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RESEARCH REPORT

Survival potential of the anhydrobiotic nematode *Panagrolaimus superbus* submitted to extreme abiotic stresses

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Abstract

Most organisms die when confronting extreme desiccation regimes, as observed in severe and prolonged droughts. However, some organisms are able to withstand such conditions by entering into a unique state of true suspended animation known as anhydrobiosis. Notably, anhydrobiosis also renders the organism tolerant to several other physical stresses such as extremes of temperature, pressure and radiation. Anhydrobiosis-based technologies are promising strategies to preserve crop plants as well as organs for transplant. In order to understand the relation between anhydrobiosis and tolerance to physical stresses, we submitted the anhydrobiotic nematode *Panagrolaimus superbus* to diverse abiotic stresses when alive (hydrated) and in anhydrobiosis (desiccated). Remarkably, our data revealed that hydrated *P. superbus* naturally displays considerable tolerance to ultra-low temperature (-196 °C), X-radiation (500 Gy) and ultracentrifugation (400,000x*g*) in the tested conditions. More importantly, anhydrobiosis enhances nematode tolerance to ultra-low and high temperatures (+100 °C), but not to X-radiation or ultracentrifugation. These findings may help explain the successful wide distribution of *P. superbus* on Earth, since extremes of temperature are the most common stresses confronted by this species. Finally, due to its intrinsic survival potential (hydrated or desiccated), our data evidence the potential of *P. superbus* as a model in astrobiology.

Key Words: anhydrobiosis; desiccation tolerance; X-radiation; extreme temperatures; ultracentrifugation

Introduction

The phenomenon of anhydrobiosis (from the Greek: "life without water") was first described over 300 years ago by Antonie van Leeuwenhoek and can be defined as a highly stable state of true suspended animation that certain organisms (within diverse groups including bacteria, yeasts, plants and small invertebrates) enter when exposed to very low relative humidity conditions (Tunnacliffe and Lapinski, 2003). While most organisms die in this scenario, these organisms lose 95 - 99 % of the body water content, replacing their intracellular aqueous milieu by an amorphous bioglass, composed of trehalose (Erkut *et al.*, 2011), intrinsically disordered proteins (Boothby *et al.*, 2017) and other elements, which literally arrests all biomolecules in space and time. This ametabolic state is stable for long periods of time; life is resumed when the organism is rehydrated (Crowe *et al.,* 1992, Clegg, 2001; Tunnacliffe and Lapinski, 2003; Rebecchi *et al.*, 2007).

Notably, several anhydrobiotic organisms (or anhydrobionts) are able to tolerate different types of stress when desiccated (*e.g.,* ionizing radiation, vacuum, extreme temperatures, high hydrostatic pressures, hipogravity, etc) (Tunnacliffe and Lapinski, 2003; Hengherr *et al.,* 2009; Horikawa *et al.,* 2009; Jönsson *et al.,* 2008; Beltrán-Pardo *et al.,* 2013). Therefore, anhydrobiotic organisms are extremotolerant, *i.e.*, they may live in conditions similar to those 'suitable for human life', but can tolerate conditions of extreme abiotic stress when necessary (Rampelotto, 2013). The phenomenon of anhydrobiosis displays an immense biotechnological potential in agriculture and biomedicine: the preservation of crops during severe droughts as well as organ preservation at room temperature for transplant. Recently, an anhydrobiosis-based strategy for vaccine storage at room temperature was developed (Alcock, 2010), illustrating the great potential behind this natural phenomenon.

