswatun et al. (2020) reported the effect of cryopreservation on the ultrastructure of catfish albinos (*Pangasius hypophthalmus*) with fresh spermatozoa that had intact cell membranes, mitochondria, and flagella. Meanwhile, post-equilibration spermatozoa appear to have a longer head width than fresh, but the structure is still intact. There was damage to cell membranes in the head and mitochondria, breaking the flagellum in preserved spermatozoa. The morphology aspects, such as midpiece size and flagella length, can be used as an index to assess the ability to fertilize (Bitencourt da Costa et al. 2022). Any abnormalities in these characteristics can affect the ability of spermatozoa to fertilize (Holt and Comizzoli 2022). Therefore, morphology plays an important role in fertilization and embryo development. Ultrastructural studies of spermatozoa provided information on physiological functions for optimizing storage protocols. Those studies aim to obtain sufficient numbers for in vitro fertilization (Díaz et al. 2019). Ultrastructure refers to the finest biological structure of an organism that requires high magnification obtainable with electron microscopy (Schneider et al. 2010). It plays a vital role in biology, including components of cell architecture, which is performed using an electron microscope with high magnification capabilities (Narida et al. 2022). Electron microscopy is also used in diagnostic pathology, comparative animal and plant anatomy, functional morphology, cellular integrity, and mitochondrial membrane potential (Figueroa et al. 2014). A comprehensive study of ultrastructural observations can understand the mechanisms underlying the failure of the cellular response to preservation. The morphological and ultrastructural changes of post-preserved spermatozoa have been studied in fish species such as Giant Grouper (*Epinephelus lanceolatus*) (Narida et al. 2022), Atlantic Salmon (*Salmo salar*) (Figueroa et al. 2016), and Soho salmon (*Oncorhynchus kisutch*) (Sandoval-Vargas et al. 2022). These studies showed several inter-specific morphological changes marked by the plasma membrane, head swelling, and flagellar coiling. However, findings on such changes in Dewa mahseer (*Neolissochilus soro*) spermatozoa are sparse. This study aims to evaluate different honey concentrations' effect on the characterization of morphological and ultrastructural changes in *Neolissochilus soro* spermatozoa, maintained the temperature at 4°C storage for 48 hours.

MATERIALS AND METHODS

Procedures

Semen collection

Semen was collected using the stripping technique with a 3 mL syringe and placed in a microtube filled with stock solution and honey. The stock solutions consist of a fish ringer extender and protectant. Fish ringer extender made from 3.25g NaCl, 0.125g KCl, 0.175g $CaCl_2.2H_2O$, and 0.1g $NaHCO_3$ with distilled water until the volume reaches 500 mL (Draper et al. 2008). The ratio of semen to the stock solution was 1:10, and the stock solution preparation was based on Table 1.

Storage at 4°C

The Semen microtubes were stored in a refrigerator at 4°C for 48 hours. Next, left at room temperature for 35 seconds and fixed using 2.5% glutaraldehyde in cacodylate. Subsequently, the semen was observed through transmission electron microscopy (TEM).

Ultrastructure analysis

All spermatozoa samples were fixed with 1 mL of 2.5% glutaraldehyde and cacodylate buffer, following Luo et al. (2011). Next, the samples were dehydrated, described, and embedded in pure resin. Ultra-thin sections of 60 to 100 nm thickness were collected using glass knives, and sections were placed on copper grids, then stained with uranyl acetate and lead citrate. The ultrastructure of spermatozoa was screened by TEM. Meanwhile, a sample from each treatment was examined using TEM, so the total sample observed was 5. Micrographic analysis was performed using ImageJ v1.50i software (Luo et al. 2011).

Table 1. Stock solution dilution

Honey concentration(%)	Extender (µL)	Protectant (µL)	Sperm (µL)
0	450	-	50
0.5	440	10	50
1	430	20	50
1.5	420	30	50
2	410	40	50

Data analysis

Images from TEM and morphometric data were processed using NIH ImageJ. In each sperm, the length, width, and area of the flagellum-shaped head were scored. Sperm head length is defined as the longest area of the head. Meanwhile, the width is the widest width of the head perpendicular to the length measurement (Gu et al. 2019). The results are expressed as a percentage (mean± standard deviation). Furthermore, the data is tabulated, then the normality test is performed using the Shapiro-Wilk, and the homogeneity test is continued using the Levene test. Finally, if the data obtained is normally distributed and varies homogeneously, the one-way analysis of variance test (ANOVA) is continued to compare the percentage values of the width, length, and area of the head of spermatozoa and proceed with the Tukey test.

