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Comparison of the relative tolerance of *Colpoda* resting cysts and vegetative cells to electrostatic exposure

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ABSTRACT

The soil protist ciliate *Colpoda cucullus* forms resting cysts that have extreme tolerance to many environmental stresses. In this study, we show that *Colpoda* resting cysts, unlike vegetative cells, can survive electrostatic exposure. The viability of vegetative cells fell with the number of electrostatic exposures; cyst viability did not show this response. The morphology of exposed cysts appeared to be essentially normal at the optical microscope level; excysted cells from exposed cysts retained a similar proliferative ability to those of non-exposed cysts, whereas most vegetative cells were fatally damaged by electrostatic exposure.

Keywords: cryptobiosis; ciliate; Wimshurst machine; environmental stress; survival strategy

INTRODUCTION

The resting cysts of the soil ciliate *Colpoda* show extreme tolerance to adverse environmental conditions, enabling them to survive in unstable environments (Verni and Rosati, 2011). Resting cysts are a typical feature of cryptobiosis (Gutiérez et al., 1990); in *Colpoda*, these cysts are able to tolerate desiccation (Müller et al., 2010; Corliss and Esser 1974), high and low temperatures (Taylor and Stickland 1936), freezing (Uspenskaya and Lozina-Lozinsky 1979; Matsuoka et al., 2020), acidic and alkaline media (Sogame et al., 2011; Nakamura et al., 2020), ultraviolet radiation (Matsuoka et al., 2017; Yamane et al., 2020), and even gamma radiation (Saito et al., 2020). The tolerances of *Colpoda* cysts to a wide range of stresses have been reported; however, their responses to electrostatic exposures sufficiently high to fatally damage cells have not yet been elucidated.

Although the effects of electrostatic exposure of *Colpoda* cysts are unknown, the responses of cells from other organisms to electrostatic stimulation have been well documented. For example, electroporation, which is mediated by electrostatic exposure and

is a membrane phenomenon, has many applications in biology, biotechnology, and medicine (Weaver and Chizmadzhev, 1996). In addition, the response to electrical stimulus, i.e., galvanotaxis, has been well studied in many types of cells (Robinson, 1985; Ogawa et al., 2006). Furthermore, the responses to electrical stimulus have been extensively investigated in the protist *Paramecium* (Naito and Eckert, 1968; Eckert and Naito, 1970; Naito et al., 1972; Naito and Sugino, 1984; Machemer and de Peyer, 1977; Ogawa et al., 2006).

Here, we report the novel finding that *Colpoda* resting cysts can tolerate exposure to electrostatic shock, and discuss the biological significance of the tolerance.

MATERIALS AND METHODS

Cells, culture, and induction of encystment and excystment

Colpoda cucullus R2TTYS strain was cultured in 0.05% (w/v) rice leaf infusion medium supplemented with 0.05% (w/v) NaH_2PO_4 at 25°C. Encystment was induced by suspending cells at high density (>10,000 cells/mL) in an encystment-inducing medium [1 mM Tris-HCl (pH 7.2), 0.1 mM $CaCl_2$; En-medium]. Excystment was induced by replacing the En-medium with an excystment-inducing medium [0.2% (w/v) rice leaf infusion medium supplemented with 0.05% (w/v) NaH_2PO_4; Ex-medium].

Sample preparation

Vegetative cells were washed twice with En-medium by centrifugation (1,500 × *g*, 1 min), and suspended at 10,000 cells/mL. For the preparation of cyst samples, vegetative cells were washed twice with En-medium by centrifugation (1,500 × *g*, 1 min), suspended at >10,000 cells/mL, and incubated for more than 1 week on a sample rotator to make floating cyst samples.

Electrostatic shock

The electrostatic shock was supplied to vegetative cells and cysts using a handmade power generator (Wimshurst machine) with a 90 mm diameter electrode (Fig. 1). This generator can charge static electricity in Leyden jars with a capacitance of 3 pF measured at 1 kHz by an LCR meter ZM2353 with 2325A test leads (NF Corporation, Kanagawa, Japan). The dielectric breakdown voltage is 85 kV (theoretical value assuming that the diameter of both sphere electrodes is 10 mm).