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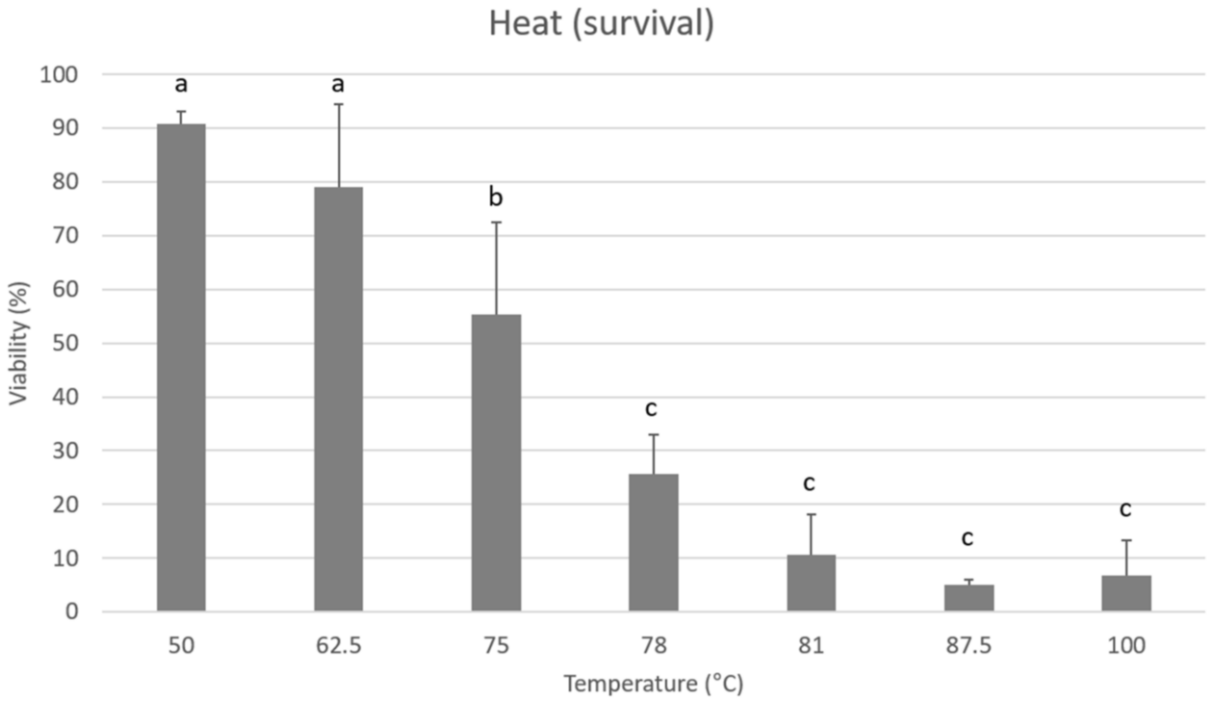

Fig. 1 Tolerance of dessicated *P. superbus* to heat. N = 600 per group, per technical replicate. Different letters indicate statistically significant differences ($p < 0.05$).

Panagrolaimus superbus is a free-living anhydrobiotic nematode of approximately 1 mm in length, dioic, which was first described by Fuchs (1930). Members of the genus *Panagrolaimus* occupy several different niches, from Antarctic, volcanic islands, temperate and semi-arid soils to terrestrial mosses (Shannon *et al.*, 2005; Mcgill *et al.,* 2015). It is closely related to the well characterized nematode *Caenorhabditis elegans*. Here we investigated the tolerance profile of *P. superbus* to extremes of temperature (-196 ºC and +100 ºC), X-radiation (100 and 500 Gy) and hypergravitational force (400,000x*g*) in order to uncover the survival potential of this species in the hydrated and desiccated (anhydrobiotic) states, as well as the role of anhydrobiosis on these abilities.

Material and Methods

Nematode maintenance

Panagrolaimus superbus, kindly provided by Prof. Tunnacliffe A (University of Cambridge, UK), was maintained in the dark, at 20 ºC, on NGM (Nematode Growth Medium) agar plates and fed with a layer of *Escherichia coli* (OP50 strain). Mixed populations, composed of all developmental stages, were used in all experiments.

Desiccation, rehydration and viability assay

NGM agar plates were rinsed with M9 buffer in order to dislodge and collect worms, which were subsequently washed three times with M9 buffer to remove excess of bacteria. Worms were then immobilized on 0.45 µm Supor filter membranes by vacuum filtration using a Sartorius funnel. These membranes were placed in a sealed chamber containing a saturated solution of $CuSO_4$, for 24 h [pre-conditioning in 98 % relative humidity (RH)]. Then, they were transferred to another chamber containing regenerated silica gel, for 24 h (desiccation in 10 % RH). Desiccated worms were then submitted to different stresses (temperature, X-radiation or ultracentrifugation), as described below. Thereafter, membranes were placed in a chamber with distilled water for 24 h (pre-rehydration in 100 % RH) and then the membranes are immersed in M9 buffer for 3 h for worm rehydration. Subsequently, we performed a survival assay using a modified version of protocol which has been used for isolated cells (Krause *et al.*, 1984). Briefly, the supernatant was removed and erythrosin B dye was added (0.4 % w/v in M9 buffer). After 1 h, samples were washed three times with M9 buffer to remove excess of dye. Dead worms stained in pink, live worms remained unstained.

Liquid Nitrogen (-196 ºC)

The exposure procedure to liquid nitrogen (N_2) was based on previous experiments performed in tardigrades with some modifications (Horikawa *et al.,* 2008). After 24 h in regenerated silica gel chamber, desiccated worms (immobilized on Supor membranes and placed inside closed 1.5 ml microtubes without paraffin) were immersed for 15 min, 30 min, 1 h, 1 week or 1 month directly in liquid N_2 (-196 ºC). At the end of exposure, worms were thawed at room temperature for 5 min and submitted to the subsequent steps (pre-rehydration, rehydration and viability assay). Negative control group (NC) was composed of hydrated worms

immobilized on membranes, placed inside microtubes and directly immersed in liquid N_2. Positive control group (PC) was composed of desiccated worms immobilized on membranes, placed inside microtubes but not immersed in liquid N_2. Experimental group (EG) was composed of desiccated worms immobilized on membranes, placed inside microtubes and directly immersed in liquid N_2. Three biological replicates were performed (N = 600 per group, per technical replicate. Each technical replicate comprised all three groups: NC, PC and EG. The only exceptions were PC for 15 min, 30 min and 1 h, which are the same, since they are functionally equivalent). Statistical analyses (One Way ANOVA) were performed for each time point (comparing the corresponding PC, NC and EG).