RESULTS AND DISCUSSION

Transmission electron microscopy (TEM) with the Cell Block Preparation protocol revealed differentiation between the heads and center (neck) of spermatozoa preserved with honey and without honey (Figures 1.A-B) to fresh spermatozoa (Figures 1.C-D). While scanning transmission electron microscopy (TEM) with a negative staining protocol showed that all spermatozoa that were preserved without honey (the control) were in a coiled condition (Figure 1.A), while spermatozoa that were given honey had wavy tails (Figure 1.B).

Membrane damage on several parts of the spermatozoa head was observed from the transmission electron micrograph by a cross-section of the spermatozoa head. The mitochondria observed in the control were severely damaged, characterized by incomplete cell membranes and incomplete chromatin formation (Figure 2.A). Some damage also occurred to the spermatozoa head's cell membrane, which was preserved using a honey concentration of 0.5% (Figure 2a) and 0% concentration (2.B). That damage causes deformity of the spermatozoa head, likely affecting their main function. At a concentration of 1% honey (Figure 2.C) the condition of the cell membrane on the head is still intact. However, at a concentration of 2% honey (Figure 2.D) the cell membrane of the head was still intact, but there were sperm nuclear vacuoles (NV) in the chromatin area, indicating DNA fragmentation.

Based on the Vanderzwalmen criteria (2008), 0% honey treatment; honey 0.5%; honey 1%; and fresh sperm belong to class I (no vacuoles). Whereas 1.5% honey is included in the class II group in which only two small vacuoles (which occupy <4% of the head area). Meanwhile, the 2% honey treatment was in class III because there were more than two small vacuoles or one large vacuole (which occupied between 13% and 50%).

Furthermore, an important structure of every cell is the presence of a cell membrane which defines the cell boundaries and the various internal cell components. The membrane functions as a transport protein to facilitate and regulate the movement of substances between cells and compartments. Therefore, the spermatozoa head membrane damage and changes in the condition of the spermatozoa tail observed in this study, tend to cause adverse effects on spermatozoa function. That damage could decrease spermatozoa's viability and motility, leading to decreased egg hatchability.

Table 2 shows the measurement data and the spermatozoa stored in honey and without honey. The head size of spermatozoa stored in honey shows larger sizes than those without honey. Meanwhile, Figure 3 shows that fresh spermatozoa (1.66±0.06) µm and those stored without honey (1.66±0.12) µm had the same head width.

Figure 3 shows spermatozoa stored without honey had the largest head length (1.93±0.33) µm compared to those (1.72±0.09) µm stored with honey. Compared to fresh spermatozoa, those stored with honey had a larger head width (1.87±0.08) µm. Meanwhile, spermatozoa preserved with honey had the largest head size (2.88±0.07) μm^2 than those fresh (2.29±0.21) μm^2 and stored without honey. This study indicated that there was damage to the cell membrane in the treatment group that did not add honey (control). However, a decrease in damage level to the cell membrane in sperm given honey 1%, followed by vacuolization of spermatozoa head area, with high honey concentrations (> 1%). The changes found in the study could explain the decreased membrane integrity, motility and kinetic parameters, mitochondrial membrane potential, and DNA damage in *Neolissochilus soro* spermatozoa.

