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Effects of sodium fluoride on the gametogenesis of the tubificid oligochaete *Branchiura sowerbyi* Beddard

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Abstract

Fluoride concentration is significantly increasing in many aquatic ecosystems because of human activities (agrochemical, pharmaceutical, refrigerant, pesticide and surfactant compounds). This study aims to examine the effects of fluorides on gametogenesis by exposing *Branchiura sowerbyi* (Oligochaeta) to sodium fluoride concentrations 50 to 100 times above normal ones in freshwater (0.1–0.3 mg/L) and observing the effects over time. Because non-toxic concentrations determined in short-term experiments could induce effects as exposure times increase, *B. sowerbyi* were exposed to sodium fluoride from the end of February 2012 to the end of July 2012. They were continuously monitored until the cocoon deposition stage to investigate any possible effects. Weekly observations were conducted on treated and untreated specimens to monitor gamete maturation in both male and female germinal lines. The results revealed temporal differences in the gametogenesis phases of treated specimens, such as incomplete spermatozoa maturation. At the end of the experiment, the exposed animals were thinner and shorter than the controls and cocoon deposition did not occur.

Key words: fluoride effects, gametogenesis, *Branchiura sowerbyi,* freshwater oligochaete

Introduction

Many authors have previously studied the life cycle and gametogenesis of *Branchiura sowerbyi* and the organism's reaction to xenobiotic exposure (Aston 1966, Casellato 1984; Casellato et al. 1987; Casellato et al. 1992; Casellato & Negrisolo 1989; Hirao 1973; Lobo & Alves 2011; Naqui 1973; Reynoldson et al. 1991). Fluoride (F^-) concentration is significantly increasing in many aquatic ecosystems as a consequence of human activities. These activities include the manufacture and use of agrochemicals, pharmaceuticals, refrigerants, pesticides and surfactant compounds. The main goal of this study is to examine the effects of fluorides on gametogenesis by exposing *B. sowerbyi* to fluoride concentrations in water 50 to 100 times above normal (0.1–0.3 mg/L) and observing the effects over time. A recent study by Del Piero et al. (in this issue) showed that *B. sowerbyi* is more resistant to fluoride than other freshwater invertebrates (Camargo, 2003; Gonzalo et al.,2010; Del Piero et al., 2012), especially in the presence of sediment. These studies indicated NOEC (no-effect concentration) and LOEC (lowest effect concentration) values to be between 80 and 120 mg/L for a short exposure time (96 h).

Considering that non-toxic concentrations in short- and medium-term experiments could induce effects as exposure time increases, numerous *B. sowerbyi* were exposed to fluoride concentrations of 15 mg/L and 30 mg/L (lower than the NOEC), and were monitored weekly beginning late February 2012, to investigate the possible effects of fluoride on male and female gamete maturation.

Materials and methods

At the end of February, many *B. sowerbyi* specimens were collected in a water lily tank in the Botanical Garden of Padua University. The tank was filled with thermal water that was maintained at a temperature range

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between 17°C and 24°C throughout the year. The period of late winter was chosen for the beginning of the experiment because the sexual apparatus of *B. sowerbyi*, which degenerates in mid-summer after cocoon deposition, partially regenerates in the following autumn when the ovaries and testes increase in size and show clear signs of germ cell renewal. The organisms are without a clitellum during this period. At the beginning of winter, the testes and ovaries undergo a quiescence period for about two months and normal gonad activity resumes with an increase in testis and ovary size at the end of winter (Casellato 1984; Casellato et al. 1987). In this period it is easy to distinguish the two-year-old specimens (9–12 cm long) with a completely regenerated sexual apparatus from the one-year-old specimens (5–6 cm long) that will reach maturity for the first time. Only animals of the first cohort (the larger specimens) were considered for the experiments.

Small aquaria (10 L) were prepared to mimic the conditions of the original sampling site. These conditions included using the original water at its normal temperature range, the original sediment at a depth of 8 cm, and the original population density. Numerous specimens (n = 50) were reared and exposed to water containing either 15 or 30 mg/L of F^- (prepared with NaF solution), which was renewed weekly, and compared with a control without fluoride. We chose to perform the experiment at 23 ± 1°C because this temperature is optimal for gamete maturation. The treated and untreated specimens were checked weekly to monitor gamete maturation in both male and female germinal lines. To observe gamete maturation, individual samples were numbed with drops of 70% alcohol and segments 5 to 17, which contain the entire sexual apparatus, were dissected using micro-scissors. The sexual apparatus was placed on a slide and gently compressed with a coverslip to extract germinal cysts from the coelomatic fluid, which could be observed outside the body wall. In this manner, cytomorphological changes during the transformation of spermatogonia to mature spermatozoa, and of oogonia to mature eggs could be observed under a light microscope (Leica Application Suite software with a Leica HQ DFC480 camera) over the course of five months. This method was used to observe changes in the entire sexual apparatus.

