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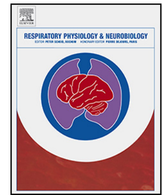

Respiratory adaptations to a combination of oxygen deprivation and extreme carbon dioxide concentration in nematodes

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ABSTRACT

To examine physiological adaptations to the two combined stressors O_2 deprivation and extreme CO_2 concentrations, we compared respiratory responses of two nematode species occurring in natural CO_2 springs. The minimum O_2 concentration allowing maintenance of respiration in both species was 0.0176 μmol O_2 ml^{-1} (corresponds to 1.4% O_2 in air). After exposure to anoxia, individuals resumed respiration immediately when O_2 was added, but on a lower level compared to control and without showing a respiratory overshoot. A species-specific response was found in respiration rate during 20% CO_2: the more tolerant species maintained respiration rates, whereas the sensitive species showed a decreased respiration rate as low as after anoxia. The results indicate that during 20% CO_2 the sensitive species undergo a survival state. We conclude, that the ability to maintain respiration even under low oxygen and high CO_2 concentrations may allow the better adapted species to occupy an ecological niche in the field, where others cannot exist.

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1. Introduction

Hypoxia occurs in organisms, tissues and cells when oxygen supply does not correspond to the demand (McLellan and Walsh, 2004; Celeste Simon, 2006). Different evolutionary conserved mechanisms exist to cope with oxygen deprivation (e.g., Bunn and Poyton, 1996; Hochachka et al., 1996; Hochachka and Lutz, 2001; Storey and Storey, 2004). Often the metabolic rate is decreased drastically, which is a widespread adaptation of animals to stress (metabolic rate depression; e.g., Childress and Seibel, 1998; Storey, 1998; Guppy and Withers, 1999). In a number of species, organisms' metabolism is reduced below the limit of detection and they survive inactively in a cryptobiotic state (Keilin, 1959). By undergoing a form of cryptobiosis, which is caused by oxygen deprivation (anaerobiosis), some species may endure long periods of anoxia, e.g. 4 years in *Artemia franciscana* (Clegg, 1997). However, most eukaryotes are essentially aerobic; among other factors, they need oxygen to complete their life cycle (Bunn and Payton, 1996; Clegg, 2001; DiGregorio et al., 2001; Hermes-Lima and Zenteno-Savín, 2002). Cryptobiotic organisms, accordingly, get active again as soon as possible when environmental conditions improve and O_2 is available again (Clegg, 1997; Padilla et al., 2002). Often species need some minutes up to several hours to recover from cryptobiotic states ("lag phase", Wharton, 2002). Some then show a respiratory overshoot (Herreid, 1980), an enhanced respiration rate necessary for O_2 consuming processes like repairing damage, rebuilding ATP stores and disposing of the accumulated end products of anaerobic metabolism (Ellington, 1983). Little is known about the underlying mechanisms of cryptobiosis caused by lack of oxygen, and even less about the "awakening" and subsequent processes.

In the field stress factors are often interrelated and individuals face a combination of different stress factors, for example dehydration and freezing or dehydration and osmotic stress. Few studies exist dealing with a combination of different stress factors which cause the transition into survival states (e.g. Wharton and To, 1996; Wharton et al., 2003) and even less is known about hypoxia. The present study focuses on soil-dwelling nematodes from an extreme habitat, a so called mofette field. A mofette field is a natural, cold-degassing CO_2 spring, characterized not only by hypoxic/anoxic conditions but also by extreme CO_2 concentrations of up to 100%. Degassing of CO_2 reduces O_2 and in soil a pattern of spatially and temporally variable CO_2 concentrations is formed. Species living in such soils are able to cope with two stressors at once: First, the effects of anoxia, which lead on the cellular level to a shortage of ATP, and second, high concentrations of CO_2, which lead to signs of paralysis (Nicholas, 1975; Hajeri et al., 2010). But not all nema-

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tode species are equally adapted: Hohberg et al. (2015) found that some are more tolerant and occur in the field in sites with up to 62% CO_2, e.g. *Acrobeloides* cf. *buchneri*, whereas others are more sensitive and occur only in sites containing at maximum 20% CO_2, e.g. *Acrobeloides nanus*. Despite the different species distribution pattern of the two closely related species, in the laboratory they both survived 100% CO_2 in an inactive state at least for 5 days (Pilz and Hohberg, 2015).

In the present study, we investigated respiratory adaptations of the two species, one tolerant and one sensitive, and the effects of two combined, mutually dependent stressors: O_2 deprivation and extreme CO_2 concentrations. The aim of the study was to investigate the importance of physiological adaptations for niche building processes in the field.

