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Experimental Evidence for Non-encysted, Freeze-resistant Stages of Terrestrial Naked Amoebae Capable of Resumed Growth after Freeze-thaw Events

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Abstract. Experimental evidence is presented to support a hypothesis that terrestrial naked amoebae, collected during late autumn from cold, moist temperate soil, develop a non-encysted, freeze-thaw resistant stage that is capable of surviving winter frozen soil. Therefore, in addition to cyst formation, naked amoebae may survive harsh, frozen winter soil in a dormant or resting stage that is capable of rapid resumed growth in spring, thus gaining an immediate competitive advantage in exploiting food and other environmental resources early after the winter thaw.

Key words: Cold adaptations, cryoprotective dormancy, resting cells, winter freeze resistance.

INTRODUCTION

Protists have evolved a variety of adaptations to survive harsh or unfavorable environmental conditions. Among these, cyst formation is an effective means of avoiding desiccation, or to avoid starvation when food or other resources are limited. This is particularly notable among freshwater and terrestrial protists, including naked amoebae. However, cyst formation usually requires substantial reorganization of the protist cell, and in many cases involves the synthesis and deposition of cyst wall material. Hence, it is energy and resource expensive. Moreover, current experimental evidence indicates that the process of encystment as well as excystment requires hours (Chambers and Thompson 1974, Kaushal and Shukla 1977), for example, minimally nine hours in *Vermamoeba vermiformis* (Fouque *et al.* 2014), and therefore may not provide a supple, survival response mode for relatively rapid environmental changes such as freezing and thawing. Other responses to unfavorable environments, less harsh than desiccation, involve various forms of resting stages where the cell undergoes reorganization, often with cytoplasmic condensation and modification of major organelles leading to a dormant state, but not necessarily with deposition of an enclosing protective envelope or wall. Among other examples of resting stages in protists (e.g.

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Kawecka and Eloranta 1986, Osafune and Schiff 1984, Scheer *et al*. 1986), diatom resting cells that are formed in conditions of extreme cold and limited light have been extensively documented in marine and freshwater environments; and their fine structure and physiological characteristics are well known (e.g. Anderson 1975, 1976; Hargraves and French 1983; Sicko-Goad *et al*. 1986; Souffreau *et al*. 2013). Some protists, in environments with below freezing temperatures, have physiological adaptations that prevent cell damage due to ice formation during the freeze-thaw cycle. These include membrane protective modifications and synthesis of cryoprotective (anti-freeze) substances that reduce or eliminate ice crystal formation that disrupts vital cell structures (e.g. Bayer-Giraldi *et al*. 2010, Gwak *et al*. 2010, Jung *et al*. 2014, Laybourn-Parry 2002). Some terrestrial diatom resting cells also are reported to be freeze-thaw tolerant and are likely winter hardy (Souffreau *et al*. 2013). Presently, there appears to be no previously published evidence for freeze-thaw protective stages of terrestrial amoebae, other than cysts. Given the significant ecological role of naked amoebae in terrestrial environments, especially as predators on bacteria (e.g. Anderson 2012, Clarholm 2002, Geisen *et al*. 2014), evidence of amoeba survival adaptations that provide greater advantages in exploiting the environment may have particularly important consequences for terrestrial microbial food webs and soil fertility.

This is a report of laboratory experimental evidence indicating that some terrestrial amoebae, dwelling in temperate terrestrial environments, are capable of surviving soil freeze-thaw cycles in a non-encysted state, with relatively rapid resumption of growth when soil is thawed and warmed above freezing temperatures. The hypothesis evaluated is: "Some terrestrial amoebae have evolved freeze-resistant stages during late autumn chilling of moist soil containing active trophonts that permit them to survive the freeze-thaw winter seasonal cycles in a viable, probably resting state with the capacity to resume relatively rapid growth upon thawing of the frozen soil and arrival of more favorable growth conditions."

Two experimental approaches were used as explained more fully in the Materials and methods section.

