Chapter 3
Dormancy in Freshwater Tardigrades

Roberto Bertolani, Roberto Guidetti, Tiziana Altiero, Diane R. Nelson, and Lorena Rebecchi

Abstract For more than two centuries, tardigrades have been well known for their ability to undergo dormancy. However, this capability has been well studied mainly in the so-called limnoterrestrial species, i.e., in the species colonizing moist terrestrial habitats, such as mosses, lichens, and leaf litter. In these kinds of substrates, tardigrades are active only when a film of water is available around their body so in this condition they behave like aquatic animals. When the substrate dries or freezes, tardigrades achieve dormancy (quiescence) by entering cryptobiosis, specifically anhydrobiosis or cryobiosis, respectively. In freshwater habitats, both forms of cryptobiosis have been verified only in species able to live both in freshwater and terrestrial habitats. In the truly freshwater (or limnic) species, anhydrobiosis has not been verified, while cryobiosis has been confirmed in a few species. Another dormancy phenomenon bound to diapause is frequent in freshwater species: encystment (sometimes found even in limnoterrestrial species). The cyst state, which involves deep structural and physiological modifications, has been known from the beginning of the past century, but only recently has its morphology and inducing factors been studied in depth. Although data on molecular mechanisms allowing cryptobiosis are available, this information does not exist for encystment.

Keywords Tardigrades · Diapause · Encystment · Cryobiosis · Anhydrobiosis

---

R. Bertolani (✉)
Department of Education and Humanities, University of Modena and Reggio Emilia, Modena, Italy
e-mail: roberto.bertolani@unimore.it

R. Guidetti · L. Rebecchi
Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy

T. Altiero
Department of Education and Humanities, University of Modena and Reggio Emilia, Modena, Italy

D. R. Nelson
Department of Biological Sciences, East Tennessee State University, Johnson City, TN, USA

V. R. Alekseev, B. Pinel-Alloul (eds.), *Dormancy in Aquatic Organisms. Theory, Human Use and Modeling*, Monographiae Biologicae 92,
https://doi.org/10.1007/978-3-030-21213-1_3

3.1 Introduction

Together with rotifers and nematodes, tardigrades have been well known for more than two centuries for their capability to undergo dormancy at any stage of their life history, from the egg up to the adult. In tardigrades, dormancy has been mainly studied in the so-called limnoterrestrial species, i.e., those colonizing terrestrial habitats, such as bryophytes, lichens, grassland, and leaf litter, in which they are active only when surrounded by at least a film of water. Tardigrades, rotifers, and nematodes do not have specific structural adaptations for living as terrestrial organisms. When the substrate dries or freezes, these animals become dormant (quiescent) by entering cryptobiosis: anhydrobiosis or cryobiosis, respectively (Rebecchi et al. 2007; Guidetti et al. 2011b). In freshwater habitats, cryptobiosis has been verified only in a few tardigrade species, e.g., *Hypsibius dujardini* (Doyère 1840), which can also colonize terrestrial habitats (Boothby et al. 2017). In the truly limnic tardigrade species, anhydrobiosis has never been verified, while cryobiosis has been identified in a few species (Guidetti et al. 2011a) and for the marine heterotardigrade *Echiniscoides sigismundi* (Schultze 1865) (see Clausen et al. 2014). Another dormancy phenomenon, encystment, can be present in freshwater species, bound to diapause. To date, this type of dormancy has been verified only in a limited number of limnic species, but it is also known for some limnoterrestrial and marine species (see Guidetti and Møbjerg 2018). Moreover, in tardigrades, another phenomenon bound to diapause has been identified: resting eggs, which were described in the species *Paramacrobiotus fairbanski* (Schill et al. 2010) that mainly colonize leaf litter, but sometimes are also found in freshwater (Altiero et al. 2010).

