Comparative Biochemistry and Physiology, Part A 199 (2016) 8–13

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Earthworms accumulate alanine in response to drought

Martin Holmstrup [a,*], Stine Slotsbo [a], Per G. Henriksen [b], Mark Bayley [b]

[a] Department of Bioscience, Aarhus University, Vejlsøvej 25, 8600 Silkeborg, Denmark
[b] Department of Bioscience, Aarhus University, C.F. Møllers Allé, Universitetsparken, 8000 Aarhus, C, Denmark

ARTICLE INFO

Article history:
Received 25 February 2016
Received in revised form 11 April 2016
Accepted 14 April 2016
Available online 21 April 2016

Keywords:
Aporrectodea
Compatible osmolytes
Desiccation
Lumbricus
Oligochaeta
Osmotic stress

ABSTRACT

Earthworms have ecologically significant functions in tropical and temperate ecosystems and it is therefore important to understand how these animals survive during drought. In order to explore the physiological responses to dry conditions, we simulated a natural drought incident in a laboratory trial exposing worms in slowly drying soil for about one month, and then analyzed the whole-body contents of free amino acids (FAAs). We investigated three species forming estivation chambers when soils dry out (*Aporrectodea tuberculata*, *Aporrectodea icterica* and *Aporrectodea longa*) and one species that does not estivate during drought (*Lumbricus rubellus*). Worms subjected to drought conditions (< −2 MPa) substantially increased the concentration of FAAs and in particular alanine that was significantly upregulated in all tested species. Alanine was the most important FAA reaching 250–650 μmol g^{-1} dry weight in dehydrated *Aporrectodea* species and 300 μmol g^{-1} dry weight in *L. rubellus*. Proline was only weakly upregulated in some species as were a few other FAAs. Species forming estivation chambers (*Aporrectodea* spp.) did not show a better ability to conserve body water than the non-estivating species (*L. rubellus*) at the same drought level. These results suggest that the accumulation of alanine is an important adaptive trait in drought tolerance of earthworms in general.

1. Introduction

Earthworms are ecosystem engineers performing important functions in the soil. By their tunneling through the soil, earthworms increase soil porosity, and by ingesting considerable amounts of soil and dead plant material, they contribute to the mixing of organic matter and mineral soil. Altogether, these functions of earthworms improve aggregate stability, soil aeration and increase microorganism decomposition processes (Edwards and Bohlen, 1996; Lavelle et al., 1997; Parmelee et al., 1998). Although earthworms are terrestrial animals, they are sensitive to drying of the soil (Edwards and Bohlen, 1996). Earthworms have only very moderate morphological or physiological means for reducing water transport through the cuticle (Carley, 1978) and they are active only if free water is available in the soil (Lee, 1985). During dry periods, earthworms may move to deeper soil layers where conditions are more favorable (Gerard, 1967; Rundgren, 1975), but if droughts are long-lasting, earthworms are forced to cope with desiccation stress by physiological means. Since earthworms are ecologically significant in many ecosystems, it is important to understand the physiological and biochemical adaptations of these animals to extreme drought events.

The earthworm *Aporrectodea caliginosa* and other species of this genus enter diapause during summers if the water potential becomes too low (Gerard, 1967; Nordström, 1975). In this process, the worm excavates a spherical cell in the soil, lined with mucus and egested gut contents. By coiling itself into a ball in the soil, water loss may be reduced during drought. Nevertheless, earthworms can generally survive extensive water loss for several days and water losses up to 80% of the normal water content are tolerated (El-Duweini and Ghabbour, 1968; Grant, 1955). As a consequence of water loss, earthworms must be able to tolerate deleterious effects of osmotic stress caused by high concentrations of inorganic ions such as Cl^- and Na^+. Invertebrates faced with osmotic stress, whether it is caused by high salinity, desiccation or freezing, often provide protection by producing compatible osmolytes that may "dilute" inorganic ions, help in cell volume regulation and protect cellular membranes and proteins (Crowe et al., 1992; Storey, 1997; Yancey, 2005). Compatible osmolytes are small organic molecules such as sorbitol, trehalose, betaine or certain free amino acids (e.g. alanine) that can be present in high concentrations with insignificant effects on cellular processes (Hochachka and Somero, 2002). In earthworms, glucose is an important osmolyte in relation to freeze tolerance (Holmstrup et al., 1999; Holmstrup and Overgaard, 2007), but apparently not in desiccation tolerance (Friis et al., 2004), whereas earthworm embryos of dehydrated egg capsules accumulate the osmolyte sorbitol in high concentrations (Holmstrup, 1995; Petersen et al., 2008).

