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Influence of ultraviolet radiation on selected physiological responses of earthworms

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Summary

The purpose of this study was to investigate the adverse effects of ultraviolet (UV) radiation on earthworms. Earthworms that crawl out of the soil may die within a few hours after sunrise. This study shows that UV exposure can be lethal. In general, UV-B had a stronger damaging effect than UV-A. Different species of earthworms had different tolerances to UV exposure. In this study, *Pontoscolex corethrurus* showed the highest tolerance of the three tested species to UV radiation, while *Amynthas gracilis* was the most sensitive. UV radiation induced both acute and chronic responses. The acute response, which occurred immediately on or after UV exposure, was characterized by the appearance of abnormally strong muscle contractions, including S-shaped movements and jumping behavior, possibly caused by bad coordination between the circular and longitudinal muscles. The chronic response included damage to the skin and muscle cells, which resulted in a high mortality rate. Oxygen consumption by *A. gracilis* was significantly decreased after exposure to UV-A or UV-B. Since the circulation in earthworms is mediated by muscle contraction and the skin is the main organ of respiration, it is reasonable to expect that abnormal muscle contraction and a damaged epithelium could cause suffocation. Because of their sensitive responses, we propose that some earthworms, such as *A. gracilis*, could serve as a new model for studying UV-induced photodamage.

Key words: invertebrate, ultraviolet, earthworm, crawling behavior, oxygen consumption.

Introduction

Ultraviolet (UV), one of the components of solar radiation, is divided into UV-A (320–400 nm), UV-B (280–320 nm), and UV-C (200–280 nm). Normally, stratospheric ozone reflects UV-C and most of the UV-B, so only UV-A and a little of the UV-B reach the Earth (Steeger et al., 2001). Organisms on Earth are therefore evolutionarily adapted to UV-A, but might not be adapted to UV-B. Recently, due to atmospheric ozone depletion, a great deal of attention has been focused on UV-B-induced photodamage to organisms (Misra et al., 2002). A 10% decrease in the total stratospheric ozone would increase the amount of UV-B reaching the Earth's surface by 20% (Misra et al., 2002). Under normal conditions, many vertebrates and invertebrates can protect themselves from UV by skin pigments or integuments, such as feathers, hairs or scales (Charron et al., 2000). However, the increasing UV-B radiation has influenced organisms and ecosystems on Earth (Pool, 1991; Rozema et al., 2002; Sommaruga, 2001; Urbach, 1989). Effects caused by UV-B, including DNA damage (Davies, 1995), melanogenesis, skin erythema, skin cancer, immunosuppression and damage to the eyes, have been well studied (Iyengar, 1994; Noonal and Lewis, 1995; Rapp and Ghalyini, 1999; Urbach, 1989). UV-B also affects egg hatching and causes deformities in amphibian and fish embryos (Hunter et al., 1979; Savage and Danilchik, 1993; Blaustein et al., 1998; Epel, 1999; Steeger et al., 2001).

Although the damaging effect of UV-A on organisms is less than that of UV-B, the influences of UV-A also need to be considered. In the past, UV-A was ignored because its intensity reaching the earth does not increase or decrease. However, there is evidence that it can alter cell membrane components (Banerjee et al., 2005), induce DNA–protein crosslinking (Marrot et al., 2005), and increase reactive oxygen species (ROS). Thus, it is equally important to know the effect of UV-A and UV-B on organisms.

Generally, earthworms do not expose themselves to sunlight and only extend their anterior region outside the soil for feeding or for reproduction at night. Normally they return to the soil when the sun comes up. However, sometimes after heavy rain, earthworms do not crawl back into the soil when the rain stops and can be found dying in puddles (Darwin, 1881; Tsai, 1964). Merker and Brauning [cited by Edwards and Bohlen (Edwards and Bohlen, 1996)] suggested that UV radiation is an important factor causing damage to earthworms when they are on the soil surface. Up to now, only a few reports have demonstrated that

UV is harmful to earthworms (Albro et al., 1997; Misra et al., 2005). In the present study, we tried to test the above hypothesis of Merker and Brauning (see Edwards and Bohlen, 1996) and used three species of earthworms, *Amynthas gracilis*, *Metaphire posthuma* and *Pontoscolex corethrurus*, to investigate whether UV radiation is a key factor influencing earthworm crawling, mortality and respiration. *A. gracilis* usually remains on the soil surface after heavy rain (Tsai, 1964), *Metaphire posthuma* is the species that was found crawling on the soil after the 1999 '9-21 Chichi earthquake' in central Taiwan (Liaw and Lee, 2002) and, according to our long-term observations, *P. corethrurus* never exposes its entire body on the soil surface in normal situations.

Materials and methods

Earthworms

Amynthas gracilis (Kinberg 1867) (Megascolecidae) was collected from Taipei County, northern Taiwan. *Pontoscolex corethrurus* (Müller 1856) (Glosscloedidae), an exotic species, which is now widespread in Taiwan (Tsai et al., 2000), was collected from the main campus of National Taiwan University, Taipei, northern Taiwan. *Metaphire posthuma* (Vaillant 1868) (Megascolecidae) was purchased from fishing tackle stores. These earthworms were kept in a photoperiod-controlled room on a 12 h:12 h light:dark cycle. The room temperature was maintained at 22–25°C, and the humidity of the culture soil maintained at 70–80%. The earthworms were fed with green bean sprouts and rice husks. In order to exclude interference due to body mass, only mature earthworms weighing 0.8–1.0 g were used.