3.2 Diapause in Tardigrades

The first case of encystment in tardigrades was described at the beginning of the past century (Lauterborn 1906) and confirmed a few years later (Murray 1907a, b, c; Heinis 1910; von Wenck 1914). The species cited by Lauterborn (1906), *Macrobiotus macronyx* Dujardin 1851 (currently *Dactylobiotus macronyx*), is difficult to identify, leading some to question its existence. Subsequent studies involved more easily identifiable species. One year after Lauterborn's paper, Murray (1907a, c) described encystment in *Macrobiotus dispar* (Murray 1907a), a species still accepted and currently named *Dactylobiotus dispar* (Murray 1907a). Wanda von Wenck (1914) described in detail the encystment in *Macrobiotus lacustris* Dujardin, a species no longer accepted, but from the drawings of the claws and the placoids, this species is easily attributable to *Pseudobiotus megalonyx* (Thulin 1928). As reported by Murray (1907c) and later by many other authors (for review, see Guidetti and Møbjerg 2018), cyst formation is reported for other species, both eutardigrades and heterotardigrades. Encystment is very rare among moss-dwelling tardigrades (except for the species in the genus *Bertolanius*) but more frequent in species dwelling in substrates such as leaf litter, grassland, and especially freshwater sediments, in which heterotardigrades are extremely rare (such as the true limnic genus *Carphania* Binda 1978). Encystment is probably occasional in species from mosses and lichens surrounding freshwater basins. The limnic species that frequently enter encystment belong to different genera and families: *Dactylobiotus* (Murrayidae), *Pseudobiotus* and *Isohypsibius* (Isohypsibiidae), *Hypsibius* (Hypsibiidae), and *Bertolanius* (Eohypsibiidae).

Rahm (1925) noted the difference between the “tun” (anhydrobiotic state) and the cyst, verifying that the tun had a single cuticle and was absolutely unable to move, while the cyst had at least two cuticles (one thickened) within which the animal could also move. Contrary to what was reported by Murray (1907c), von Wenck (1914) concluded that histolysis does not occur during encystment. The absence of histolysis was confirmed by Węglarska (1957) and Szymańska (1995), both working on reared specimens of the freshwater eutardigrade *D. dispar*, and by Guidetti et al. (2006), working with the freshwater *Dactylobiotus parthenogeneticus* Bertolani 1982a and the limnoterrestrial *Bertolanius volubilis* (Durante Pasa and Maucci 1975).

Węglarska (1957) was the first author to compare cyst formation with molting. In particular, she noted that both phenomena begin by expelling the sclerified parts of the buccal-pharyngeal apparatus. In comparison, during molting, the old cuticle is subsequently abandoned, while during encystment, the old cuticle is retained. Węglarska (1957) and Szymańska (1995) reported information on the histochemistry and ultrastructure, respectively, of the encysted tardigrades. Other authors examined the steps involved in encystment. Westh and Kristensen (1992) and Hansen and Katholm (2002) analyzed the steps of cyst formation, revealing the presence of two types of cysts in two species of *Bertolanius* (Eohypsibiidae) collected in freshwater (submersed or bank mosses in Greenland springs): *Bertolanius nebulosus* (Dastych 1983) and *Bertolanius weglarskae* (Dastych 1972). A complete picture of the various steps of encystment was provided by Guidetti et al. (2006), who considered two species belonging to different superfamilies and living in different habitats: *D. parthenogeneticus* (Macrobiotoidea: Murrayidae) (Fig. 3.1), an exclusively limnic species, and *B. volubilis* (Eohypsibioidea: Eohypsibiidae) (Fig. 3.2), a moss-dwelling species. Cysts are also known in a related limnic species, *Bertolanius smreczynskii* (Węglarska 1970), in addition to the two species found both in freshwater and in terrestrial habitats: *B. weglarskae* and *B. nebulosus* (Bertolani 1982b; Dastych 1983; Westh and Kristensen 1992; Bertolani and Rebecchi 1996). *Dactylobiotus* has only one type of cyst (Guidetti et al. 2006, 2008), whereas *Bertolanius* has two types of cysts that Westh and Kristensen (1992) called “white” and “red” and correlated with seasonal environmental changes. In both genera, the cyst is the result of a series of successive morphological changes that, as in molting, begins by discharging the sclerified parts of the buccal-pharyngeal apparatus (but not the body cuticle, as during molting), reducing the animal size by contracting the body longitudinally, and decreasing the body movements to a complete standstill (Guidetti et al. 2006, 2008). At this stage, any openings of the animal (i.e. mouth and cloaca) are closed. Including the steps before and after encystment, Guidetti and Møbjerg (2018) recognized nine

