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Physiology

Naturally occurring fluorescence protects the eutardigrade *Paramacrobiotus* sp. from ultraviolet radiation

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Naturally occurring fluorescence has been observed in multiple species ranging from bacteria to birds. In macroscopic animals such as birds, fluorescence provides a visual communication signal. However, the functional significance of this phenomenon is unknown in most cases. Though photoprotection is attributed to fluorescence under ultraviolet (UV) light in some organisms, it lacks direct experimental evidence. Here, we demonstrate naturally occurring fluorescence under UV light in a eutardigrade belonging to the genus *Paramacrobiotus*. Using a natural variant that lacks fluorescence, we show that the fluorescence confers tolerance to lethal UV radiation. Remarkably, the fluorescent extract from *Paramacrobiotus* sp. could protect the UV-sensitive tardigrade *Hypsibius exemplaris* and nematode *Caenorhabditis elegans* from germicidal UV radiation. We propose that *Paramacrobiotus* sp. possess a protective fluorescent shield that absorbs harmful UV radiation and emits harmless blue light.

1. Background

Tardigrades are microscopic (0.5 to 1 mm in length) animals with four pairs of legs. About 1300 species have been reported worldwide from various habitats [1–4]. The phylum Tardigrada is included in the superphylum Ecdysozoa along with its sister phyla: Arthropoda, Onychophora and Cycloneuralia [5]. Terrestrial tardigrades, in cryptobiotic state, can tolerate harsh conditions such as extreme temperature and pressure (high and low), ionizing and ultraviolet (UV) radiations, osmotic stress, and even space vacuum at low Earth orbit [6–19].

Molecular and cellular mechanisms behind the extraordinary stress tolerance of tardigrades are poorly understood. However, there has been an increase in the molecular studies focused on the stress tolerance of tardigrades in recent years [20–22]. For example, the eutardigrade *Ramazzottius varieornatus* Bertolani & Kinchin, 1993 produces damage suppressor protein (Dsup), which contributes to its radiotolerance by binding to nucleosomes [11,22]. Tardigrade-specific intrinsically disordered proteins, AMP-activated protein kinase and protein phosphatase 1/2A contribute to the anhydrobiosis in some tardigrades [23–25].

Autofluorescence has been reported in parrots (e.g. *Melopsittacus undulatus* Shaw, 1805), scorpions (e.g. *Centruroides vittatus* Say, 1821), chameleons (e.g. *Calumma* spp.), frogs (e.g. *Hypsiboas punctatus* Schneider, 1799), nematodes (e.g. *Caenorhabditis elegans* Maupas, 1900), cnidarians (e.g. *Anemonia sulcate* Pennant, 1777) and tardigrades [26–31]. Functional significance of this phenomenon is unclear although visual signalling towards potential mates has been attributed in parrots [26]. Here, we show that a eutardigrade, *Paramacrobiotus* sp., exhibits tolerance to germicidal UV radiation for up to 1 h. This eutardigrade also shows autofluorescence under UV light. We demonstrate that this fluorescence contributes to its exceptional tolerance to UV radiation.

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2. Methods

(a) Tardigrade cultures

Tardigrades were isolated from a moss sample grown on a concrete wall in Bengaluru, India. They were cultured in KCM solution (7 mg KCl, 8 mg $CaCl_2$ and 8 mg $MgSO_4 . 7H_2O$ in 1 l of water) in 35 mm Petri dishes coated with 2% agarose (Lonza, Switzerland) at 20°C (protocol in [32]). Cultures were kept in the dark and *C. elegans* were provided as a food source. Tardigrades were 633 µm long on average with reddish-brown pigmentation. Morphology of their buccopharyngeal apparatus, claws and eggs suggests that they belong to the family Macrobiotidae (electronic supplementary material, figure S1 and table S1) [33,34]. Analysis of the nucleotide sequence of *COI* (mitochondrial cytochrome c oxidase gene (i) and *ITS-2* (internal transcribed spacer region (ii) using BLAST revealed that the isolated specimens belonged to the genus *Paramacrobiotus* (electronic supplementary material, tables S2 and S3) [35,36]. This strain was named as *Paramacrobiotus* sp. BLR. The generation time (days between deposition and oviposition [37]) of this strain was 64 ± 2 days. They reproduced by parthenogenesis under laboratory conditions.

