VIE ET MILIEU - LIFE AND ENVIRONMENT, 2021, 71: 29-34

ADAPTATION OF GRASSLAND SPRINGTAILS (COLLEMBOLA) TO DRY AND HOT ENVIRONMENTAL CONDITIONS

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ANHYDROBIOSIS
DIAPAUSE
COLLEMBOLA
SPHAERIDIA PUMILIS
ECOLOGICAL HEATING

ABSTRACT. – Some springtails (Collembola) are known to be tolerant to drought. They adapt to extreme environmental conditions by going into diapause or anhydrobiosis. The purpose of the present study was to assess the desiccation and the heat tolerance of an epedaphic springtail community of a grassland habitat (lawn). Survival at high temperatures with regard to these parameters has also been investigated. Out of the seven collected species (*Ceratophysella cavicola*, *Entomobrya multifasciata, Lepidocyrtus cyaneus, Sminthurinus aureus, Sminthurinus elegans, Sminthurus viridis,* and *Sphaeridia pumilis*) diapause was observed in *S. elegans* (up to 60 days), *S. viridis* (90 days), *S. pumilis* and *C. cavicola* (246 days). Only *C. cavicola* showed anhydrobiosis. *Sphaeridia pumilis* survives extreme heating up to 120 °C.

INTRODUCTION

Many organisms from diverse taxa of life have the remarkable ability to survive extreme desiccation in nature by entering a metabolic state known as anhydrobiosis (life without water) (Erkut & Kurschalia 2015). Anhydrobiotic organisms survive thermal extremes in addition to drought, switching metabolic activities on and off, according to the needs of the moment. It is achieved by the reversible loss of almost all the organism's water (Clegg 2001, Salehian *et al*. 2011). Some organisms have cryptobiotic/anhydrobiotic potential throughout their entire life cycle, whereas some others exhibit it only at a certain developmental stage (Erkut & Kurschalia 2015). Some arthropods, mainly insects and crustaceans, can undergo anhydrobiosis at certain stages of life as eggs and larvae. Egg diapause is a programmed state of developmental arrest that is accompanied by the suppression of the metabolic rate, serving to dramatically reduce energy consumption (Hahn & Denlinger 2011). Termination of dormancy can be triggered by desiccation/inundation or dehydration/hydration cycle and the presence of favorable environmental conditions, such as adequate light, temperature, salinity, pH, oxygen, and so on (Brendonck 1996, Saengphan *et al*. 2005, Beladjal *et al*. 2007, Atashbar *et al*. 2014).

Cold hardiness is an adaptation to an extreme temperature gradient (Holmstrup & Bayley 2013, Teets & Denlinger 2014). The adaptation to hot conditions (being another extreme) has less attention. Terrestrial life in humid environments is frequently subjected to hot conditions during periods of water deficit, especially in semi-arid and arid habitats. Most of the soil dwelling species disappear before water loss is reduced to the limit. In our previous work, we stated that anhydrobiotic stages, such as seeds, spores, diapause eggs, and cryptobiotic animals (*e.g*., tardigrades) survive up to 130° C, but only if they are subjected to slow heating ("ecological heating"), in agreement with their ecological environment, whereas rapid heating (a 'heat shock') results in a dramatic reduction of survival (Mertens *et al*. 2008).

The literature on metabolic pathways in anhydrobiotic eukaryotes is quite well documented (Clegg 2001, 2005, Rebecchi 2013, Erkut & Kurzchalia 2015). The ability to regulate osmotic pressure of body fluids is based on the accumulation of compatible osmolytes, such as sugars and free amino acids (Holmstrup & Bayley 2013). However, the endurance against drought of a species in the community is less well known. Alvarez *et al*. (1999) examined the effects of a 4-month simulated drought on the emergence of diapause eggs of Collembola collected from arable fields. The time scale and the variability of survival of anhydrobiotic springtails are supposed to be very different. It is usually assumed that edaphic springtails are prone to desiccation, while epigeal *Symphypleona* have become adapted to periodically dry environments. We hypothesize that some species, as well as *Poduromorpha* and *Symphypleona*, not only tolerate extreme dry and hot environmental conditions, but that the survival time can vary greatly between species of the same community.