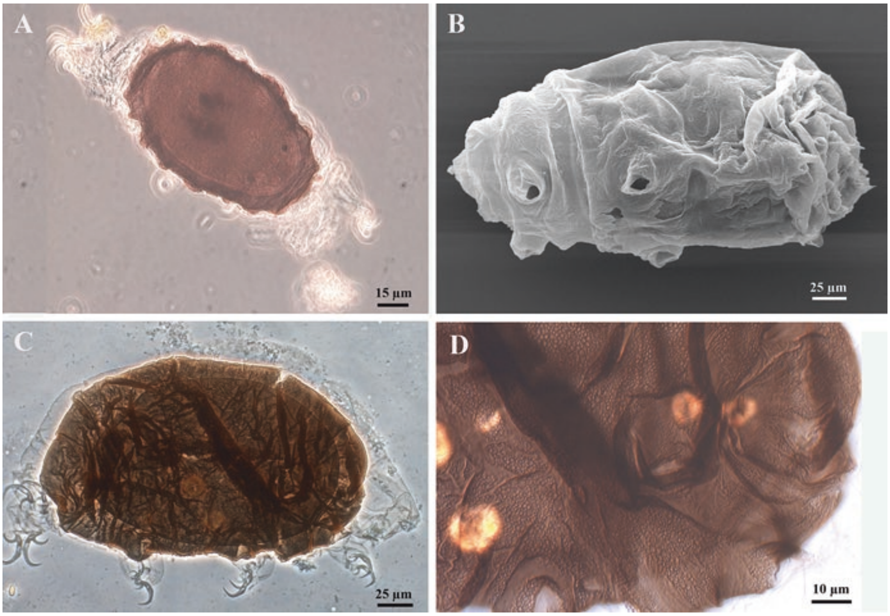

Fig. 3.1 *Dactylobiotus parthenogeneticus*. (**a**) cyst with the old cuticle; eye spots are still visible (phase contrast); (**b**) cyst (SEM); (**c**) cyst containing the mummy cuticle (phase contrast): D: detail of the cyst cuticle (differential interference contrast)

steps of the encystment process in *D. parthenogeneticus*, seven in the “type 2” cysts of *B. volubilis*, and five in the “type 1” cysts of the same species. After the discharge of the sclerified parts of the buccal-pharyngeal apparatus and the body contraction, the synthesis of cuticular material begins with the production of the so-called sarcophagus cuticle with a folded surface, common to all the three types of cysts and characterized by the absence of claws (Guidetti et al. 2006). In *Dactylobiotus*, the sarcophagus cuticle becomes harder and darker. Its formation is followed by the synthesis of a new, highly modified and nonfunctional buccal-pharyngeal apparatus. Successively, the animal synthesizes another wrinkled cuticle, the mummy cuticle (name proposed by Westh and Kristensen 1992, for a step in the formation of *Bertolanius* cysts), without both legs and claws. The subsequent step in the *Dactylobiotus* cyst is the discharge of the sclerified structures of the buccal-pharyngeal apparatus, followed by a new synthesis of them, but always nonfunctional. Further subsequent steps include yet another discharge of the buccal-pharyngeal sclerified structures, followed by the synthesis of a new body cuticle with legs and claws and the cuticular parts of a functional buccal-pharyngeal apparatus, like those of the non-encysted animals. At this time, the cyst is ovoid and the external involucra are dark-brown, almost black; the animal is ready to leave the cyst by breaking all the cuticular coats (onion or matryoshka-like stage; Guidetti et al. 2006).

