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Effect of Waterborne Copper on the Microstructures and Ultrastructure of the X-Organ Sinus Gland Complex in *Eriocheir sinensis*

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Abstract The effects of different water-borne copper (Cu^{2+}) concentrations on the microstructures and ultrastructures of the X-Organ sinus gland (XO/SG) in *Eriocheir sinensis* were studied. Under a light microscope, no significant differences were found between the treated and control groups. Under Transmission Electron Microscopy (TEM), the mitochondrial cristae in the neuroterminal of the XO of the 5.00 mg/L Cu^{2+} treated group disappeared, or even only a few vacuoles left. No other differences were found in SG between the 0.01, 0.10, and 1.00 mg/L Cu^{2+} treated and the control group under light microscopy, except for the 5.00 mg/L Cu^{2+} treated groups, in which the boundary between the SG hemocoels and glial cells became unclear, and some central hemocoels closed and even disintegrated. In addition, in the 5.00 mg/L Cu^{2+} treated groups the hemocytes mixed with the glial cells, axon terminal disordered arranged, and furthermore, under the TEM glial cells ruptured, mitochondrial cristae disappeared, or even remained only a vacuoles. Such a change also occurred in the mitochondria in the axon terminal. In some of the glial cells the structure of the membrane disintegrated, the cytoplasm disappeared, and the nucleus entered the haemocoels being piled together with the hemocytes. The axon terminal and neurosecretory granular decreased near the amorphous figurationling of the haemolymph. The results indicate that the damage to the microstructure and ultrastructure of the XO/SG was relevant to the elevated water-borne copper concentration in *E. sinensis.*

Keywords *Eriocheir sinensis* · Copper (Cu^{2+}) · X-Organ sinus gland · Microstructure · Ultrastructure

Copper is an essential nutrient essential for crustaceans in the synthesis of hemocyanin and a requirement for the normal biological function of many enzymes necessary for growth and development (Liu et al. 1990; Méndez et al. 2001). However, excess copper has serious toxicity to normal biological functions in crustaceans at the molecular, cellular, and tissue levels (Liu et al. 1988; Brouwer et al. 2002). The Chinese mitten crab, *Eriocheir sinensis*, is a commercially important decapod crustacean cultured extensively in China. For this aquaculture, the application of copper sulfate in ponds is very effective in reducing the abundance of *Microcystis* and other blue-green algae. However, the increase in water-borne copper negatively correlated with the molting rate in *E. sinensis* (Yang et al. 2005). The process of ecdysis of decapod crustaceans is an antagonistic interaction by ecdysone and the molting inhibition hormone (MIH), which originates from the Y-organ and X-organ/sinus gland (XO/SG) complex, respectively. The X-organ/sinus gland complex is located within the eyestalks. SG is the bulge of the axon terminal of the XO neurosecretory cell which is located in the

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eyestalk optic nerve, and is an important nerve organ in Crustacea. Besides MIH, several other kinds of neurohormones are secreted by the XO, such as the gonad inhibiting hormone (GIH) and crustacean hyperglycaemic hormone (CHH), which are also stored and released by the XO/SG complex to adjust the growth of the gonads and molting and reaction against the environment (Cooke and Sullivan 1982; Keller 1992). Up to now, there have been a number of studies on the microstructure and ultrastructure of the XO/SG complex in a variety of crustaceans, including *E. sinensis*, *Scylla serrata*, *Penaeus orientalis*, *Homarus gammarus*, *Carcinus maenas* and *Cardisoma carnifex* (Shivers 1976; Weatherby 1981; Dircksen 1992; Shangguan and Li 1994, 1995; Castany 1997; Kang and Wang 1998; Qiu 1999; Sun et al. 2001; Guo et al. 2002; Wei et al. 2002). However, little is known about the adverse effect of heavy metals on the structure or function of the XO/SG complex. In this paper, we selected copper to study the effects of waterborne copper toxicities on the microstructure and ultrastructure of the XO/SG complex in *E. sinensis*. The mechanism of these toxicities is also discussed.