Heat (50 - 100 ºC)

Desiccated worms (immobilized on Supor membranes and placed in 0.6 mL microtubes) were subjected to heating at different temperatures (50 ºC, 62.5 ºC, 75 ºC, 78 ºC, 81 ºC, 87.5 ºC or 100 ºC) using a thermal cycler (mastercycle Eppendorf). Treatment of samples started at 25 ºC with an increase rate of 5 ºC every 2 min until reaching the desired temperature (previously indicated), in which they remained for 5 min. This period of time (5 min) was chosen since it may represent acute 'peaks of stress' that occur in nature. Since survival percentage of desiccated worms at 50 ºC is high, we assume that temperatures below it may not represent stressing conditions. In a second experiment, worms were exposed to 50 ºC for 15 min, 30 min or 1 h. NC was composed of hydrated worms immobilized on membranes and exposed to heating. PC was composed of desiccated worms immobilized on membranes but not exposed to heating. EG was composed of desiccated worms immobilized on membranes and exposed to heating. Three biological replicates were performed (N = 600 per group, per technical replicate. Each technical replicate comprised all three groups: NC, PC and EG). Statistical analyses (One Way ANOVA) were performed comparing all groups (Fig. 1) or within each group separately (NC or EG) (Fig. 2).

X-Radiation

In order to measure the tolerance of *P. superbus* to X-radiation, desiccated worms were immobilized on membranes and placed in Petri dishes which were irradiated (100 or 500 Gy) using the RS 200 Biological Research irradiator (Rad Source) located in the radiology section of the University of São Paulo Hospital - FMRP/USP. After rehydration worms were divided into 10 equal samples and population sizes were determined throughout 10 time points (the following 10 days after stress). Population growth (in percentage) was determined by dividing the final number of living worms (output) by initial number of worms (input). NC was composed of hydrated worms immobilized on membranes and exposed to radiation. PC was composed of desiccated worms immobilized on

