

How To Culture Limnoterrestrial Heterotardigrades: A Case Study In The *Echiniscus*, *Pseudechiniscus* And *Viridiscus* Genera

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Methodology

Keywords: Tardigrades, Heterotardigrades, Culturing, Viridiscus, Echiniscus, Pseudechiniscus, Tun-formation, Desiccation, Moss, Algae

Posted Date: June 11th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-578089/v1>

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Abstract

Background: Tardigradologists have long tried to culture limnoterrestrial heterotardigrades without success. This is likely because they depend on specific conditions in their microhabitats. Most limnoterrestrial heterotardigrades are found associated with bryophytes and lichens (collectively cryptogams). In contrast to eutardigrades, many of which are easily cultured, heterotardigrades are found in cryptogams that prefer drier and sunnier habitats and that tend to grow on the vertical surfaces of boulders, cliffs, or tree trunks. By carefully observing heterotardigrades in the *Echniscus* and *Viridiscus* genera over many months, we determined that these organisms feed on chloroplasts and cytoplasm of both moss cells (typically moss protonema) and single-celled green algae associated with the moss (typically *Chlorella vulgaris*). We also determined that the cryptogams they associate with, and hence the heterotardigrades themselves, spend more time in a dried state than most eutardigrade species. Taking these observations into account we varied food, water, and desiccation cycle conditions with the aim of developing a culturing protocol for heterotardigrades.

Results: We have maintained laboratory cultures of several generations and counting of *Echiniscus*, *Pseudechiniscus* and *Viridiscus* genera tardigrades using the following experimentally derived protocol: Both moss and algae from their natural habitats are required as food sources and a small layer of rain or spring water is added every morning and allowed to evaporate some overnight. Furthermore, the organisms are more likely to lay eggs on a dense mat of moss protonema, grown by inserting tips of moss branches into a solidified KCM / agar medium. The medium also provides a walking substrate for the tardigrades, and possibly a source of cations. Crucially, the cultures must be allowed to dry out completely every ten days for a period of at least three days. Moss in the culture dish significantly improves the chances of the tardigrades coming out of their desiccation-resistant states successfully.

Conclusions: We conclude that periodic drying out, moss and algae are all required to successfully culture heterotardigrades. Furthermore, drying must occur slowly, and the moss protonema enables this by retaining moisture thereby slowing evaporation. We suspect our protocol will work for most limnoterrestrial heterotardigrades with minor tweaking of culturing conditions.

Background

Tardigrades are small, aquatic invertebrates that are ubiquitous in all marine, freshwater, and limnoterrestrial habitats on earth. They are categorized into two main classes, the unarmored eutardigrades, and the armored heterotardigrades and the number of documented species is ca. 1300 and counting (1). Whereas some tardigrade species are cosmopolitan, others have endemic distributions (2, 3). Tardigrades are often called the “toughest animals on earth” due to their abilities to survive extreme environmental conditions well beyond the limits of other animals (4). In response to desiccation and other types of rapidly deteriorating environments, many freshwater and limnoterrestrial tardigrade species are able to enter a volume reduced, ametabolic state known as a “tun” exemplified by the near complete

loss of all intracellular water and a number of molecular modifications to protect cellular components in this state (5, 6).

The ability to rear tardigrades under laboratory conditions enables replicable experiments to study various aspects of their fascinating biology (7). There is however, no unified protocol for culturing all tardigrade taxa, and all available protocols proposed to date concern eutardigrades only (3, 8). The published methods differ depending on the specific environmental requirements of different species (9–11). Furthermore, many culturing protocols have been proposed by different authors for different tardigrade species or even different methods for the same species (2, 12–15). These methods differ primarily in the type of food they use, walking substrate, photoperiod, and ambient temperature (8, 16). Aside from having a suitable, oxygenated water source, the most important requirement for successfully culturing tardigrades appears to be their food source (2, 17). However, ecological studies concerning their dietary habits and other behaviors are rare (3, 18). Current studies have indicated that carnivorous tardigrades mostly feed on nematodes, rotifers, other small invertebrates, and even other tardigrades, while a herbivorous diet consists of algae or bryophyte leaf cells (1, 19). Some tardigrade species are omnivorous, and others detritivores (3). Studying tardigrade feeding behavior furthermore allows us to understand their distributions and roles in the food web, e.g. prey-predator interactions (17).