Materials and Methods

Eriocheir sinensis (weighing 100.00 ± 10.11 g) were collected from the Yangtze River of China. Two-hundred crabs were selected for the experiment, and were kept temporarily in pools (4 m × 10 m), each containing 50 crabs, for 7 d. The water for the experiment was obtained from Dianshan Lake, Shanghai, China, and kept for a week to deposit and filter the undissolved-copper as well as any other impurities. The measured background copper concentration in this water was 0.001 mg/L, as determined with a Plasma 2000 type ICP-AES (Perkin-Elmer Company, USA).

The control group was prepared with no additional copper. $CuSO_4 \cdot 5H_2O$ was used to formulate the experiment solutions with different copper content (0.01, 0.10, 1.00 and 5.00 mg/L, nominal concentration). The crabs were fed twice daily (9:00 and 18:00 h) at 5% of their body weight. Uneaten diet and excreta were removed daily at about 8:00–9:00 h when the water was exchanged. The crabs were randomly put into fifteen 80 cm × 50 cm × 50 cm polyethylene tanks (containing 50-l test solutions). Each tank contained ten crabs (five males and five females). The tanks were continuously aerated by air stones. During these experiments, the crabs were fed similarly to the previous method. The water temperatures ranged from 20.2°C to 25.5°C, dissolved oxygen (DO) was at 8.3 ± 0.4 mg/L, ammonia ranged from 0.013 to 0.039 mg/L and pH was at 7.2 ± 0.3. The crabs were divided into three treatment groups, which were comparable with the controls, respectively.

According to our pre-experiment, there was no significant difference in the microstructures of XO/SG between the female and male crabs. Therefore, after 10 days of copper exposure, five crabs randomly selected from each group were sampled. The XO/SG were dissected out and fixed immediately in Bouin's solution. The tissue of the XO/SG were cut into flat pieces approx 6 μm thick, stained with hematoxylin-eosin, and observed under an OLYMPUS BH-2 light microscope (OLYMPUS, Japan).

Small pieces of XO/SG complex (1.0 mm^3) were fixed in 2.5% glutaraldehyde (with 0.2 M PBS buffer, pH7.4) for 3 h at 4°C, and then were postfixed in 2.0% (m/m) osmium-acid followed by rinsing three times (15 min each) with 0.2 M PBS buffer. The specimens were then dehydrated in graded ethanol, permuted in acetone, and embedded in Epon812 resin (SERVA Company, USA). Seventy nm-thin sections were cut on a LKB2088 (Sweden) with a diamond knife, mounted on copper grids, double-stained with uranyl acetate and lead citrate, then photographed under a HITACHI-600 transmission electric-microscope (TEM) (HITACHI, Japan).

Results and Discussion

XO is located at the outside of the neuro-terminal in the optic nerve of the eyestalk. Its axon passes through the medulla terminalis to the back-side and crosses the medulla interna in the optic nerve. There its terminal forms a bulge SG (Fig. 1A). Hemocoels form SG central and the SG wall consists of axons and bugle neuro-terminal and glial cells. Mitochondrial structures are in the glial cells and axon-terminals (Fig. 2B, C), and the bugle terminal has plenty of neurosecretory granules (Fig. 2D). The outer wall, consisting of the glial cells and processes, covered the whole gland and separates SG from the end-marrow, medulla interna and other organs (Figs. 1B, C; 2A). The inner wall of the SG is covered by an amorphous non-cell membrane (Figs. 1C, 2E), twisted by the axon, axone-terminal and glial cells, which form a solid structure to rivet the shape of the SG. The axone terminal continues along the central hemocoels radially. Many small hemocoels in the SG wall extend from the central hemocoels to the outer wall of the SG, which made the gland emerge in the hemocoels (Fig. 1B, C) Hemocytes can be seen in the hemocoels (Figs. 1F, 2E).

Under light microscopy, no differences were found between all Cu^{2+} treated groups and the controls in XO. However, under TEM the mitochondrial cristae disappeared, or only a few vacuoles remained in the neuro-terminal of the XO of the 5.00 mg/L Cu^{2+} treated group. No differences were found in the XO between the 0.01,

