Pathological Effects of Environmental Pollution on the Ultrastructure of Spermatogenic Stages and the Mature Sperm of *Brachydontes variabilis* (Krauss, 1848) (Bivalvia-Mytilidae)

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**Abstract:** The present field study was conducted to investigate the effect of environmentally-relevant pollutants on the ultrastructure of the spermatogenic stages and the mature sperm of the mussel, *Brachydontes variabilis*. For this purpose, two mussel populations were collected from two different sites subjected to different contamination-exposure stresses: a contaminated site (Lake Timsah) and a reference site from Suez Canal. The spermatogenic cells and sperm structure were compared in both populations using light and electron microscopy. Based on TEM observations, the sperm of the investigated mussels is of the primitive type. It is characterized by a head with an ovoid nucleus and a cup-shaped acrosome, a midpiece with a ring of 5 rounded mitochondria encircling two centrioles and a long tail flagellum. The sperm of the present species differs in some structural details from other mytilids. This indicates that sperm morphology is species specific within Mytilidae and thus may be helpful in resolving the taxonomic or phylogenetic relationships within the family. On the other hand, microscopic examination revealed significant adverse effects on testes of the mussels collected from the polluted sites compared to that of the reference site. The most dramatic toxic effect was the presence of some degenerating male follicles. It was also noticed that pollution induced many malformations in the sperm developmental stages including pathological alteration in spermatogonia, spermatocytes and spermatozoa. These results revealed that *B. variabilis* is sensitive to environmentally relevant levels of pollutants. Based on this finding, it is suggested that this species may be chosen as a model for studying the effects of pollutants on gametogenesis. The adverse toxic effects induced by the environmentally relevant levels of pollutants on the testis of the investigated species may reflect the extent to which the level of pollutants has been reached in Lake Timsah. This may be the main cause of decreasing the success of bivalve communities in the lake in recent years.

**Key words:** *Brachydontes variabilis*; Suez Canal; Pollution; Gametogenesis; Sperm ultrastructure.

**INTRODUCTION**

Bivalves play an important role in the ecosystem equilibrium and constitute an important member of the near-shore biota, contributing significantly to the food chain and to the modification of the substrata. Among the biota of Lake Timsah (Suez Canal), bivalves were considered as the most dominant species both in number of species and individuals (Ghobashy *et al.*, 1992). In order to guarantee an increasing production, it is important to identify stress factors and their impact on the species of ecological and commercial interests. The effect of pollutants in different aspects of bivalves has been well characterized, (Mona *et al.*, 1994., Desouky and Mohammadein, 2001., Pytharopoulou *et al.*, 2006., Angelo *et al.*, 2007., Banni *et al.*, 2007). Peterson *et al.* (1993) reported that structural alterations in the male reproductive system were not common in species; however, functional alterations such as Leydig cell function, daily sperm production and sperm maturation were sensitive signs of developmental toxicity. Therefore, less attention has been focused on the possible influence of various anthropogenic pollutants on histopathology of bivalve gametogenesis. For instance, Lowe (1988) found an increase in gamete alterations and degenerations in the reproductive tissue of *Mytilus edulis* exposed to various contaminant concentrations (PAHs, PCBs, heavy metals). Moreover,
some studies on marine bivalve molluscs have shown alterations in the structure and composition of reproductive tissue associated with adverse environmental conditions due to anthropogenic activities (Timmermans et al., 1996., Brown et al., 2003., Aarab et al., 2004., Darriba et al., 2004).

On the other hand, the ultrastructural and morphometric characteristics of mature spermatozoa in Mollusca have been correlated with the different reproductive strategies of organisms, including the adaptation to egg structure of the species, and were also shown to be of great value for tracing taxonomic relationships between species (Popham, 1979., Healy, 1995., Hodgson, 1986., 1995., Morse and Zardus, 1997). In view of the aforementioned, the present study was designed to undertake two goals: (1) to describe the spermatogenesis and sperm ultrastructure of the marine mytilid, Brachydontes variabilis (Krauss, 1848) (2) to examine the effect of pollution on the ultrastructure of spermatogenic stages and sperm of this species.

MATERIALS AND METHODS

Study Sites:

In this study, two populations of the mussels, Brachydontes variabilis with similar gonadal maturation stages were selected from two sites that were subject to different contamination-exposure stresses. The two populations were collected from sites of relatively short distances from each other to ensure that the gonad status will not be affected by the change in environmental conditions. Lake Timsah (Fig., 1) was chosen as a “polluted site”. It is a shallow body of water with a surface area of about 15 km². It is almost half way the Suez Canal and represents a barrier or an area of settlement of biota, which migrate between the Mediterranean Sea and Red Sea via Suez Canal. The lake receives several kinds of pollutants coming from freshwater inputs, treated sewage of Ismailia Town in addition to oil and other industrial byproducts coming from platforms of ship construction and maintenance. Samples were collected from “ship maintenance platform” which is a highly polluted site.