1) Moist winter soil samples were collected and frozen for one week to ensure that the total soil mass was fully frozen solid. Each soil sample was allowed to thaw at room temperature, and immediately analyzed for the proportion of viable, active amoebae relative to encysted amoebae using a standard culture observation method (COM). Evidence that the number of active amoebae exceeded the number that was encysted would support the hypothesis that viable non-encysted amoebae survived the freeze-thaw event.
2) Moist early winter soil samples were collected and maintained at 5°C for two days to ensure they were cold adapted. Each soil sample was divided into two subsamples, one was a control and retained at 5°C, the other was frozen for at least one hour to ensure that it was solidly frozen. Both were brought to room temperature and immediately prepared for COM analysis for active and encysted stages. Upon thawing, comparable evidence of active amoebae in the frozen sample and the control would support the hypothesis that there were freeze-resistant, viable stages of the amoebae, comparable in number to those in identical soil samples that were not frozen.

MATERIALS AND METHODS

Sampling sites and soil sample collection

Soil samples were obtained from three sites on the Lamont-Doherty Earth Observatory campus (Torrey Cliff, Palisades, N. Y.). Sample 1 was obtained from soil in the understory of a stand of deciduous trees (organic content: 25%, moisture content 38%). Sample 2 was taken from a sparse grassy site with more mineralized soil at the margin of the stand of trees (organic content: 9%, moisture content: 31%). Sample 3 was obtained from a marshy margin of a pond near dense stands of *Spartina* grass (organic content: 10%, moisture content: 35%). The geographic coordinates are: Sample 1 (41.004147, –73.90871), Sample 2 (41.004149, –73.908653), and Sample 3 (41.003608, –73.907593). Soil samples were taken using a 2.5 cm diam. Lamotte® Soil Sampler to a depth of ~ 5 cm. Samples from sites 1 and 2 were obtained in January 2015 immediately before the first hard freeze, and samples from site 3 were taken in March 2015 just as the soil was thawing but still at a temperature close to zero degrees Celsius. Samples were deposited in plastic bags and returned to the laboratory for immediate processing as described below.

Experimental procedure 1

This experiment examined the effects of controlled laboratory freezing of soil samples on the densities of freeze-resistant active amoebae compared to encysted amoebae in the soil, when assessed immediately after thawing of the soil. Each experiment was done in duplicate from each of the three sampling sites (1–3) and labeled accordingly: 1A, 1B; 2A, 2B; and 3A, 3B, respectively). A thoroughly mixed portion of each soil sample was deposited in closed Nalgene bottles and placed in a deep freeze for one week prior to additional processing. After one week, each Nalgene bottle was removed from the freezer, examined to ensure that the soil was fully frozen, and prepared for further analysis. Each bottle was allowed to come to

room temperature at 25°C (less than 30 min) before further analysis. The frozen sample was immediately prepared for enumeration of active amoebae and of cysts using a standard culture observation method (COM), including a drying stage for cyst assessment, as widely published previously with various modifications (e.g. Anderson 2000, 2004; Butler and Rogerson 1995; Geisen *et al*. 2014). The COM procedure, including the dried preparation for cyst enumeration, is presented below. Based on the COM analyses, the densities (no. g^{-1} soil dry wt.) of active amoebae, encysted amoebae, and percent encysted were reported for each of the freeze treatments and controls for the six samples (1A–3B).

Experimental procedure 2

This experiment examined the effects of controlled laboratory freezing on the densities of freeze-resistant active amoebae compared to encysted amoebae assessed immediately after thawing of the soil. Each experiment was done in duplicate for soil taken from sampling sites 1 and 2. The soil was kept at 5°C for three days to ensure that the amoebae were cold adapted before beginning the freezing experiment. Each of the soil samples was thoroughly mixed and divided into two portions, one that was used as the treatment condition to be frozen and the other as the control maintained at 5°C. Ten g were taken for the frozen treatment sample and placed in a plastic zip-lock bag, compressed into a flattened mass to ensure rapid freezing, and placed directly on the surface of the freezing coils of the freezing compartment of a refrigerator for at least 60 min to ensure that the soil was solidly frozen. The remaining soil in a zip-locked bag served as a control sample and was maintained at 5°C for the duration of the 60 min. Thus, the only variable in the experiment was the presence or absence of freezing. At the end of 60 min, the frozen sample was removed and examined to be certain it was solidly frozen. Both samples were allowed to come to room temperature (less than 30 min) before further analysis. The frozen sample and the control sample were immediately prepared for enumeration of active stage amoebae and of cysts using the standard culture observation method (COM), that was modified to include a drying stage for cyst assessment. Densities of active amoebae in the frozen treatment were compared to densities of active amoebae in the control to determine the relative effects of freezing on the active state of the amoebae. An additional extension of the experiment was done to determine changes, if any, in the densities of active amoebae during freezing for an additional time of two weeks and eight weeks. One of three replicates of soil samples for the frozen treatment were placed in the freezer for one hour on the initial day the experiment was begun, and two others were retrieved at two weeks and eight weeks later, respectively, to assess the densities of active amoebae using the COM after an extended time of freezing.