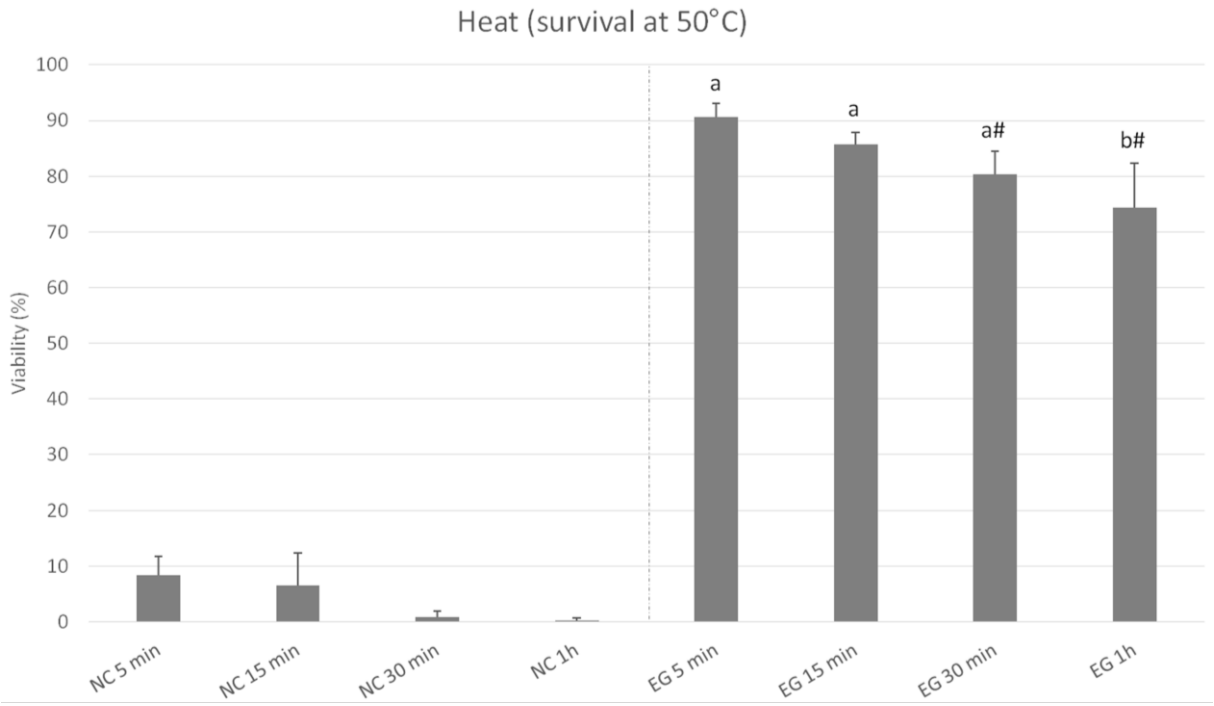

Fig. 2 Tolerance of *P. superbus* to 50°C for different periods of time. Negative Control (NC): hydrated worms immobilized on membrane and exposed to stress; Experimental Group (EG): desiccated worms immobilized on membrane and exposed to stress. N = 600 per group, per technical replicate. Different letters indicate statistically significant differences ($p < 0.05$). #: marked groups are not statistically different.

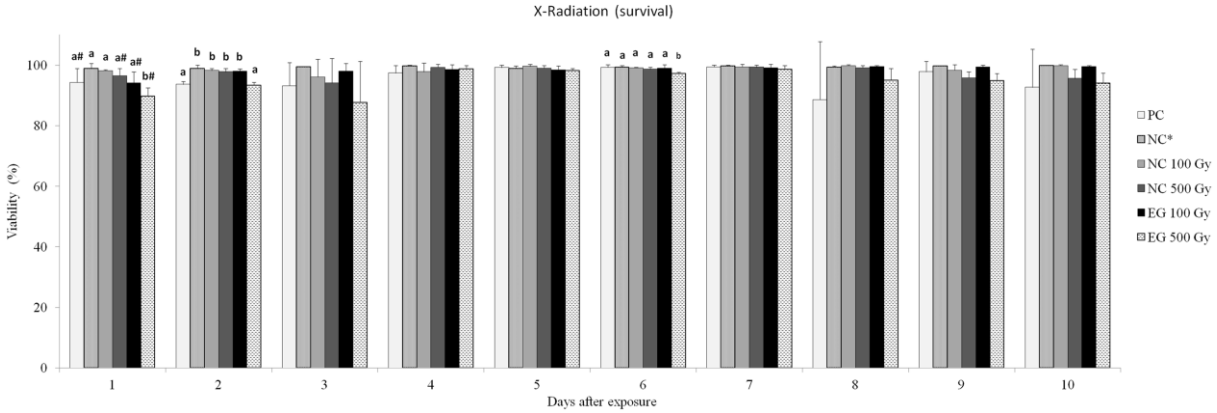

Fig. 3 Tolerance of *P. superbus* to X-radiation. PC (Positive Control): desiccated worms immobilized on membranes but not exposed to stress; NC* (Negative Control*): hydrated worms immobilized on membranes but not exposed to stress; NC 100 Gy (or 500 Gy) (Negative Controls): hydrated worms immobilized on membranes and exposed to 100 Gy (or 500 Gy); EG 100 Gy (or 500 Gy) (Experimental Groups): desiccated worms immobilized on membranes and exposed to 100 Gy (or 500 Gy). N = input of 100 per group, per day, per technical replicate. Different letters indicate statistically significant differences ($p < 0.05$). #: marked groups are not statistically different.

membranes but not exposed to radiation. EG was composed of desiccated worms immobilized on membranes and exposed to radiation. An additional negative control group (NC*) consisting of immobilized hydrated worms not exposed to radiation was used in order to reproduce 'population dynamics' close to which is observed under normal conditions. Three biological replicates were performed (N = 1,000 per group, per technical replicate. Each technical replicate comprised all three groups: NC, PC and EG). Statistical analyses (One Way ANOVA) were performed comparing only the groups within the same time point (Figs 3, 4).

Ultracentrifugation (hypergravitational force)

P. superbus worms were centrifuged at 400,000×*g* at 4 ºC (the working temperature of the equipment) for 5, 15, 30 min or 1 h using MAX-XP Ultracentrifuge. Desiccated immobilized worms, submitted to centrifugation were considered as the EG. Hydrated immobilized worms, submitted to centrifugation were considered as the NC. Non-immobilized worms centrifuged while immersed in M9 buffer were considered as 'M9 negative control' (M9-NC). Hydrated, non-immobilized worms, not centrifuged but kept at 4 °C for 1 h were considered as the 'PC4 °C'. Two positive controls were used. Positive control 1 (PC1): desiccated, immobilized worms, kept at 4 ºC for 1 h but not centrifuged and then rehydrated. Positive control 2 (PC2): non-immobilized worms kept in M9 at 4 ºC for 1 h but not centrifuged. Three biological replicates were performed, each one consisting of three technical replicates (each one comprising all three groups: NC, PC and EG). N = 600 worms/technical replicate. Statistical analyses (One Way ANOVA) were performed comparing only the groups within the same time point (Fig. 5).