Table 2. Spermatozoa ultrastructure measurement (a and b show a statistical difference)

Parameter	Fresh spermatozoa	Spermatozoa (preservation without honey)	Spermatozoa (preservation with honey)
Head width (µm)	1.66^{a}±0.06	1.66^{a}±0.12	1.85^{b}±0.08
Head length (µm)	1.78^{a}±0.09	1.93^{a}±0.33	1.89^{b}±0.11
Head area/shape (μm^2)	2.29^{a}±0.21	2.70^{a}±0.80	2.88^{b}±0.07
Flagella/tail	Straight	Coiled	Wavy
Whole	Intact	Non-intact	Intact

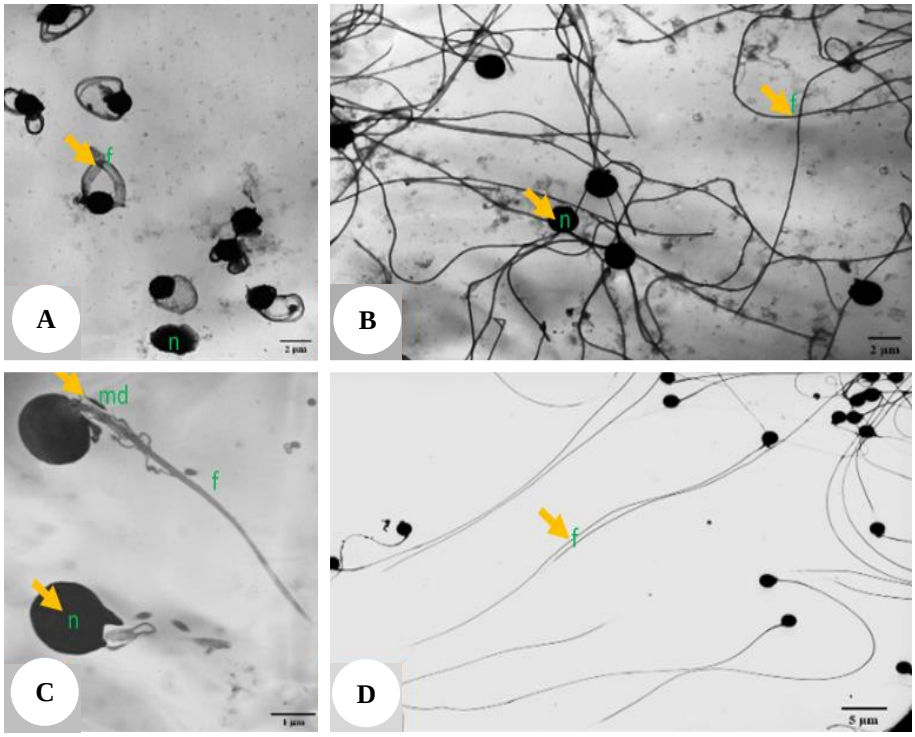

Figure 1. Ultrastructural changes that occur in the spermatozoa tail after 48 hours of preservation. A. Preservation without honey (control), B. Preservation with 1% honey, C-D. Fresh sperm. N: nucleus; (f) Flagella/tail; ms: midpiece