The animals were exposed to the nominal fluoride concentrations in the water, which were maintained by weekly renewal for all treatment periods. Moreover, fluoride accumulation in the sediment was periodically tested because it was important to quantify the real exposure level of the endobenthic animals to the substance over time. The fluoride concentration was determined from aliquots of water from the aquaria at the beginning of the experiment, and before every weekly renewal, using an ion-selective electrode ISE F800 (0.02 mg/L to saturation, accuracy 0.1%). The sediment was also monitored by taking 0.5 mg samples and analysing them using the alkali fusion method as described by McQuaker & Gurney (1977).

During the experiment the temperature of the aquaria was continuously monitored and the data showed an almost constant temperature during the 5-month period (24 ± 1°C). Chemical analyses were conducted periodically (monthly) during the experiment to ensure optimal fluoride content in the water and sediment, and to measure organic matter content and dissolved oxygen concentration.

Results

By monitoring the F^- concentration range in the water tanks during the experiment, variations from 15 mg/L to 19 mg/L, and from 30 mg/L to 39.56 mg/L, respectively, were observed throughout the week, suggesting a portion of the fluoride was absorbed by the sediment and then released back into the water. The fluoride concentration in the sediment increased from an initial value of 2.59 ppm to 95.87 ppm in the tank filled with 15 mg/L of fluoride, and reached 122.01 ppm in the tank with 30 mg/L of fluoride. This observation indicates that the animals were exposed to a higher concentration of fluoride than the nominal value in water during the experimental period. The amount of dissolved oxygen varied between 3.9 mg/L and 5.3 mg/L, which corresponded to an average saturation percentage of 68% at 25°C. The mean organic matter content never exceeded 4%, which is consistent with the original sampling site.

When *B. sowerbyi* individuals (average length 9–12 cm) were first exposed to fluoride at the end of February, they revealed normal gonad activity, indicated by increased volume in the testes and ovaries, as well as by the appearance of 4–8 spermatogonial cysts in the sperm sacs and large oogonia in the ovaries (Fig. 1). None of the specimens had developed a clitellum yet, which only appeared after a month of fluoride exposure in both treated animals and controls. At the beginning of March, the first 16-cell spermatogonial cysts (22 µm) (Fig. 2A) appeared in the control specimens, which was a month before than in the treated animals. On the contrary, free oocytes in the coelom (51 µm long) were found in all treated and untreated specimens at the

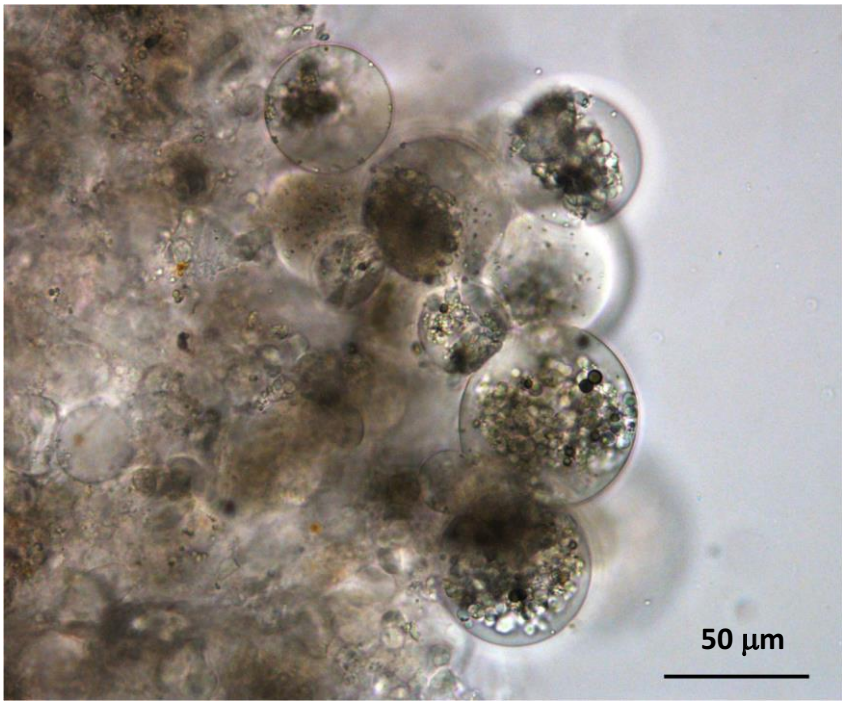

FIGURE 1: Large peripheral oogonia in the ovary, sign of germ cell renewal.