On the other hand, the main channel of the Suez Canal was chosen as “reference site”. Samples were collected from a clean site just after the Ferryboat No 6 (Fig. 1) which was relatively exempt from any direct source of pollution.

Water Analysis:

The methods discussed in the American Public Health Association (APHA, 1995) were used for the determination of the chemical parameters except where noted. Water alkalinity (CO₂ and HCO₃⁻) was determined immediately after samples collection using phenol-phthalein and methyl orange indicators. Sulphate determined using turbidimetric method. Calcium and magnesium were determined by using complexometry method by direct titration using EDTA solution. Ammonia was determined by phenate method. Nitrite was determined using colorimetric method. Nitrate was determined by cadmium reduction method. Orthophosphate and total phosphorus were determined by using ascorbic acid molybdate method. The TDS and TSS were determined by direct gravimetric methods (APHA, 1995).

Ten elements of heavy metals were measured by ICP Optima 3000 (Inductively Coupled Plasma Optical Emission Spectrometry, ICP-OES). These elements included aluminum, antimony, arsenic, barium, copper, iron, lead, selenium, tin and zinc.

Light and Electron Microscopy:

Samples were collected from the two sites at the same time during September 2007. Gonadal samples from five male individuals (males were identified by their mantle colour) from both sites were freshly dissected out and then fixed for 24 h in 2% glutaraldehyde in 0. 1M phosphate buffer. Post-fixation was completed in 1% OsO₄ using the same buffer for 60 min on a rotating plate at room temperature. The samples were rinsed in buffer, and then dehydrated in ethanol solutions (70, 80, 95, and 100%). Samples were placed on a rotating plate for a total of 30 min for each progressive ethanol solution. Samples were cleared in acetone, placed in a 1:1 mixture of epon : araldite in ethanol (100%) for 1 h, placed in a 1:3 mixture of epon : araldite in acetone for 1 h, and samples were finally placed in 100% epon : araldite and polymerization was completed at 60 °C for 24 h. Semi-thin (1μm) and ultra-thin (60–70 nm) sections were cut with glass and
diamond ultramicrotome knives, respectively. The semi-thin sections were stained with 0.5% toluidine blue for light microscopy (LM). The ultra-thin sections were contrasted with uranyl acetate, lead citrate stains, and examined using a JOEL JTM. 1200 EXII electron microscope, Ain Shams University, Cairo, Egypt. All examined testes samples were chosen from the same developmental stage (stage III, ripe).

RESULTS AND DISCUSSION

Water Analysis:

The data of water quality (Table 1) generally displayed high values of the parameters analyzed in station A (polluted site) than station B (reference site). The values of hardness and nutrients in station A were also higher than those measured in station B.

During the course of this study, ten heavy metal elements were measured. Four of these elements were recorded values below the detection limits. These are antimony (<30 μg l⁻¹), arsenic (<10 μg l⁻¹), selenium (<30 μg l⁻¹) and tin (<30 μg l⁻¹).

The values of aluminum, barium, copper, iron, lead, and zinc that measured at station A were significantly higher than at station B (Table 2), especially iron. However, all values of the measured heavy metals were within the permissible limits of law 48/1982 for water body protection in Egypt except iron and lead, which exceed the permissible limits.

Table 1: Water quality analysis for the studied stations (n=5±SD)