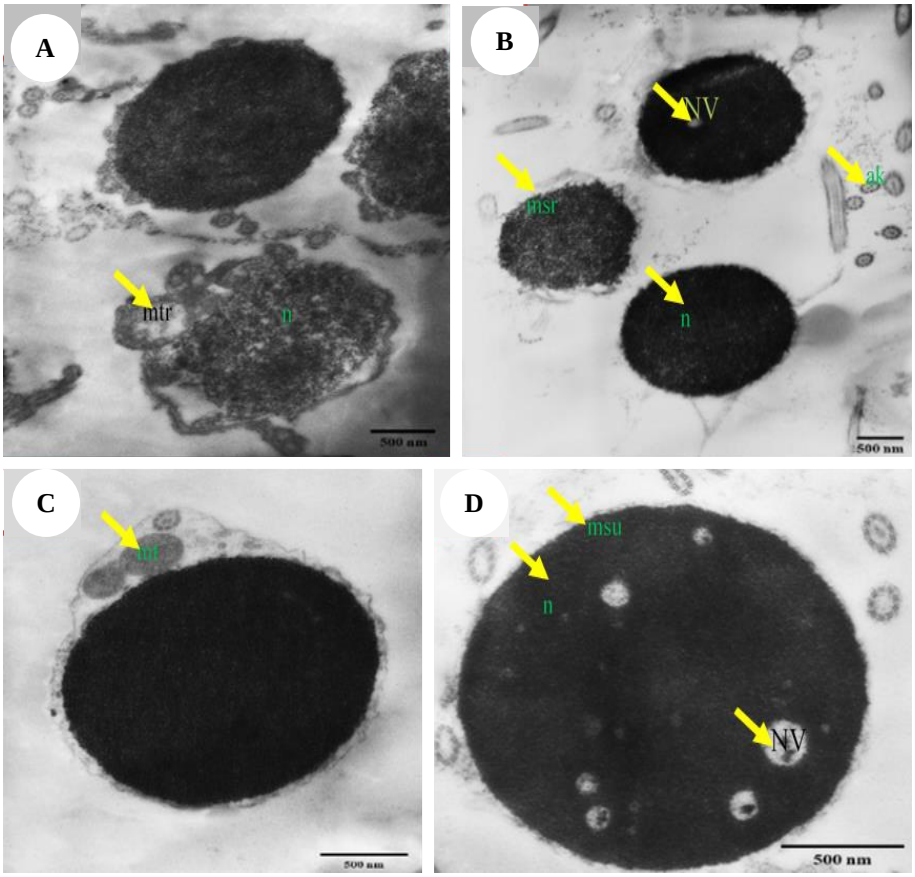

Figure 2. Changes occurred in the ultrastructure of *Neolissochilus soro* spermatozoa heads after 48 hours of preservation. A. Control, B. Honey concentration 1.5%, C. Honey concentration 1%, D. Honey concentration 2%. Mt: mitochondria; NV: sperm nucleus vacuoles; ak: axoneme; msr: damaged mitochondria

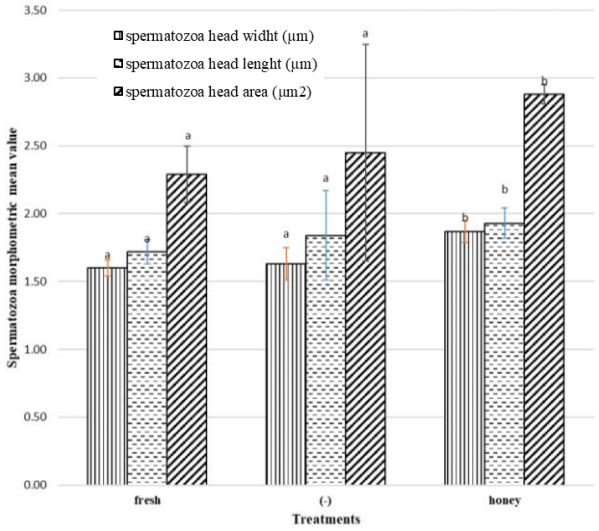

Figure 3. The sperm head width and length are based on the ultrastructure of the post-preserved *Neolissochilus soro*. The results are expressed as a percentage (mean±standard deviation)

Discussion

Transmission electron microscopy (TEM) analysis with Cell Block Preparation protocol showed differentiation between the head and the middle (neck) of the sperm preserved with and without honey. Meanwhile, TEM with negative staining protocol showed that all tails of the control were coiled, while sperm with honey had a wavy-shaped tail. In addition, the cross-section shows several membrane damage areas on the sperm head and non-protected sperm with honey.

The results also indicated that the sperm preservation of *Neolissochilus soro* fish was under severe stress. Viveiros et al. (2012) highlighted that the electron microscopy approach is very important in assessing the adverse effects of environmental stress. Damage to the head and neck and shortening of sperm tails after preservation proves that using honey has a protective effect at a specific concentration and harms the sperm at high concentrations (Lestari et al. 2021). The sperm head transfers genetic material localized in the nucleoplasm to the egg. Therefore, the optimal shape and size are a prerequisite for proper spermatozoa penetration through egg micropyle (Psenicka et al. 2007).