We observed variants of these tardigrades without reddish-brown pigmentation at a frequency of 10–20% during isolation from the same location. Morphological features of pigmented and hypopigmented variants were highly similar (figure 1; electronic supplementary material, table S1 and figure S1). Both variants appeared in the same clade in the phylogenetic tree. Genetic distance between them is also very small (*ITS2*, 0% and *COI*, 1.7%; electronic supplementary material, figure S2). Moreover, hypopigmented variants develop pigmentation within a week in laboratory conditions. These findings show that the hypopigmented and pigmented tardigrades belong to the same species of the genus *Paramacrobiotus*.

Hypsibius exemplaris Gąsiorek, Stec, Morek & Michalczyk, 2018 (previously known as *Hypsibius dujardini*) were obtained from Professor Bob Goldstein, University of North Carolina at Chapel Hill, USA. They were cultured in Chalkley's medium with algae (*Chlorococcum* sp.) as a food source [38]. Prior to experiments, tardigrades were washed thoroughly using reverse osmosis (RO) water (pure water with the resistivity of 18.2 MΩ.cm at 25°C) and starved for 24 h in RO water containing ampicillin (0.5 mg ml^{-1}).

(b) Microscopy

A CXL Plus (LABOMED, USA) microscope was used for isolation of tardigrades. Differential interference contrast (DIC) and fluorescence images were acquired using an AxioObserver.Z1 inverted fluorescence microscope (Carl Zeiss) equipped with an HBO 100 lamp. The DAPI channel was used to capture fluorescence images (exposure time, 1 s). G365 and BP 445/50 bandpass filters were used for excitation and emission, respectively. Bright-field images were captured using an Axioscope upright light microscope (Carl Zeiss).

(c) Ultraviolet irradiation

Three groups with ten tardigrades in each were transferred to 35 mm Petri dishes coated with 2% agarose. The animals remained well hydrated for the duration of the experiment as they were in contact with the moist agarose surface. Tardigrades were exposed to UV radiation (peak wavelength 253 nm; duration 15 min to 1 h). The source of UV radiation was a germicidal lamp (LT-T8 30 W/UV-C HRA, Narva). The lamp was kept at a distance of 50 cm perpendicularly above the Petri dishes. The UV output of the lamp was 0.111 mW cm^{-2} as measured by a UV radiation meter (UVITEC, RS 254) at the same distance from and angle to the lamp as the Petri dishes. Because 1 W is 1 J s^{-1}, 0.111 mW cm^{-2} is equal to 1.11 J m^{-2} s^{-1}. Hence, the dose delivered in 15 min will be 1 kJ m^{-2} [12]. After the exposure, specimens were transferred to fresh agarose-coated Petri dishes and cultured. Tardigrades were monitored daily. Their movements and numbers of eggs laid and hatched were documented.

To test if *H. exemplaris* or *C. elegans* can be protected against the lethal effects of UV radiation in the presence of the fluorescent extract of *Paramacrobiotus* BLR strain, 300 individuals of *Paramacrobiotus* BLR strain (pigmented or hypopigmented) were homogenized in 120 µl of RO water using a tissue grinder. This was followed by centrifugation of the lysate at 20 000*g* for 15 min. The supernatant showed fluorescence under UV light (wavelengths 254 nm and 365 nm; source VL-6.LC, Vilber Lourmat). Extract (40 µl) from pigmented or hypopigmented *Paramacrobiotus* BLR strain or photobleached extract was added to a single well of a 96-well plate. Water (40 µl) was used as control. Twenty individuals of *H. exemplaris* or 50 of *C. elegans* were added to those wells and exposed to UV radiation (1 kJ m^{-2}) as described above. These assays were performed in triplicate (three wells for each group).

Tardigrades with a stretched out flaccid body without any visible movements after 24 h were considered dead. *Caenorhabditis elegans* were considered dead when they did not respond to external mechanical stimulus (a poke using a microtip). Photobleaching of the aqueous extract of *Paramacrobiotus* BLR strain was performed by exposing it to 254 nm UV light (VL-6.LC, Vilber Lourmat) for 10 min from 5 cm distance (5 kJ m^{-2}).

