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Diapause in tardigrades: a study of factors involved in encystment

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SUMMARY

Stressful environmental conditions limit survival, growth and reproduction, or these conditions induce resting stages indicated as dormancy. Tardigrades represent one of the few animal phyla able to perform both forms of dormancy: quiescence and diapause. Different forms of cryptobiosis (quiescence) are widespread and well studied, while little attention has been devoted to the adaptive meaning of encystment (diapause). Our goal was to determine the environmental factors and token stimuli involved in the encystment process of tardigrades. The eutardigrade *Amphibolus volubilis*, a species able to produce two types of cyst (type 1 and type 2), was considered. Laboratory experiments and long-term studies on cyst dynamics of a natural population were conducted. Laboratory experiments 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. The dynamics of the two types of cyst show opposite seasonal trends: type 2 cysts are present only during the warm season and type 1 cysts are present during the cold season. Temperature represents the environmental factor involved in induction, maintenance and termination of the cyst. We also obtained evidence that *A. volubilis* is able to perform both diapause and cryptobiosis, even overlapping the two phenomena. The induction phase of tardigrade encystment can be compared to the induction phase of insect diapause, also indicating an involvement of endogenous factors in tardigrade encystment. As in insect diapause, tardigrade encystment can be considered a diapausing state controlled by exogenous and endogenous stimuli.

Key words: dormancy, diapause, encystment, token stimuli, stress, Eutardigrada, *Amphibolus volubilis*.

INTRODUCTION

In many organisms, stressful environmental conditions limit survival, growth and reproduction, or these conditions induce resting stages generally indicated as dormancy (Càceres, 1997). Dormancy involves a temporary suspension of active life, a reduced or suspended metabolism and a developmental standstill. It can have profound ecological and evolutionary implications, affecting rates of population growth and potential rates of adaptation to a varying environment (Hunter and McNeil, 1997). In addition, it can allow the synchronization of reproductive cycles and the regulation of population structure and dynamics (Gilbert, 1974; De Stasio, 1989), contributing to the maintenance of biodiversity and genetic variability within populations (Ellner and Hairston, 1994; Boero et al., 1996). According to the cues required for its induction, maintenance and termination, dormancy can be subdivided into quiescence and diapause (Hand, 1991). Quiescence is under exogenous control, being directly induced and maintained by adverse environmental conditions. Conversely, it can immediately be reversed by removal of the external stimuli (Hand, 1991). The concept of diapause is not always univocal, depending on the animal groups considered (Daniel, 1970; Alekseev and Sterobogatov, 1996; Càceres, 1997; Belmonte and Rossi, 1998; Denlinger and Tanaka, 1999; Ricci, 2001; Sommerville and Davey, 2002). According to Hand, diapause is under endogenous control, being not directly induced by environmental conditions and maintained by an internal physiological response (Hand, 1991). Its termination requires a specific cue that may not correspond to favourable environmental conditions. According to Sommerville and Davey (Sommerville and Davey, 2002) and Koštál (Koštál, 2006), diapause should be better viewed as a process, rather than a status, whose induction and maintenance are related to external (environmental) and internal (physiological) stimuli. In insects, diapause can be schematized in several phases (Koštál, 2006): (a) the induction phase, which occurs during a sensitive period when token stimuli from the environment reach some critical level; (b) the initiation phase in which development ceases, a regulated metabolic suppression occurs and physiological preparations for adversities take place; (c) the maintenance phase in which token stimuli may help to maintain diapause, metabolic rate is relatively low and physiological processes lead to an increase in sensitivity to terminating conditions; and (d) the termination phase in which specific changes in environmental conditions stimulate a decrease of diapause intensity.

In tardigrades, microinvertebrates found worldwide in a variety of habitats, dormancy is very well documented. Tardigrades represent one of a few animal phyla in which different forms of resting stage occur (Bertolani et al., 2004). With regard to quiescence, the different forms of cryptobiosis are very widespread in terrestrial tardigrades. Under desiccation or cooling stresses, each stage of the tardigrade life cycle is able to escape the stressful condition by entering anhydrobiosis or cryobiosis (Bertolani et al., 2004). Anhydrobiotic or cryobiotic tardigrades are able to colonize environments exposed to rapid and unpredictable desiccation or freezing. They show extraordinary resistance to physical and chemical extremes far exceeding the tolerance ranges of other active organisms (Rebecchi et al., 2007). These abilities allow cryptobiotic

tardigrades to persist in environments from which most other organisms are excluded and to reduce competition (Bertolani et al., 2004).