<table>
<thead>
<tr>
<th>Item</th>
<th>St. A (Polluted)</th>
<th>St. B (Ref)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC (mS/Cm)</td>
<td>53.90±3.4</td>
<td>48.80±2.8</td>
</tr>
<tr>
<td>TDS (gL⁻¹)</td>
<td>35.9±1.2</td>
<td>29.6±1.8</td>
</tr>
<tr>
<td>Chloride (gL⁻¹)</td>
<td>19.62±0.8</td>
<td>18.46±0.7</td>
</tr>
<tr>
<td>SO₄ (gL⁻¹)</td>
<td>2.49±0.3</td>
<td>2.15±0.2</td>
</tr>
<tr>
<td>Calcium (mg/l⁻¹)</td>
<td>601.00±6.0</td>
<td>561±5.0</td>
</tr>
<tr>
<td>Magnesium (mg/l⁻¹)</td>
<td>1940.00±13.6</td>
<td>1569±9.8</td>
</tr>
<tr>
<td>Potassium (mg/l⁻¹)</td>
<td>635.00±5.8</td>
<td>545±9.4</td>
</tr>
<tr>
<td>Sodium (gL⁻¹)</td>
<td>10.16±0.4</td>
<td>9.86±0.7</td>
</tr>
<tr>
<td>NH₃ (μg/l⁻¹)</td>
<td>63.00±3.9</td>
<td>51.00±2.4</td>
</tr>
<tr>
<td>NO₂ (μg/l⁻¹)</td>
<td>45.40±2.0</td>
<td>34.40±1.7</td>
</tr>
<tr>
<td>NO₃ (μg/l⁻¹)</td>
<td>7.00±0.4</td>
<td>4.50±0.4</td>
</tr>
<tr>
<td>PO₄ (μg/l⁻¹)</td>
<td>21.30±1.1</td>
<td>15.60±0.9</td>
</tr>
<tr>
<td>TP (μg/l⁻¹)</td>
<td>52.3±3.0</td>
<td>41.80±2.0</td>
</tr>
</tbody>
</table>

Table 2: Results of heavy metals values (μg l⁻¹, n=5±SD) measured at stations of study and their permissible limits (PL, μg l⁻¹) of low 48/1982 for water body protection in Egypt.

<table>
<thead>
<tr>
<th>Metals</th>
<th>St. A (Polluted)</th>
<th>St. B (Ref)</th>
<th>Permissible limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aluminum</td>
<td>1557.2±15.4</td>
<td>1004.0±11.2</td>
<td>3000</td>
</tr>
<tr>
<td>Antimony</td>
<td>&lt;30</td>
<td>&lt;30</td>
<td></td>
</tr>
<tr>
<td>Arsenic</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>Barium</td>
<td>149.0±6.4</td>
<td>48.8±5.8</td>
<td>2000</td>
</tr>
<tr>
<td>Copper</td>
<td>452.6±6.6</td>
<td>291.8±8.4</td>
<td>1000</td>
</tr>
<tr>
<td>Iron</td>
<td>10382.0±23.0</td>
<td>1932.0±14.0</td>
<td>1000</td>
</tr>
<tr>
<td>Lead</td>
<td>54.2±2.2</td>
<td>48.8±2.2</td>
<td>50</td>
</tr>
<tr>
<td>Selenium</td>
<td>&lt;30</td>
<td>&lt;30</td>
<td></td>
</tr>
<tr>
<td>Tin</td>
<td>&lt;30</td>
<td>&lt;30</td>
<td></td>
</tr>
<tr>
<td>Zinc</td>
<td>170.8±5.1</td>
<td>112.2±5.9</td>
<td>1000</td>
</tr>
</tbody>
</table>

Description of Testicular Tissues of Samples from Reference Site:

Anatomy of the Male Reproductive System:

Although no external signs of sexual dimorphism could be noticed, it is possible to determine the sex of individuals by their mantle colour. Female mantle tends to be pinkish orange, whereas that of the male is creamy white. The gross morphology of the testis consisted of many branched ducts or follicles that mainly ramify inside the mantle lobes (Fig., 2A) and some follicles appear within the mesosoma under the pericardium. Each follicle is delimited by a connective tissue sheath containing granular adipose cells (Fig., 2A).
Spermatogenic Stages:

The testicular follicles are occupied with crowded germinal cells at different developmental stages. The cell of a particular stage can be identified by their shape, size and staining properties. Spermatogonia, the largest male germinal cells, lie in clusters against the inner wall of the follicles (Fig. 2B). Primary spermatogonia are oval and have relatively little cytoplasm. Under TEM, heterochromatin in these cells appears in clumps scattered in the nucleoplasm and one or two nuclei are present, as are mitochondria in the cytoplasm along with RER (Fig. 3A). Secondary spermatogonia have oval nuclei with a single nucleolus and chromatin clumped around the periphery (Fig. 3B).

Fig. 1: Study area showing the location of sampling sites: "A" polluted site, "B" reference site.
Fig. 2: Light micrographs of sections of testicular follicles of *Brachydontes variabilis*, collected from reference site of Suez Canal, stained toluidine blue showing sperm in various stages of spermatogenesis.

**(A):** Low power of testicular follicles. ct: connective tissue., F: follicle. 200X.

**(B):** Enlarged portion of "A" showing early spermatogenic stages developing from the follicle wall. Sg: primary spermatogonia., Sg: secondary spermatogonia. 1200X. **(C):** Enlarged portion of "A" showing the spermatocytes (sp) and spermatids (st). ct: connective tissue. 1200X.