In the moss-dwelling *B. volubilis*, the “type 2” cyst (Fig. 3.2a), corresponding to the “red” cyst of the limnic species *B. nebulosus* and *B. weglarskae*, after the forma-

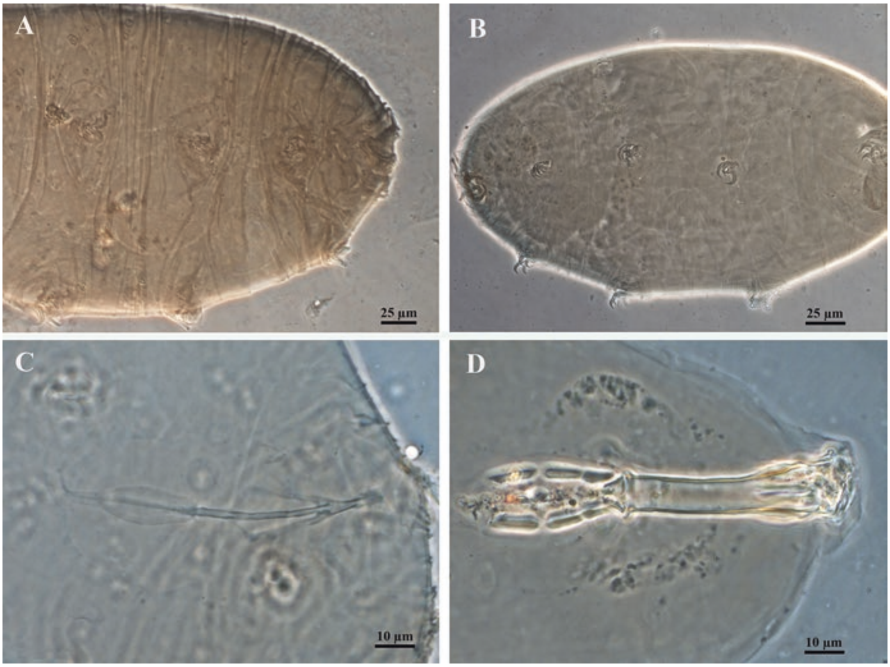

Fig. 3.2 *Bertolanius volubilis*. (**a**): red or type 2 cyst (phase contrast); (**b**): white or type 2 cyst (phase contrast); (**c**): modified buccal-pharyngeal apparatus (phase contrast); (**d**): functional buccal-pharyngeal apparatus (phase contrast)

tion of the sarcophagus cuticle (however absent in *B. nebulosus* and *B. weglarskae*; see Westh and Kristensen 1992; Hansen and Katholm 2002), a mummy cuticle is formed together with modified and nonfunctional circular parts of the buccal-pharyngeal apparatus (Fig. 3.2c). These parts are subsequently discharged, and a fourth cuticle and a functional buccal-pharyngeal apparatus (Fig. 3.2d) are produced (matryoshka-like stage). This step is followed by the exit of a complete and non-encysted animal. After the sarcophagus cuticle formation, the "type 1" cyst of *B. volubilis* (Fig. 3.2b) produces a third cuticle (mummy cuticle) and a complete and functional buccal-pharyngeal apparatus. This step is followed by the exit of a complete and non-encysted animal. For a more detailed description of the encystment steps, see Guidetti et al. (2006, 2008) and Guidetti and Møbjerg (2018).

In laboratory conditions, encystment was induced in limnic species by Węglarska (1957) and Szymańska (1995) (both using *D. dispar*) and by Guidetti et al. (2006) (in *D. parthenogeneticus*). Guidetti et al. (2006, 2008) also induced encystment in the moss-dwelling species *B. volubilis* (which has related limnic species). Węglarska (1957) and Szymańska (1995) reared their animals with decomposed vegetable materials. Węglarska's observations led her to believe that encystment was caused by gradually deteriorating environmental conditions, whereas Szymańska concluded that a decrease in environmental pH was an important factor leading to encystment. Nelson et al. (2016) stated that the factors inducing cyst formation were unknown, even though stressful environmental conditions (e.g., reserve or oxygen depletion, pH alteration, temperature variation, and pollution) have been suggested. Węglarska (1957) noted that *D. dispar* needed approximately 12 days to produce a cyst, but once formed, it could stay for more than 9 months in this state. She also observed that the placement of an encysting *D. dispar* specimen in clean water induced an active normal state rather than the completion of encystment, whereas when left in water with decaying leaves, it completed encystment. Węglarska (1957) also noted that cysts placed in water containing a tangle of algae did not encyst, but when they were subsequently placed in tap water, the animals left the cyst within 6–48 h.