Little attention has been devoted to the adaptive meaning of other forms of tardigrade resting stage: resting eggs, cyclomorphosis and encystment. Hansen and Katholm identified possible resting eggs in a Greenlander population of *Amphibolus nebulosus* Dastych 1983 (Hansen and Katholm, 2002), but their existence has only recently been confirmed in experimental cultures of *Macrobiotus richtersi* Murray 1911 (Bertolani et al., 2004). Cyclomorphosis is a cyclical change in morphology and physiology occurring during the tardigrade life cycle that has been recorded with certainty only for the marine eutardigrade *Halobiotus crispae* Kristensen 1982 (Kristensen, 1982; Møbjerg et al., 2007). Cyclomorphosis is characterized by four distinct states. Particularly interesting is the resting state called pseudosimplex 1 because it represents an adaptation to withstand stressful conditions. According to the geographic distribution of different populations of *H. cripsae*, the pseudosimplex 1 state can withstand low temperatures (in Greenland) or can tolerate periods of oxygen depletion and heat stress during the Danish summer (Møbjerg et al., 2007).

Encystment has been found in some freshwater, moss-dwelling and soil tardigrades (Murray, 1907a; Murray, 1907b; Węglarska, 1957; Szymańska, 1995; McInnes and Pugh, 1999; Hansen and Katholm, 2002; Guidetti et al., 2006). Encysted tardigrades exhibit a contracted and oval form, with a thickened external envelope made up of several cuticular layers, and eventually produce a modified buccal–pharyngeal apparatus and claws. Encystment begins with the discharging of the sclerified parts of the buccal–pharyngeal apparatus (simplex stage), as in the molting process. Then, two or three new cuticles are serially synthesized, according to the type of cyst (Hansen and Katholm, 2002; Guidetti et al., 2006). Some species of the genus *Amphibolus* produce two types of cyst, called type 1 and type 2 (Westh and Kristensen, 1992; Hansen and Katholm, 2002; Guidetti et al., 2006). In *Amphibolus volubilis*, type 1 cysts are structurally simpler than type 2 cysts and require a lower number of steps for their production (Guidetti et al., 2006). In an Arctic population of *A. nebulosus*, the production of the two types of cysts is strictly related to season, and a correlation between the two types of cyst and the production of resting eggs could exist (Hansen and Katholm, 2002).

Some field or lab observations have not clearly identify the stimuli involved in the cyst production of tardigrades (Von Wenck, 1914; Marcus, 1929; Marcus, 1936; Węglarska, 1957; Szymańska, 1995; Westh and Kristensen, 1992; Hansen and Katholm, 2002). Starting from these premises, our goal was to determine the role of the environmental factors involved in cyst formation. Consistent with this goal, laboratory experiments and a long-term study on the cyst dynamics of a natural population of *A. volubilis* sampled for two consecutive years were carried out. In addition, the cryptobiotic abilities of cysts were evaluated, since there was no information on their anhydrobiotic ability and only a short citation on their cryobiotic ability (Westh and Kristensen, 1992).

MATERIALS AND METHODS

Non-encysted or encysted specimens of a boreo-alpine tardigrade species, *Amphibolus volubilis* (Durante Pasa and Maucci 1975), were extracted from mosses [*Racomitrium sudeticum* (Funck) Brunch and Schimp, and *Racomitrium elongatum* (Ehrh.) ex Frisvoll] growing on sandstone located in a post-glacial valley of the Northern Apennines (Rondinaio Mountain, Modena, Italy; N44°7.421′/E010°35.222′; 1670 m above sea level). They belong to a gonochoric amphimictic population already studied with regard to its reproductive mode (Rebecchi and Bertolani, 1994) and to the ultrastructure of its two types of cyst (Guidetti et al., 2006). By immersing the moss in tap water and sifting it repeatedly, non-encysted animals and cysts were extracted and collected using a stereoscope. Other than active animals, two types of cyst (type 1 and type 2) were recognized.

Induction of cyst state

Two experiments were performed to evaluate the involvement of temperature in cyst formation. For these experiments, non-encysted active animals and encysted animals were used. Specimens were extracted from moss samples collected in the wild in November 2001, April 2002 and July 2002. The samples collected in November 2001 and April 2002 were completely desiccated in the lab at room temperature and then stored at –80°C until use (May 2002). Specimens with movement of legs or of their internal organs were considered alive.

Experiment 1

Specimens extracted from samples collected in November 2001 and April 2002 were used. Most tardigrades extracted from these samples were non-encysted. Non-encysted animals with different body sizes were used. Each animal was singly placed in a covered glass cap containing 3 ml of mineral water and then kept at 6, 14 or 20°C with a constant photoperiod: 12 h/12 h light/dark. In particular, 14 animals collected in April and 19 collected in November were kept at 6°C; 11 animals collected in April and 24 collected in November were kept at 14°C; and 14 animals collected in April and 13 collected in November were kept at 20°C. Every 3 days the state of each animal was checked under a light microscope (objective ×40) and the mineral water of each cap was partially changed.