Von Wenck performed the first attempt of culturing tardigrades in 1914 by keeping them in an aquarium (20). His study provided useful information about their mating behavior and embryology. Some of the other early studies during the 19th century used algae and agar for providing food and a walking substrate in glass petri dishes, distilled, tap, or demineralized water as a medium, and *Chlorella*, *Chlorococcum*, blue-green algae, nematodes, or diatoms as their food (21–25). More recent studies contain more detailed culturing protocols for eutardigrades and provide information about their life cycles. Kagoshima et al used 1.8% agar, 1x Bold Modified Basal Freshwater Nutrient Media (BMBFN), and 5 µg mL⁻¹ cholesterol with distilled water in petri dishes for culturing *Acutuncus antarcticus*. Cyanobacteria and green algae isolated from the moss they were found in were used as their food. Tsujimoto et al cultured *A. antarcticus* in individual wells of tissue culture plates coated with 1.5% agar gel on the bottom using *Chlorella sp.* algae as a food source, a 2 mm layer of Volvic water, and keeping the cultures at 15°C in the dark (26). For culturing *Ramazzottius varieornatus*, 2% Bacto agar (Difco) gel, Volvic mineral water, and *C. vulgaris* algae were used. Animals were transferred to new petri dishes every week, and eggs and juveniles were examined to study life-history traits (27). Tumanov cultured *Hypsibius pallidoides* for a redescription study. He used a mixture of distilled and filtered tap water as a medium. Instead of agar gel, the petri dishes were scratched with sandpaper to provide a suitable substrate for tardigrade locomotion. They were fed with unicellular *Chlorella sp.* algae and kept at 16°C in the lab (28). For culturing *Paramacrobotus sp.*, Suma et al used 2% agarose with KCM solution as a medium. Cultures were kept in the dark at 20°C and animals were fed with *C. elegans* (29). *Paramacrobotus* tardigrades were also cultured with different protocols in Sugiura et al study by using a 1.2% agar gel for coating the bottom of 30 or 90 mm petri dishes. *Lecane inermis* rotifers and *C. vulgaris* algae were used as their food source and mineral water as a medium (30).

Culturing heterotardigrades has proved more challenging, and as far as we are aware, there is no published culturing protocol available for them despite decades of culturing attempts. This is likely because they are more dependent on the specific conditions of their microhabitats to survive, thrive and reproduce. The limnoterrestrial heterotardigrades studied here are generally found in bryophytes or lichens growing in drier and sunnier habitats (Pienaar, pers. obs.). Whereas all of the existing tardigrade culturing protocols are wet cultures with a continuous film of water, and animals are always active in these protocols (8, 19, 31), limnoterrestrial heterotardigrades appear to spend a lot of their time in a dried state. Studying heterotardigrades diet preferences will also help to identify suitable food sources for them under laboratory conditions. For example, Schill et al. identified the moss *Grimmiaceae* in the gut of *Echiniscus granulatus* (31). Some other studies recorded moss chlorophyll organelles as the *Echiniscidae* family's main source of food; however, both algae and fungi could be part of their diet (19). Here, based on observations of natural feeding habits and wet / dry conditions, and making use of what has been learned from the eutardigrade culturing literature, we aimed to experimentally determine a viable culturing protocol for limnoterrestrial tardigrades.