The ultrastructure of spermatozoa was previously studied in zebrafish (*Danio rerio*) (Sáez-Espinosa et al. 2022). The results showed that the head of *Neolissochilus soro* was round. TEM observations indicated marked decondensation with less dense genetic material after preservation. Furthermore, there is a strong ultrastructural distortion of the spermatozoa head due to nucleus and plasma membrane swelling. In the group that was not given honey, plasma membrane fragmentation was added to the coiling of the spermatozoa flagellum. A concentration of 1% has an intact spermatozoa head ultrastructure, but at above 1%, vacuolization has occurred. Vacuoles are cavities and holes on the surface of the sperm. Vacuoles can be induced due to air drying, leading to significant overproduction (Rothmann and Bort 2018).

The sperm preserved using a honey concentration of 0.5% and 0% (without honey) caused damage to the cell membrane and deformity of the head, most likely affecting the main function. Furthermore, the important structure of every cell is the presence of a membrane which determines the boundaries and various internal components. This membrane functions as protein transportation that facilitates and regulates the movement of substances in and out of the cell and its compartments (Díaz et al. 2019). Therefore, the sperm head membrane damage and changes in the condition of the tail are likely to cause adverse effects on the function. According to Shan et al. (2021), the cell membrane plays a very active role in the fertility capacity of spermatozoa.

The results showed that the preservation of *N. soro* fish spermatozoa was under pressure. Morphological and ultrastructural changes occurred after storage at 4°C for 48 hours. That damages the cell membrane of the spermatozoa head, the release of flagella and mitochondria, and the flagellar plasma membrane. Similar effects were observed in *Eleginops maclovinus*, including damage to the plasma membrane in the head and flagella and mitochondrial damage during 7-day storage (Ulloa-Rodríguez et al. 2019). Changes in sperm during preservation are associated with decreased cellular function, resulting in reduced motility and fertilization rates (Cheng et al. 2022). The head of the spermatozoa has the function of transferring genetic material localized in the nucleoplasm to the egg. Therefore, the optimal shape and size of the spermatozoa head is a prerequisite for proper penetration through the egg micropyle (Psenicka et al. 2007). Post-curing membrane destabilization can occur due to physical damage that causes oxidative stress (Shaliutina-Kolešová et al. 2022). Cold storage and thawing processes induce lipid phase transitions of sperm membranes, affect spatial redistribution, destabilize membranes, reduce motility and viability, and increase lipid peroxidation (Martínez-Páramo 2012). Structural changes and membrane destabilization consequences are functional modifications associated with ion transport, water balance, fertilization signal receptors, fluidity regulation, and permeability (Cejko et al. 2022).

Cellular osmoregulation may also be impaired, resulting in swelling of the head and tail and secondary lesions that decrease mitochondrial function (Shaliutina-Kolešová et al. 2022). Furthermore, cell membrane damage can be attributed to the concentration and toxicity of the barrier, as well as the time of its exposure, possibly due to the rapid penetration of the barrier in sperm and its interaction with membrane phospholipids (Gárriz and Miranda 2013). Dilution of carp semen (*Cyprinus carpio*) with a protective medium resulted in changes in head shape, decondensation of chromatin and vacuoles in some cells, and vesiculation of head and tail regions (Viveiros et al. 2012).

The head of the spermatozoa has the function of transferring genetic material localized in the nucleoplasm to the egg. Therefore, the optimal shape and size are prerequisites for proper penetration through the egg micropyle (Psenicka et al. 2007). Some damage to the

sperm head cell membrane is preserved with 0.5% honey and 0% concentration, deforming spermatozoa. Furthermore, the important structure of every cell is the presence of a membrane that defines the boundaries and various internal components. The membrane functions as a transport protein to facilitate and regulate the movement of substances between cells and compartments. Therefore, the head membrane damage and changes in the tail condition are likely to cause adverse effects. Shan et al. (2021) reported that sperm cell membranes play an important role in fertility.

Even though some morphological and ultrastructural changes occur up to some extent in *Neolissochilus soro* spermatozoa at 4ºC for 48 hours, preservation without honey causes severe head damage and undergoes morphological distortions, such as swelling of the nucleus, rupture of the plasma membrane, and changes in genetic material. The midpiece undergoes cell membrane rupture and mitochondrial lysis. Furthermore, most of the spermatozoa show circular flagella and disaggregated plasma membranes. These findings indicated that the observation of spermatozoa leads to substantial morphological and ultrastructural changes.

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