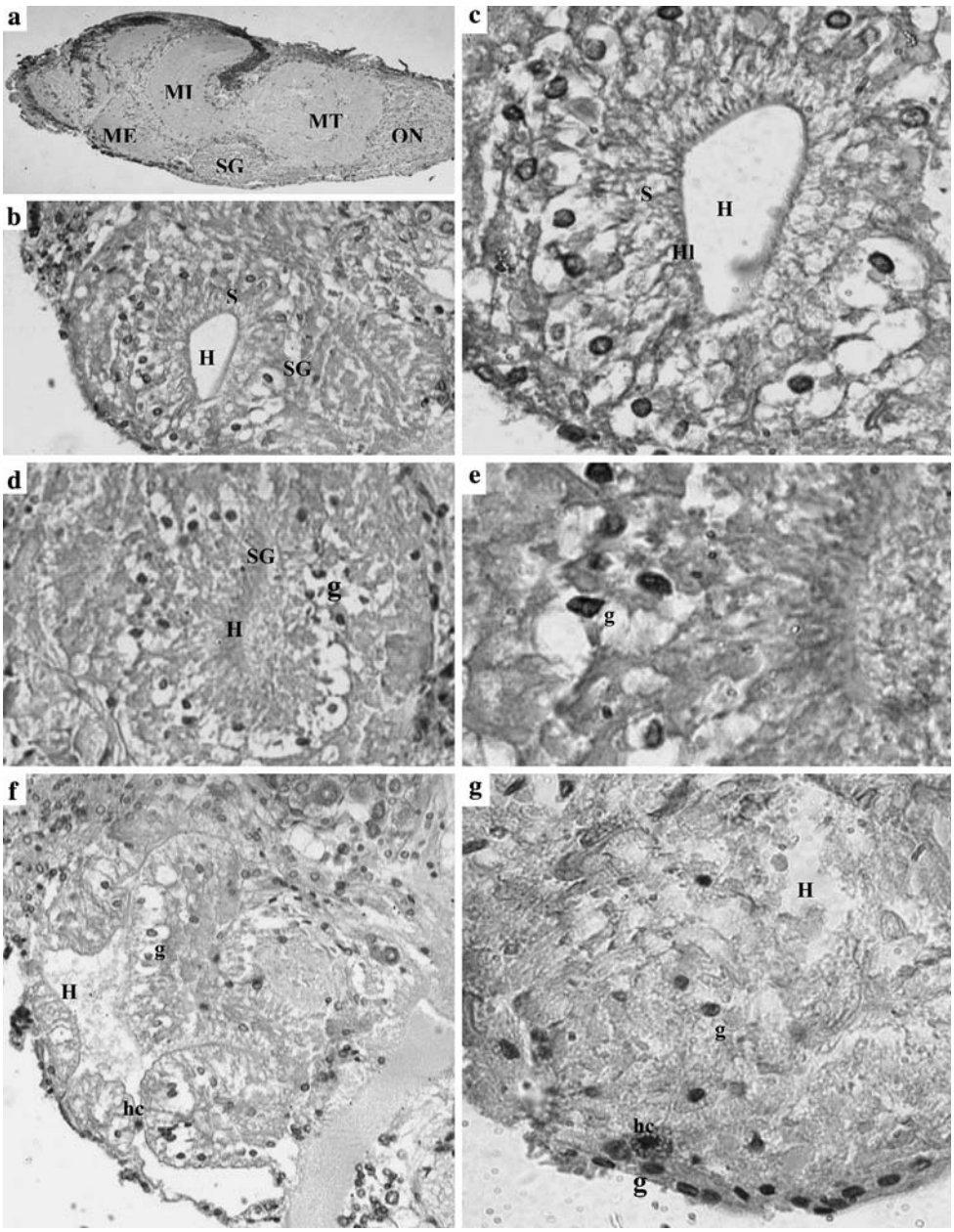

Fig. 1 The effect of Cu^{2+} on the microstructure of the X-Organ sinus gland of *Eriocheir sinensis*. (**a**) optic ganglion in control group, ×100; (**b**) sinus gland in control group, ×400; (**c**) sinus gland in control group, ×1,000; (**d**) the boundary between the SG hemocoels and glial cells became unclear (5.00 mg/L Cu^{2+}), ×400; (**e**) the SG central hemocoels hemosinus closed (5.00 mg/L Cu^{2+}), ×1,000; (**f**) the SG central hemocoels disintegrated (5.00 mg/L Cu^{2+}), ×400; (**g**) the SG central hemocoels disintegrated, the hemocytes mixed with the glial cells, and the axon terminal disordered arranged, ×1,000. AT: An enlarged terminal; g: Glial cell; H: Hemocoel; hc: Hemocyte; Hl: haemocoels lining; ME: Medulla externa; MI: Medulla interna; MT: Medulla terminatia; ON: Optic nerve; S: small haemocoels; SG: Sinus gland

0.10, and 1.00 mg/L Cu^{2+} treated groups and the controls (Figs. 2D, 3A).