Results

We report for the first time a viable culturing method for heterotardigrades in the *Echiniscus*, *Pseudechiniscus*, and *Viridiscus* genera, and to date have successfully maintained healthy cultures of these organisms for close to two years (> ten generations for all species).

Pilot culturing experiments - For observation purposes, *Echiniscus* sp. and *Pseudechiniscus ramazottii* tardigrades isolated from *Pylasia selwynii* mosses growing on the upper surface of *Juniperus virginiana* branches and *Viridiscus viridianus* tardigrades, isolated from a mix of *Andreae* and *Grimmia* sp. mosses and *Xanthoparmelia* sp. lichens growing on sun-exposed boulders were initially placed in 10cm petri dishes coated with a 1.2% KCM medium / agar gel, along with samples of the cryptogams they were found in. Following eutardigrade culturing protocols, these were maintained as wet cultures for a period of three months by placing vented lids on the Petri dishes to minimize evaporation. The water was replaced every three days by carefully pipetting the old water out and adding new, locally collected rainwater, and. Our reasoning was to provide fresh oxygen and reduce microbial loads. During this three month period, *Echiniscus*, *Pseudechiniscus*, and *Viridiscus* tardigrades were observed to be feeding both on small single-celled algae identified as *Chlorella vulgaris* and moss protonema which started to grow on the agar (**Fig. 1A**). They use their piercing stylets and feeding straws (**Fig. 1C, D**) (32, 33) for sucking up the chloroplasts inside the algae and moss protonema (3, 34). These attempted cultures would invariably yield three generations of tardigrades at most before dying out.

Additional experiments - when experimentally drying our pilot cultures in the presence or absence of the moss branches they were found in, we recorded that 84% of tardigrades in the tun state were specifically found in the adaxial base of moss phyllids (see **Fig. 2B**), and 96% of these become active again 30 minutes after addition of water. Of the 16% of tardigrades that form tuns elsewhere, only 48% became active again upon the addition of water, suggesting that the moss may play a role in modulating

successful tun formation and or /exit. We also noticed however, that upon rewetting the dried out cultures with rainwater, egg production increased as compared to the cultures that were maintained constantly wet.

Optimizing culturing conditions - Based on the initial observations and additional experiments outlined above, a wet / dry cycle of allowing partial evaporation over 24 hours before replenishing the water substrate (by leaving the vented lids off the Petri dishes in an airconditioned room at 55% relative humidity and maintained between 20 to 25°C) and allowing for near-complete dehydration over 3 days every 10 days was found to yield the highest increase in heterotardigrade numbers for all three species. Lower temperature led them to become inactivated and to enter encystment (a form of dormancy typically used to overwinter). Although all the tardigrades studied here can extensively feed on *C. vulgaris*, the presence of growing mats of moss protonema in the culture dishes is critical and their absence has a negative impact on their survival. Light cycles of 12:12 light / dark yielded better culturing results as compared to 8 hours of light. Both rain and mineral water were found to be suitable media, whereas deionized and tap water or mixes containing these water sources very quickly killed the cultures. The size of the culturing container (within the range of 2.5 cm to 10cm Petri dishes) does not seem to have an effect on culturing efficiency

Reproductive Process - As mentioned earlier, moss protonema is an important factor for successful culturing (35). Observations of the cultures reveal that they provide a preferred environment for heterotardigrades to lay eggs (**Fig. 3**). *Viridiscus* and *Echiniscus* heterotardigrades were observed to lay between 1–4 eggs inside of their cuticles, and these cuticles invariably stick to the moss protonema until the larva hatch. Most of the individuals under laboratory conditions only laid one egg before shedding their cuticles (**Fig. 4**). These species appear to be mostly parthenogenic, but we note that males have recently been observed amongst *V. viridianus* cultures (Momeni pers. obs)