In the present work, we investigate the adaptation to desiccation from the opposite direction. Springtails, sampled from a humid environment, were gradually dried in culture containers, allowing the adaptable springtail species of the community to enter anhydrobiosis, or to die if active dispersion is the only option to escape the dry conditions of the sampled habitat.

MATERIAL AND METHODS

Surviving dry conditions: The springtails were collected from a lawn in the Botanical garden of Ghent University (51°02'27"N-03°43'20"E, Gent, Belgium) using a hand vacuum cleaner. After several minutes vacuuming, the reservoir of the vacuum cleaner was emptied into culture boxes. This process was repeated until a rich sample was obtained, in order to start the experimental set-up. The culture boxes were kept for a few minutes without a lid in order to let the most arthropods (spiders, ants etc.) escaping the boxes. Other unwanted species (*e.g.*, mites) were subsequently removed under a stereomicroscope (Wild M5), leaving the springtails on the substrate.

Prior to the experiment, a water saturated mixture of plaster/charcoal (9/1) was applied to the bottom of culture containers (plastic boxes 100 × 70 × 35 mm) and left for several days to dry. A filter paper (water saturated by impregnation on the plaster/charcoal substrate) was added to the culture boxes. The collected sample was distributed at random over 25 culture containers. No food was added, since the collected material had a rich diversity of appropriate food items for the springtails. All species had the opportunity to reproduce during 5 days at 100 % relative humidity, 20 °C (± 5 °C) and a day/night regime (12 h/12 h). After this period, the lids of the boxes were removed, allowing the substrate to dry slowly. One week later, the dried filter papers at the bottom were gently removed (horizontally, in order to not lose any material) and were cut in two pieces (50 × 70 mm). The fifty (50) paper strips were air dried.

Once a week two paper strips, chosen at random among the stock, were put on a fresh wet substrate (plaster/charcoal) in two culture boxes. This process lasted 8 months (Fig. 1). The boxes were checked every two days for living springtails under a stereomicroscope (Wild M5) during 3 weeks. Active animals were removed, counted and identified. When dealing with juveniles, we assumed that they hatched from eggs in the sample, thus they were representative for diapause eggs.

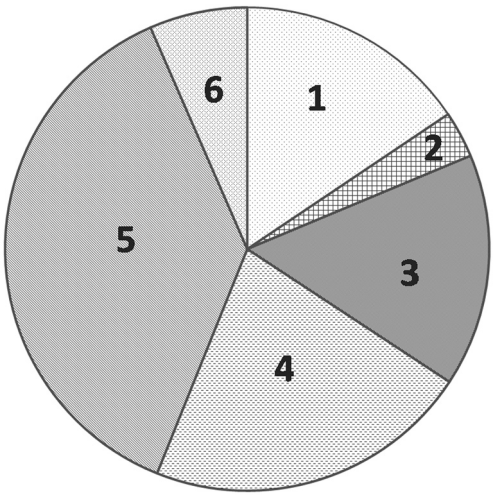

Fig 1. – Circular diagram summarizing the different stages of the experiment time.
(1): Preparation of the culture containers with plaster/charcoal bottom (5 days); (2): Sampling the springtails in the field: start of the experiment (1 day); (3): Reproduction period in humid environment (5 days); (4): Dehydration period (7 days); (5): Stock of samples as dried filter papers (min. 7 days max. 240 days); (6): Rehydrated environment (21 days).

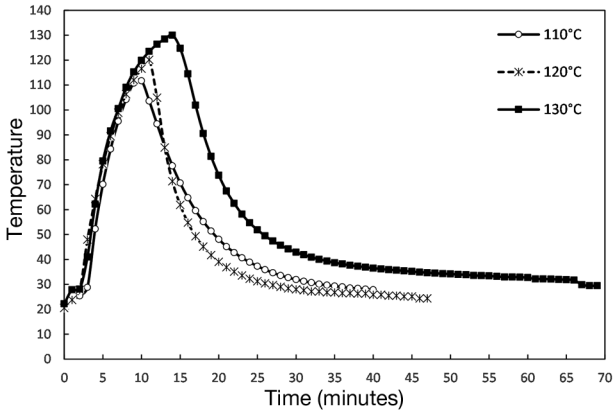

Fig. 2. – Rate of temperature increase (°C) of the samples during the heating experiment as a function of incubation time (minutes).