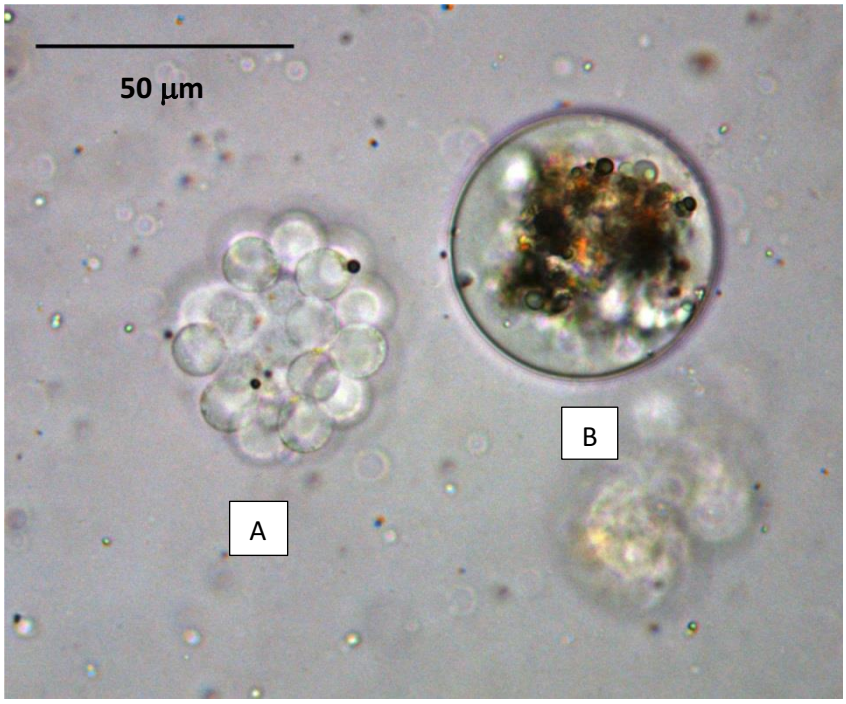

FIGURE 2: Spermatogonial cysts of 16 cells (A) and a free oocyte in coelomatic cavity (B).

beginning of March (Fig. 2B). Free oocytes appearing greatly enlarged (56 µm), showed more englobing and more yolk globules in their cytoplasm (Fig. 3). The first 32-cell spermatocyte cysts (42 µm) (Fig. 4) appeared at the beginning of April in the controls, but were a month late in the specimens treated with either 15 mg/L or 30 mg/L of fluoride. The first mature eggs were observed in both the controls and the specimens treated with 15 mg/L of fluoride (Fig. 5), but were observed a month later for those treated with 30 mg/L of fluoride. The development of the male germinal line occurred normally in controls. The 64-cell spermatocytes and the 128-cell spermatids cysts (Fig. 6) were present in large numbers in the coelom cavity. At the beginning of June, free spermatozoa were present in some of the controls (Fig. 7). However, the first spermatid cysts were

observed a month late in the treated specimens, and presented certain deformities (Fig. 8). There were also no free spermatozoa found in these specimens. At the end of May, mature sperm could be observed in the seminal funnels of the control specimens and a month later, spermatozeugmata were found in the spermathecae of these animals, which confirmed they had copulated (Fig.9). These structures have been described for other tubificid species (Braidotti & Ferraguti 1982; Ferraguti et al. 1989). Mature spermatozoa and spermatozeugmata were never observed in the treated specimens, which also presented fewer mature eggs when compared with the controls.

At the end of the experiment, the treated animals appeared to be shorter and thinner than the controls.