Discussion

The “recipe” for successfully culturing limnoterrestrial heterotardigrades can be summarized as follows: insert live moss shoot tips into a KCM / 1.2% agar gel. Mist them daily with rain / mineral water and allow them to grow protonema (this process typically takes a month or more but can be sped up by growing them under 24 hrs of light). Once the protonema medium almost fully covers the petri dish, add a drop of water containing pregrown *C. vulgaris* algae (see methods), add a 1-2ml layer of a 50:50 mix of rainwater and mineral water (or one or the other) and introduce the heterotardigrades into the Petri dishes. Allow the water to partially evaporate overnight, and rehydrate each morning with 1–2 ml of water and add 20–60 µl of *C. vulgaris* to each petri dish. Adjust the light cycle to a 12:12 light / dark cycle. Half of the tardigrades should be transferred to new petri dishes after 2–3 months (by gently sucking them up with a pipette) to prevent overcrowding. The KCM / agar substrate also needs to be inspected every month as it can disintegrate (possibly due to agar eating bacteria). In this case, all tardigrades should be transferred to a newly made Petri dish with moss protonema as they cannot survive for long without the medium (a week at most). This protocol results in healthy, multiple generations of growing tardigrade populations.

Drying down overnight and adding water every morning for ten days is an important factor in this protocol and we suspect it is required so as to provide enough oxygen for these armored tardigrades. Many of the eutardigrade protocols mentioned in the introduction rely on lowering the water temperature to maintain sufficient oxygen levels, but as shown here, this induces long term encystment in heterotardigrades, thus another mechanism is needed. Although an aquarium style oxygen pump may perform the same function, we suspect there are other benefits to the drying / rewetting cycle, and this most likely mimics their natural cycle more closely where dew formation in the morning could provide moisture that dries out during the day in the sunny areas these tardigrades tend to be found in. Every ten days, we let the petri dishes dry out completely for a period of three days as our previous culturing attempts revealed that we could not get more than three generations of tardigrades if they are maintained constantly wet (see also 17). We suspect that drying out periodically like this may allow the very slow-moving heterotardigrades to reduce pathogen loads, especially fungal growth (e.g. see Loefelholz et al. in review). However, even though we found dehydration to be an essential factor for heterotardigrades to reproduce, we also do not recommend less than one week between periods of dehydration since that will also decrease the survival rate (36). In this case, the active time for foraging and reproducing may be too short. These factors will likely vary for different species of heterotardigrade, and we recommend that they be experimented with when attempting to culture other species.

Our experiments with and without moss (see additional experiments in results and methods sections) revealed that the presence of the moss is an important factor that allows the tardigrades to successfully exit their tun state again. Mosses can slow down the desiccation process for tardigrades by acting as a moisture-retaining mulch that provides a moist substrate for tardigrades (37, 38). This likely allows them to enter their tun state in a slow and controlled fashion which seems necessary for these species to successfully enter the active state again with rehydration. Spallanzani indicated in 1776 that slow desiccation allowed for successful tun formation and exit and that their substrates aid in this process (39). Other studies also investigated the desiccation process in microorganisms and showed the importance of slow drying during anhydrobiosis (37). It is also possible that tardigrades and mosses communicate chemically during phases of desiccation and rehydration, a possibility that we are currently pursuing in our laboratory. Thus far, for example, we have found that dilute apigenin, a flavonoid compound known to be produced by many moss species when stressed (e.g. desiccated) (40, 41), can induce encystment at very low concentrations and tun formation at higher concentrations in *V. viridianus* tardigrades.

Conclusions

Having the ability to culture limnoterrestrial heterotardigrades in the laboratory significantly increases the armory of the tardigradologist. In particular, it opens these organisms up for experimentation and the application of various modern "omics" techniques. We are currently in the process of sequencing the *V. viridianus* genome for example, which is made possible by being able to grow mostly parthenogenic cultures containing thousands of genetically identical / similar individuals. We hope that the protocol

presented here will be useful to other tardigradologists interested in uncovering the numerous secrets of the enigmatic limnoterrestrial heterotardigrades.