Exposure to UV radiation

The UV light boxes (UV crosslinker, Spectrolinker XL-1000, Spectronics Co., Westburg, NY, USA) used in this study had peak of intensities at 312 nm (UV-B) or 365 nm (UV-A). The UV dose was measured by a sensor in the UV light box. The average intensity of UV-A or UV-B in the UV light box was, respectively, 4.5±0.5 or 1.2±0.5 mW cm^{-2}. The dose was calculated as dose (J m^{-2})=10×intensity (mW cm^{-2})×time (s). The UV-B dose of 500 J m^{-2} generated by the UV-B box is equivalent to the incident UV-B dose over a period of 1 h on a cloudy day in Taiwan. According to our observations, it takes several hours for an earthworm crawling on the soil surface after heavy rain to die, so the dose of UV-B was set to 500–1500 J m^{-2}. Before exposing the earthworms to UV, they were allowed to settle in the plastic dish for 15 min. They were then placed in the UV light box and exposed to different doses. To exclude the effect of heat produced by UV radiation, ice was placed in the UV light box to maintain the temperature at 25°C.

Abnormal behaviors of earthworms exposed to UV-A or UV-B

On exposure to UV-A or UV-B, two unusual behaviors, S-shaped movement and jumping behavior, were recorded using a digital camera (JVC, GR-DVM96U, Yokohama, Japan), and their frequencies counted.

Influence of UV-A or UV-B exposure on crawling activity

Soil (400 g) with a humidity of 70% and pH 7.0 (soil moisture and pH meter; Soil Tester, Takemura Electric Works, Tokyo, Japan) was packed into a circular container of radius 10.5 cm and height 5 cm. The final soil density was approximately 0.72 g cm^{-3}. Crawling time was counted from when the earthworm first contacted the soil surface to when it was again completely embedded in the soil. The relative increase in crawling time was defined as the crawling time after UV exposure/crawling time before UV exposure ratio, a higher ratio indicating a greater effect on crawling time.

Oxygen consumption

Oxygen consumption was measured using oxygen equipment (YSI5300, YSI5301) as described previously (Chuang et al., 2004). Briefly, an earthworm was placed in a water chamber containing 12 ml of air-saturated water (ASW), the test period was 30 min, and data were recorded every 5 min. A chamber with no earthworm was used as the negative control and the oxygen content was found to decrease by 5–7% in 2 h.

ASW at normal atmospheric pressure and a given temperature contains X mg O_2 ml^{-1}, which was recorded using a digital oxygen meter (Lutron, Do 5510, Taipei, Taiwan), so Y ml contains XY mg O_2. When an earthworm weighing G g causes the oxygen concentration of the water to drop by $Z\%$ (percentage in the chamber with a earthworm minus that with no earthworm) in t min, the rate of oxygen consumption is $(XYZ\%)t/60$ mg h^{-1} and the oxygen consumption rate of a unit weight (g) of earthworm (mg h^{-1} g^{-1}) is $[(XYZ\%)t/60]/G$.

Because earthworm oxygen consumption has a rhythm (Chuang et al., 2004), we tested the effect of UV on oxygen consumption at the same time of day (13:00–16:00 h). Because the soil temperature in northern Taiwan is around 25°C the experimental temperature was maintained at 25°C.

Mortality of earthworms exposed to UV-A or UV-B

After exposure to UV-A or UV-B, the earthworms were gently transferred to a 90 mm plastic dish containing moist no. 1 filter paper at 25°C. When the prostomium showed no response to probe contact, the earthworm was recorded as having died and the time of death recorded.

Tissue damage to earthworms after UV-A or UV-B exposure

After irradiation with 1500 J m^{-2} UV-A or UV-B for different time periods, earthworms were anesthetized with 10% ethanol, and then small tissue blocks were dissected from the body wall, fixed in Bounid's fixative, dehydrated by an ethanol series, and embedded in paraffin. The tissue samples were then sliced into 3 μm sections, which were stained with Mayer's Hematoxylin/Eosin, observed under light microscopy (Leica), and recorded using a digital camera (Nikon, Coolpix 990).

Statistical analysis

Each experiment was repeated at least 5 times. Differences in jump behavior were tested using the χ^2 distribution. The significance of differences between the control and treatment groups was examined using ANOVA or two-way ANOVA. Differences in the means for the different treatment groups were tested using Duncan's new multiple-range test. A level of significance of $P<0.05$ or 0.01 was accepted as significant or highly significant, respectively.

Results

Abnormal behaviors of earthworms exposed to UV-A or UV-B

When the worms were exposed to UV-A or UV-B, only *A. gracilis* showed the abnormal behaviors of S-shaped movement and jumping. As the dose of UV-A or UV-B was increased, the frequency of S-shaped movements increased (Fig. 1). In addition, individuals exposed to UV-A or UV-B exhibited jumping behavior (Fig. 2). As the dosages increased, the cumulative percentage of individuals jumping was significantly increased (χ^2 test, $P<0.001$).