The culture observation method of enumerating encysted and total active amoebae

One g of soil to be assessed is suspended to a total volume of 6 ml using 0.45 µm pore-size filtered pond water (Carolina Biological, Burlington, North Carolina). Hence, the dilution is 1/6. This suspension is further diluted with the filtered pond water to be in the range of either 1/20 or 1/30 dilution for purposes of assessing the densities of active amoebae by the COM as explained at a later point. The initial suspension at 1/6 dilution is used for the cyst enumeration. For cyst enumeration, a 30 µl aliquot is micropipetted into the base of each dry well in a 24-well Falcon culture dish. The aliquots are immediately dried by exposure to flowing air at room temperature from an electric blower. This rapid drying kills all of the non-encysted amoebae but should not kill mature cysts that are adapted to withstand soil desiccation. When the drops of suspension are completely dried (typically within 10 to 20 min), each well of the Falcon dish is emended with a small drop of malt/yeast nutrient agar (Page 1988) that is placed opposite to the side of the well where the inoculum was placed. The nutrient agar is used to support bacterial growth as food for the excysting amoebae. Two ml of micropore-filtered pond water are added to each well, and the Falcon dish is covered and allowed to incubate at 25°C for 10 to 14 days. At that point, each well is inspected using an inverted compound microscope with phase optics to identify each morphotype of amoeba that excysted in the well from the dried aliquot (typically no more than one or two different morphotypes are detected per well, if any). The assumption is that no more than one cyst of a particular amoeba morphotype is included in the 30 µl aliquot, and therefore each instance of a morphotype indicates that at least one cyst of that amoeba was introduced in the original 30 µl aliquot. Based on the number of amoebae morphotypes tallied in the total of 24 wells, the number per ml of original suspension is calculated on the basis that there are 720 µl of inoculum all totaled in the Falcon dish (i.e. 24 wells × 30 µl inoculum per well). The number of amoeba morphotypes per ml is converted to number g^{-1} soil dry weight, based on the equivalent amount of dry soil that was suspended in 720 µl of inoculum.

The total number of viable amoebae in the soil suspension is enumerated by a modification of the method described above for the encysted amoebae. Instead of drying the aliquot initially, each well of the 24-well Falcon dish that has an added drop of nutrient agar is filled with two ml of the micropore filtered pond water before the 30 µl of inoculum is added. This allows all viable amoebae to proliferate from the 30 µl aliquot added to each well (active forms and excysting forms). Then, the dish is covered and incubated at 25°C for 10 to 14 days to permit proliferation of each morphotype before microscopic examination. The densities of amoebae are calculated as explained for the cyst preparation, and expressed as no. g^{-1} soil dry weight. This yields an estimate of the total number of viable amoebae, active and those that excyst from the suspension added. To obtain the number of active trophonts in the original suspension, the density of amoebae estimated from the encysted analysis is subtracted from the number obtained from the total viable amoebae. The percent encysted is calculated as (density of encysted amoebae/density of total amoebae) × 100.