Vegetative cell and cyst samples were supplemented with 0.3% NaCl (w/v final conc.) as an electrolyte and 70 μL of each sample were transferred into an electroporation cuvette [Gene Pulser/MicroPulser Electroporation Cuvettes 2 mm gap #1652086 (Bio-Rad Laboratories, Inc., CA., USA.)] just before exposure to the electrostatic shock. Charged Leyden jars were discharged 10, 30, or 50 times with a 30 mm distance between electrodes. The Leyden jars were recharged if needed. For control, non-electrostatic shocked samples, the cell suspensions were prepared as above, transferred into the cuvettes, and then incubated for the duration of the experiment.

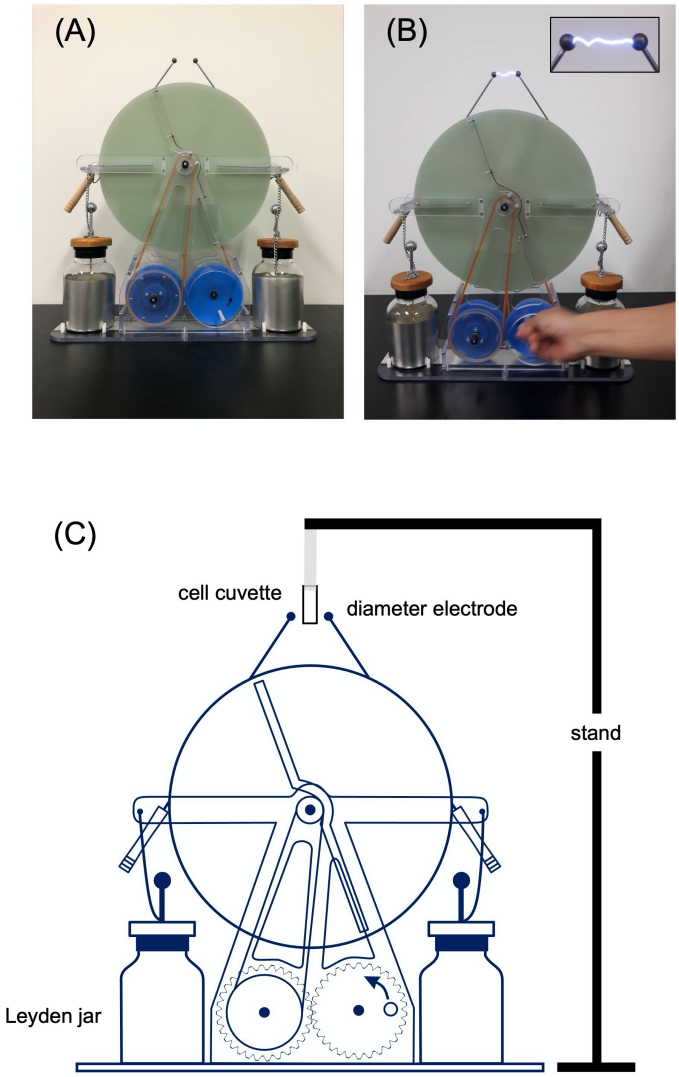

Fig. 1. Images of the handmade Wimshurst machine. A. The completed machine. B. An electrical discharge by the machine. C. A schematic illustration of the experimental set-up.

Bioassay and microscopic observation

In the bioassay of vegetative cells, 10 μL samples (control and electrostatic exposed) were transferred from cuvettes into 100 μL of fresh Ex-medium, and the numbers of live (moving) cells were directly counted using an optical microscope (Carl Zeiss Stemi 305). The survival rate of vegetative cells was calculated as follows:

survival rate of vegetative cells (%) = [living cell number / 100 (theoretical number of cells transferred)] ×100.

In the bioassay of cyst samples, a 10 μL sample from each cuvette was transferred to 100 μL of fresh Ex-medium; theoretically, each transfer should have 100 cells. Prior to suspension, the actual cell number in each sample was counted under a microscope and, if necessary, was adjusted to about 100 cells. Excystment was induced for 6 h, and the number

of excysted vegetative cells was counted under the optical microscope. The survival rate of cysts was calculated as follows:

survival rate of cysts (%) = [number of excysted vegetative cells / the number of suspended cysts] × 100.

All values are shown as the mean of six identical samples. Statistical analyses were performed by the Mann-Whitney U test using the Bell Curve for Excel software (Social Survey Research Information Co., Ltd., Japan).