Statistical analyses

All experiments were performed in biological triplicates (each one consisting of technical triplicates) and data are presented as mean values and standard deviations. Statistical analyses were performed using "One Way ANOVA" (with Student Newman post-hoc or Student-Newman-Keuls for ultracentrifugation). Statistical differences were considered significant when $p \leq 0.05$. Identical letters indicate those groups are not statistically different. Distinct letters indicate those groups are statistically different. In some cases, one group may be statistically not different from only one specific group, within a larger set of groups indicated with a different letter. In these specific cases, those groups indicated with hashtag (#) are not statistically different.

Results

Tolerance to liquid nitrogen

Remarkably, a considerable percentage of hydrated *P. superbus* is tolerant to liquid nitrogen (196 °C) in the absence of any cryoprotectants for up to one month (22.6 % on the average of the five time points) (Fig. 6). Survival is 30.2 % after 15 min, decreasing to 9.8 % after four weeks submitted to ultracold conditions.

More importantly, desiccated worms exposed to liquid nitrogen always displayed much higher, statistically significant, survival percentages (80.9 % on the average of the five time points) than NC group - similar or higher than the PC group in all treatments (75.0 % on the average). Therefore, hydrated *P. superbus* presents a natural tolerance to ultra-low temperature (indicated as NC), which is enhanced by anhydrobiosis in the long term (indicated as EG).

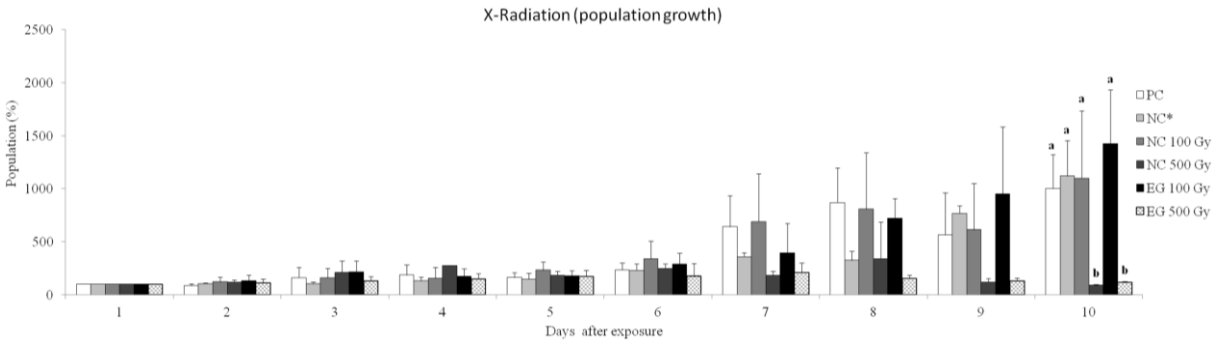

Fig. 4 Population growth of *P. superbus* exposed to X-radiation. PC (Positive Control): desiccated worms immobilized on membranes but not exposed to stress; NC* (Negative Control*): hydrated worms immobilized on membranes but not exposed to stress; NC 100 Gy (or 500 Gy) (Negative Controls): hydrated worms immobilized on membranes and exposed to 100 Gy (or 500 Gy); EG 100 Gy (or 500 Gy) (Experimental Groups): desiccated worms immobilized on membranes and exposed to 100 Gy (or 500 Gy). N = input of 100 per group, per day, per technical replicate. Different letters indicate statistically significant differences ($p < 0.05$).

Tolerance to heating (50 ºC - 100 ºC)

The survival curve (Fig. 1) evidenced that desiccation rendered worms tolerant to heating. Viability percentages obtained after exposure to temperature gradient revealed a nearly linear, inverse correlation, with a significant decrease observed from 75 ºC above. This data also evidences that a small fraction of the desiccated population seems to be thermostable from 80 ºC to 100 ºC (7.5 % on the average of the three time points). As expected, worms in almost all NC (hydrated exposed to heating) died (data not shown). Therefore, by comparing both groups (hydrated versus desiccated), our data evidences that anhydrobiosis confers partial heat tolerance.

Although high temperatures are lethal to hydrated *P. superbus*, a few worms were still alive at 50 ºC for 15 min (6.5 %; Fig. 2, NC), a situation abolished by 1 h. Remarkably, a high percentage (74.3 %) of desiccated worms remained viable after one hour at 50 ºC, evidencing a protective effect of anhydrobiosis to heat. However, this tolerance gradually diminishes.

X-Radiation

Viability analysis of irradiated worms throughout ten days revealed small but statistically significant decreases on the first, second and sixth days (differences observed among groups within the same day), especially in desiccated worms exposed to 500 Gy (Fig. 3). Therefore, according to these experiments, X-ray doses of 100 and 500 Gy were not lethal to worms within the period of analysis.