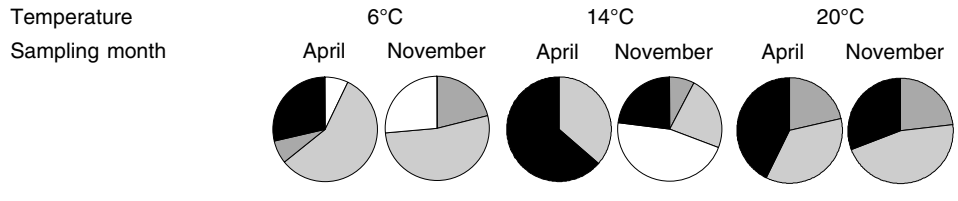
Experiment 2

Eighty non-encysted animals were used. These animals were derived from type 2 cysts, which were extracted from a moss sample collected in July 2002. After 13 days in water and moss debris at 14°C these animals had left the cyst state. Non-encysted animals were placed in a cap containing mineral water and kept at 14°C with a photoperiod of 12 h/12 h light/dark. Every 3 days the state of each animal was checked until it formed a cyst or died.

Cyst dynamics

Moss samples were collected almost every month from March 2003 to March 2005. In the coldest months (from January to April), the rock was covered with snow. At each sampling date, five samples of moss were randomly collected. In the lab, all specimens of *A. volubilis* were extracted from 0.5 g of each sample, divided into non-encysted animals, type 1 cysts and type 2 cysts, and then counted.

Meteorological data (air temperature, rainfall and air relative humidity – RH) were considered for all sampling dates. The data came from the CAMM station of the Italian Air Force situated on Cimone Mountain (2165 m above sea level), 12 km from the sampling site. The mean air temperature and the mean RH recorded in the period between two consecutive sampling dates were considered. For the first sampling date, we considered the mean air temperature and RH recorded the 30 days previously. For rainfall, the sum of the millimetres of rain that fell 20 days before each sampling was considered. The hours of daylight (recorded in Modena) during the two sampling years were also considered.



Type 1 cyst		7.1%	26.3%	0%	46.2%	0%	0%
Type 2 cyst		28.7%	0%	63.6%	23.0%	42.9%	30.8%
Dead in simplex		57.1%	52.6%	36.4%	23.1%	35.7%	46.2%
Dead		7.1%	21.1%	0%	7.7%	21.4%	23.0%

Fig. 1. Response of non-encysted *Amphibolus volubilis* to different temperatures (6, 14 and 20°C) in relation to the sampling period. The percentage of type 1 cysts, type 2 cysts, animals dead in simplex stage or animals dead without any morphological modification is reported.

Analysis of the relationships between the dynamics of type 1 and type 2 cysts and non-encysted specimens of *A. volubilis* during the two sampling years and the meteorological data were performed with Kolmogorov–Smirnof and Pearson correlation tests. The software program SPSS version 14.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

Survival ability

To test the ability of encysted and non-encysted animals to withstand desiccation or freezing, and therefore to perform anhydrobiosis or cryobiosis, we exposed tardigrade specimens to stressful conditions.

Freezing

Fifty-three type 2 cysts were used to evaluate their freezing survival ability. They were extracted from the moss sample collected in July 2002. All cysts were put in a covered glass cap containing 3 ml of mineral water, transferred to –9°C for 24 h and then to –80°C. The cysts were kept frozen at –80°C for 61 days. After this time, the cap containing cysts was directly transferred to 20°C for thawing.

For comparison, 20 non-encysted active animals were frozen and thawed using the same protocol as for the cysts.

Immediately after the complete melting of the ice, all cysts and non-encysted animals were examined under a microscope to verify whether they were alive or dead.

Desiccation

Sixteen type 2 cysts still alive after the freezing experiment were used to evaluate their desiccation survival ability. Cysts were placed on wet and defauned moss leaves (microcosm) inside a small plastic Petri dish and desiccated at room humidity (RH about 60%) and temperature (about 22°C). Then, this microcosm was kept dry for 7 days at room temperature (about 22°C).

For comparison, one microcosm with 48 non-encysted animals was desiccated using the same protocol indicated above for cysts.

After these treatments, microcosms were rehydrated. Twenty-four hours after re-hydration, all cysts and non-encysted animals were examined under a microscope to verify whether they were alive or dead.

Thermal stress

Type 2 cysts still alive after the freezing experiment were used to evaluate their thermal tolerance. Sixteen cysts were put in a covered glass cap with 6 ml of pre-heated mineral water and then kept for 3 h at 60°C [a temperature used previously for similar experiments (Rahm, 1925)]. Right after this period the cysts were observed under a microscope and their survival was evaluated.