3. Results

(a) *Paramacrobiotus* BLR strain shows tolerance to ultraviolet radiation

We tested specimens of *Paramacrobiotus* BLR strain for the ability to tolerate UV radiation in the active state. All specimens survived 30 days after 15 min exposure to germicidal UV radiation (1 kJ m^{-2}). By contrast, specimens of *H. exemplaris* died within 24 h after the same treatment, consistent with a previous report (figure 1*a*(i)) [12]. There was no difference in the survival of these two tardigrade species when they were not treated with UV radiation (figure 1*a*(ii)). Furthermore, 60% of *Paramacrobiotus* BLR specimens survived 1 h exposure to germicidal UV radiation (4 kJ m^{-2}) (electronic supplementary material, figure S3A). After UV radiation treatment, tardigrades were observed daily for signs of life—active movement and egg laying. There was no significant change in the number of eggs laid, their hatchability and the hatching time, between UV-treated and untreated *Paramacrobiotus* BLR specimens (electronic supplementary material, table S4).

Controlled dehydration of *Paramacrobiotus* BLR strain resulted in 'tun' or anhydrobiotic state. Slow rehydration revived them back to an active state with 90% efficiency, showing that these tardigrades possess desiccation tolerance in addition to UV tolerance (electronic supplementary material, figure S3B).

(b) Naturally occurring fluorescence of *Paramacrobiotus* BLR strain is necessary for its tolerance to ultraviolet radiation

Incidentally, we observed strong fluorescence under UV light in specimens of *Paramacrobiotus* BLR strain. This fluorescence was absent in specimens of *H. exemplaris*, which were sensitive to UV radiation (figure 1*b*). Extract from *Paramacrobiotus* BLR specimens obtained after homogenizing in tissue lysis buffer also showed strong fluorescence under UV light. This fluorescence was absent in the extract from specimens of *H. exemplaris* (figure 1*c*).

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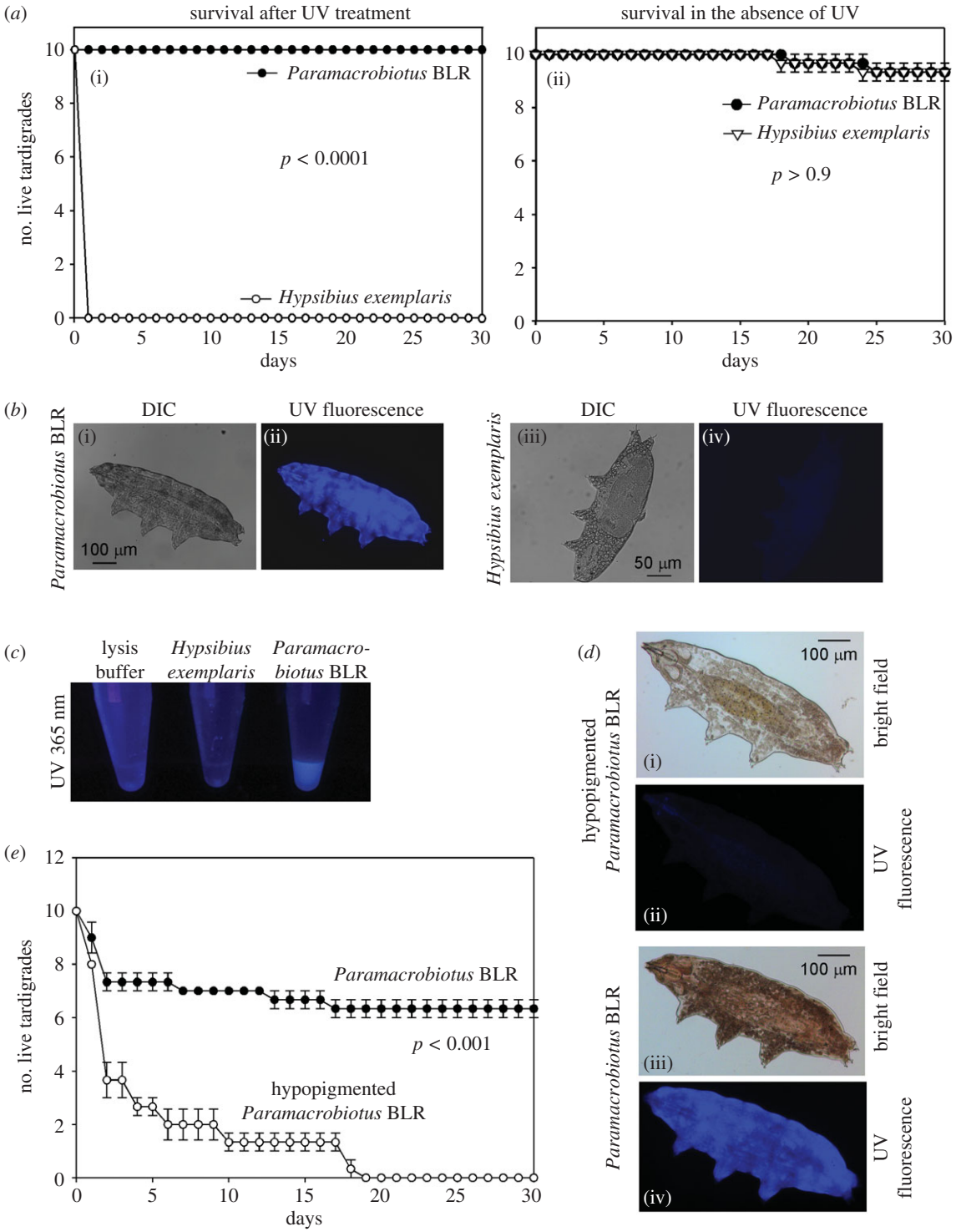