Because an accurate estimation of the density of encysted amoebae using the drying technique is critical to the validity of the conclusions of this research, a check on the accuracy of the method was made. If the drying method, for example, caused loss of some of the encysted amoebae, this would bias the results of the experiments by indicating an erroneously low density of encysted amoebae. To check the method, a sample of soil from site 1 was allowed to dry slowly at room temperature, while enclosed in a lightly sealed zip-lock bag, until the soil was completely desiccated and all amoebae remaining would have to be encysted. This soil sample was prepared for analysis by suspension in micropore-filtered pond water, and 30-µl aliquots were analyzed both by the drying method and by the non-drying set up; where in the latter, the aliquots of the suspended soil were added directly into the wells containing two

ml of pond water and nutrient agar. If the drying step altered the estimation of the encysted amoebae, we would expect to find a difference in the counts in the dried preparation compared to the wet preparation, since both received identical aliquots of a suspension containing only cysts. The analysis was repeated in triplicate. The results, expressed as the mean percent agreement between counts in the dried and wet preparations (mean percent ± SE), was 88 ± 3 percent. This indicates that the drying method, widely used previously (e.g. Anderson 2000, 2002, 2004), was sufficiently reliable in estimating the number of encysted amoebae. In general, the error in enumerating amoebae using COM is ± 4% (Anderson 2002). All microscopic observations were made using a Nikon Diaphot inverted microscope with phase contrast optics.

RESULTS

Experimental procedure 1

Densities of active (trophont) naked amoebae, encysted amoebae, and percent encysted amoebae obtained by COM analysis of thawed-soil samples are presented in Table 1. All six of the samples had substantial densities of active amoebae recovered from the thawed soil, varying from 700 to 3000 amoebae g^{-1} soil dry wt., and percent encysted varying from 7 to 29%. These data support the hypothesis that active amoebae in sufficiently moist soil that is frozen and subsequently thawed are capable of surviving the freeze-thaw event and resume active growth. This experiment, however, does not provide evidence if there are any differences compared to equivalent soil samples that were not frozen. Results from a control experiment to address this question are presented in the next section.

Experimental procedure 2

The data in Table 2 show the results of four replicates of a controlled experiment comparing the densities of amoebae in short-term (one-hour) frozen samples compared to control samples of the same soil that were not frozen. The experiments were limited to one hour to ensure that the control samples maintained at 5°C would not likely proliferate and bias the results. While the data in Table 2 show that there were some variations in the densities of the amoebae between the experimental and control groups among the individual experiments (in some cases with more in the treatment group), the means of the experimental and control groups (860 and 840, respectively) are sufficiently comparable, within the range of the error of the COM method (± 4%), to conclude that the freeze-thaw treatment apparently did not adversely affect the densities of viable amoebae when compared to the non-frozen control soil samples. The mean percent encysted amoebae in treatment and control samples was 18 and 20%, respectively, indicating as reported in the first experiment, that the preponderance of amoebae in both the control and treatment conditions were active rather than encysted. These data further support the hypothesis that winter chilled soil, when frozen, contains active amoebae, possibly in a resting state, that are capable of surviving freeze-thaw events and resume active growth.

To further determine the survival capacity of the freeze-tolerant amoebae, a subsequent experiment was added to examine the densities of amoebae that were viable after freezing for one hour, and then for two and four weeks later, all using subsamples from the same soil sampled at site 1. The results are shown in Table 3. While there appeared to be a lower number (500) in the two-week sample compared to the initial densities, the four-week sample showed nearly identical densities (600) to the initial sample, thus suggesting that approxi-

Table 1. Densities (no. g^{-1} dry wt.) of active trophonts, encysted amoebae and percent encysted terrestrial amoebae in frozen-treated samples from soil at three sites, with duplicate samples taken at each sampling site

Site	Active	Encysted	Encysted (%)[a]
1A	1400	180	12
1B	1800	140	7
2A	1000	400	29
2B	3000	450	13
3A	854	300	26
3B	700	130	15

[a]Percent encysted = [no. encysted/ (no. active + no. encysted)] × 100.

Table 2. Densities (no. g^{-1} dry wt.) of total amoebae obtained from freeze-thaw treated and non-frozen controls taken from the same soil sample in short-term freezing experiments

Sample[a]	Treatment	Control
1	2000	2030
2	750	630
3	500	400
4	180	300
Means	860	840

[a]Mean percent encysted amoebae in treatment and control samples was 18 and 20, respectively. Soil samples 1 and 2 are from site 1 and samples 3 and 4 are from site 2.