Surviving ecological heating: The methodology used for the heating experiment was the same as described in Mertens *et al*. (2008). The samples were transferred to a glass test tube of 8 mm diameter (VWR, article 212-0011) and heated in a dry air oven (Jouan-EU170). A thermocouple wire probe (connected to a data logger, Testo 175-T3) was placed directly into the sample holder in contact with the eggs, assuring that the actual temperature of our samples (dormant eggs) was measured and not the surrounding one. Ecological heating ('slow heating') refers to samples at room temperature (~22 °C), heated at a rate of 4 °C/min to the final temperature of 110 °C. (Fig. 2).

To investigate the heat resistance of aestivating springtails, the same sampling methods and experimental set-ups as described above were applied. Here too, every dry filter paper was cut into two pieces: one piece for the heating experiment, the other one as a control (no heating). One hour after the heating experiment, the viability of eggs and springtails was assessed in the same way as for surviving the dry conditions.

Hot hardiness of Sphaeridia pumilis: Grass and moss clippings containing eggs of *S. pumilis* were collected from the same lawn as for 'surviving dry conditions', during early spring (March) and air dried at room temperature. These eggs were exposed to negative temperatures during different nights before sampling since ground frost is very frequent at this period of the year. After a final drying during 3 days in a desiccator over silica gel, the samples were slowly heated in the dry-air oven up to 110 °C as described previously. The temperature was measured by a thermo-couple placed directly among the sample in the aluminium sample holder and connected to a data logger (Testo 175-T3). After cooling down, the samples were immediately assayed for viability. Subsequently they were transferred into culture boxes (100 × 70 × 35 mm) on a wet plaster/charcoal substrate, and regularly checked for neonate springtails under a stereomicroscope (Wild M5) for 3 weeks. The experiment gives

only a qualitative indication of the survival of the embryos, since it was impossible to count all the eggs attached into the collected vegetation.

Good visible eggs on an appropriate substrate are needed for quantitative data. Small glass tubes (Ø 2 cm, height 3 cm, filled 1/3 with a substrate of plaster/charcoal) were appropriate for the breeding tests. The top of each of the tubes was closed with parafilm. About 5 to 10 females and 5 males were housed per tube. A few days later enough eggs were laid on the wall of the glass tubes just above the plaster/charcoal substrate for an experiment. Eggs were easy to observe and to count under a stereomicroscope (Wild M5).

After air-drying, the tubes with eggs were incubated for 3 days over silica gel in a desiccator. The samples were ecological heated (slowly) to 110 °C, 120 °C and 130 °C, respectively. The probe of a wire thermocouple (connected to a data logger, Testo 175-T3) was placed directly in the sample holder, in order to measure the actual temperatures experienced by the eggs. After cooling down, the substrates of the tubes were subsequently rewetted and the top of the tubes recovered with parafilm. A few days later and over a week, the juveniles were counted and removed.

Statistical analysis: The statistical analysis was done using the SPSS software package (version 25; SPSS Inc.). The Mann-Whitney U-test was used as non-parametric analysis. $p < 0.05$ was chosen as significance level.

RESULTS

In total, seven (7) Collembola species springtails species were collected and identified: *Ceratophysella cavicola* (Börner, 1901), *Entomobrya multifasciata* (Tullberg, 1871), *Lepidocyrtus cyaneus* Tullberg, 1871, *Sminthurinus aureus* (Lubbock, 1836); *Sminthurinus elegans* (Fitch, 1863), *Sminthurus viridis* (Linnaeus, 1758), and *Sphaeridia pumilis* (Krausbauer, 1898).

The neonates hatched from the diapausing eggs after they underwent the dry and the wet period were identified as *C. cavicola, S. aureus, S. elegans, S. viridis,* and *S. pumilis*. No juveniles were identified as *E. **multifasciata*** and *L. cyaneus* after wetting the substrate. These species are typical to the humid environment and did not survive the dry conditions.

Active adults as well as colored juveniles of one species (*Ceratophysella cavicola*) occurred in the samples after rewetting from the first day on. They survived as cryptobiotic individuals.

Only *C. cavicola* showed anhydrobiosis. The difference between neonates, hatching from diapause eggs, and the cryptobiotic survival of juvenile and adult instars was obvious since active purple adults, as well as reddish juveniles were present one day after inundation, while dormant eggs needed at least 5 days to hatch into transparent/white neonates.