Figure 1. *Paramacrobiotus* BLR strain shows tolerance to UV radiation and exhibits fluorescence under UV light. (*a*) (i) Survival of *Paramacrobiotus* BLR strain after UV radiation exposure for 15 min (1 kJ m^{-2}). *Hypsibius exemplaris* was used as control. (ii) Their comparable survival in the absence of UV radiation. (*b*) Fluorescence microscopy images of *Paramacrobiotus* BLR strain and *H. exemplaris* taken under identical microscope settings. (*c*) Fluorescence images of the lysates of *Paramacrobiotus* BLR strain and *H. exemplaris* under UV light (365 nm). (*d*) Images of hypopigmented *Paramacrobiotus* BLR strain showing reduced fluorescence under UV light compared with the pigmented ones. Images were taken under identical microscope settings. (*e*) Survival of hypopigmented *Paramacrobiotus* BLR strain after UV radiation exposure for 1 h. Each point in the graphs (*a*,*e*) shows mean ± s.e., $n = 10 \times 3$ individuals. Graphs are representative of three independent experiments. Statistical significance was calculated using log-rank test.

Interestingly, hypopigmented variants showed much less fluorescence under UV light (figure 1*d* and electronic supplementary material, figure S1F). When they were exposed to UV radiation for 1 h (4 kJ m^{-2}), hypopigmented tardigrades showed significantly less UV tolerance compared with pigmented ones. All hypopigmented specimens died within 20 days after UV exposure, whereas 60% of the pigmented ones survived more than 30 days (figure 1*e*).

(c) Fluorescent extract of *Paramacrobiotus* BLR strain is sufficient to provide UV tolerance to UV-sensitive tardigrades and *C. elegans*

We then investigated the role of UV fluorescence of *Paramacrobiotus* BLR strain in its ability to tolerate UV radiation. The UV-sensitive *H. exemplaris* were covered with the fluorescent aqueous extract of *Paramacrobiotus* BLR strain and