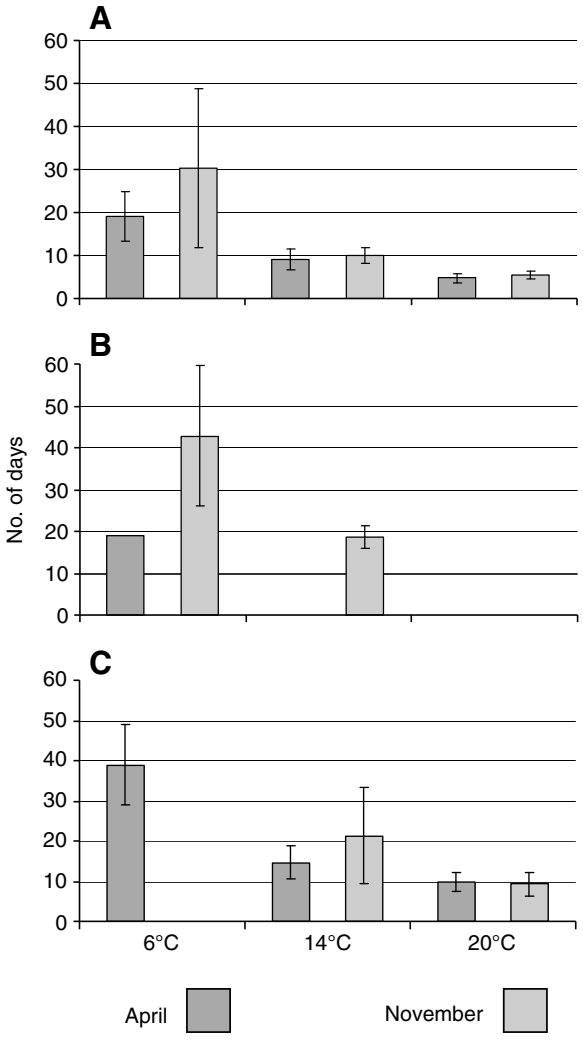


Fig. 2. Number of days spent by specimens of *Amphibolus volubilis* in entering simplex stage (A) and producing type 1 (B) or type 2 (C) cysts in relation to temperature (6, 14 and 20°C) and sampling month (April, November).

For comparison, 17 non-encysted animals were handled in the same way as indicated above for encysted specimens.

In addition, two microcosms (see desiccation protocol), one with 14 type 2 cysts and the other with 13 non-encysted specimens, were

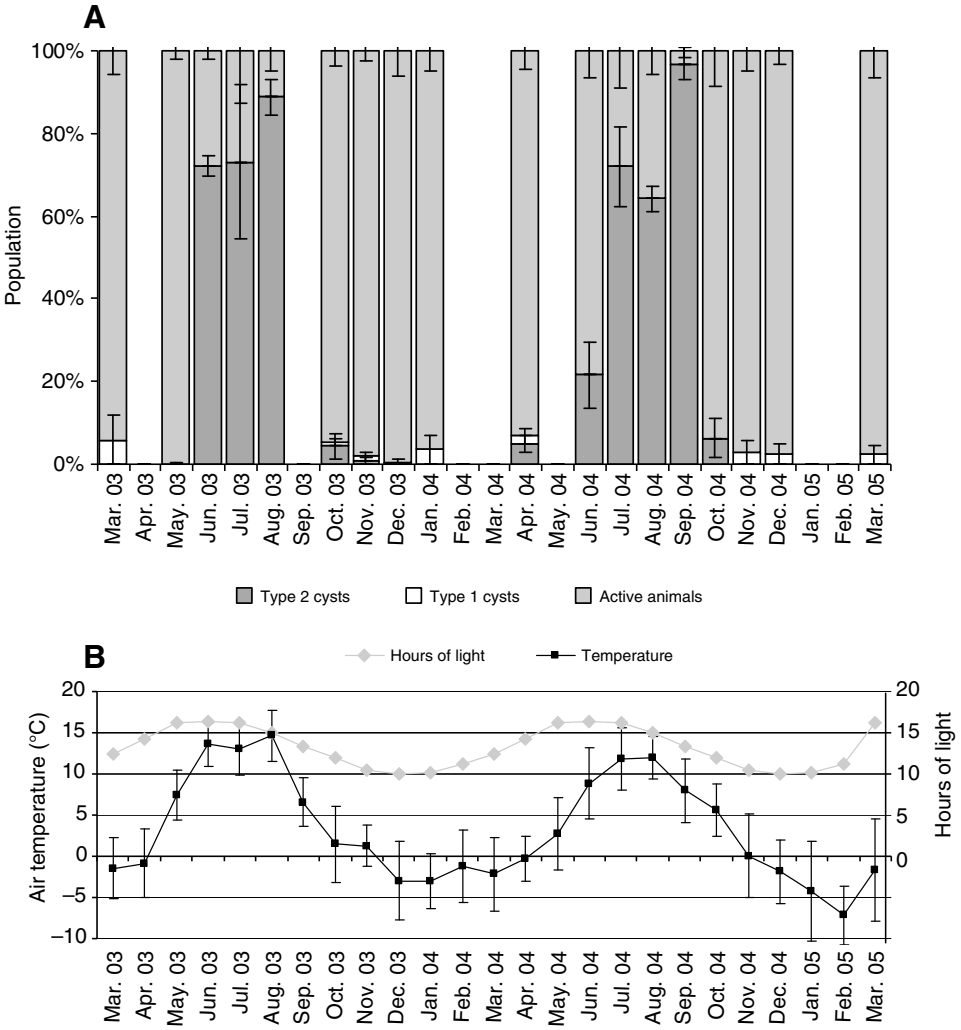


Fig. 3. (A) Dynamics of type 1 cysts, type 2 cysts and non-encysted animals in a population of *Amphibolus volubilis* during two sampling years. Each column reports the mean percentage and standard deviation (s.d.) of each state within the population recorded in the samples. The empty columns indicate the months in which samples were not collected. (B) Dynamics of mean (±s.d.) air temperature recorded between two sampling dates and hours of daylight from March 2003 to March 2005.

desiccated at room temperature and RH and then kept at 60°C for 3 h. After this treatment both cysts and animals were rehydrated and examined under a stereoscope to verify their viability.