Conversely, analysis of population growth indicated an apparent negative effect of X-rays from the seventh day on, which is statistically significant at day ten. During this period, both hydrated and desiccated worms exposed to 500 Gy presented stagnation of population growth (Fig. 4) (on average both groups - NC 500 Gy and EG 500 Gy - halted at 103.9 % at day ten, compared to the average of 1,162.6 % of the other groups).

Therefore, our data evidence that anhydrobiosis does not confer tolerance against X-radiation in any tested condition, since there were no differences in survival or population growth between experimental groups and their respective NCs.

Ultracentrifugation

Experiments with hypergravitational forces revealed unexpected findings (Fig. 5). Notably, a high percentage (41.3 %) of desiccated *P. superbus* is tolerant to extreme hyperacceleration (400,000x*g*) for 1h. However, more surprisingly is the fact that hydrated worms (immobilized on filter membranes) presented a similar result (39.1 %), thus uncovering a natural tolerance of this nematode to extreme *g*-forces.

Since the filter membrane, used as immobilization substrate for both previous groups, often collapsed during ultracentrifugation (potentially damaging the worms), we decided to evaluate non-immobilized *P. superbus*. Remarkably, such hydrated and freely swimming worms in liquid medium are virtually fully tolerant to such *g*-force (5 min - 96.7 %; 15 min - 97.2 %; 30 min 98.3; 1 h - 98.0 %) (Fig. 5).

Discussion

P. superbus is an anhydrobiotic nematode able to thrive at diverse enviromental conditions. Our data reveals that anhydrobiosis confers considerable resilience to high temperatures for short periods, a situation that might take place in natural environments as semi-dry soils (Shannon *et al*., 2005). We also observed that *P. superbus* tolerates up to 1 h in a relatively high temperature (50 ºC). Taken together, these data indicate that anhydrobiosis guarantees the perpetuation of the desiccated population when exposed to high temperatures for varying periods of time in natural environments.

Notoriously, *P. superbus* can be stored at -80 ºC for 24 h without compromising viability (Mcgill *et*

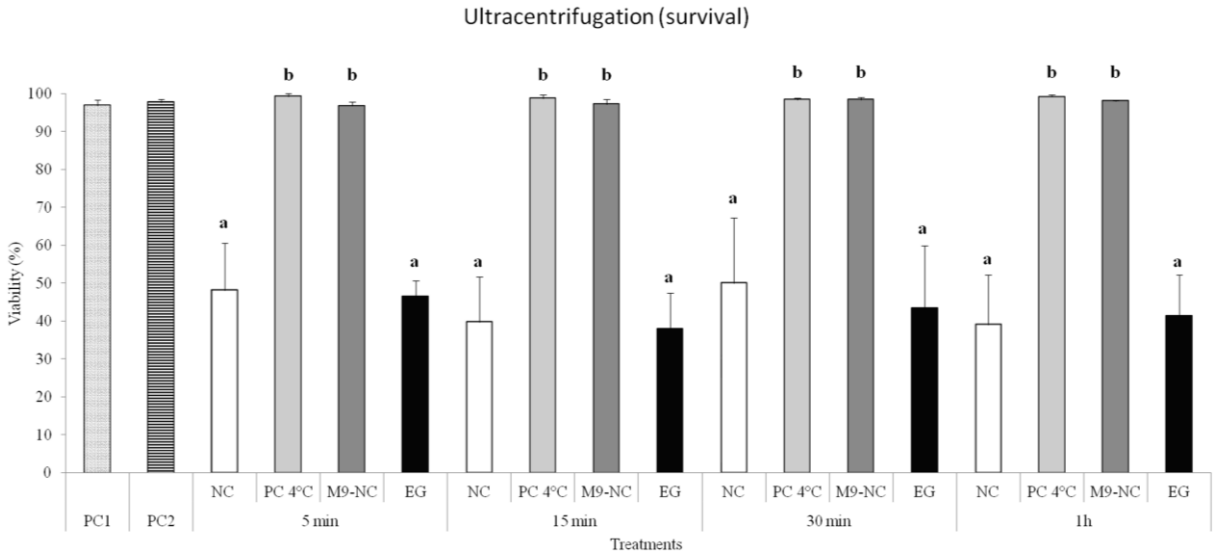

Fig. 5 Tolerance of *P. superbus* to ultracentrifugation. N = 600 per group, per technical replicate. Different letters indicate statistically significant differences ($p < 0.05$). PC1: positive control group 1, desiccated worms immobilized on membranes but not centrifuged, kept at 4 °C for 1 h. PC2: positive control group 2, worms kept in M9 buffer but not centrifuged, kept at 4 °C for 1 h. NC: negative control group, hydrated, immobilized worms, centrifuged at 4 °C for 1 h. PC4 °C: hydrated, non-immobilized worms, not centrifuged but kept at 4 °C for 1 h. EG: experimental group, desiccated worms, immobilized on membranes, centrifuged at 4 °C for 1 h. M9-NC: hydrated worms immersed on M9 buffer, centrifuged at 4 °C for 1 h.