Except for the 5.00 mg/L Cu^{2+} treated groups, in the 0.01, 0.10, and 1.00 mg/L Cu^{2+} no differences were found in all Cu^{2+} treated groups compared to the controls on the SG micro- and ultra-structures. In the 5.00 mg/L Cu^{2+} treated groups, the boundary between the SG hemocoels and glial cells became ambiguous. Some central hemocoels were closed (Fig. 1D, E) or even disintegrated. Hemocytes mixed with the glial cells and the axon terminal locally disordered (Fig. 1F, G). Under TEM imaging, the glial cells ruptured and the mitochondrial cristae disappeared, or remained only a vacuole (Fig. 2F). Such a change also occurred in the mitochondrion of the axon terminal (Fig. 2G). The cell membranes of some glial cells disintegrated. The cytoplasm disappeared from the cells. The nucleus could enter into the haemocoels, where it piled together with the hemocytes (Fig. 3B). The axon terminal and neurosecretory granulars decreased near the amorphism membrane of the haemolymphs (Fig. 3C).

Molting is necessary to growth among crustaceans (Du 1993). This process is controlled by ecdysone and the molting inhibition hormone (MIH) alternately, which are excreted from the endocrine gland Y-organ and XO/SG complex, respectively. MIH is generated by XO and then transported to the SG by axons.