RESULTS

Induction of cyst state

Experiment 1

In general, the non-encysted specimens of *A. volubilis* exhibited different responses to the test temperatures: (a) they formed type 1 cysts; (b) they formed type 2 cysts; (c) they died in simplex stage (beginning of the molting or encystment process); or (d) they died without any evident morphological change (Fig. 1). The number of animals showing the different responses was related to the sampling period of specimens. If tardigrades were collected in April, type 2 cysts were always formed at all tested temperatures (6, 14 and 20°C), whereas only one cyst of type 1 was formed at 6°C. If specimens were collected in November, type 2 cysts were formed only at 14 and 20°C, whereas type 1 cysts were formed at 6 and 14°C. Independently of the sampling month and at all three tested temperatures, a high number of animals either died in simplex stage, or they died without any evident morphological change (Fig. 1).

The number of days spent in forming type 1 cysts (P=0.021) and type 2 cysts (P<0.001) was inversely related to the temperature at which the non-encysted animals were kept: the higher the temperature, the faster the process (Fig. 2).

With regard to the duration of cyst states, under laboratory conditions animals stayed in type 1 cysts for about 20 days (at 14°C) or 40 days (at 6°C). The termination of the type 1 cyst state took place spontaneously; nevertheless some animals died before exiting from this state. Under laboratory conditions, animals spent up to 60 days in the type 2 cyst state. No animals were able to leave the type 2 cyst state and most of them died in this condition. Nevertheless, if the most external thick involucres were accidentally broken in the lab, the animal emerged from the cyst state after a few days. In these experiments, we did not find evidence of spermatozoa and mature oocytes within the gonads of type 2 cysts.

Experiment 2

Among the 80 non-encysted animals, 26 formed type 1 cysts and 33 died during the simplex stage. Twenty-one specimens died 13 days after the beginning of the experiments without any morphological modifications. Type 2 cysts were never formed. At 14°C, the time spent by non-encysted animals to form type 1 cysts ranged between 13 and 35 days (mean 17.8 days; s.d. 8.4 days). The specimens remained in the type 1 cyst state for 10–15 days, and spontaneously left it.

Cyst dynamics

Fig. 3 reports the percentage of non-encysted animals, type 1 cysts and type 2 cysts of *A. volubilis* at each sampling date. In both years, non-encysted animals were found in all samplings. Cysts were found mainly in limited periods, and very rarely the two types were found together. Type 2 cysts were abundant from June to October, whereas type 1 cysts appeared from November to April. Type 2 cysts were more abundant than non-encysted animals during summer periods (reaching 90.7% of the total), whereas type 1 cysts were generally in lower numbers than non-encysted animals, reaching not more than 12.4% of the total. We found no evidence of spermatozoa or mature oocytes within the gonad of *A. volubilis* type 2 cysts. For type 1 cysts, we did not have sufficient information.

The dynamics of the non-encysted animals were inversely related to air temperature values (P<0.001; Fig. 4) and hours of daylight (P=0.03) and directly related to air RH values (P<0.001; Fig. 5). The dynamics of type 2 cysts were directly related to air temperature (P<0.001; Fig. 4) and hours of daylight (P=0.02), and inversely related to air RH (P<0.001; Fig. 5). The dynamics of type 1 cysts