For the cell proliferation assay, cyst samples that had been electrostatically exposed 30 times and control, non-exposed cysts were induced to excyst in Ex-medium. After 6 h incubation, excysted cells were resuspended at 500 cells/mL in fresh Ex-medium; cell numbers were counted under a microscope in 20 μL samples at 0 h, 24 h, and 48 h after resuspension.

The morphologies of non-exposed cells and of cells that had been exposed to 30 electrostatic shocks were compared under an Axiovert A1 microscope system (Carl Zeiss Co. Ltd., Tokyo, Japan). We also compared actin filament (F-actin) distribution in non-exposed and exposed cells. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1h, washed twice with PBS, treated with 0.1% Triton x-100 in PBS for 1h and then with 1% Nonidet NP-40 (NP-40) in PBS for 1 h. Double fluorescence staining of the cells was performed using Alexa Fluor 594 Phalloidin (Thermo Fisher Scientific K.K., Tokyo, Japan) and 4',6-diamidino-2-phenylindole (DAPI, DOJINDO LABORATORIES Co., Ltd., Kumamoto, Japan). The cells were stained twice with Alexa Fluor 594 Phalloidin for 1h following the manufacturer's instructions. They were then stained using 1 μg/mL (final concentration) DAPI in 0.001% (final concentration) DMSO for 15 min. The stained cells were washed twice with PBS and then analyzed using an Axio scope Al microscope system with a 555 nm LED laser for Phalloidin and 385 nm LED laser for DAPI.

RESULTS AND DISCUSSION

The relative survival rates of cysts were not affected by the number of electric exposures to electrostatic shock; in contrast, that of vegetative cells was reduced (Fig. 2A). The relative survival rates of cysts in the control (no exposure), 10, 30, and 50 electrostatic shock groups were 97.2%, 120.6%, 104.5%, and 103.8%, respectively. Thus, survival tended to be elevated by exposure (Fig. 2A, 'Cyst'). The survival rate of cysts was significantly elevated after 10 exposures but was not significantly greater than the control cells after 30 or 50 exposures (Fig. 2B). By contrast, the relative survival rates of unexposed vegetative cells and those exposed to 10, 30, or 50 electrostatic shocks were 96.9%, 56.5%, 3.8%, and 0.4%, respectively. Thus, survival was reduced with the increased number of electrostatic shocks (Fig. 2A, 'Vegetative cell'). The survival rates of vegetative cells were also significantly reduced by 10, 30, or 50 electrostatic exposures (Fig. 2C).

When vegetative cells were exposed to 30 electrostatic shocks, only a few cells survived (i.e., showed movement); however, almost all the cells were fatally damaged and either did not maintain their cell shape or disappeared (Fig. 3, compare A-1 and A-3, and B). The damage to these cells is a consequence of exposure to an electric field, i.e., the electrical

potential reached a sufficient magnitude around the cell to irreversibly damage cell membranes and cause cell death as described by Sale and Hamilton, (1967; 1968) and Hamilton and Sale (1967). Mechanical breakdown of the membrane is irreversibly induced by local electro-mechanical compression, i.e., it becomes locally unstable, which leads to the formation of pores on the cell membrane (Zimmermann et al., 1981). The cell membrane therefore loses its function as a semipermeable membrane (Zimmermann et al., 1981), causing leakage of cell contents and lysis of protoplasm (Hamilton and Sale, 1967). Electrical exposure of *Colpoda* vegetative cells causes similar damage to cell membranes, i.e., physical disruption of the cell membrane leading to loss of semi-permeability, leakage of cell contents, lysis of protoplasm, and cell death. In addition, electrically-exposed *Colpoda* vegetative cells change shape and become round. It is possible that this change occurs due to alterations in actin dynamics influenced by electrical activity. Phalloidin staining of exposed cells showed some indication of partial depolymerization of F-actin by the electrostatic exposure in vegetative cells (Fig. 4). Similar subcellular cytoskeletal changes have been reported to be induced by electrostimulation in HeLa cells (Yaoita et al., 1989).

By contrast, resting cysts were intact after electrostatic exposure (Fig. 3, compare A-2 and A-4). Although electrostatic exposure altered cell membranes in vegetative cells and caused fatal damage, the multilayered cyst wall may be able to maintain its shape even if the cell membrane is damaged.

The proliferative ability of excysted cells from cysts exposed to 30 electrostatic shocks was the same as that of non-exposed cysts: excysted cells from both non-exposed (control) and exposed cysts proliferated more than two-fold in 48 h (Fig. 5). Hence, resting cysts tolerate electrostatic exposure and can proliferate after excystment.