al., 2015). When comparing survival of hydrated versus desiccated *P. superbus*, both submitted to ultralow or high temperatures, the second group displayed higher viability in both stressing scenarios. Therefore, acute tolerance of desiccated *P. superbus* to extremes of temperature is due to anhydrobiosis rather than a natural adaptation of this species to extreme cold environments (a common habitat) or to heat (which in fact is lethal). The comprehension, at the genetic, biochemical and physiological levels, of how anhydrobiosis renders the organism tolerant to -196 ºC for such long periods or to high temperatures may not only help the advancement of cryobiology and anhydrobiotic engineering, but also to understand the limits of life confronting physical stresses.

When comparing survival percentages of desiccated worms to ultralow versus high temperatures, it is clear that anhydrobiosis confers higher tolerance for long periods at ultralow temperatures rather than at high ones. This may seem counterintuitive since anhydrobiosis is a phenomenon directly related to high temperatures (when dehydration/desiccation naturally takes place). This lower tolerance to higher temperatures is probably due to the transition point of the bioglass, an amorphous matrix composed in some species of non-reducing disaccharides and other proteins which seems to stabilize the structure and cellular constituents during anhydrobiosis (Buitink *et al*., 2004; Sakurai *et al*., 2008; Hengherr *et al*., 2011). If the external temperature raises up to values above the glass-transition point, its own integrity is compromised, thus decreasing viability (Hengherr *et al*., 2009).

Curiously, different anhydrobiotic organisms seem to present distinct glass-transition points (or other heat-stabilizing elements), as judged by the fact that they tolerate higher temperatures. These are the cases of some rotifers, tardigrades and nematodes which survive to brief exposure to +150 ºC (reviewed in Tunnacliffe and Lapinski, 2003; Eisenback *et al.,* 2013).

Radiation exposure is possibly the most studied stress in suspended animation in scientific literature (Horikawa *et al.,* 2006; Watanabe *et al*., 2006; Gladyshev and Meselson, 2008; Nilsson *et al.,* 2010; Beltrán-Pardo, 2013, 2015). These studies have focused on the understanding of eukaryote's resilience to ionizing radiations and can be compared to other radiation analyses in non-anhydrobiotic animal models such *Drosophila melanogaster*. As previously observed in *C. elegans* (Onodera *et al*, 2010), doses of 100 and 500 Gy caused no viability decrease in *P. superbus* ten days after exposure to X-rays, probably due to eutely (low degree of somatic cell divisions in the adults), thus less susceptible to the harmful effects of radiation.

However, the stagnation in population growth at 500 Gy dose possibly reveals a sterilizing (Chang *et al.,* 2015) or egg-lethality effect of X-rays. More importantly, our data evidence that anhydrobiosis does not confer tolerance against X-radiation in any tested condition. Other anhydrobiotic organisms, when exposed to intense radiation regimes, suffer extensive DNA damage. However, they survive by activating unique DNA repair systems, able to reconstruct all the genomic landscape (Zahradka *et al*., 2006). Our data suggest that such mechanisms