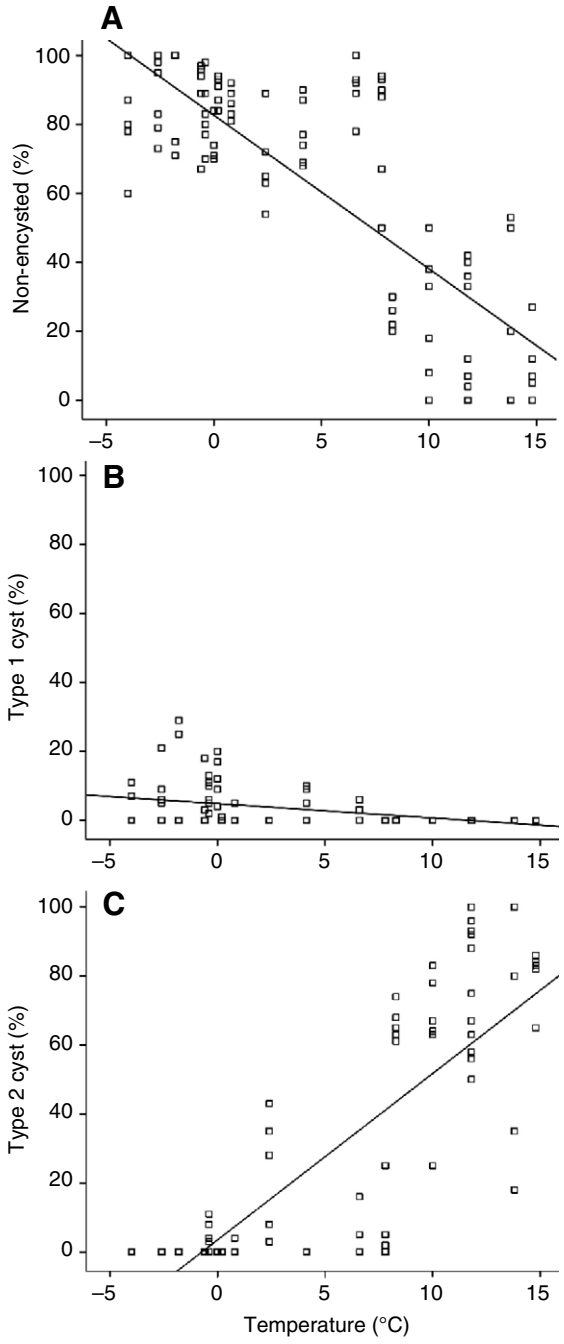


Fig. 4. Relationships between the dynamics of each state of *Amphibolus volubilis* and the mean air temperature during the two sampling years. (A) Non-encysted animals; (B) type 1 cysts; (C) type 2 cysts. Lines represent linear curves of adaptation to the total data. *N*=90.

were inversely related to air temperature ($P<0.001$; Fig. 4) and hours of daylight ($P=0.01$). No relationships were found between rain fall and the dynamics of the non-encysted animals, or of either type of cyst.

Survival ability

The survival to freezing of 48 cysts (out of a total of 53) and 18 non-encysted animals (out of a total of 20) demonstrates that both type 2 cysts and non-encysted animals are able to withstand freezing. Similarly, the desiccation experiments showed that both type 2 cysts and non-encysted animals are able to survive desiccation. In fact, seven type 2 cysts (out of a total of 16) and 34 non-encysted animals (out of a total of 48) were alive after 7 days in a desiccated state at room temperature. Moreover, six desiccated type 2 cysts (out of a total of 14) and seven desiccated non-encysted animals (out of a total of 13) were alive after 3 h at 60°C.

All type 2 cysts and all non-encysted animals died after 3 h at 60°C in water.

DISCUSSION

The most common factors inducing diapause in insects and nematodes are temperature, dehydration, photoperiod, and food quantity and quality (Denlinger and Tanaka, 1999; Sommerville and Davey, 2002; Koštál, 2006). In insects the induction phase of diapause occurs during a sensitive period when the token stimuli from the environment reach a critical level (Koštál, 2006). In tardigrades, reserve depletion, low oxygen tension, pH alteration, temperature variation and generic environmental deterioration have been related to encystment processes (Von Wenck, 1914; Marcus, 1929; Marcus, 1936; Węglarska, 1957; Szymańska, 1995; Westh and Kristensen, 1992; McInnes and Pugh, 1999; Hansen and Katholm, 2002) but it is still unclear whether these factors must be considered as the 'inducing factors' of encystment or the 'environmental adversities' that the cyst state has to withstand.

Our first experiment with cyst induction in *A. volubilis* led us to hypothesize an involvement of temperature as a token stimulus. Moreover, even the sampling period seemed to be involved in cyst production: tardigrades collected in April formed mainly type 2 cysts, whereas animals collected in November formed mainly type 1 cysts. The analyses of cyst dynamics confirmed that temperature is directly or indirectly involved in the induction and maintenance of the cyst state. In the two sampling years, the dynamics of the two types of cyst of *A. volubilis* followed seasonal variations. Type 2 cysts were present during warm and dry periods increasing suddenly in number around June and similarly decreasing suddenly in October. Type 1 cysts were present in cold periods, but always in low numbers. In these periods, factors such as temperature induced animals to form and successively to leave type 2 or type 1 cyst states. A large percentage of type 2 cysts (from 60.0 to 90.7%) suddenly appeared when the mean air temperature reached or exceeded 12°C, while type 1 cysts were present only when the mean air temperature was below 0°C. The token stimulus involved in the termination of the type 2 encystment process was probably the same stimulus involved in aestivating insects, where diapause terminates when temperature decreases (Denlinger and Tanaka, 1999). The population percentage of type 2 cysts of *A. volubilis* quickly decreased from 90.7% to 5.4% (Fig. 3) when the mean air temperature decreased below 8°C. This verifies a synchronous process within the population. In insects, the termination of diapause is also a synchronous process induced by specific changes in the environment that may or may not correspond to favourable conditions (Càceres, 1997; Koštál, 2006). Further support for temperature involvement as a token stimulus in cyst production in tardigrades is given by a study of a Greenland population of *A. nebulosus* in which a warm period induced type 2 cysts and a cold period induced type 1 cysts (Hansen and Katholm, 2002).