Here, we showed that *Colpoda* resting cysts were more tolerant to electrostatic exposure than vegetative cells. The exact value of the electric current or voltage that each cell received could not be measured in our experiments; nevertheless, our data clearly show that *Colpoda* resting cysts have tolerance to electrostatic exposure. We are unable to completely exclude the possibility that our data include some effects of the low (0.3%) NaCl concentration. However, even if this level of salinity contributes to the response, the most important fact is that *Colpoda* resting cysts clearly tolerate electrostatic exposure.

The electrostatic tolerance is probably a by-product of the evolution of resting cyst formation as a strategy for adaptation to terrestrial environments. Under the present terrestrial environments, *Colpoda* rarely encounters electrostatic exposure or electricity discharge, such as during thunderstorms (Fig. 6A). Alternatively, we possibly hypothesize that the electrostatic tolerance might have been a way to survive in the primitive environment for early life on Earth (Fig. 6B). The assembly and break up of supercontinents may have involved increased volcanic activity (Nance et al., 2014) and associated volcanic lightning (Aizawa et al., 2016). Based on the molecular clock described in Wright and Lynn (1997), Ciliophora and Colpodaea originated more than 2200 million years ago (Mya) and 1600 Mya, respectively. The ability to form resting cysts may have preceded the origin of the ciliates because various members of the monophyletic clade SAR group (Burki et al., 2007; 2020) also have this ability. It has been lost in some lineages during evolution; nevertheless, retained by the clade class *Colpoda*. Our suggestion implies a possible hypothesis, i.e.,

resting cyst formation is the ultimate result of single-celled organisms such as *Colpoda* surviving severe environmental changes during the long history of the Earth.