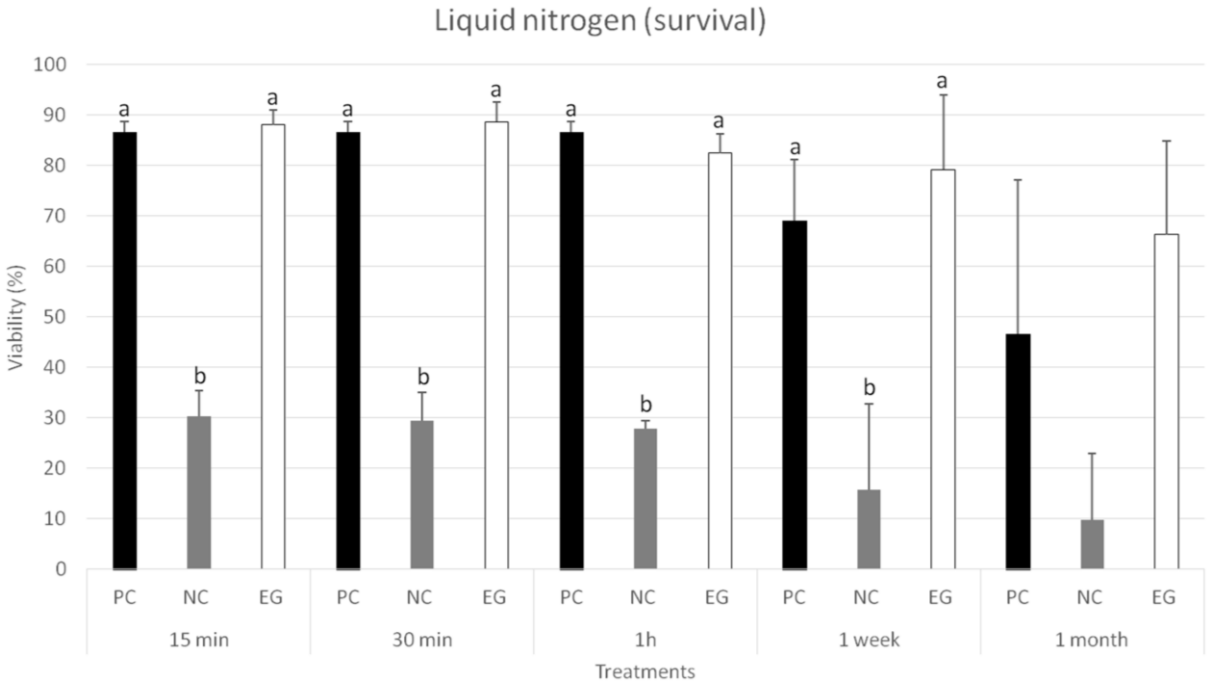

Fig. 6 Tolerance of *P. superbus* to liquid nitrogen. Positive Control (PC): desiccated worms immobilized on membrane but not exposed to stress; Negative Control (NC): hydrated worms immobilized on membrane and exposed to stress; Experimental Group (EG): desiccated worms immobilized on membrane and exposed to stress. N = 600 per group, per technical replicate. Statistical analyses were performed for each time point separately. Different letters indicate statistically significant differences ($p < 0.05$).

are not present in *P. superbus*, or if so, they are not effective within the tested conditions (period and dose).

Deguchi *et al.* (2011) revealed that single-celled microorganisms are able to withstand (and even discretely thrive) when subjected to 400,000x*g*. Since this experimental condition seemed to be an interesting physical stress to test anhydrobiosis' protective effect in a multicellular organism, we submitted desiccated *P. superbus* to ultracentrifugation.

We initially hypothesized that such extreme forces would be lethal, to both hydrated and desiccated worms, leading to sedimentation of intracellular components, affecting internal structures and body morphology. However, unexpectedly, we observed that hydrated worms are completely tolerant to such extreme physical stress.

After a deep and extensive search in the literature we noted that Beams and King (1936) showed that eggs of the nematode *Ascaris suum* were able to withstand identical hypergravitational forces, presenting dividing cells 48 h later. The only other report is of Morey-Holton (2003), who mentioned in a review that '*nematodes tolerate 10^5xg for brief periods*' (without reference), leading us to believe that it refers to the study with nematode eggs (Beams and King, 1936). Therefore, to our knowledge, this is the first time that an adult animal is shown to tolerate 400,000x*g*, orders of magnitude above conventional studies (1-100x*g*) (Kim *et al*. 2007; Sasagawa *et al*., 2003; Qiao *et al*. 2013).

Curiously, desiccated worms display a much lower tolerance than hydrated ones. This may be due the fact that the bioglass, as a solid matrix (Hengherr *et al.*, 2009), is susceptible to damages in its structure and/or integrity due to the tensional forces experienced during the hyperaccelerations, and/or the movement/collapse of the membrane on which the worms are immobilized. All these stressing physical forces probably cause ruptures within the bioglass, thus decreasing the viability of the desiccated worms.

The fact that adult *P. superbus* withstands hyperaccelerations may have implications in diverse and fundamental aspects of biology. Perhaps the most important involves the physical limits that constrain the existence of life (Rothschild and Mancinelli, 2001). The present evidence that a multicellular organism tolerates such condition extends the range of possible inhabitable planets, thus setting the foundation to consider the existence of biological activities also in much more massive celestial bodies, which display much higher gravitational forces.

It is also important to highlight that the natural and continuous exchange of mass among planets (Pizzarello and Cronin, 1998) also involves equivalent hyperacceleration forces for short

periods, either during the ejection of a rock caused by intense eruption or the impact of a meteorite. Therefore, organisms located on such rocks might survive such events, helping understand the origin and distribution of life in the universe.

Conclusions

Our data evidence that *P. superbus* presents remarkable survival potential to freezing, radiation and hyperacceleration even in the hydrated state. However, anhydrobiosis potentiates its survival in extremes of temperature, providing the additional capacity needed to withstand severe droughts or freezing observed in its natural environments. The surprising observation that hydrated *P. superbus* is fully tolerant to such hyperacceleration (400,000x*g* for 1 h) extends the known limits of tolerance to *g*-forces for adult animals in orders of magnitude, raising new and fundamental questions about the limits of life. Finally, due to its intrinsic survival potential (in the hydrated and desiccated states), *P. superbus* might also be exploited as a model in astrobiology.

Acknowledgments

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