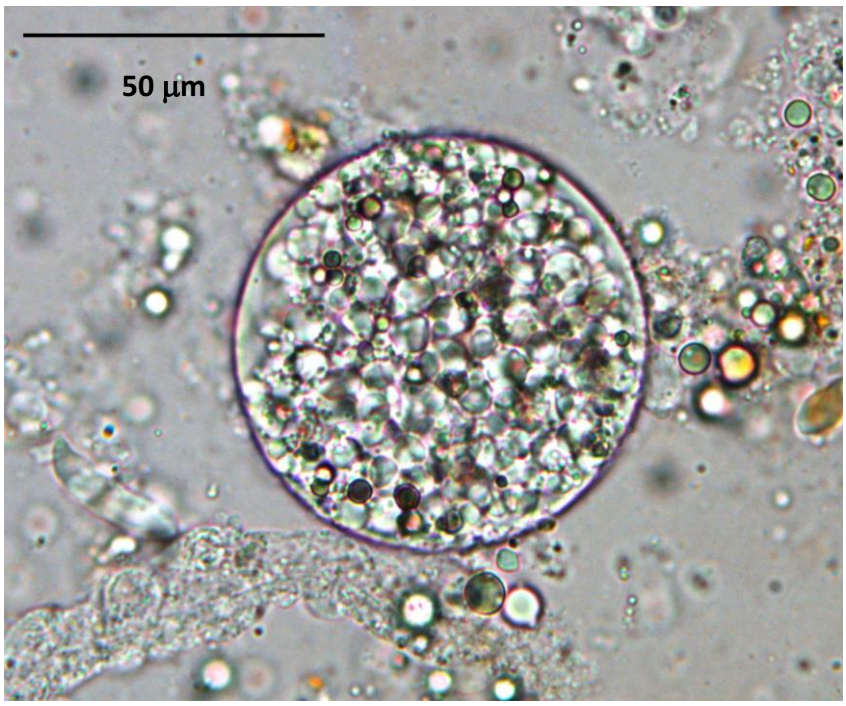

FIGURE 3: Free oocyte in advanced stage of maturation.

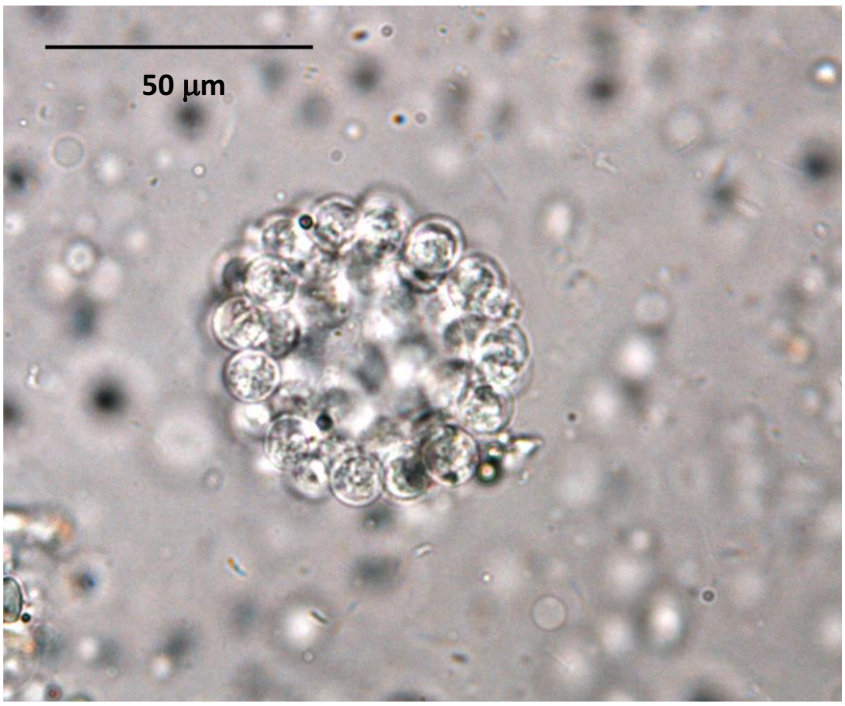

FIGURE 4: 32 cell spermatocyte cyst in a control individual.