Methods

Source material - *V. viridianus* tardigrades were collected from a mix of lichens and mosses growing on the vertical surface of boulders in and around Tuscaloosa, Alabama. Two of the boulders are from the shore of Lake Nicole (33°17'23" N, 87°29'1" W); (33°17'22" N, 87°29'0" W), and one on a hillside abutting Lake Harris (33°15'54" N, 87°28'5" W). *Echiniscus sp.* and *Pseudechiniscus* tardigrades were collected from mosses growing on the upper side of *Juniperus virginiana* tree branches from Munny Sokol Park, Tuscaloosa, Alabama (33°15'44" N, 87°31'47" W); (33°15'85" N, 87°31'50" W). All samples were stored in acid-free paper envelopes and allowed to dry out for at least 24 hours in an air-conditioned room at 20°C, with 55% relative humidity. The dried mosses were then suspended overnight over deionized water using the custom-built Bauerman pan described by Davison to extract tardigrades from the mosses and lichens (35). The tardigrades were identified to genus or species level (when possible) using the dichotomous keys (34, 42) and primary literature (43–45).

Pilot culturing experiments – 60 mm petri dishes were coated with 1.2% agar in KCM solution (7mg KCl, 8 mg CaCl, and 8mg MgSO₄ in 1L DI H₂O) gel to provide traction (and possibly cations) and a 2mm deep layer of locally collected rainwater was added as a medium. We kept all the meiofauna and small particles extracted from the moss under these conditions in the lab at 20°C and 55% relative humidity, and a 12:12 light-dark cycle for one year.

Additional experiments - Initial observations indicated that having the moss growing along with the tardigrades in the culture chambers is an important requirement for maintaining successful cultures. To investigate this phenomenon further, experimental desiccation and observation of a subset of our joint tardigrade moss pilot cultures (n = 122 tardigrades across ten different petri dishes) were performed to determine if the mosses played a role in enhancing the tardigrades ability to survive the wet / dry cycles.

Optimizing culturing conditions - We next attempted to optimize culturing conditions by varying the wet / dry cycles, food, and water sources (the KCM / agar substrate was used for all experiments as earlier observations revealed that the slow-moving heterotardigrada cannot get to their food sources without it). We varied: 1) the food source by using either the faster growing *C. vulgaris* algae as the sole food source (Soil and water media was used for culturing the algae (Fig. 1.B) according to Belcher & Swale protocol (46), by pre growing mats of moss protonema from the moss shoot tips identified above on KCM / agar gels as a sole food source or by supplementing pregrown mats of moss protonema with *C. vulgaris* as an additional food source; 2) the water source between tap, deionized, mineral, rain and combinations of these; 3) the size of the petri dishes 60 mm, 90 mm, and 120 mm; 4) the length of wet / drying out cycles; 5) the temperature between 16, 20 and 25°C and; 6) the length of the light cycles between 8 and 12 hours of light (Fig. 2).

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and / or analyzed during the current study are available in the [NAME] repository, [PERSISTENT WEB LINK TO DATASETS]

Competing interests

The authors declare that they have no competing interests

Funding

This work was supported by an NSF CAREER award (no: 2045035) to Jason Pienaar.

Authors' contributions

SM performed culturing experiments, maintained the cultures, and wrote the paper. JFG performed pilot culturing experiments, desiccation survival with and without moss experiments, and helped write the paper. JP designed the experiments, performed some of the culturing experiments, made the mistake of leaving the lid of a culture dish and allowing it to dry for two weeks, managed the project, and helped write the paper.

Acknowledgments

We would like to thank Caleb Turberville for identifying the moss species and Jacob Loeffelholz for helping to collect some of the initial samples used to start the cultures. Paul Davison provided many useful tips and tricks for isolating and culturing tardigrades and mosses and we are grateful for his prescient advice.