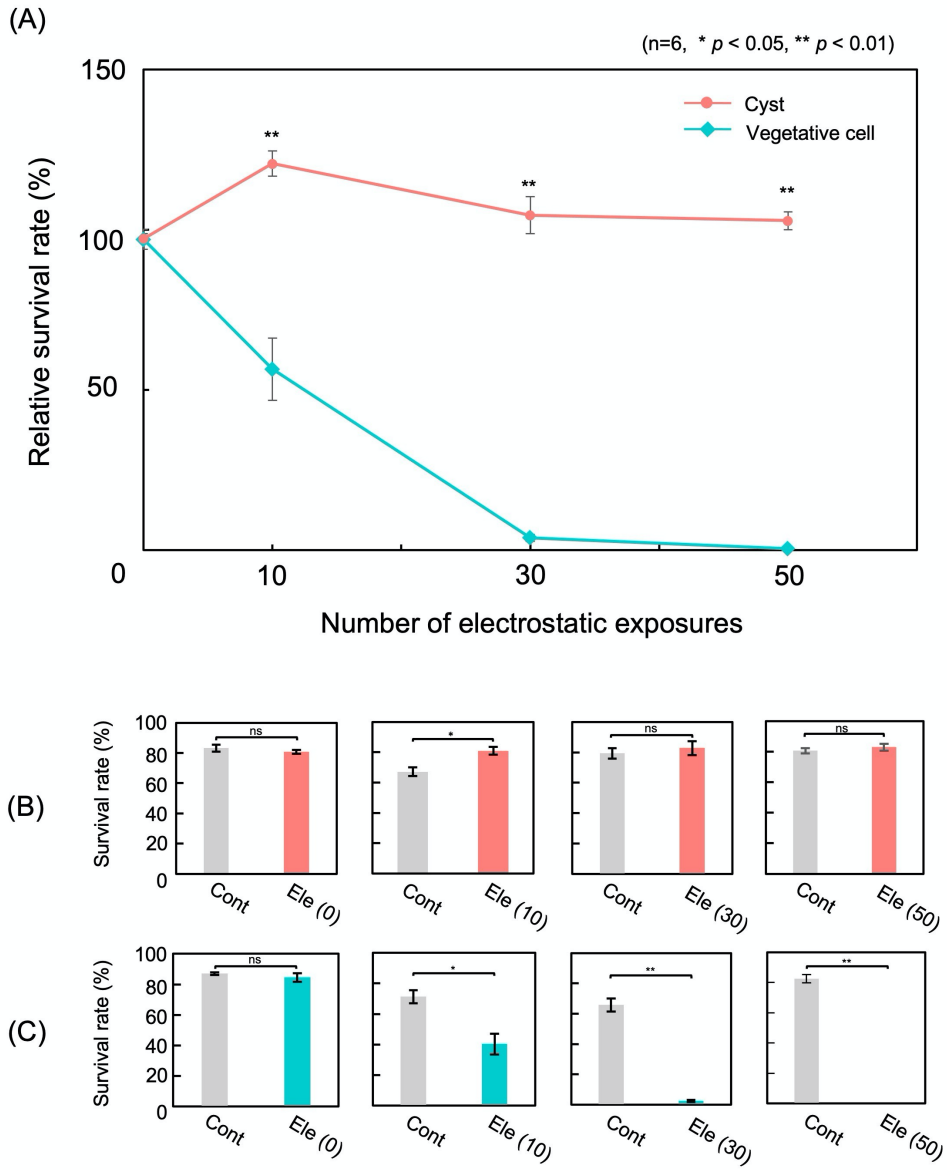

Fig. 2. Tolerance of *Colpoda* resting cysts and vegetative cells to electrostatic exposure: 0 (no electrostatic exposure), 10, 30, and 50 electrostatic shocks. A. Relative survival rates between non-exposed cells (control) and exposed cysts or vegetative cells. B and C. Effect of electrostatic exposure to survival rate of *Colpoda* resting cysts (B) and vegetative cells (C). Survival rates of control, no exposure cells (Cont) and electrostatically-exposed cells (Ele): 0 (no exposure), 10, 30, and 50 electrostatic shocks. Relative survival rates were calculated from the same data as survival rates of resting cysts (B) and vegetative cells (C). Points and bars (A) and columns and bars (B, C) show the means and SE of six identical samples. Asterisks and double asterisks indicate significant differences at $p < 0.05$ and $p < 0.01$, respectively, and ns indicates no significant difference. The significant differences shown in A are between cyst samples and vegetative samples.

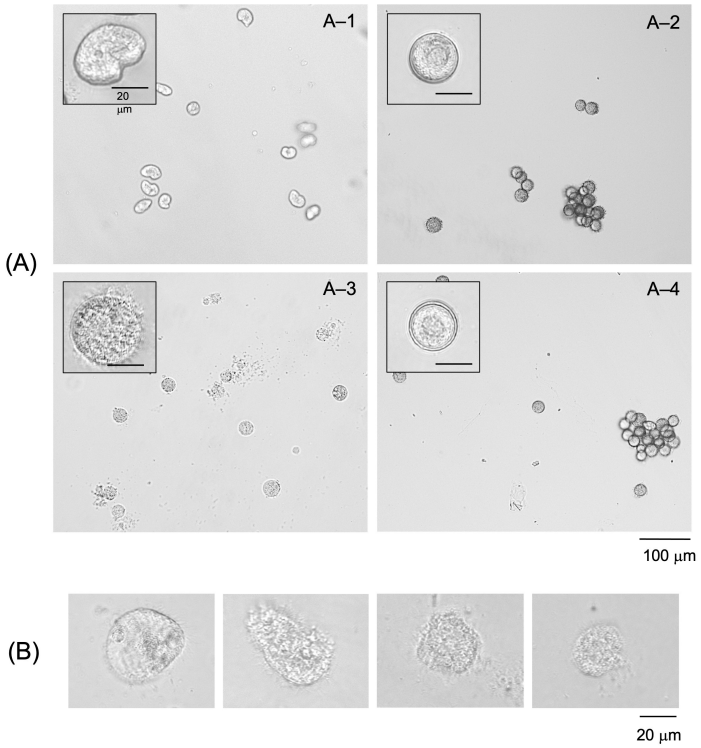

Fig. 3. Microscopical observation of non-exposed (control) vegetative cells (A-1) and cysts (A-2), electrostatically-exposed vegetative cells (A-3) and cysts (A-4). A high-magnification image of a representative cell in each condition is shown separately. The bars are 20 μm. The morphological features of some vegetative cells, exposed 30 times, are shown (B).