Discussion and conclusions

The experiment indicated that prolonged exposure to fluoride damages the gametogenic cycle, particularly the spermatogenic cycle, in *B. sowerbyi*, even if the animals are exposed to concentrations that do not show an effect in the short- or medium-term. These results revealed temporal differences in the gametogenetic phases of treated specimens when compared with controls. Treated animals did not complete spermatozoan maturation in either the 15 or 30 mg/L NaF concentrations, and the number of mature eggs produced was greatly reduced. The male line stopped maturation at the spermatid stage, which appeared abnormal and regressed. Moreover, the treated animals were thinner and shorter than the controls at the end of the experiment and cocoon deposition did not occur.

No previous studies have been performed to study the effects of fluoride on aquatic Oligochaeta. Only data for some mammals are available in the literature. For example, El-lethey et al. (2011) demonstrated that the relative weights of reproductive organs were lower in adult rat males that were administered sodium fluoride (NaF). Histopathological examination showed degenerative changes in the testes, seminal vesicles and prostate glands of NaF-exposed males. The severity varied according to the dose, which was administered in the drinking water (0.50 ppm to 100 ppm). Other studies have reported delayed maturation of male cells in relation to testicular oxidative stress, with decreased testicular testosterone in the plasma of fluoride-exposed rats when compared with controls. Ghosh et al. (2002) and Bataineh & Nusier (2006) investigated the effects of sodium fluoride on adult male rats that ingested the substance in drinking water (100 ppm and 300 ppm) for 12 weeks. The rats' spermatozoa showed decreased motility and density, and females impregnated by these males showed increased foetal resorption. Susheela & Kumar (1991) administered 10 mg of fluoride per kg of body weight in rabbits for 29 months and observed the disruption of spermatogenic cells in the seminal funnels. Reddy et al. (2007) indicate that exposure to NaF during gestation and lactation affects the reproduction of male rats born from treated females by decreasing spermatogenesis and steroidogenesis. Other authors (Elbetieha et al. 2000; Inkielewicz-Stepniak & Czarnowski 2010; Kour & Singh 1980; Jhala et al. 2004) report abnormal development of both male and female germinal lines in animals treated with fluoride, which confirms that NaF can adversely affect fertility in mammals. Other published studies report that fluoride can induce oxidative stress and modulate intracellular redox homeostasis, lipid peroxidation and protein carbon content, as well as alter gene expression and cause apoptosis (Ahotupa & Huhtaniemi 1992; Barbier et al. 2010; Casellato et al. 2012). These data may help explain the anomalies observed in the gametogenic processes of *B. sowerbyi* that were exposed to NaF for a long time.

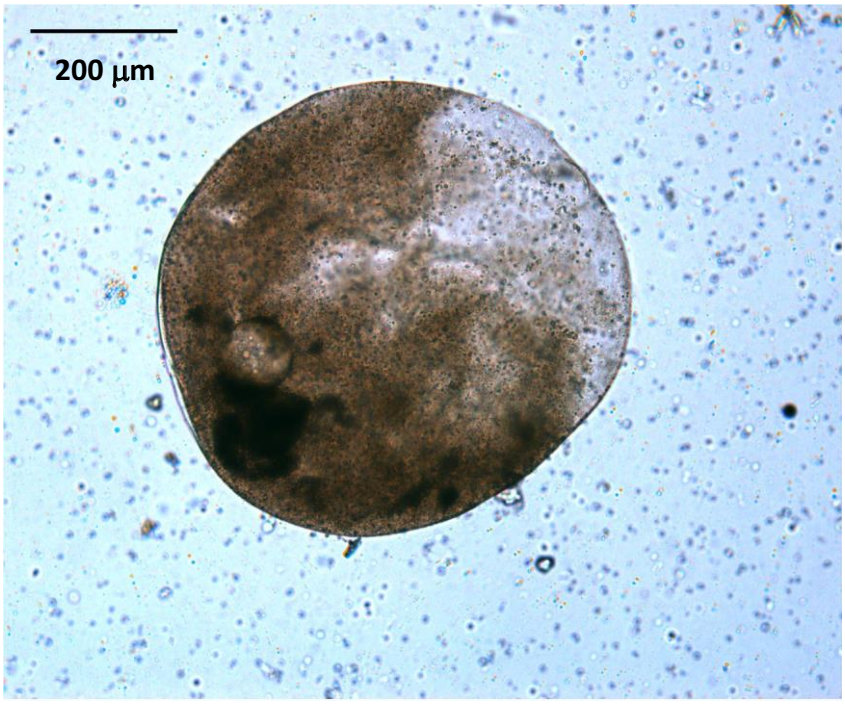

FIGURE 5: Ripe egg in a control individual.