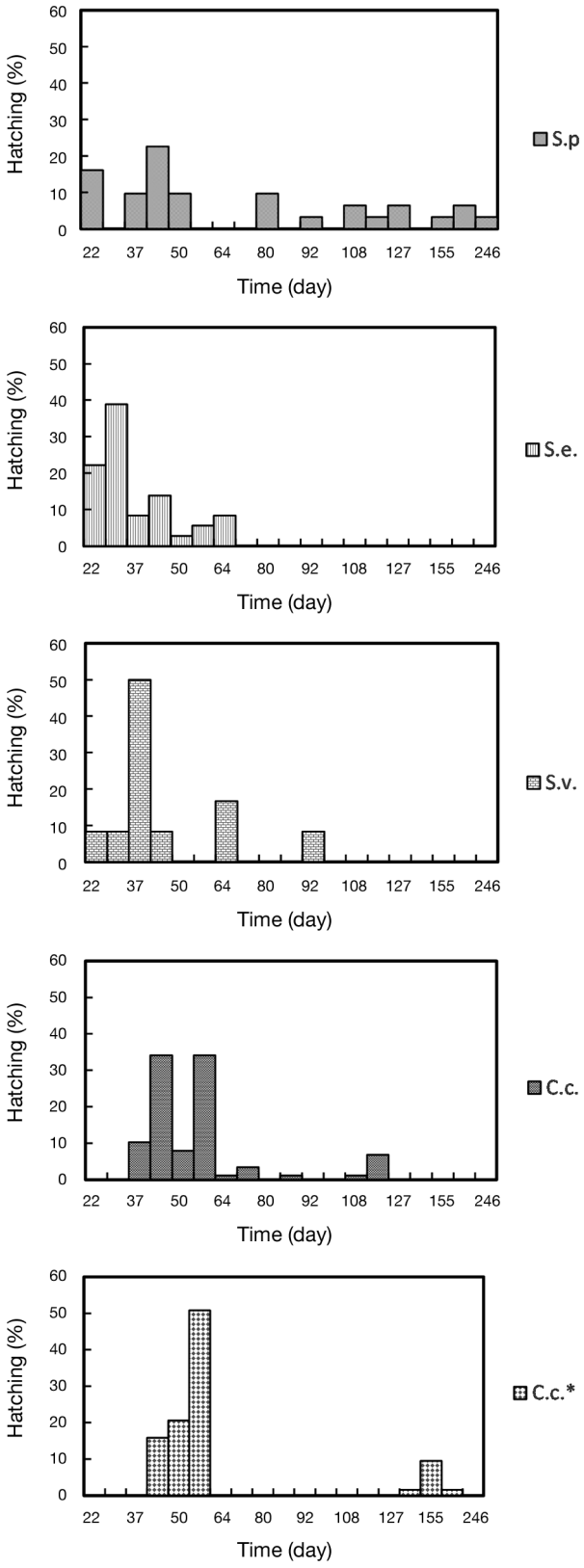

Fig. 3. – The percentage (%) of springtail's neonates that survive a dry period. (S.p: *Sphaeridia pumilis*; S.e.: *Sminthurinus elegans*; S.v.: *Sminthurus viridis*; C.c.: *Ceratophysella cavicola* from cryptobiosis ; C.c.*: *C. cavicola* from eggs).