**(D):** Enlarged portion of "A" showing mature spermatozoa (S) filling the lumen of the follicles. The sperm flagella (Fl) are found in clusters. 1400X.

Spermatocytes are smaller than the spermatogonia. Primary spermatocytes are spherical with a large homogeneous nucleus (Fig., 3C). However, they have no visible nuclear membrane and their nucleoli are not clearly detected (Fig., 3C). Secondary spermatocytes are polygonal or irregular and have spongy-looking electron-dense chromatin. Several mitochondria are present in the cytoplasm along with centrioles and small dense granules and vacuoles (Fig., 3C).

Spermatids form clusters of small cells distributed in the centre of the lumina of the follicle (Fig., 2C). Under TEM, the spermatids appear irregularly rounded; have a spherical rather homogeneous nucleus. The spermatids are also endowed with few dense granules in the cytoplasm, SER and a variable number of mitochondria (Fig., 3D).

**Ultrastructure of Mature Sperm:**

The mature spermatozoon of *B. variabilis* consists of three distinct regions: a head, midpiece and flagellum. The head includes the nucleus and the anteriorly positioned cap-like acrosome. It contains fine electron-dense homogeneous chromatin (Fig., 3E) and measured 1.99±0.2μm long when measured from the tip of the acrosome to the distal end of the nucleus and 1.4±0.1μm wide (n=10) while the cytoplasm is much reduced. The acrosome is somewhat elongated cap-like, membrane bound and invaginates on the adnuclear side (Fig., 3E, 3G). It measures 0.79±0.1μm in length and 0.77±0.1μm in maximum diameter at the base (n=10). The subacrosomal space

The short midpiece around the centrioles arranged cristae (Fig., 3H).

The flagella are very of the typical 9 + 2 diameter of the flagellum microtubular pattern axoneme and the ensheathing plasma membrane (Fig., 3I). The

Under EM, It consists well-developed randomly

of five mitochondria

the typical 9 + 2 microtubular pattern axoneme and the ensheathing plasma membrane (Fig., 3I). The

The flagella are very of the typical 9 + 2 diameter of the flagellum microtubular pattern axoneme and the ensheathing plasma membrane (Fig., 3I). The
**Histopathological Alterations in the Testicular Tissues of Mussels from Polluted Site:**

The mussels collected from the polluted site showed altered gonadal development represented by the disorganization of the developing follicles and the surrounding supportive gonadal tissue. The interfollicular connective tissue of all examined samples from the polluted site was enlarged, very loose and contained numerous granular adipose cells (Figs. 4A, 4B). Accumulation of haemocytes was also seen in contact with follicles (Figs. 4D, 5A). A wide space was demonstrated between the spermatogenic cells and the tubule epithelium (Fig. 4D).

The testes of four out of five examined samples from polluted site displayed some degenerating follicles indicative of dramatic toxic effect (Fig.4A). Moreover, some tubules show unsynchronized spermatogenesis (i.e. (1) sperms, (2) spermatids, (3) spermatocytes, and (4) spermatogonia are seen in same area of tubule (Fig.4D).

At the ultrastructural level, several pathological alterations were observed in sperm developing stages of mussels collected from the polluted site compared to that collected from the reference site. The spermatogonia exhibited peri-nuclear vacuolization, more condensed chromatin and degenerated cytoplasm (Fig. 4C, 5B). In the spermatocytes, the chromatin materials became condensed and the nucleoplasm appeared granulated. A large number of small electron-dense granules as well as dilated SER appeared in the cytoplasm. Moreover, the mitochondria had lost their a cristae (Fig. 5C). In some spermatocytes the nucleoplasm has lost its density and wide degenerative portion of the nucleus could be seen with some vacuoles scattered here and there (Fig. 5D).

Some structural changes were also observed in the mature spermatozoa of the animals collected from the polluted site. The nuclear chromatin was less condensed than that of the animals collected from reference site (Fig. 5E). Moreover, mitochondrial cristae appeared intact (Fig. 5F) and damage of the plasma membrane and nuclear membrane became apparent. (Fig. 5E).

**Discussion:**

In this paper, the main cellular features of spermatogenic stages and sperm have been described in *Brachydontes variabilis*. The various types of germ cells were characterized at the light and electron microscopy levels, largely based on nuclear detail. The sperm developmental stages described here are similar to those described in other bivalves such as *Crassostrea gigas* (Franco *et al.*, 2008). Based on TEM
observations, the current study showed that the sperm of this mytilid, as in other bivalves, is of primitive type. It is characterized by a head with an ovoid nucleus and a cap-shaped pointed acrosome, a midpiece with a ring of 5 rounded mitochondria encircling two centrioles and a long tail flagellum. Although this morphological features of the sperm is conservative characteristics among Mytilidae, nevertheless, the sperm of the present species differs in some structural details from other mytilids such as *Modiolus difficilis* (Drozdov and Reunov, 1986), *Mytilus galloprovincialis* (Hodgson and Bernard, 1986) and *Bathymodiolus azoricus* (Kádár et. al., 2006). This indicates that sperm morphology is species specific within Mytilidae and thus may be helpful in resolving the taxonomic or phylogenetic relationships within the family.