Influence of UV-A or UV-B exposure on crawling time

As shown in Fig. 3, UV-A caused a significant relative increase in crawling time of *A. gracilis* and *P. corethrurus* ($P<0.01$), but not of *M. posthuma*, and the effect was dose-dependent. Muscle contractions of the head and tail in *A. gracilis* were extraordinarily uncoordinated. As shown in Fig. 4, after exposure to 1000 and 1500 J m^{-2} of UV-B, the crawling time of *M. posthuma* and *P. corethrurus* was significantly increased ($P<0.01$). The crawling time of *A. gracilis* showed no significant increase after UV-B exposure (Fig. 4), but distinct abnormal muscle contractions appeared, which were not shown by *M. posthuma* or *P. corethrurus*.

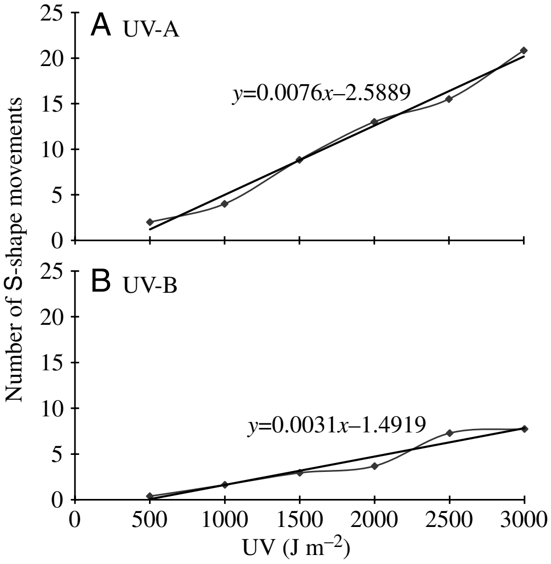

Fig. 1. S-shaped movements of *Amynthas gracilis* exposed to UV-A or UV-B. (A) UV-A exposure. As the dose of UV-A increases, the frequency of the S-shaped movements increases. (B) With UV-B exposure, the same phenomenon is seen.

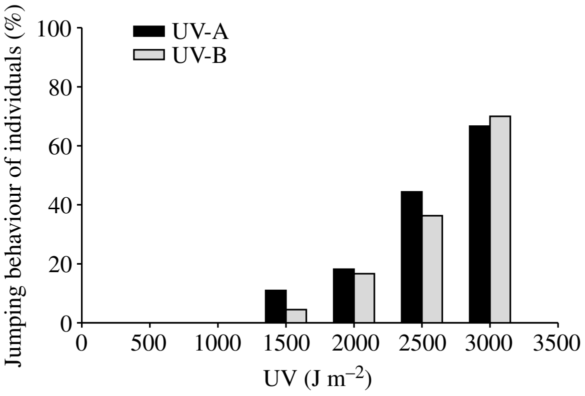

Fig. 2. Jumping behavior of *Amynthas gracilis* exposed to UV-A or UV-B expressed as the percentage of individuals showing this behavior. When the earthworms were exposed to less than 1000 J m^{-2} of either UV-A or UV-B, no individuals jumped. But after exposure to higher doses, abnormal jumping behavior appeared. As the dosages increased, the cumulative percentage of jump individuals was significant increased (χ^2 test, $N=30$, $P<0.001$).

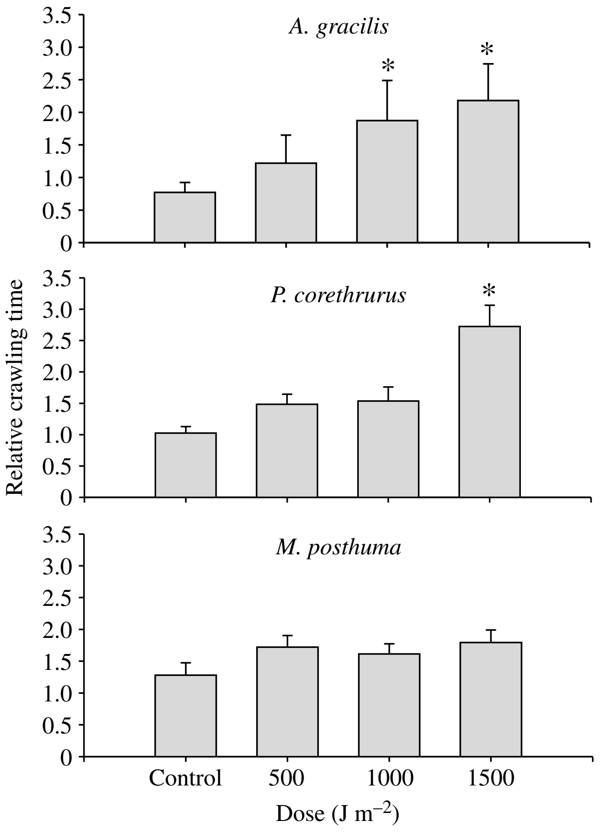

Fig. 3. Relative crawling time of earthworms after exposure to either UV-A. The crawling time of *P. corethrurus* and *A. gracilis* exposed to 1500 J m^{-2} of UV-A was significantly increased compared to controls (two-way ANOVA, $N=6$, $*P<0.01$), but that of *M. posthuma* shows no significant difference.