Recently, Bayley et al. (2010) discovered that dehydrated adult individuals of *A. caliginosa* estivating in dry soil for a month had drastically increased the concentration of alanine to more than 80 mOsm, suggesting that alanine is an important player in the drought tolerance of this species. This observation encouraged us to investigate if

* Corresponding author.
E-mail address: martin.holmstrup@bios.au.dk (M. Holmstrup).

http://dx.doi.org/10.1016/j.cbpa.2016.04.015
1095-6433/

Table 1
Body water content (mean ± SE) of earthworms at control conditions and at the end of a 32-day drought exposure. The estimated soil water potential (mean ± SE) of the desiccated soil is also shown. See text for further descriptions.

Species		Control	Drought	Stat. significance
A. tuberculata	Soil water potential (MPa)	≈0	−2.36 ± 0.69	
	Body water content (g g^{-1} dry mass)	3.67 ± 0.12	2.26 ± 0.42	$p = 0.024$
A. icterica	Soil water potential (MPa)	≈0	−3.04 ± 0.19	
	Body water content (g g^{-1} dry mass)	4.56 ± 0.42	1.07 ± 0.02	$p < 0.0001$
A. longa	Soil water potential (MPa)	≈0	−4.64 ± 0.76	
	Body water content (g g^{-1} dry mass)	4.46 ± 0.43	1.23 ± 0.13	$p < 0.0001$
L. rubellus	Soil water potential (MPa)	≈0	−1.73 ± 0.15	
	Body water content (g g^{-1} dry mass)	3.77 ± 0.23	2.65 ± 0.05	$p = 0.0038$

accumulation of free amino acids is also a response to drought in other earthworm species. We simulated a natural drought incident in a laboratory trial exposing worms in slowly drying soil for about one month, and then analyzed the whole body contents of free amino acids. In this experiment, we determined the responses of three species forming estivation chambers when soils dry out (*Aporrectodea tuberculata*, *Aporrectodea icterica* and *Aporrectodea longa*) and one species that does not estivate during drought, namely, *Lumbricus rubellus* (Nordström, 1975).

2. Methods

2.1. Earthworms

Earthworms were collected from garden soil in Aarhus, Denmark, in late November 2012, where soils were moist and earthworms were active. We collected large immature and adult specimens of four species: *A. tuberculata* Savigny, *A. icterica* Savigny, *A. longa* Ude and *L. rubellus* Hoffmeister. The worms were kept in moist garden soil in aerated plastic containers and brought to the laboratory where they were held at 15 °C for a week before being used in experiments.

2.2. Drought exposure

Drought exposure followed a modified version of the method described by Friis et al. (2004). The soil used for the drought exposure experiment was an organically farmed loamy sand consisting of 32% coarse sand (>200 μm), 48% fine sand (20–200 μm), 9% silt (2–20 μm), 7% clay (<2 μm), 4% organic matter and a pH of 5.9 as described by Holmstrup (2001). The soil was dried at 80 °C, sieved through a 4 mm sieve, and then re-wetted to a water content of 19% of dry weight at the beginning of the experiment. About 160 g of this moist soil was added to a polystyrene cylinder (inner diameter 6 cm; height 5 cm) together with one earthworm (except for *L. rubellus* where two worms were added). Both ends of the cylinder containing moist soil and worm(s) were closed with two layers of gauze to confine the worm(s), but allowing the soil to slowly desiccate. The cylinder with soil and worm(s) was placed horizontally in a 1-litre plastic pot (height 135 mm; lid diameter 120 mm; bottom diameter 95 mm). The lid had 12 holes (diameter 3.5 mm) allowing slow evaporation of water from the moist soil through the holes in the lid. All pots were kept in a climate room at 14.8 ± 0.2 °C. The relative humidity (RH) of the climate room could not be controlled but ranged between 30% and 60% with an average of 41.8% RH.