To obtain cysts, Guidetti et al. (2006) kept *D. parthenogeneticus* in water with algae at 14 °C for at least 90 days, whereas they kept *B. volubilis* in water at 14 °C for at least 20 days. Further laboratory experiments were conducted by Guidetti et al. (2008) using *B. volubilis*. The study of cysts and animals collected in nature (Italy) and kept at 6, 14, or 20 °C and at 12 h/12 h light/dark demonstrated that active tardigrades collected in April produced mainly "type 2" cysts, whereas animals collected in November produced mainly "type 1" cysts, indicating that the different responses are functions of the physiological state at the time they were collected. Photoperiod did not seem to affect the type of cysts formed by *B. volubilis*. The dynamics of the two types of cysts in nature show opposite seasonal trends: "type 2" cysts are present only during the warm season, and "type 1" cysts are present during the cold season (Guidetti et al. 2008). These data confirmed what was observed in nature for the congeneric limnic species *B. nebulosus* from Greenland (Westh and Kristensen 1992; Hansen and Katholm 2002). Temperature was the environmental factor involved in induction, maintenance, and termination of the cyst. Guidetti et al. (2008) hypothesized that endogenous factors, due to exogenous factors, can be involved in tardigrade encystment. Moreover, animals and cysts of *B. volubilis* survived freezing (18 of 20 animals and 48 of 53 cysts) after the following protocol: frozen at −9 °C for 24 h, then placed at −80 °C, kept frozen at −80 °C for 61 days, and then directly transferred to 20 °C for thawing (Guidetti et al. 2008). Importantly, *B. nebulosus* overwinters as frozen cysts or, in a few cases, as eggs or adults (Westh and Kristensen 1992).

We have unpublished data on cysts of tardigrades living in submerged mosses (sample labeled as C2573) from a spring located in Val de la Mare, Pian Venezia (Italian Alps, Stelvio National Park, Trentino, NE Italy, at 2270 m a.s.l.). The samples collected in November 2004 were frozen at −20 °C and maintained in this state up to October 2008. The species present in the sample (all eutardigrades) were *Borealibius zetlandicus* (Murray 1907b), *Mixibius saracenus* (Pilato 1973), *Murrayon pullari* (Murray 1907b), and *Isohypsibius granulifer* (Thulin 1928). After thawing, all the specimens of the first three species were extended and did not show any movement. Only two specimens, recognizable as *I. granulifer* by the typically granulated cuticle, placoids, and claws, were present as live cysts (Fig. 3.3a). After putting them in a

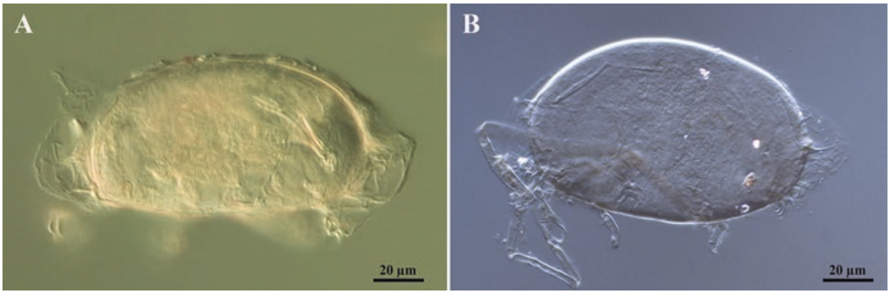

Fig. 3.3 (**a**): cyst of *Isohypsibius granulifer* from Pian Venezia that survived freezing (differential interference contrast): (**b**): cyst of freshwater *Thulinius ruffoi* from type locality (phase contrast)