Authors' information (optional)

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Figures

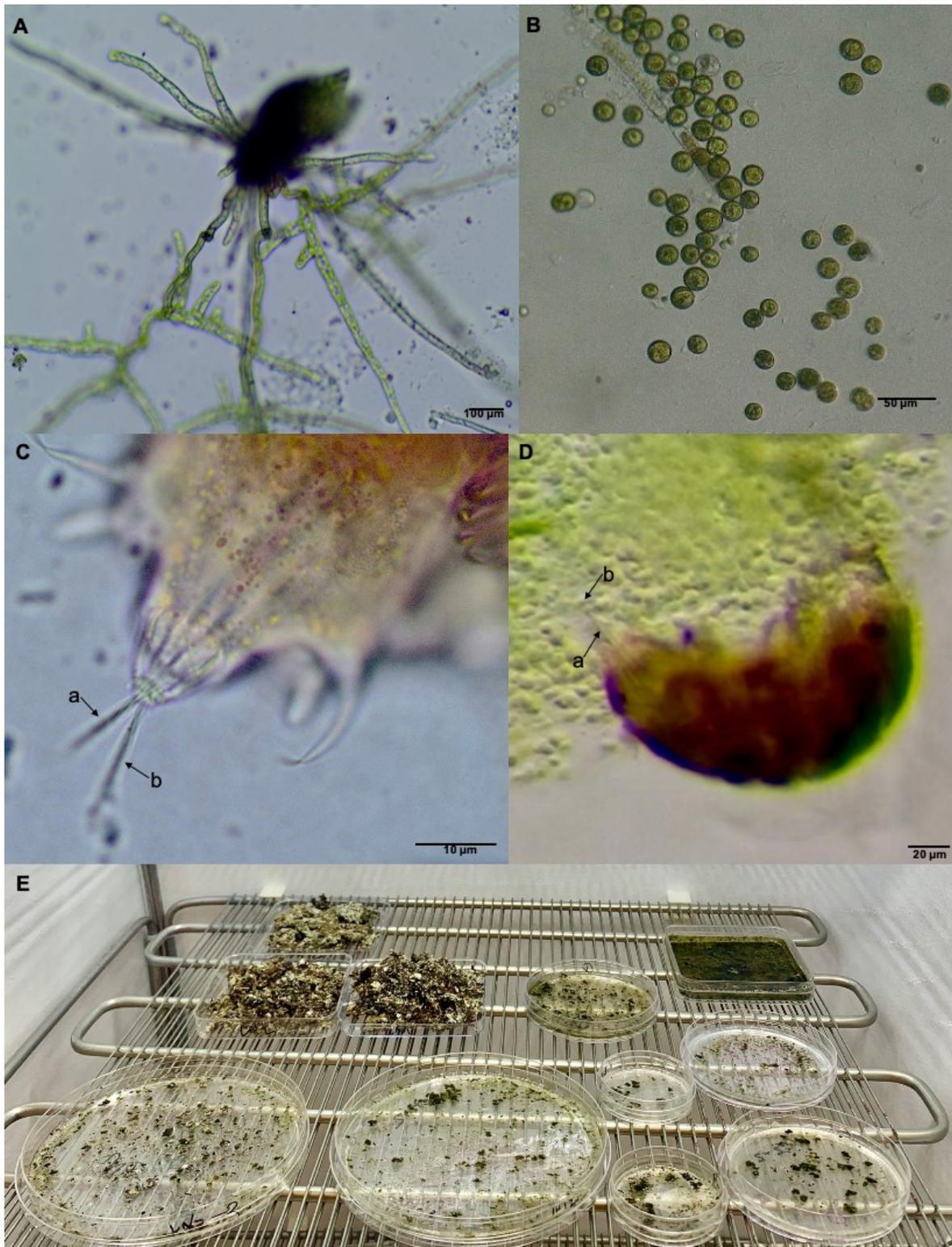


Figure 1

Culturing process, A. Moss protonema growing on the agarose / KCM gel from a moss leaf tip. B. single-celled *C. vulgaris* cultured as tardigrades food. C. image showing the *V. viridianus* buccal apparatus, a. Piercing stylets extended from the mouth b. Feeding straw. D. The animal Feeding on *C. vulgaris*, a. piercing stylets and feeding straw used for making a hole and eating the algae, b. Single-celled *C. vulgaris*. E. Different size petri dishes containing moss protonema, algae cells, and tardigrades.