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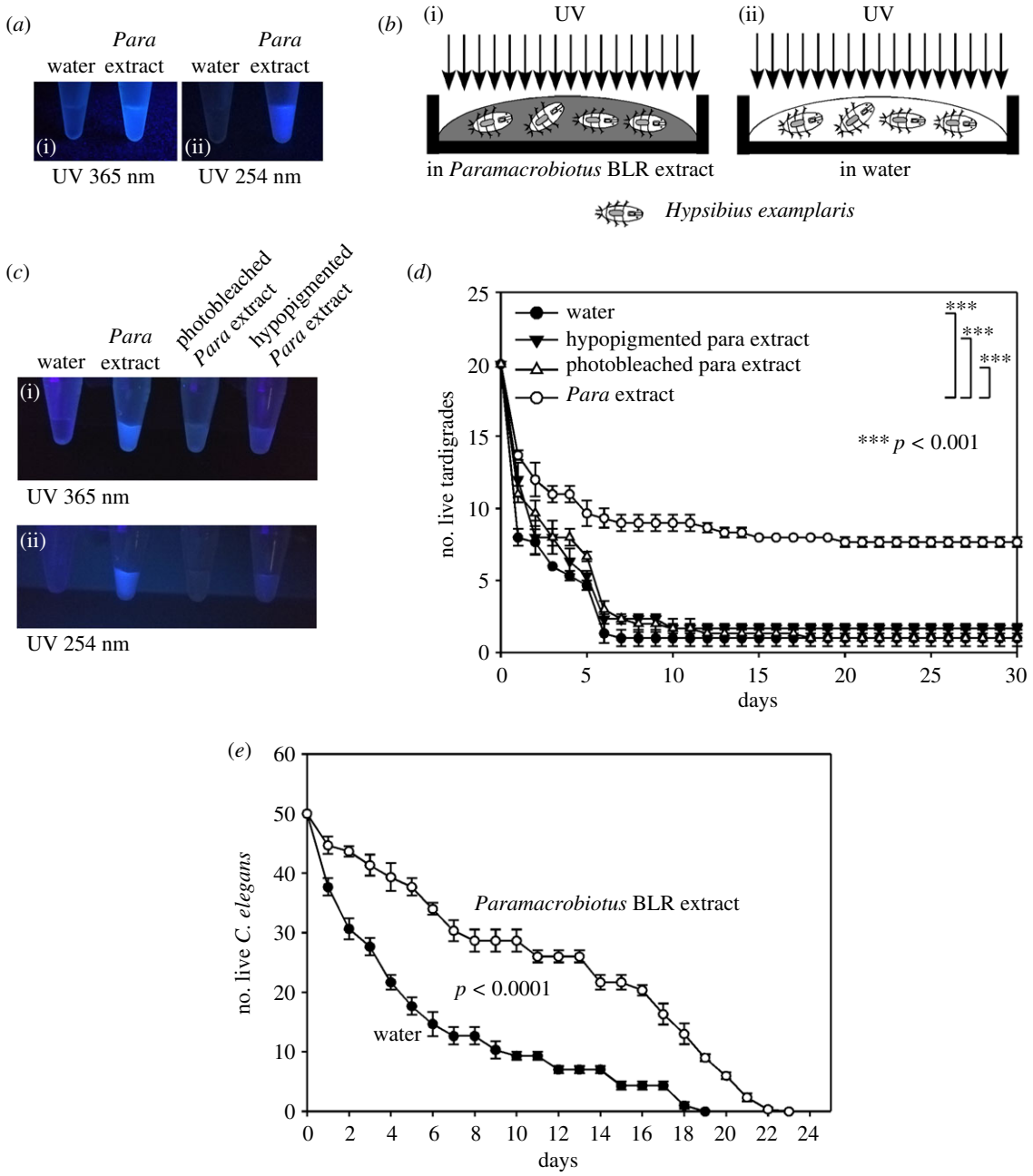

Figure 2. Transfer of UV tolerance property from *Paramacrobiotus* BLR strain to *H. exemplaris* and *C. elegans*. (*a*) Fluorescence of aqueous extract from *Paramacrobiotus* BLR strain under UV light. (*b*) Schematic of the experimental setup. (*c*) Fluorescence of aqueous extract from *Paramacrobiotus* BLR strain, photobleached extract and extract from hypopigmented strain. (*d*) Survival of *H. exemplaris* tardigrades incubated with extracts shown in (*c*) after UV radiation exposure for 15 min (1 kJ m^{-2}). (*e*) Survival of *C. elegans* incubated with fluorescent aqueous extract from *Paramacrobiotus* BLR strain after UV radiation exposure for 15 min (1 kJ m^{-2}). Each point in the graphs shown in (*d*) and (*e*) represents mean ± s.e., $n = 20 \times 3$ (in *d*) or $n = 50 \times 3$ (in *e*). Graphs are representative of three (*d*) or two (*e*) independent experiments. Statistical significance was calculated using log-rank test. *Para*, *Paramacrobiotus* BLR strain.