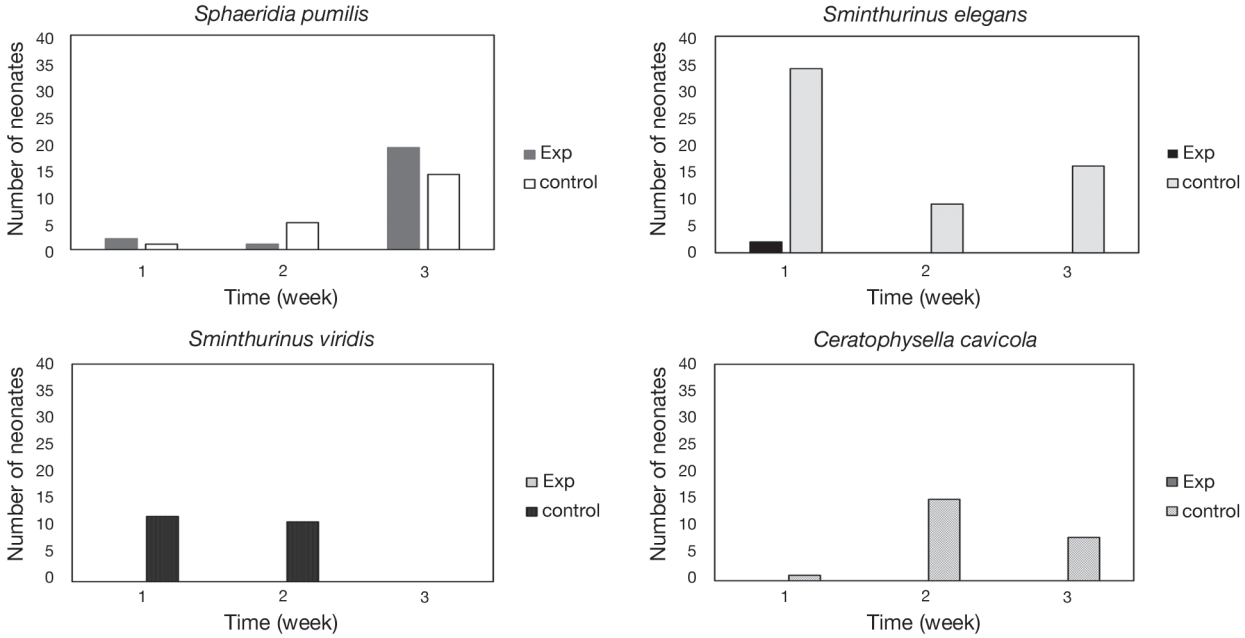

Fig. 4. – Neonate springtails hatched on week 1, 2 and 3 after heating (110°c) and rewetting the substrate compared to the control (not heated).

Surviving dry conditions differs from one species to another (Fig. 3). *S. elegans* tolerated no more than 2 months of desiccation, while *S. viridis* endured a drought period of 3 months. However, *S. pumilis* and *C. cavicola* survived up to 8 months (246 days exactly), having only 4 months for a reproductive season. This is only valid to the dormant eggs of *C. cavicola*. Specimens recovered after a period of cryptobiosis are restricted to a dry period of 4 months.

The hatching of *S. pumilis* eggs after slow heating up to 110 °C is presented in Fig. 4, together with the data of all other diapausing species. Some diapause eggs of *S. elegans* (± 1 %) and all diapause eggs of *S. pumilis* survived the extreme heating. All the other species did not survive these temperatures.

No significant difference ($p < 0.05$) was observed in *S. pumilis* when comparing hatching time and the number of neonates between the experimental data and the control groups. Furthermore, the eggs on the grass clippings, collected during the period of night frost, did not only survive 110 °C, but also the negative temperatures outside during the weeks before the sampling and the heating experiment. This means that an embryo tolerates, within a period of 2 weeks, 100 % relative humidity in the lawn of its natural environment, as well as ± 0 % relative humidity in a desiccator in the laboratory, the freezing temperatures of the lawn at night (–5 °C minimum), as well as and 110 °C in the oven.

Some *S. pumilis* juveniles hatched from eggs heated up to 120 °C, being the upper thermal limit tolerated by this species. The high temperatures have no influence on the development of an individual afterwards. Juveniles grow into adult males and females that reproduce and give a normal offspring.

A female coats her diapause egg immediately after the oviposition with a fresh faecal pellet that is molded around the egg using her end legs (Fig. 5). In dry conditions, the oval eggs shrivel into a cup form, recovering within hours after rewetting. Non-diapause eggs are mostly not coated.

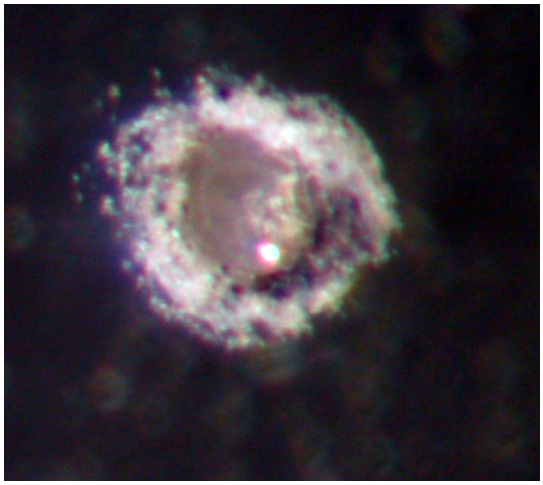

Fig. 5. – Diapause egg of *Sphaeridia pumilis*. The coat is partly removed in order to expose the egg.