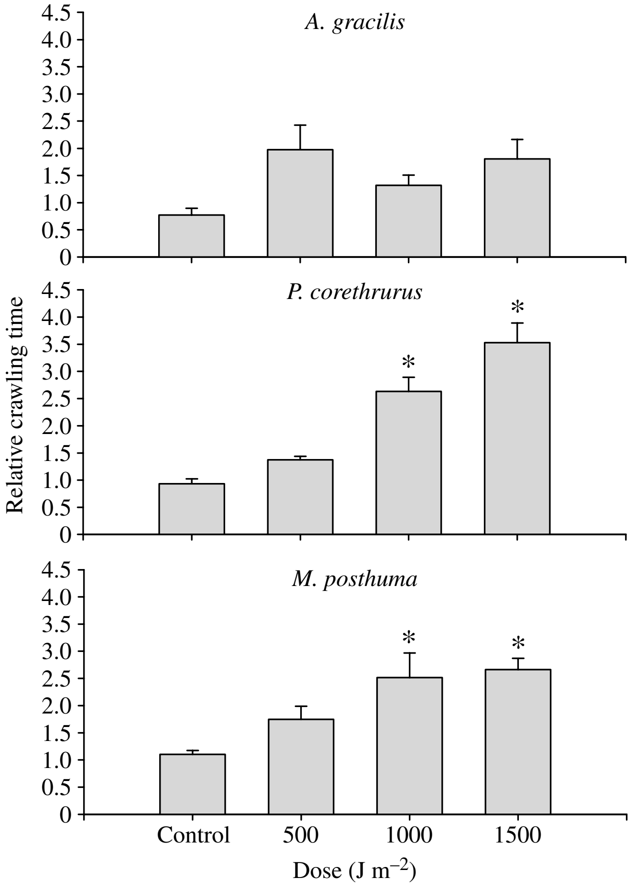

Fig. 4. Relative crawling time of earthworms after exposure to either UV-B. After UV-B exposure, *P. corethrurus* and *M. posthuma* show a significantly increased crawling time compared to controls (two-way ANOVA, *N*=6, **P*<0.01), but *A. gracilis* does not.

Respiration of earthworms after exposed to UV-A or UV-B

As shown in Fig. 5A, under normal conditions, oxygen consumption of *A. gracilis* was higher than that of *M. posthuma* ($P<0.01$). After exposure to 1500 J m^{-2} of UV-A, *A. gracilis* oxygen consumption was significantly decreased ($P<0.01$), whereas that of *M. posthuma* was unaffected. Similar results were obtained after UV-B exposure (Fig. 5B).

Mortality of earthworms after exposed to UV-A or UV-B

As shown in Fig. 6A, when *M. posthuma* was exposed to 1000 J m^{-2} of UV-B, the mortality was about 60% in 48 h, and all tested individuals died within 120 h, by which time their skin had turned black. All earthworms died within 72 h after 1500 J m^{-2} UV-B exposure. This means that the UV-B dosage exposure and the mortality of *M. posthuma* showed a positive correlation ($P<0.05$). As shown in Fig. 6B, significant mortality of *A. gracilis* was seen in 12 h of exposure to 1500 J m^{-2} of UV-B ($P<0.01$), and all earthworms died within 48 h in this study. Surprisingly, no *P. corethrurus* died after UV-B exposure. Strikingly, none of the three species died after UV-A exposure (data not shown).