We conclude that the spring animals (used in experiment 1) were already sensitive (programmed) to approach to a warm season, while the autumn animals were sensitive (programmed) to a cold season. Therefore, the different responses were functions of the physiological state of the animals at the time they were collected. This condition could be compared with the sensitive period of insects

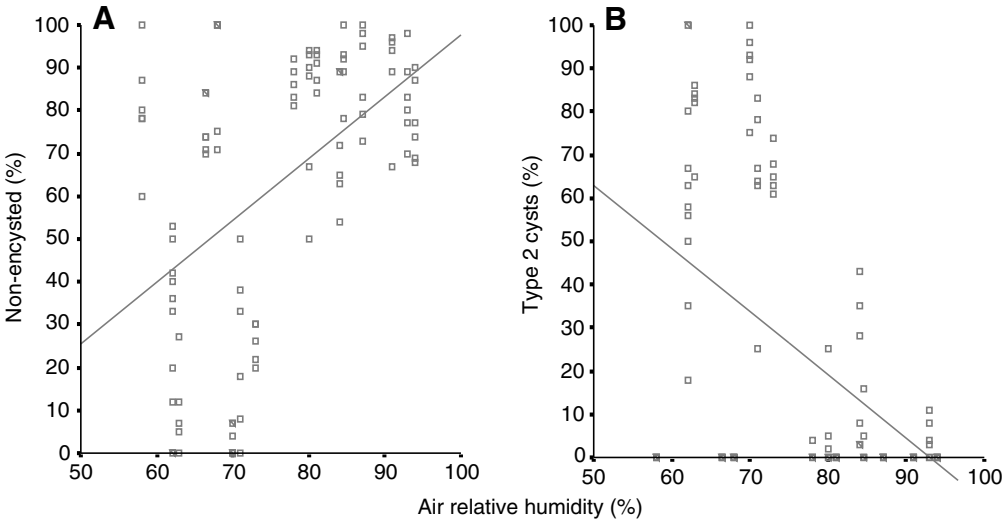


Fig. 5. Relationship between the dynamics of non-encysted animals (A) and type 2 cysts (B) of *Amphibolus volubilis* and the mean air relative humidity data during the two sampling years. Lines represent linear curves of adaptation to the total data. *N*=90.

(Koštál, 2006), indicating the involvement of endogenous factors in tardigrade encystment. In insect diapause, responses to the token stimuli can be modified by environmental factors during the induction phase (Koštál, 2006). Similarly, the results of experiment 1 indicate that high temperature (i.e. 20°C) can modify the response of the specimens collected in November, leading them to produce only type 2 cysts. A further demonstration of the involvement of endogenous factors in the encystment processes of *A. volubilis* comes from the univocal response of the animals under experiment 2. Non-encysted animals that previously were in the type 2 cyst state formed only type 1 cysts, whereas non-encysted animals collected from the wild (experiment 1) were able to form both cyst types. These two cyst types were formed under the same photoperiod. Therefore, photoperiod does not represent an indispensable direct token stimulus for cyst induction in tardigrades.

Encystment in tardigrades has to be viewed as a diapausing process controlled by exogenous (e.g. temperature) and endogenous (not yet identified) stimuli that lead to successive phases of morphological and physiological transformations. In other invertebrates, diapause is induced in advance of the advent of environmental adversities (Koštál, 2006). To protect themselves from these environmental adversities, diapausing animals minimize exchanges with their environment by producing cocoons [e.g. Nemertea and Annelida (Càceres, 1997; Diaz Cosin et al., 2006)] or a thicker cuticle [e.g. dauer larva of nematodes (Càceres, 1997)], or remaining within the cuticle of the previous instar [e.g. pharate state of insects (Gullan and Cranston, 2005)]. Similarly, encysted tardigrades (or pseudosimplex 1 state of cyclomorphosis in the marine eutardigrade *Halobiotus crispae*) use their old cuticle (exuvia), and in some cases further cuticular involucres, to isolate or protect themselves from the environment (Węglarska, 1957; Kristensen, 1982; Hansen and Katholm, 2002; Guidetti et al., 2006; Møbjerg et al., 2007). During type 2 encystment, *A. volubilis* produces several cuticular structures, undergoing deep