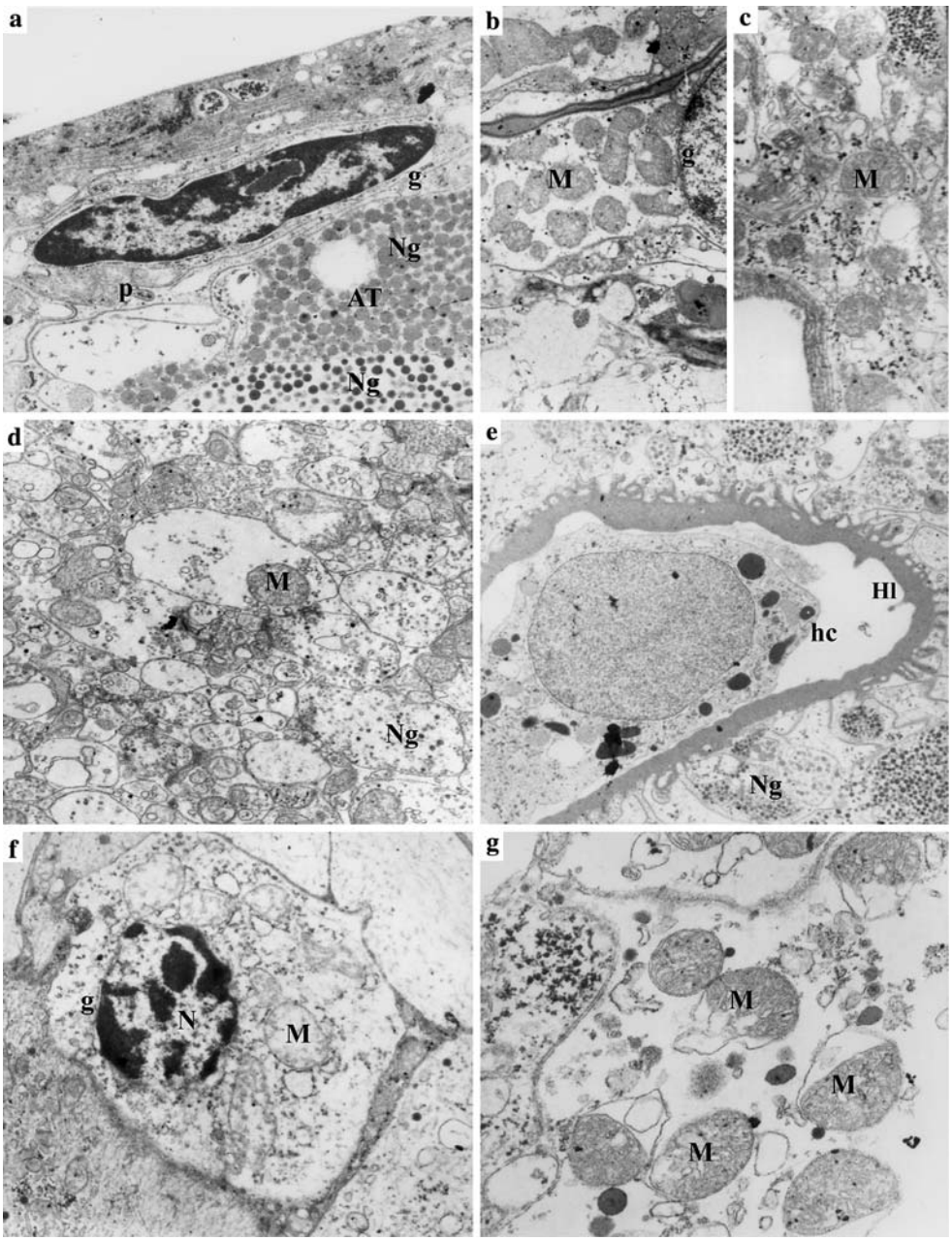

Fig. 2 The effect of Cu^{2+} on the ultrastructure of XO/SG of *Eriocheir sinensis*. (**a**) glial on the SG wall and plenty of neurosecretory granular in the control group. ×10,000. (**b**) mitochondrial in the glial cells of SG in the control group. ×10,000. (**c**) Mitochondrial in axon terminal in control group, ×10,000. (**d**) plenty of neurosecretory granular in XO axon terminal in control group, ×10,000. (**e**) hemocytes in hemocoels of SG in the control group, ×6,000. (**f**) glial cell membrane disintegrated, nucleic-membrane was damaged, and mitochondrial cristae disappeared and even left a vacuole (5.00 mg/L Cu^{2+}), ×10,000. (**g**) mitochondrial cristae disappeared from the axon terminal of SG and a lot of vacuoles were seen in the mitochondrial, ×17,000. AT: An enlarged terminal; g: Glial cell; hc: Hemocyte; Hl: haemocoels lining; M: Mitochonsria; N: Nucleus; Ng: Neurosecretory granules; P: Processes

The present study demonstrated that the mitochondria of XO glial cells and SG axons were badly damaged by the high level of waterborne Cu^{2+}. The plausible explanation is the toxicity of Cu^{2+} absorbed in the XO/SG complex exceeded the tolerable Cu^{2+} range for the hemocoels, and thus impaired the cellular structure of the membrane and function, causing the disappearance of the mitochondrial cristae (Zhang et al. 1993). Since the mitochondrial cristae play an important role in cellular energy metabolism and ion transfer, damage to the mitochondrial membrane will cause a drop in its capacity for synthesizing ATP (Bubel et al. 1976). Consequently, the cellular energy metabolism in the XO/SG complex will be weakened and the synthesizing ability of MIH will probably be blocked and even