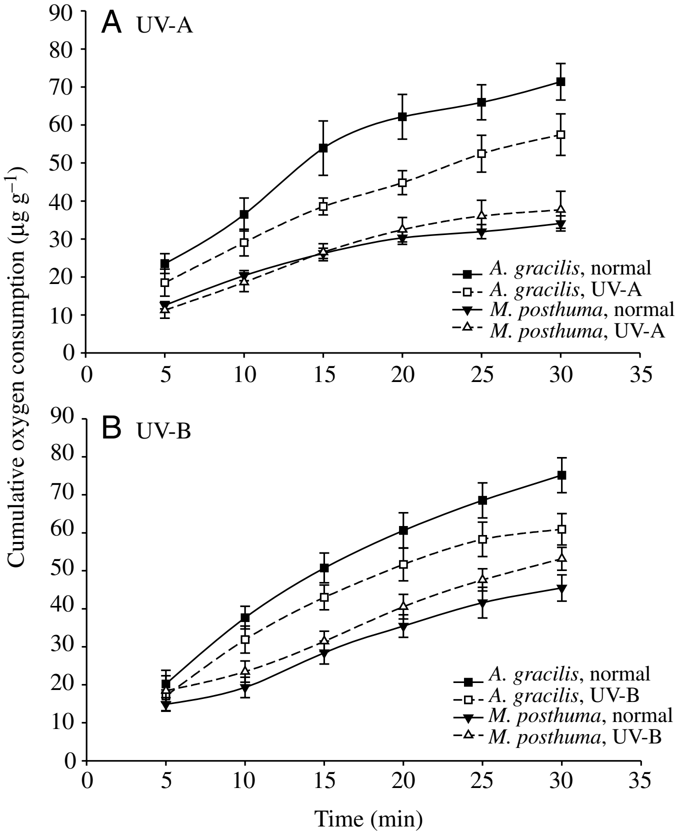

Fig. 5. Oxygen consumption of *Amynthas gracilis* and *Metaphire posthuma* after UV exposure. (A) After UV-A exposure (1500 J m^{-2}), the oxygen consumption of *A. gracilis* is lower than that of controls (NEST design, *N*=6, *P*<0.01), but that of *M. posthuma* is not affected. (B) After UV-B exposure (1500 J m^{-2}), the oxygen consumption of *A. gracilis* is lower than in controls (NEST design, *N*=6, *P*<0.01), but that of *M. posthuma* is not affected.

Tissue damage after exposure to UV-A or UV-B

The skin of earthworms exposed to UV-A did not show any obvious differences (data not shown). However, clear damage was seen on exposure to UV-B. As shown in Fig. 6, when exposed to 1500 J m^{-2} of UV-B, the cuticle of *A. gracilis* swelled, and its epidermis immediately showed necrosis (Fig. 7A,B). After 2 h of exposure, the cuticle had begun to break down and the epidermis and circular muscles were seriously necrotic (Fig. 7C), while, after 18 h of exposure, the epidermis and circular muscles were completely destroyed and only the longitudinal muscles remained (Fig. 7D).

As shown in Fig. 7, in *M. posthuma*, no obvious damage to the cuticle or epidermis was seen immediately after exposure to 1500 J m^{-2} of UV-B (Fig. 8A,B), but, after 36 h of exposure, the cuticle and epidermis were pleated and a few epidermal cells showed necrosis (Fig. 8C). After 48 h of UV-B exposure, serious damage was seen, with the epidermis being destroyed and some of the circular muscles deformed (Fig. 8D).

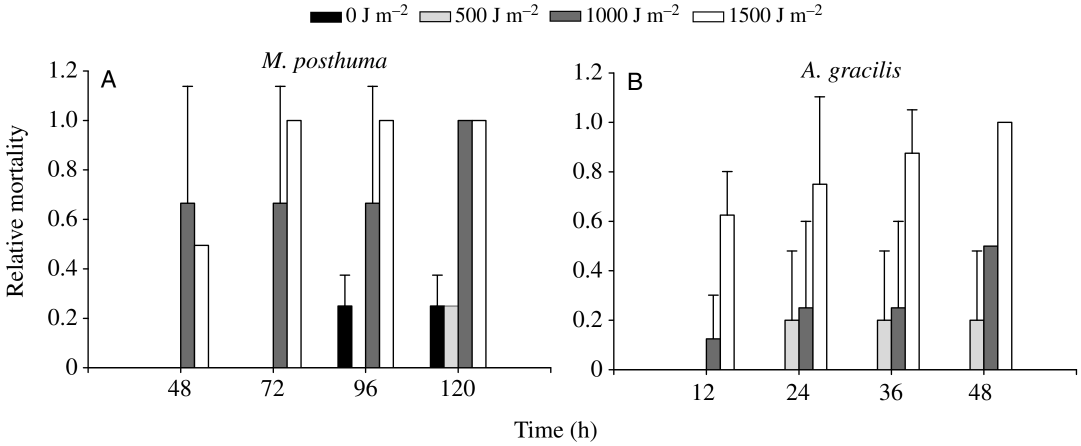

Fig. 6. Mortality of earthworms after UV-B exposure. (A) *M. posthuma* shows significant mortality after exposure to UV-B (1000 or 1500 J m^{-2}; two-way ANOVA, *N*=20, *P*<0.01), the effect being dose-dependent. (B) *A. gracilis* only shows significant mortality after exposure to UV-B (1500 J m^{-2}; two-way ANOVA, *N*=20, *P*<0.01). In addition, the survival time of *A. gracilis* was shorter than that of *M. posthuma* after UV-B exposure (ANOVA, *P*<0.01).