Table 3. Densities (no. g^{-1} dry wt.) of active trophonts, encysted amoebae and percent encysted terrestrial amoebae in frozen-treated samples during a longitudinal study at the initial day of freezing for one hour and after two and four weeks of continuous freezing

Day	Active	Encysted	Encysted (%)
1	620	180	23
14	500	150	23
28	600	200	25

mately one-month of freezing had negligible effect on the viability of the freeze-resistant stages of the soil amoebae. A wide variety of naked amoeba taxa were observed in the freeze-treated soil samples, including *Acanthamoeba*, *Cochliopodium*, hartmannellids, vahlkampfiids, *Vannella*, and large reticulate amoebae (> 100 µm in some cases) resembling vampyrellids.

DISCUSSION

Two lines of evidence support the hypothesis that terrestrial naked amoebae survive freeze-thaw cycles as non-encysted, freeze-resistant cells capable of resuming active growth when favorable environmental conditions resume. The evidence from Experiment 1 shows that non-encysted amoebae survive freezing and thawing of moist soil, and that the number of active amoebae that resume growth with densities ranging from 700 to 3000 g^{-1} soil dry weight are substantially greater than those encysted. Encysted amoebae in the treated soil ranged from 7 to 29% of the total amoebae capable of resuming growth, further indicating that there is a preponderance of freeze-tolerant, non-encysted amoebae. Densities of active amoebae found in the thawed soil are comparable to densities of amoebae typically found in soil at this temperate locale (e.g. Anderson 2000, 2002) further suggesting that active amoebae surviving the freeze-thaw treatment are within the range of densities typically found in moist temperate soil before freezing. The additional line of evidence in Experiment 2 (Table 2) provides evidence that the densities of active amoebae surviving a freeze-thaw treatment cycle are comparable to those in a control sample of the same soil that was not frozen, with mean densities of 860 and 840 g^{-1} soil dry weight, respectively. The percentages of encysted amoebae (18 and 20) are within the range reported for Experiment 1 (Table 1). Moreover, given current evidence that encystment of naked amoebae requires several hours, this further supports the conclusion that rapid freezing (within minutes) of the soil sample could not likely induce cyst formation, and further supports the conclusion that evidence of viable amoebae after two hours of freezing cannot be attributed to survival in the cyst stage. The extension of Experiment 2 to include a four-week-long study provides evidence of longer term survivability of the freeze-resistant amoebae. However, additional longer-term studies are needed, approximating the length of a winter season, to test the survivability of the freeze resistant stages. In this regard, it should be noted that samples 3A and 3B (Table 1) were obtained in very early spring 2015 when the soil was just thawing and near a temperature of zero °C after a very cold northeast U.S.A. winter. The densities of active, freeze-resistant amoebae, though lower than those in the other two sites (1 and 2), were substantially larger than the number that were encysted at site 3. This suggests that there was a fairly substantial residual pool of freeze-resistant amoebae in the soil after the 2014–2015 winter freeze. The percent encysted are not too different from those of the other two samples. The marshy habitat at site 3, moreover, was very different from the wooded area and grassy location at sites 1 and 2, which may also account for some of the differences in densities of freeze-resistant amoebae between site 3 samples and those in sites 1 and 2.

Although this is one of the first studies to make a more extensive examination of possible freeze-resistance of soil amoebae, Chang (1958) reported that freezing of the heterolobosean *Naegleria gruberi* in the flagellate stage had no adverse effect on its ability to return to an amoeboid form when the freezing time did not exceed 30 min. At present, it is not possible to determine the morphology or the cellular organization of the freeze-resistant soil amoebae examined in this study. Soil-dwelling amoebae are closely attached to soil particle surfaces, or dwell deeply in the interior of soil particles. Therefore, it is not possible to view them by direct light microscopic examination in the living state. And it is even more difficult, if not impossible, to observe them in solid frozen soil. The current evidence for the densities of the amoebae surviving the freeze-thaw cycle is based on a widely adopted culture observation method that permits microscopic enumeration of the number of morphospecies that proliferate from a very small aliquot of soil suspension that is added into the culture wells of a Falcon culture dish. The aliquots of soil suspension used in the COM are taken imme-

diately after the soil thaws; therefore, there is no possibility that the amoebae have proliferated before the enumeration technique was begun.