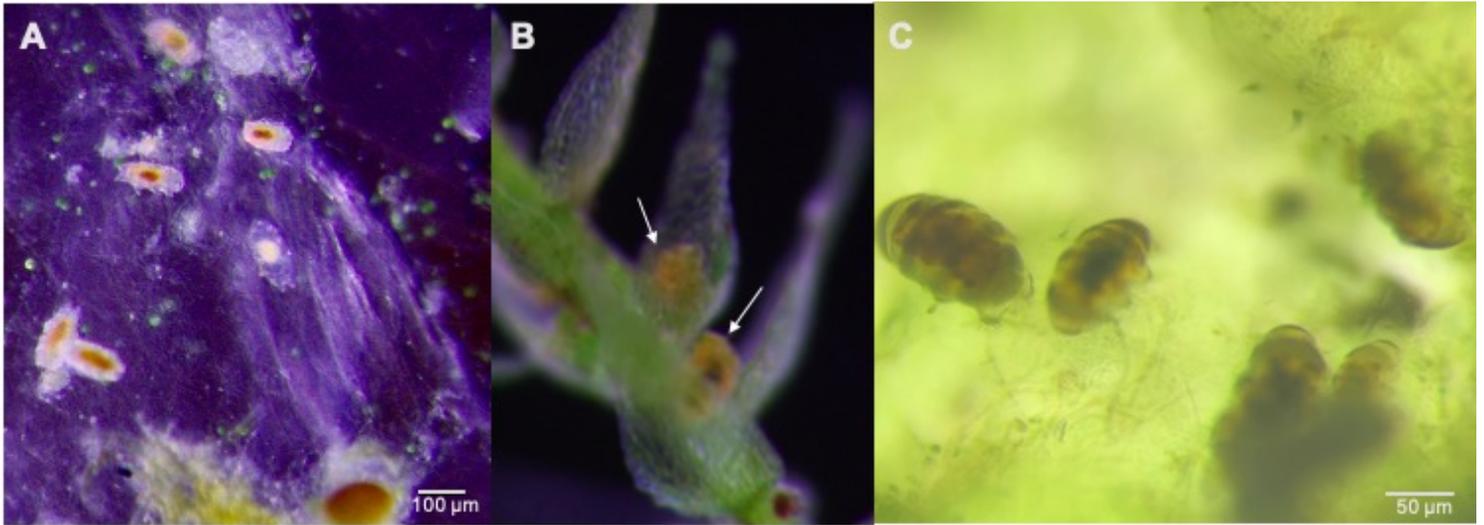


Figure 2

Heterotardigrades culture, A. *Echiniscus* sp. Growing on a culture petri dish coated with agar gel, moss *P. selwynii* protonema, and algae *C. vulgaris*. B. *Pseudechiniscus ramazzotii* Tardigrades forming tuns in the adaxial base of *P. selwynii* moss. Arrows indicate the animals. C. Culture of *V. viridianus* on a petri dish that contains the moss *Andreaea* sp. protonema and *C. vulgaris*.