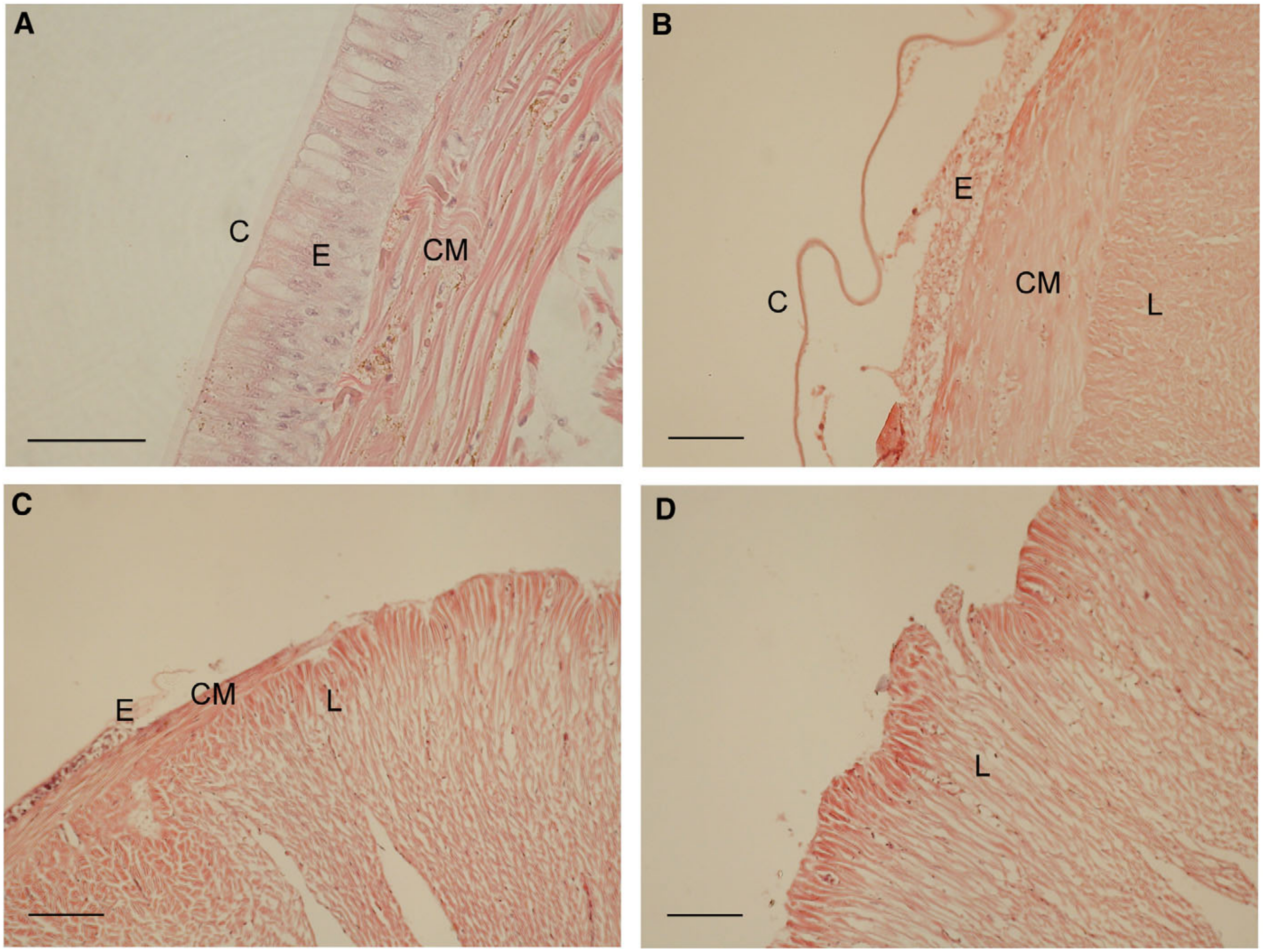

Fig. 7. Tissue structure of *A. gracilis* before and after exposure to 1500 J m^{-2} of UV-B. (A) Cross-section of *A. gracilis* without UV-B exposure, clearly showing the cuticle (C) and epidermis (E). (B) Section of *A. gracilis* immediately after exposure to UV-B. The cuticle swelled and the epidermis necrosed. (C) After 2 h exposure, the cuticle shows breakdown, and the epidermis, circular muscle (CM) and longitudinal muscle (L) show necrosis. (D) After 18 h exposure, the epidermis and circular muscle are destroyed and only the longitudinal muscle is present. Scale bar, 50 µm.