The viable, non-encysted amoebae in the frozen soil are very likely in a dormant state similar to the resting cells found in other protists as cited in the Introduction, including freeze resistant stages that contain cryoprotectant proteins to prevent ice crystal formation and cell damage (e.g. Gwak *et al*. 2010, Jung *et al*. 2014). However, this assumption needs to be further evaluated if appropriate experimental conditions can be devised to collect sufficient soil-free amoebae from frozen soil particles, or other preparations that simulate soil particle environments, to allow detailed microscopic and physiological examination as has been done with other protist resting cells (e.g. Anderson 1975, 1976; Sicko-Goad *et al*. 1986; Souffreau *et al*. 2013). In addition to physiological changes in the amoebae favoring freeze survival, the physics of freezing of the thin surface water films on soil particles, and the presence of organic molecules and solutes in the soil water, may alter the structure of the frozen water film, thus partially protecting the amoeba cells against excessive ice pressure that may develop when bulk phase water freezes. In this regard, additional research is needed to determine if amoebae in aquatic environments also form freeze-resistant stages, particularly in relation to where they are dwelling, either in the sediments or suspended in the water column.

There are clear survival advantages of resting cells, compared to cysts, when the environmental challenges are not so severe as desiccation. As mentioned in the Introduction, resting cells observed in diatoms typically undergo cytoplasmic transformations including condensation of the cytoplasm, reduction in metabolism, and the deposition of reserve substances but no wall or other enclosing structures are deposited (e.g. Anderson 1975). Consequently, the transformation requires less energy demand and material costs compared to cyst formation, especially cysts with substantial organic walls. Moreover, resting cells can make rapid recovery and active growth when favorable environments resume (Anderson 1976, Sicko-Goad *et al.* 1986). This rapid recovery may enhance the competitive advantage of the resting protist by gaining an early exploitation of available prey and greater reproductive potential than those in the cyst stages that are typically more delayed in becoming active, requiring several hours (e.g. Fouque *et al.* 2014). Furthermore, a resting stage may be more supple in responding to sporadic and rapid changes in unfreezing of soil; such as sporadic periods of rapid thawing during warmer spells, and possibly surface layer defrosting of frozen soil when insolated by sunlight on sufficiently exposed soils.

However, such strategies in terrestrial protists are effective only when the soil has moderate to optimal moisture prior to the onset of winter freezing, otherwise most of the desiccation-resistant protists will have encysted. In many temperate regions, late autumn precipitation that is accompanied by cooler temperatures that reduces evaporation, and early winter snows before the soil freezes, can ensure sufficiently moist soil to favor a substantial population of active trophonts. This increases the likelihood that a substantial proportion of the amoebae are in an active state before the onset of freezing. Additional research is needed to determine if chilling of the amoebae, prior to soil freezing, is a necessary stimulus to physiologically prepare the amoebae to become cryoprotected. There is substantial evidence that amoebae cysts are also freeze-resistant (e.g. Lloyd 2014, Matoba *et al*. 1989, Osato *et al*. 1991, Siddiqui *et al*. 2008), as may be expected, because the amoebae cyst cytoplasm is significantly condensed and clearly is in a dormant condition. However, most of the research on cyst survival during freezing is limited to a few species, and more studies are needed to examine this phenomenon in a broader range of species.

The exploratory research presented here on freeze-resistant stages of temperate terrestrial naked amoebae suggests that there is a fruitful field of further research to be pursued on the physiological ecology and adaptive strategies of amoebae to efficiently survive deep cold winter conditions, including how they develop protective mechanisms against potentially lethal freeze-thaw cycles, and rapidly resume growth when favorable environmental conditions return in spring. From a broader perspective, research on the role of resting cells in protist evolution, adaptation, ecology and physiology is not well developed; and among other topics, more attention is needed to better document resting cell occurrence across a broader taxonomic range of protists as well as exploring the range of varied environments where resting stages are most frequently observed.

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