drop of water and observing them under a light microscope, we were able to recognize slow and continuous movement in each animal within its cyst involucra. Therefore, only one species (*I. granulifer*) survived being frozen and maintained in the cyst state for 4 years. The Greenlander freshwater specimens of *B. nebulosus* seem to survive mainly as cysts, but also as non-encysted animals and eggs (Westh and Kristensen 1992). *Isohypsibius granulifer* is a limnic species and to date encystment is the only known way for it to survive freezing. Schill et al. (2007) reported that *Thulinius ruffoi* (Bertolani 1982a, b), a species normally colonizing freshwater, was found exclusively as cysts in 46 moss samples during winter (when temperature can easily drop below 0 °C at night) on the Sinai Mountains (Egypt, 1750 m a.s.l). Cysts of this species were already found in freshwater (Fig. 3.3b). Based on these data, we conclude that the formation of a cyst is certainly a way to survive freezing, even though we cannot be sure that other ways do not exist, despite the presence of all the dead extended animals. Encystment could also be involved in overcoming other seasons without freezing temperatures, since limnic tardigrade species often disappear during the summer, even when the water is still present in lakes and rivers where they live (Bertolani 1982b).

Although we now have a better understanding of cryptobiosis (anhydrobiosis, cryobiosis) in tardigrades, the molecular mediators of desiccation tolerance are not fully known. Boothby et al. (2017) demonstrated that tardigrade-specific intrinsically disordered proteins (TDPs) are essential for desiccation tolerance in *Hypsibius dujardini*, which colonizes both freshwater and terrestrial habitats. In contrast, our knowledge of the molecular mechanisms involved in encystment (diapause) are unknown, although the morphological stages of cyst formation have been described thoroughly (Guidetti et al. 2006, 2008; Guidetti and Møbjerg 2018). Encystment is thought to be induced by exogenous factors (temperature, oxygen) and possibly endogenous stimuli, but further studies are essential to confirm the ability of various tardigrade species in different habitats to form cysts and to determine the molecular mechanisms involved.

References

Altiero T, Bertolani R, Rebecchi L (2010) Hatching phenology and resting eggs in tardigrades. J Zool 280:290–296

Bertolani R (1982a) A new genus and five new species of Italian fresh-water tardigrades. Bollettino del Museo Civico di Storia Naturale di Verona 8:249–254

Bertolani R (1982b) *Tardigradi (Tardigrada)*. Guide per il riconoscimento delle specie animali delle acque interne italiane. Quaderni CNR, AQ/l/l68 15:1–104.

Bertolani R, Rebecchi L (1996) The tardigrades of Emilia (Italy). II. Monte Rondinaio. A multihabitat study on a high altitude valley of the northern Apennines. Zool J Linnean Soc 116:3–12

Binda MG (1978) Risistemazione di alcuni Tardigradi con l'istituzione di un nuovo genere di Oreellidae e della nuova famiglia Archechiniscidae. Animalia 5:307–314

Boothby TC, Tapia H, Brozena AH, Piszkiewicz S, Smith AE, Giovannini I, Rebecchi L, Pielak GJ, Koshland D, Goldstein B (2017) Tardigrades use intrinsically disordered proteins to survive desiccation. Mol Cell 65:975–984

Clausen LKB, Andersen KN, Hygum TL, Jørgensen A, Møbjerg N (2014) First record of cysts in the tidal tardigrade *Echiniscoides sigismundi*. Helgol Mar Res 68:531–537

Dastych H (1972) *Isohypsibius weglarskae* sp. n. a new species of Tardigrada from Poland. Bull Acad Pol Sci Ser Sci Biol 11:761–764

Dastych H (1983) Two new Eutardigrada species from West Spitsbergen and the Tatra Mts. Bull Soc Amis Sci Lett Pozn 23:195–200

Doyère M (1840) Mémoire sur les Tardigrades. Annales Des Sciences Naturelles 14:269–362

Dujardin F (1851) Sur les Tardigrades et sur une espèce a longs pieds dans l'eau de mer. Annales Des Sciences Naturelles 15:160–166

Durante Pasa MV, Maucci W (1975) Descrizione di tre nuove specie di Tardigradi della Scandinavia. Atti Soc Ital Sci Nat Mus Civ Stor Nat Milano 116:244–250

Guidetti R, Møbjerg N (2018) Environmental adaptations: encystment and cyclomorphosis. In: Schill RO (ed) Water bears: the biology of tardigrades, zoological monographs, vol 2, pp 249–271

Guidetti R, Boschini D, Rebecchi L, Bertolani R (2006) Encystment processes and the "Matryoshka-like stage" in a moss-dwelling and in a limnic species of eutardigrades (Tardigrada). Hydrobiologia 558:9–21