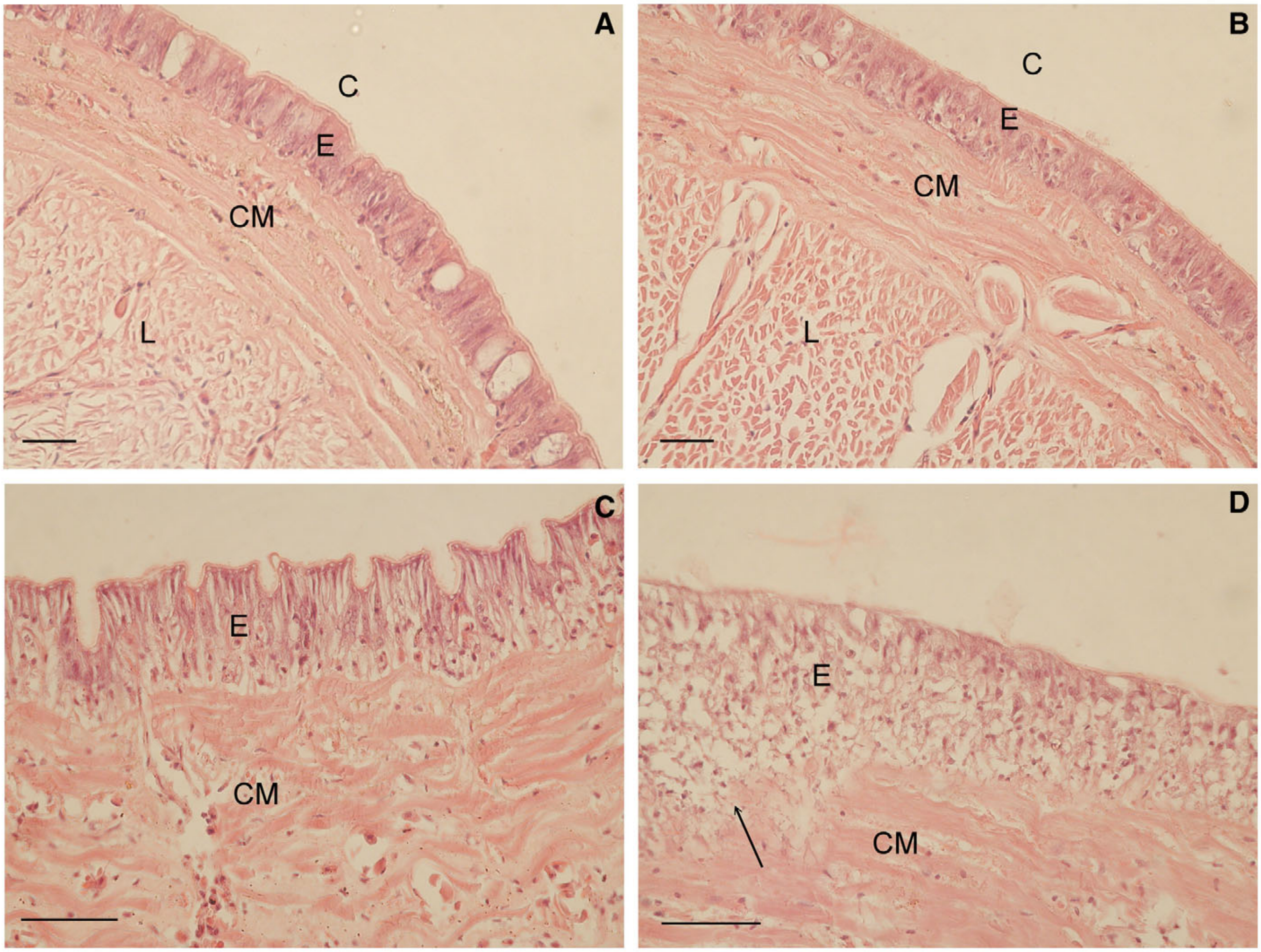

Fig. 8. Tissue structure of *M. posthuma* before and after exposure to 1500 J m^{-2} of UV-B. (A) Cross-section of *M. posthuma* without UV-B exposure, showing the cuticle (C) and epidermis (E). (B) Right after UV-B exposure, no obvious damage was seen in the cuticle or epidermis. (C) After 36 h exposure to UV-B, the cuticle and epidermis are pleated, and some epidermal cells show necrosis. (D) After 48 h exposure, the epidermis is destroyed and some circular muscle (CM) is deformed (arrow). L, longitudinal muscle. Scale bar, 50 μm.

Discussion

UV radiation has deleterious effects on many organisms (Holm-Hanseen et al., 1993; Blaustein et al., 1997; Soni and Joshi, 1997; van de Mortel et al., 1998). In this study, we demonstrate that it also caused damage to earthworms. Although *A. gracilis* did not die when exposed to UV-A, all tested individuals died when exposed to 1000–1500 J m^{-2} of UV-B, showing that the lethal effects of UV-B are stronger than those of UV-A. Because the energy in the same total photon m^{-2} of UV-A is less than that of UV-B, the deleterious effects were only slight in UV-A. *Metaphire posthuma* also died when exposed to 1000–1500 J m^{-2} of UV-B, but their survival times were, on average, longer than those of *A. gracilis*. This suggests that the tolerance of *A. gracilis* to UV-B is lower than that of *M. posthuma*. The third species of earthworm, *P. corethrurus*, survived well even when exposed to doses of UV-B as high as 3000 J m^{-2}. Thus, *P. corethrurus* presented the highest tolerance to UV-B among these three species. We suspect that it might have an unknown mechanism for protecting itself against UV-B exposure. Some chemicals or enzymes, such as mycosporine-like amino acid, flavonol quercetin or photolyase, are known to protect organisms against UV (Blaustein et al., 1994; Banaszak and Trench, 1995a; Banaszak and Trench, 1995b; Carefoot et al., 1998; van de Mortel et al., 1998; Inal and Kahraman, 2000), but which mechanism is activated in *P. corethrurus* needs further study.

Under normal conditions, earthworm peristalsis is performed by reciprocal contraction of the circular and longitudinal muscles. If muscle contraction is not coordinated, crawling activity is affected and the worm exhibits ‘fictive locomotion’ (Mizutani et al., 2002). When exposed to UV, *A. gracilis* showed an S-shape movement, which is an extraordinary behavior in earthworms in this study. The behavior is similar to the ‘fictive locomotion’, therefore, we infer that the actions of their circular and longitudinal muscles were not well coordinated. Another abnormal behavior, jumping, was seen when *A. gracilis* was exposed to UV energies of >1500 J m^{-2}. The behaviors are likely to reflect chemical irritation that in a higher animal might be considered 'pain'.