DISCUSSION

The survival limits of dried eukaryotes can be explained by the fact that they are related to the stability

of the contemporary organisms, as well as to their early evolution. It is quite possible that thermal resistance to extreme temperatures was determined very early in the evolution of terrestrial organisms and was maintained thereafter. Resistance is certainly not the result of recent adaptation, but rather reflects characteristics that were developed during an early evolutionary process on the pristine substrates of the continents, and which were subsequently maintained (Mertens *et al*. 2008).

It is possible that such thermal capacities were acquired during the Silurian and Devonian periods, before the appearance of leafy plants. There was then neither the buffering effect of the (organic) soil, nor the heat absorption by photosynthesis in shady forests (Glasspool *et al*. 2004). Anhydrobiosis evolved as an evolutionary adaptation for organisms on the continents in pre-Devonian times. The phenomenon allowed them to survive thermal extremes, as well as to activate and deactivate the metabolic activities, as needed at the time. This applies not only to terrestrial organisms, but also to aquatic species living in temporary pools and forced to survive periods of drought following evaporation and waiting for the next flood (Beladjal & Mertens 2017).

The epedaphon's diapause eggs on the surface are exposed to extreme temperature, humidity and radiation on very hot days. The crust of the topsoil layer is frequently broken into tiny pieces, leaving the resting stages to the elements. Wind can disperse these anhydrobiota as propagules to new habitats or as exchangeable elements between gene pools (Bishop *et al*. 2001).

The phenology of *Sphaeridia pumilis* is characterized by an active life ranging from a cool and humid microclimate in the fall to the end of the following spring, alternating with a long period of inactivity during the dry summer when the population survives as aestivating eggs (Blancquaert *et al*. 1982). *S. pumilis* not only survives long dry periods of summer, but also extreme temperatures above 100 °C on the hot, arid substrate, as well as freezing conditions within the same day.

Temperature has been pointed out to play an important role in regulating many aspects of springtail population dynamics (Hopkin 1997). Various mechanisms could explain the long-term impacts of temperature on the number of overwintering eggs, on the number and or fertility of overwintering adults and / or on the quality and availability of resources the following year (Wolters 1998). It is therefore not excluded that diapause eggs survive cold temperatures as they migrate through the upper layers of atmospheric air. This may explain the almost cosmopolitan distribution of the species (Bellinger *et al*. 1996-2020).

During the Carboniferous period, buffered microclimates became more abundant. As a result, most species would have abandoned the costly genetic mechanism that was necessary at that time to adapt to such extremes environment. Today, these organisms live in buffered continental habitats, but species with ancient pre-Devonian protective genes continue to resist in "hotspot" and dry places. It is conceivable that the species in the present work exhibited here the characteristics resulting from the preservation and the expression of these "ancient genes" from the Devonian period, or even earlier. Anhydrobiosis allows for adaptation of the stages of a species' life cycle to survive various environmental extremes, in addition to drought.

We do not consider the evolution of hyperthermophiles, but the need for hyperthermophile anhydrobiota to outface the dry and hot periods that existed much earlier on Earth. The vegetation structure has buffered the direct effect of climate change on aboveground microarthropods such as springtails. Soil temperature was considered as an important factor and climate change should be seen as strongly influencing the succession dynamics of springtails. (Daghighi *et al*. 2017).

Our results on ecological (slow) heating provide the basis for the hypothesis that contemporary organisms have retained "ancient genes" from the Devonian or even earlier, which were needed to protect themselves in these extremely dry and very hot continental environments.

ACKNOWLEDGEMENTS. – We thank Dr A Davis (Ghent University) for having the kindness to edit the language and F Janssens (University of Antwerp) for his useful comments on the draft version.

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Received on January 11, 2021
Accepted on April 23, 2021
Associate editor: C Battisti