exposed to UV radiation for 15 min (1 kJ m^{-2}). *Hypsibius exemplaris* in water were used as control (figure 2*a*,*b* and electronic supplementary material, figure S4). Interestingly, *H. exemplaris* tardigrades covered in the fluorescent extract from *Paramacrobiotus* BLR strain showed partial tolerance to UV radiation. To further confirm the role of fluorescence, we photobleached the aqueous extract of specimens of *Paramacrobiotus* BLR strain by exposing it to UV light. The bleached sample without fluorescence was used for the experiment described above. The survival of *H. exemplaris* covered in the photobleached, non-fluorescent extract from *Paramacrobiotus* BLR strain was comparable to their survival in water. Similarly, their survival when covered in the extract from hypopigmented variants with much reduced fluorescence was also comparable to their survival in water (figure 2*c*,*d*). Finally, we checked if the fluorescent extract of *Paramacrobiotus* BLR strain can confer UV tolerance on a non-tardigrade animal. For this, we chose *C. elegans*. Remarkably, *C. elegans* in the fluorescent extract of *Paramacrobiotus* BLR strain showed a higher survival rate after UV radiation exposure (1 kJ m^{-2}) compared with those in water (figure 2*e*).

4. Discussion

UV radiation is lethal to most organisms. Yet, some organisms resist its harmful effects using multiple mechanisms, e.g. *Deinococcus radiodurans* Brooks and Murray, 1981 has developed an efficient DNA repair pathway [39].

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Cyanobacteria and other microbes produce UV-absorbing compounds such as mycosporines and related amino acids [40]. Melanin in mammals and hipposudoric acid in hippopotamus are pigments that absorb UV radiation [41,42]. The UV radiation resistance in the eutardigrade *R. varieornatus* is proposed to be due to extensive DNA repair. Exposure to UV radiation in these tardigrades increases the expression of *phrA*, a gene involved in DNA repair [12]. Some tardigrades such as *Hypsibius klebelsbergi* Mihelčič, 1959 contain pigment organelles, which are predicted to confer protection against UV radiation [43,44]. Eutardigrades *Paramacrobiotus richtersi* Murray, 1911 and *Ramazzottius oberhaeuseri* Doyère, 1840 show resistance to UV radiation, but the mechanism is not known [9].

Photoprotection against UV radiation has been suggested as a possible function of fluorescence in some organisms such as amphioxus, comb jellies and corals [30]. In the case of corals, a strong correlation was demonstrated between fluorescence and the susceptibility to photo-inhibition and bleaching [45]. However, there is no direct experimental proof of photoprotection imparted by fluorescence in any organism.

Our study showed that specimens of *Paramacrobiotus* BLR strain exhibit natural fluorescence under UV light that protects tardigrades against the lethal doses of UV radiation. Moreover, we showed that it is possible to transfer the UV tolerance property from *Paramacrobiotus* BLR strain to the UV sensitive *H. exemplaris* and *C. elegans* using the fluorescent extract. This provides the direct experimental demonstration of photoprotection by fluorescence. The fluorescent compound forms a 'shield' against UV radiation, protecting these tardigrades from its lethal effects (electronic supplementary material, figure S5). We speculate that *Paramacrobiotus* BLR strain has probably evolved this fluorescence mechanism to counter the high UV radiation of tropical southern India (UV index can reach up to 10). The UV dose in this location (Bengaluru, India) on a typical summer day is about 4 kJ m^{-2} (see http://www.temis.nl/uvradiation/archives/v2.0/overpass/uv_Bangalore_India.dat). In addition, anhydrobiosis might also help them to survive the hot summers with high UV index of this region. The small fraction of hypopigmented, non-fluorescent variants that coexist in the same moss habitat may have other mechanisms to escape from UV radiation, for example, living deeper inside the moss where UV radiation cannot reach.

Data accessibility. The data are provided as electronic supplementary material.

Authors' contributions. S.M.E. and H.R.S. conceived the project and designed the experiments. H.R.S. and S.P. performed the experiments. H.R.S. and S.M.E. analysed the results. S.M.E. acquired funds and supervised the project. S.M.E. and H.R.S. wrote the manuscript. S.M.E., H.R.S. and S.P. read and revised the manuscript for submission. All authors approved the final version and agree to be held accountable for the work performed.

Competing interests. We declare we have no competing interests.

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