2.3. Estimation of soil water potential (SWP)

At the start of the experiment, we weighed each of the pots containing the cylinder with moist soil and worm(s). Thereafter, we weighed each pot every week to keep track of water loss and the gradual drying of the soil. Once the water content had reached the target soil moisture of approximately 4% of dry weight, we replaced the perforated lid with an intact lid drastically reducing further water loss from the soil (but not creating anoxic conditions) until all pots were harvested after 32 days. The time to reach the target soil water content varied between 11 and 29 days, but earthworms were not sampled until 32 days of drought exposure had elapsed. The final weight of the pots was noted and used to calculate the water content of the soil. Soil water content was then converted to SWP (Ψ, MPa, negative) using a retention curve determined for the test soil. The relationship between water content and SWP was determined as described by Friis et al. (2004) using Wescor C-52 sample chambers connected to a Wescor HR-33T Dew Point Microvoltmeter operated in the dew point mode (Wescor, Logan, UT, USA). In the range of soil water contents between 2% and 7% of dry weight, we found a linear relationship with log(SWP): $\log(\Psi) = -0.3208x + 4.57$ ($N = 8$; $R^2 = 0.94$), and used this relationship to estimate the SWP that each worm had experienced.

2.4. Sampling of earthworms

After 32 days of drought exposure, worms were recovered from the dry soil, and any soil particles adhering to the skin was quickly removed. The worm was considered alive if it reacted to handling. It was noted if the worm had rolled up into an estivation state. The topic of the present study was not a comparison of survival in the four species under desiccation stress, and only surviving worms were used for analysis. Each worm was flash-frozen in liquid nitrogen. The frozen worm was rapidly divided into two parts, and each part was placed in preweighed 1.5 mL centrifuge tubes, and weighed to the nearest 0.1 mg to determine fresh weight. One randomly chosen part was dried at 60 °C until constant weight (48 h) and weighed again to determine dry weight and calculate water content (g g^{-1} dry mass). The other part was stored at −80 °C until used for quantification of FAAs.

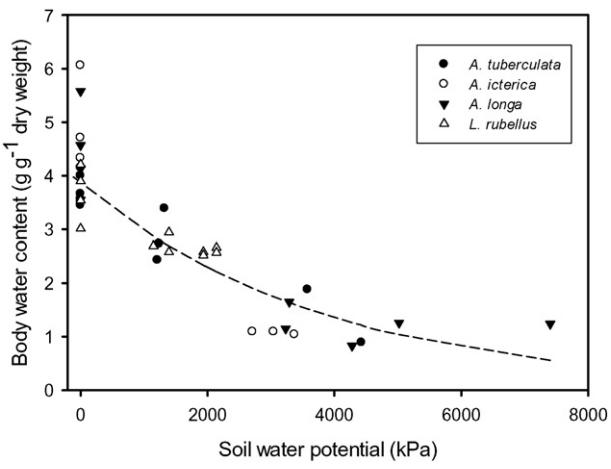

Fig. 1. Relationship between soil water potential (kPa, negative) and body water content (g g^{-1} dry weight) of worms. The line represents the best fit of a function described as log(body water content) = 1.3552521–0.000262 × SWP ($p < 0.0001$; $N = 38$). Soil water potential of control worms have been assigned the value 0 kPa.