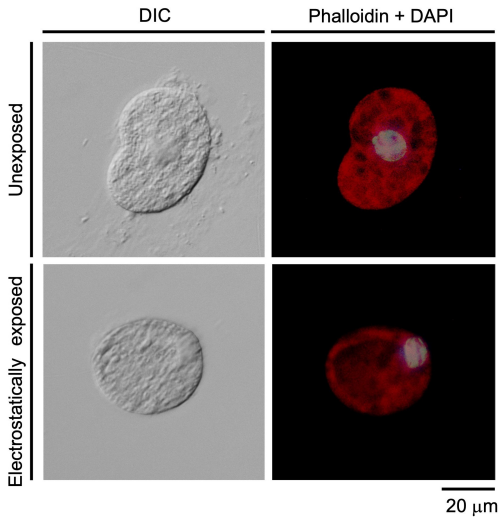

Fig. 4. Phalloidin and DAPI staining of unexposed vegetative cells (Unexposed) and of cells exposed to 30 electrostatic shocks (Electrostatically exposed). Differential interference contrast microscopic images (DIC) and overlaid images of separate photographs of phalloidin (red) and DAPI (blue) staining (Phalloidin + DAPI).

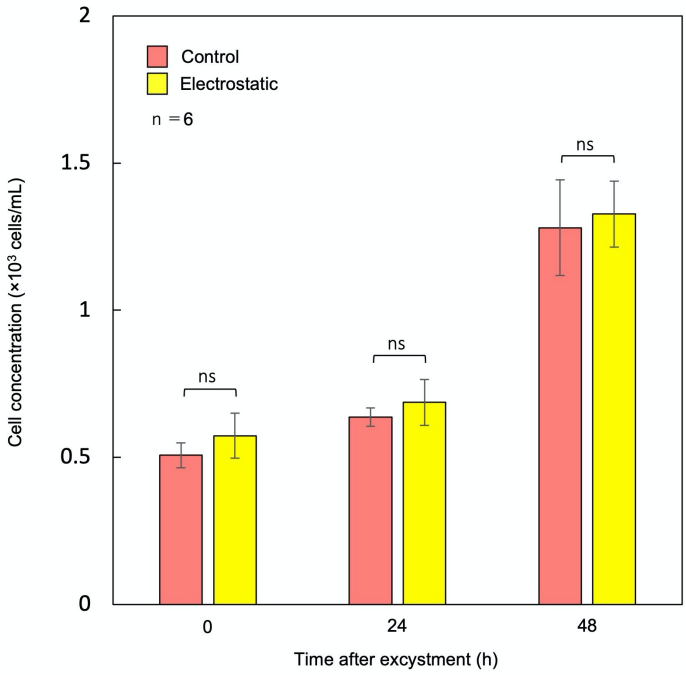

Fig. 5. Comparison of proliferation rates of excysted cells from unexposed cysts and cysts given 30 electrostatic shocks. Columns and bars show the means and SE of six identical samples, and ns indicates no significant difference.

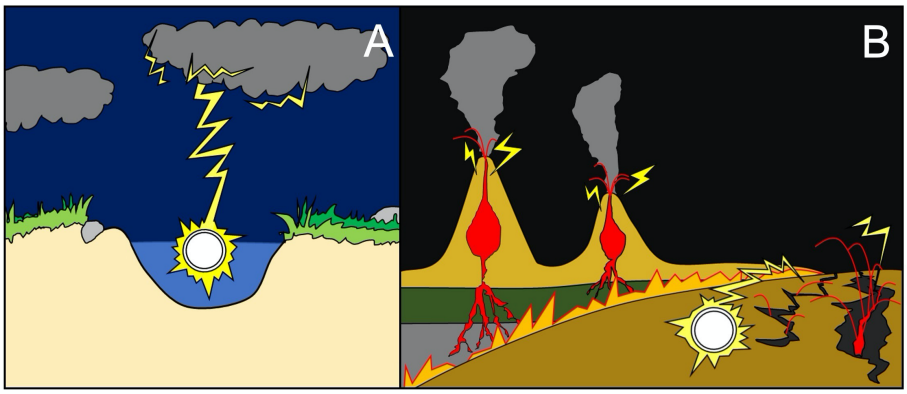

Fig. 6. Schematics of the possible biological significance of electrostatic tolerance in *Colpoda* resting cysts. This property allows survival under current environments such as thunderstorms (A) and volcanic lightning or widespread electricity around the ground due to the movement of continents (B).

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest in this study.

SUBMISSION DECLARATION AND VERIFICATION

This manuscript is original and approved by all authors. It has not been previously published, and will not published elsewhere in the same form without the written consent of the copyright-holder.

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