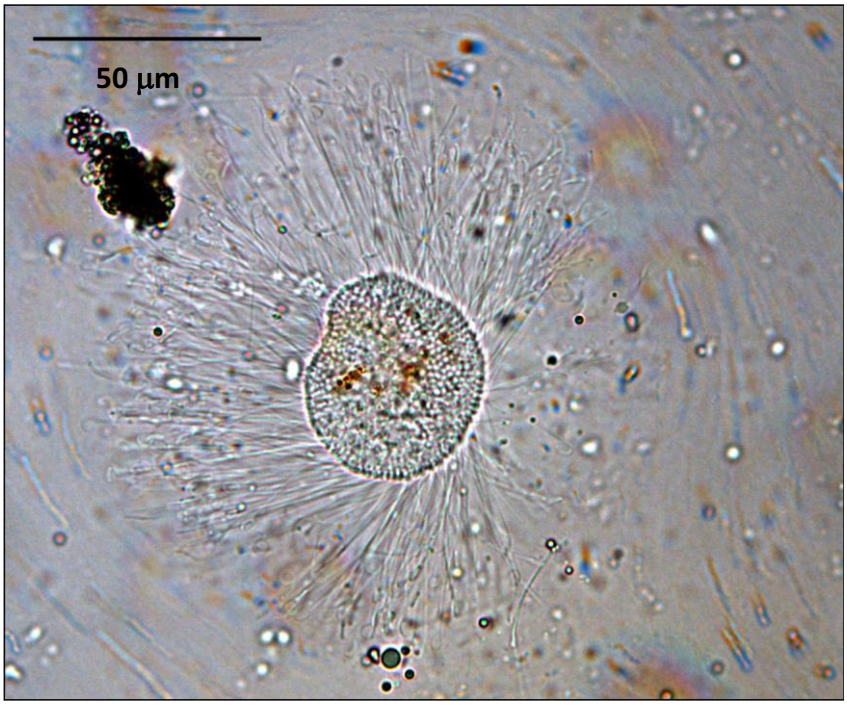

FIGURE 6: 128 spermatid cyst in a control individual.

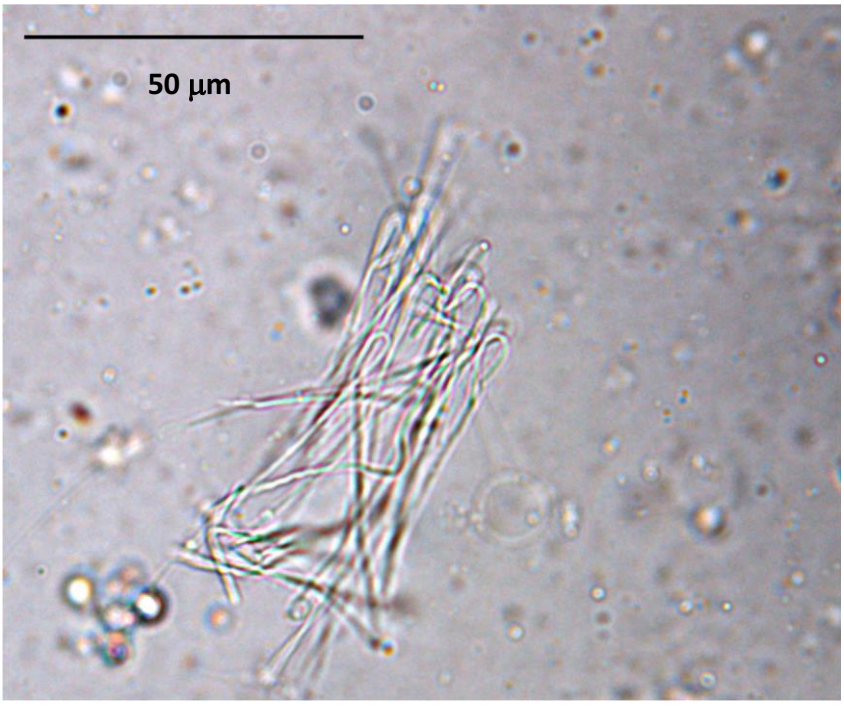

FIGURE 7: Free spermatozoa in a control individual.

Finally, our results suggest that fluoride treatment is associated with gametogenic disorders and induction of oxidative stress in the reproductive organs of both mammals and invertebrates, but while data for mammals are numerous, those for invertebrates are completely lacking. Our research emphasizes the necessity of investigating other animal groups to explain fluoride's exact mode of action and monitor its toxic potential.