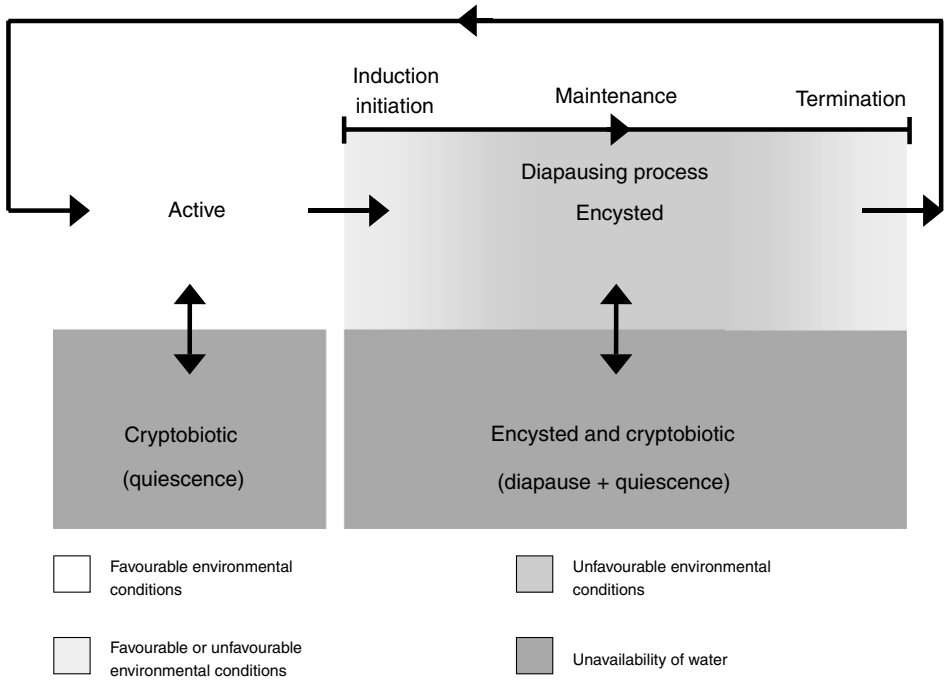


Fig. 6. Scheme showing the different states of *Amphibolus volubilis* in relation to the environmental conditions.

morphological modification (Guidetti et al., 2006). A depletion of energy supply occurs through metabolism (even though the metabolic rate is reduced) because animals cannot eat (Pigòn and Węglarska, 1953; Guidetti et al., 2006). In addition, when the animals are encysted, the population cannot increase because 90% of animals are in this state and therefore cannot reproduce. As a consequence, the production of type 2 cysts can be considered a costly process, both at individual and population levels. In comparison, production of the type 1 cyst represents a less costly process due to the lesser morphological transformations the animal undergoes, and also because only a small fraction of the population can be found in this state. Tardigrade encystment (especially type 2 cysts) represents an adaptive strategy to withstand environmental adversities, whose nature remains unknown.

Relationships between cyst production and tardigrade life cycle may exist. In an Arctic population of *A. nebulosus*, production of cysts seems obligate and is related to reproduction (Westh and Kristensen, 1992). Type 2 cysts of this species have been considered a necessary step in the production of winter resting eggs (Hansen and Katholm, 2002). The absence of mature male or female gametes within *A. volubilis* type 2 cysts, shown by our results and by Rebecchi and Bertolani (Rebecchi and Bertolani, 1994), leads us to consider that the encystment of *A. volubilis*, in contrast to that of *A. nebulosus*, is not related to reproductive function.

A possible 'environmental adversity' that animals withstand when producing cysts could be the low tolerance of animals to low oxygen tension or to high temperatures. *Isohypsius laevis* McInnes 1995, living in Antarctic lakes, passes the winter encysted as a possible response to the anoxic condition of the water (McInnes and Pugh, 1999). Even the eutardigrade *H. crispae* produces its resting state (pseudosimplex 1 state) during warm periods, probably to withstand oxygen depletion or heat stresses (Møbjerg et al., 2007). Consequently, *A. volubilis* animals withstand warm periods as type 2 cysts.

A common unfavourable environmental condition for terrestrial tardigrades is the unavailability of free water, which they withstand by entering cryptobiosis (anhydrobiosis and cryobiosis). Our experiments demonstrate that non-encysted specimens of *A. volubilis* can survive desiccation by entering an anhydrobiotic state, or they can withstand freezing by entering a cryobiotic state. In addition they demonstrate that tardigrades are able to withstand even repeated and successive cryptobiotic and diapausing states. Therefore, the lack of availability of free water related to drying or freezing cannot be considered the environmental adversity that animals withstand when producing cysts, and it cannot be the selective factor inducing the evolution or maintenance of encystment. Our experimental data demonstrate that *A. volubilis* is able to enter both diapause and cryptobiosis. Therefore, the evolution of the former adaptive strategy is not necessarily an alternative to the evolution of the latter strategy. Our results also provide evidence of an ability not known to date: the dormancy phenomena (diapause and quiescence) can occur simultaneously, as summarized in Fig. 6. In fact, type 2 cysts of *A. volubilis* are able to survive at 60°C only in a desiccated state, while they do not survive when hydrated. Moreover, type 2 cysts of *A. volubilis* are able to freeze, entering a cryobiotic state, as are both cyst types of *A. nebulosus* (Westh and Kristensen, 1992). This simultaneous ability to perform the two adaptive strategies largely increases the possibility of resistance to environmental stresses.

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