Upon viewing male gametogenesis of the investigated species using both light and electron microscopy, significant adverse effects were reported on testis of the mussels collected from the polluted sites compared to that of the reference site. The most indicative dramatic toxic effect is the presence of some degenerating male follicles. Similar anomalies, described in *Mytilus edulis* (Lowe, 1988., Aarab et. al., 2004) and clams (Navas et al., 1992., Park and Chung, 2004), have been related both to parasitic infections and environmental pollution.

![Image](image_url)

**Fig. 4:** Light micrographs of sections of testicular follicles of *B. variabilis*, collected from polluted site of Lake Timsah, stained with toluidine blue showing sperm in various stages of spermatogenesis.

- **(A):** Low power of testicular follicles showing enlarged loose interfollicular connective tissue and a degenerating follicle. ct: connective tissue., F: follicle., degenerating follicle. 160X.
- **(B):** Interfollicular connective tissue with accumulated granular adipose cells (Ad c) and granulocyte (gr). Sg: spermatogonia. 1,000X.
- **(C):** Enlarged portion of "A" showing the spermatogonia (sg) with more condensed chromatin and degenerating cytoplasm. 1,000X.
- **(D):** Enlarged portion of "A" showing unsynchronized spermatogenesis. Sperms(S), spermatids (St), spermatocytes (Sp), and spermatogonia (Sg) were seen in same area of tubule. gr: granulocyte. 10,000X.

It was also noticed that pollution induced many malformations in the sperm developmental stages including pathological alteration in spermatogonia, spermatocytes and spermatozoa. In this respect, Winternyer and Cooper (2007) reported similar pathological alterations in the testicular tissues of the eastern oyster (*Crassostrea virginica*) upon exposure to 2,3,7,8-TCDD. There are some reports of altered sperm maturation, germ cell vacuolization, and inhibition of spermatogenesis occurring in mammalian male species exposed to
some pollutants (Bjerke and Peterson, 1994., Sommer et al., 1996., Gray et al., 1997., Moon et al., 2004). These reports are in agreement with the present findings.

In several testicular follicles of the mussel collected from the polluted site, various stages of spermatogenesis were found within the same area, implying that development was not synchronized. This malformation may lead to the accumulation of premature spermatocytes and spermatids in the tubular lumen and an inhibition of sperm maturation. Rune et al. (1991) attributed this unsynchronized development to enlarged intercellular spaces between the germinal epithelium and the spermatogenic cells of the marmoset (Callithrix jacchus) upon exposure to 2,3,7,8-TCDD. Such enlarged subepithelial space was also shown in the present investigation.

The measurements of chemical properties of water in the lake and its heavy metals contents revealed that the lake is highly polluted compared to the main channel of the Suez Canal. The adverse toxic effects induced by these environmentally relevant levels of pollutants on the testis of the investigated species may also reflect the extent to which the level of pollutants has been reached in Lake Timsah, which may be the main cause of decreasing the success of bivalve communities in the lake in the recent years. The results of the present field study revealed also that the mussel B. variabilis is sensitive to environmentally relevant levels of pollutants that induce many histopathological alterations in the testis. Based on this finding, it is suggested that this species may be used as a model to use for studying the effects of pollutants on gametogenesis.

![Electron micrographs of sperm developmental stages of B. variables collected from polluted site of Lake Timsah.](image)

(A): A granulocyte (gr) aggregated around the follicular wall. N: nucleus. 6,000X
(B): Spermatogonia with condensed chromatin and perinuclear vacuolization (v). 2700X.
(C): Spermatocytes with a large number of small electron-dense granules (arrows) as well as dilated SER appeared in the cytoplasm and the mitochondria (m) had lost their cristae. 8,000X.
(D): Enlarged spermatocyte with degenerating nucleus (N). 10,000X.
(E): Mature spermatozoan with degenerating plasma membrane (arrow) and less condensed chromatin, acrosome (ac), head with oval nucleus (N) and midpiece (mp). 28,000X.
(F): Cross section of the midpiece of the sperm showing mitochondria with intact cristae. 40,000X.
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REFERENCES