Guidetti R, Boschini D, Altiero T, Bertolani R, Rebecchi L (2008) Diapause in tardigrades: a study of factors involved in encystment. J Exp Biol 211:2296–2302

Guidetti R, Altiero T, Bertolani R, Grazioso P, Rebecchi L (2011a) Survival of freezing by hydrated tardigrades inhabiting terrestrial and freshwater habitats. Zoology 114(2):123–128

Guidetti R, Altiero T, Rebecchi L (2011b) On dormancy strategies in tardigrades. J Insect Physiol 57:567–576

Hansen JG, Katholm AK (2002) A study of the genus *Amphibolus* from Disko Island with special attention on the life cycle of *Amphibolus nebulosus* (Eutardigrada: Eohypsibiidae). In: Hansen JG (ed) *Arctic biology field course Quqertarsuaq 2002*. Zoological Museum University of Copenhagen, Copenhagen, pp 129–163

Heinis F (1910) Systematik und Biologie der Moosebewohnen den Rhizopoden, Rotatorien und Tardigraden usw. Archiv für Hydrobiologie und Planktonkund 5:1–115

Lauterborn R (1906) Demostrationen aus der Fauna ders Oberreheins und seiner Umgebung. Verh Dtsch Zool Ges:265–268

Murray J (1907a) The encystment of *Macrobiotus*. The Zoologist 11:4–11

Murray J (1907b) Scottish Tardigrada, collected by the lake survey. Trans R Soc Edinb 45: 641–668 (with 3 plates)

Murray J (1907c) Encystment of Tardigrada. Trans R Soc Edinburg 45: 837–854 (with 2 plates)

Nelson DR, Guidetti R, Rebecchi L (2016) Phylum Tardigrada. In: Thorp, Covich (eds) Key to Nearctic Fauna, vol. 15, pp 277–290

Pilato G (1973) Tardigradi delle acque dolci siciliane. Nota seconda. Boll Accad Gioenia Sci Nat Catania 12:177–186

Rahm G (1925) Die Cystenbildung bei den wasserbewohnenden Tardigraden. In*:* Verh. intern. Vereinig. theoret. angew. Limnologie, Bd. 3, Moskau, pp 364–371

Rebecchi L, Altiero T, Guidetti R (2007) Anhydrobiosis: the extreme limit of desiccation tolerance. Invertebr Surviv J 4:65–81

Schill RO, Huhn F, Köhler HR (2007) The first record of tardigrades (Tardigrada) from the Sinai Peninsula, Egypt. Zool Middle East 42:83–88

Schill RO, Förster F, Dandekar T, Wolf M (2010) Using compensatory base change analysis of internal transcribed spacer 2 secondary structures to identify three new species in *Paramacrobiotus* (Tardigrada). Org Divers Evol 10:286.296

Schultze M (1865) *Echiniscus sigismundi*, ein Arctiscoide der Nordsee. Arch Mikr Anat 1:428–436

Szymańska B (1995) Encystment in the tardigrade *Dactylobiotus dispar* (Murray, 1907) (Tardigrada: Eutardigrada). Part 1: Observation of living animals and structure of cyst. Zoologica Poloniae 40:91–102

Thulin G (1928) Über die Phylogenie und das System der Tardigraden. Hereditas 11:207–266

von Wenck W (1914) Entwicklungsgeschichtliche Untersuchungen an Tardigraden (*Macrobiotus lacustris* Duj.). Zool Jahrb Abt Anat Ontog Tiere 37:465–514

Węglarska B (1957) On the encystation in Tardigrada. Zoologica Poloniae 8:315–325

Węglarska B (1970) *Hypsibius* (*Isohypsibius*) *smreczynskii* spec. nov., a new species of freshwater tardigrade. Zeszyty Naukowe Uniwersytetu Jagiellońskiego Prace zooliczne 16:107–114

Westh P, Kristensen RM (1992) Ice formation in the freeze-tolerant eutardigrades *Adorybiotus coronifer* and *Amphibolos nebulosus* studied by differential scanning calorimetry. Polar Biol 12:693–699