Until now, behavioral effects caused by UV exposure have received little attention. This study reveals striking changes in behavior after UV exposure. It can be inferred that these abnormal behaviors of the earthworm are mediated by the nervous system (Howell, 1939; Mulloney, 1970; Vining and Drewes, 1985; Hassoni et al., 1985). We therefore believe that these earthworms, especially *A. gracilis*, might be a new model animal suitable for studying the related neurophysiology, such as neuromuscular junction regulation and neurotransmitter release under UV exposure.

As regards earthworm respiration, oxygen is dissolved in the mucus on the epidermis, diffuses through the capillaries, and is transported throughout the body. Blood flow in earthworm vessels occurs through pumping by muscle contraction (Edwards and Bohlen, 1996). When muscle contractions are interrupted, the blood cannot be transported, causing anoxia, anaerobic respiration, and decreased oxygen consumption. Our results demonstrated that the oxygen consumption of *A. gracilis* significantly decreased after UV-B exposure. It is therefore reasonable to infer that the UV-induced abnormal crawling behavior and muscle contraction affected the respiration of the earthworms. In addition, when an earthworm's skin is damaged by UV, oxygen diffusion from the epithelia to the capillaries is reduced. With a low oxygen supply, it may be difficult for the muscles to contract, and crawling activity would decrease. Such a vicious cycle between behavioral activity and respiration will finally kill the UV-exposed *A. gracilis*. A similar phenomenon has been reported in tadpoles of *Bufo bufo* (Formicki et al., 2003). In addition, the oxygen consumption of the fish, *Cichlasoma nigrofasciatum*, is reduced when its gills are damaged by UV exposure (Winckler and Fidhiany, 1996).

The intensity of UV-B used in this study was 9 times higher than that used by Misra et al. (Misra et al., 2005), but the total UV-B dosage to which their earthworms were exposed was between 14 400–43 200 J m^{-2}, which is far higher than the 1500 J m^{-2} dosage used in this study. However, the pathological effect on the *M. posthuma* skin exposed to the two different energies of UV-B was similar. Several reports have demonstrated that UV induces production of reactive oxygen species (ROS) and photo-oxidation (Misra et al., 2005; Girotti, 2001; Kulms and Schwarz, 2002; Picardo, 2003; Rijnkels et al., 2003). *Lumbricus terrestris* secretes a photoreactive sterol and produces $^1O_2$ during UV-A irradiation (Albro et al., 1997). Some reports have shown that UV induces cell apoptosis and other effects (Morita and Krutmann, 2000; Norbury and Hickson, 2001; Denning et al., 2002; Zhou and Steller, 2003). We have therefore started to study whether skin damage in UV-exposed earthworms is related to ROS generation.

In the present study, we have demonstrated that UV can threaten an earthworm's existence by two different mechanisms. Firstly, an acute response immediately evoked abnormal moving behaviors and slowed the crawling activity of UV-exposed earthworms. Photoreceptors have been found in earthworms (Mulloney, 1970; Myhrberg, 1979), so we suspect that these quick responses might be mediated by a novel type of UV-sensitive photoreceptor in earthworms. Secondly, chronic responses, including skin necrosis and decreased respiration, caused the UV-exposed earthworms to die within a couple of days.

In most UV-damage studies, researchers have used cells to study the damaging effects of UV (Ichihashi et al., 2003), but little is known about its effects on animal behavior or physiology. In recent times, the generally accepted animal model for UV studies has been the hairless mouse (Van Weelden and Van der Leub, 1985), but these animals are expensive to purchase and handle, and the relevant effects occur very slowly (Lee et al., 2000). Normally, terrestrial animals have higher tolerance to UV-B than aquatic animals (Gies et al., 1995), as many protect themselves from UV-B by pigments or integuments, such as feathers, hair, a shell or scales. Earthworms have no such protective structures. Although earthworms live in the soil, UV radiation is a serious menace when they crawl out of the soil. Accordingly, the doses of UV to which the earthworms were exposed in this study were lower than those used in studies on other animals, such as the mouse, fish or frog (Blaustein et al., 1997; Steegar et al., 2001; Dissemond et al., 2003), but the effects were still obvious. *A. gracilis* was shown to be the most sensitive of the three species used in this study, while *P. corethrurus* showed no marked response to UV. Hairless mice exposed to 3000 J m^{-2} UV-B show skin erythema, but do not die (Lee et al., 2000), while *Tubefix* dies when the UV-B energy is 5 times greater (Soni and Joshi, 1997) than that which kills *A. gracilis*. *A. gracilis* thus has a higher sensitivity than these organisms. In addition, earthworms are cheaper than other model animals. We therefore suggest that earthworms, especially *A. gracilis*, could be a new animal model for studying UV-induced damage, such as erythema, free radical formation and lipid peroxidation (Taira et al., 1992; Gilchrest et al., 1996; Johar et al., 2003), while *P. corethrurus* could be used to study protection from UV.

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