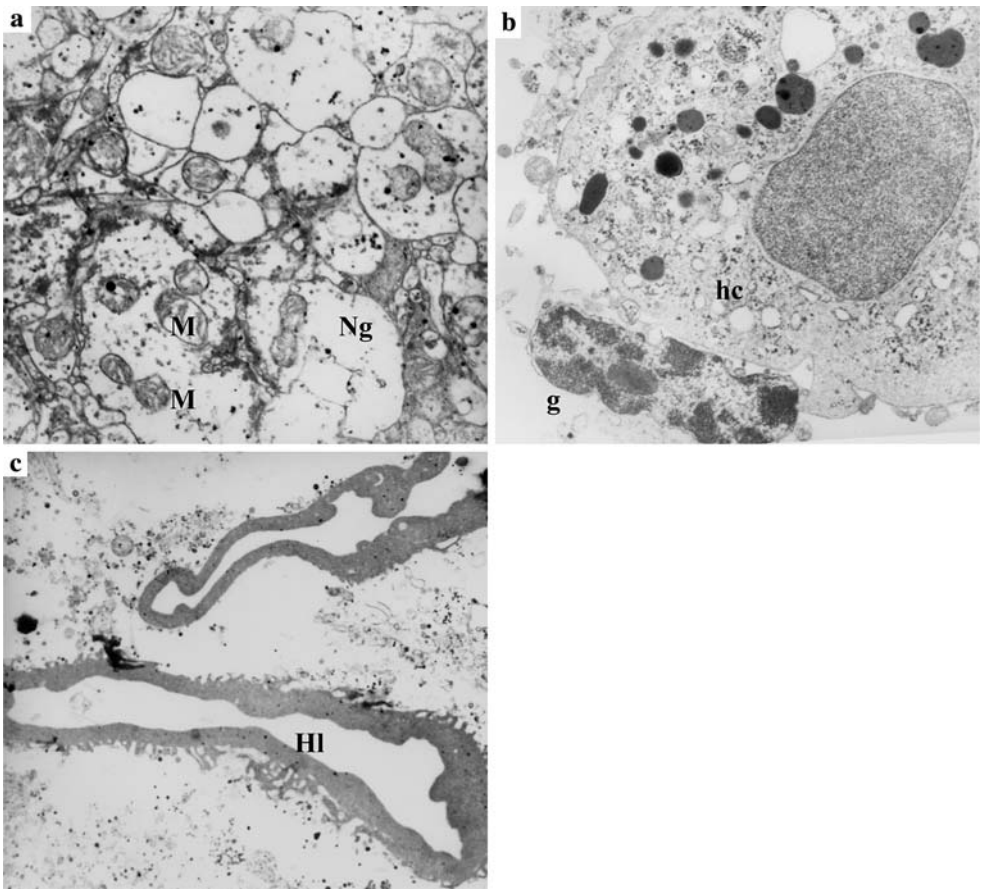

Fig. 3 The effect of Cu^{2+} on ultrastructure of XO/SG of *Eriocheir sinensis*. (**a**) the damaged mitochondrial in neuro-terminal of XO, neurosecretory granules reduced (5.00 mg/L Cu^{2+}), ×10,000. (**b**) hemocytes mixed with the glial cells in SG (5.00 mg/L Cu^{2+}), ×8,000. (**c**) neurosecretory granules and mitochondrial reduced nearby hemocoels of SG (5.00 mg/L Cu^{2+}), ×4,000. g: Glial cell; hc: Hemocyte; Hl: haemocoels lining; M: Mitochondria; Ng: Neurosecretory granules

lost. The present study suggests that a possible mechanism for this is that the hemocoels in the SG structure were destroyed by the high Cu^{2+} levels, and then, the quantity of MIH released by SG consequently declined. Namely, the major toxic effect of Cu^{2+} on the XO/SG complex seems to destroy the MIH releasing function of SG. Further study is required to confirm this hypothesis.

A reduction in MIH in the haemolymph is believed to induce molting in decapod crustaceans and stimulate the Y-organs to synthesize and secrete ecdysone, which will be converted to the active molting hormone 20-Hydroxyecdysone (20-HE) (Chang 1995; Mykles 2001). However, even exposed *E. sinensis* to the Cu^{2+} levels that do not destroy the MIH releasing function of SG, a decrease in molting rate was observed in our studies (Yang et al. 2005). Furthermore, Yang (2005) found that the YO structure of *E. sinensis* in the 5.00 mg/L Cu^{2+} treated groups also suffered considerable damage and a significantly lower level of 20-HE was recorded in the haemolymph during the interval of molting. These phenomena indicate that the decrease in the molting rate by the Cu^{2+} toxicity in *E. sinensis* seems to be mainly due to direct dysfunction of YO, e.g., by restraining the secretion of 20-HE rather than the impairment of the XO/SG complex when exposed to 5.00 mg/L Cu^{2+}. Our previous results showed that the molting rates of *E. sinensis* exposed to 0.01 mg/L Cu^{2+} were significantly decreased compared to those of the controls (Yang et al. 2005). At this level, no microstructure and ultrastructure impairment can be found in either the XO/SG complex or Y-organ. Further investigation is needed to clarify what molecular and endocrine variations of the XO/SG complex or Y-organ causes the dysregulation of ecdysis before their obvious microstructural and ultrastructural impairments.

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