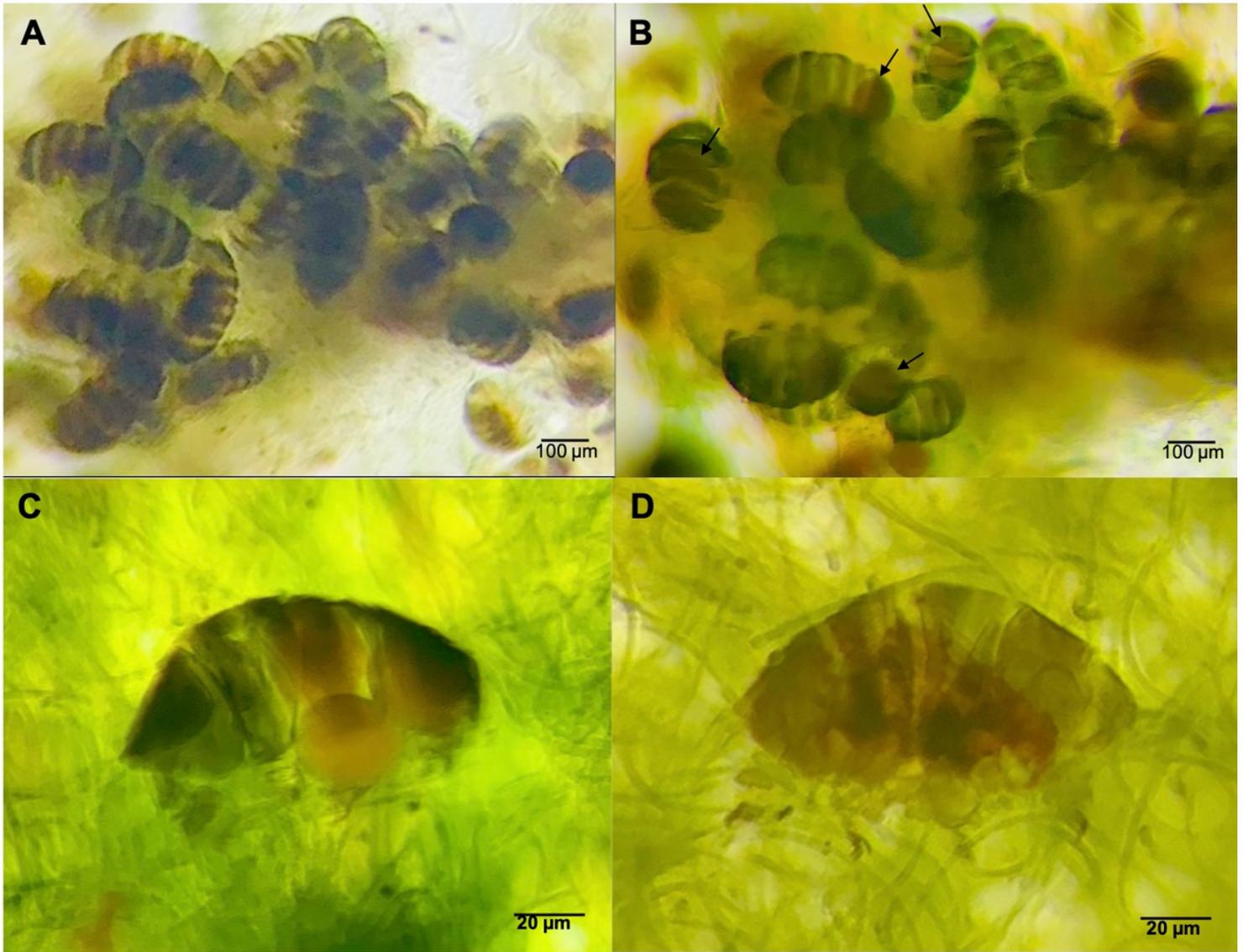


Figure 3

V. viridianus laying eggs in the cultures. A. a group of animals before laying the eggs. B. same group two days after taking the first image. Arrows indicate the eggs inside of the cuticles. C. a cuticle between moss protonema containing three eggs. D. same cuticle after hatching eggs.



Figure 4

V. viridianus walking on a shed cuticle after laying one egg inside.