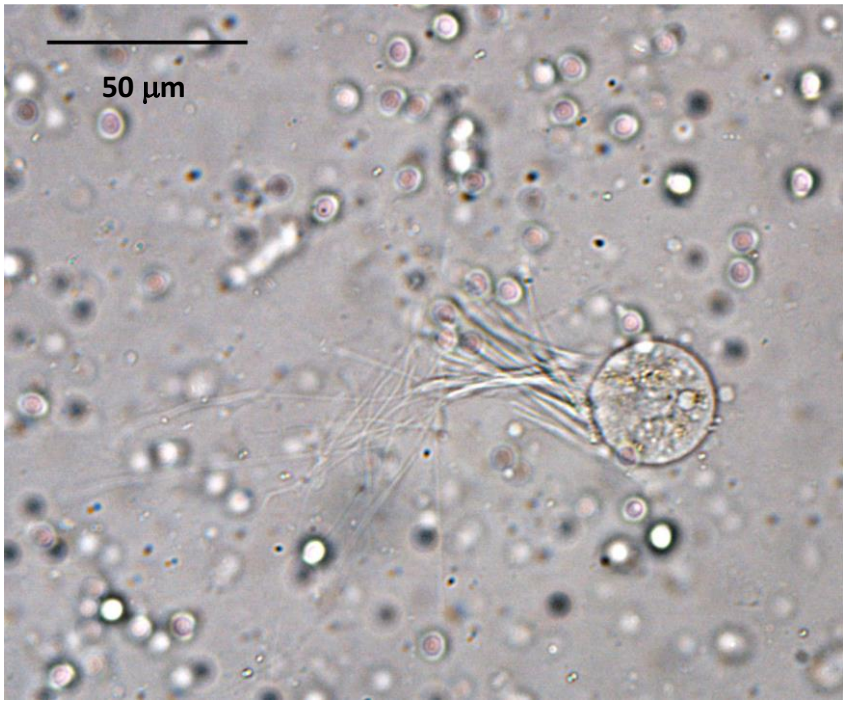

FIGURE 8: Abnormal spermatid cysts in a treated individual.

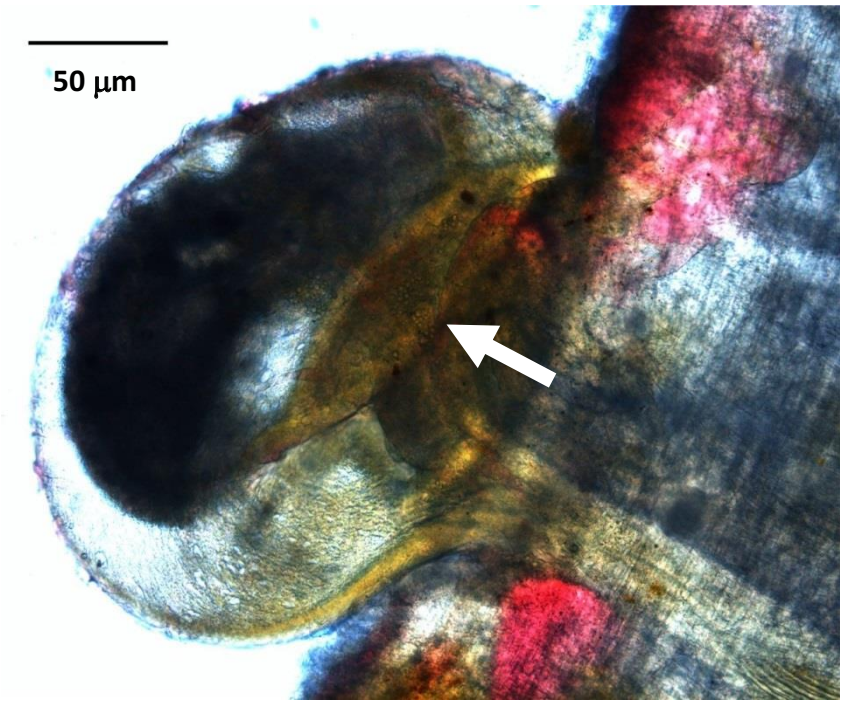

FIGURE 9: Spermatozeugma (indicated by the arrow) in the spermatheca out of the body wall obtained by squashing.

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