We hypothesize that:

1) the critical oxygen concentration down to which the nematodes are able to maintain respiration is lower in the more CO_2-tolerant species,
2) after 100% CO_2/anoxia both species need to recover from cryptobiosis and resume respiration delayed with a short lag phase, but respiration rates are enhanced ("respiratory overshoot"), and
3) during exposure to O_2 reduction/CO_2 surplus only the sensitive species reacts with a reduced respiration rate.

2. Material and methods

2.1. Nematode cultures and required preparations

Laboratory cultures of *A.* cf. *buchneri* derived from a mofette field near Hartoušov, Czech Republic (detailed site description is given in Hohberg et al., 2015), which is a cold degassing natural carbon dioxide spring and emits almost pure CO_2 (Vodnik et al., 2006). Individuals of *A. nanus* descended from a culture of the Senckenberg Museum of Natural History Görlitz. Both species are cultivated under ambient atmospheric conditions (0% CO_2, 21% O_2) on a modified, nutrient-poor LB medium (1.2 g agar, 0.33 g casein, 0.17 g NaCl, 0.17 g yeast extract, 100 ml demineralised water, adjusted with HCl to pH 4) and at a constant temperature of 20° C. As both species are bacterial feeders (Yeates et al., 1993) plates were inoculated with cultures of *Escherichia coli* MC4100 as food resource, obtained from Martin-Luther University Halle-Wittenberg (for culturing details see Pilz and Hohberg, 2015).

Per species, every treatment (control, respiration during minor stress and respiration after major stress, respectively) was measured in independent experiments on several days ($n = 13$). To gain fit nematodes in high concentrations (50,000–150,000 individuals ml^{-1}) needed for respiration measurements, every day nematodes of the two species were extracted freshly from well developed 3–4 weeks-old culture plates using modified Baermann funnels (Hohberg, 2003) and tap water (adjusted with HCl to pH 4). After extraction, nematodes were allowed to sink down for 3 h and the supernatant was removed. The remaining nematode suspension was centrifuged, again the supernatant was removed, and 0.1 ml samples were taken, from which nematodes were roughly counted under the microscope (60 x magnification). This procedure was repeated until we received per species a volume of ca. 20 ml of pH 4 water with ca. 100,000 nematodes, whereby the different suspensions contained on average 89,962 (54,827–124,000) individuals ml^{-1} and 8916 (5598–14,226) μg fresh weight ml^{-1}. The daily suspensions of the two species were each divided into the required number of 1.1 ml aliquots for respiration measurements, each of which (and thus every nematode) was only used once in a respiratory measurement. Additionally, we took three aliquots from each suspension to accurately determine nematode numbers and biomass of the respective suspension. From these 3 aliquots, body length and width of 100 individuals were measured under the microscope (100 x magnification) and individual body mass was calculated after the formula: $m = l \times d^2/1600000$, with m = mass (fresh weight) in μg, l = length in μm, d = diameter in μm (Andrassy, 1956). Translations of measured data to respiration rate per nematode fresh weight were done with the respective factors resulting from the average of the 3 respective aliquots.

Table 1
Overview of CO_2 and O_2 concentrations during the treatments.

term	gas concentration during experiments
control	ambient atmospheric conditions (0% CO_2 and 21% O_2)
during minor stress	20% CO_2 and 17% O_2 creating natural conditions in the mofette field
after major stress	atmospheric conditions, but experiments done directly after 21 h exposure to 100% CO_2 and 0% O_2

2.2. Respiration under ambient atmospheric conditions (control)

Oxygen consumption was measured using a Clark-type electrode (Oxylab, Hansatech, Norfolk, England). 0.85 ml of an aliquot was pipetted into the measuring cuvette. Per run and treatment, oxygen consumption was recorded for ca. 20 min. Respiration rates, however, were only deduced from between minute 7 and minute 15 following the start of the experiment, a time span where handling effects from inserting the sample into the analyzer are supposed to have ceased and stress effects on animals to be smallest. A magnetic stirrer ensured that oxygen was equally distributed throughout the reaction vessel and the temperature was held constant at 20 °C during the experiments. At least every fourth measurement was a control, consisting of water and bacteria from the nematode-free supernatant of the respective suspension. By subtracting the control rate from the respiration rates measured, we removed the device drift as well as the part that was respired by microbial respiration.

2.3. Respiration during minor stress (20% CO_2, 17% O_2)

For 20% CO_2 treatments, each 1.1 ml aliquot tube was set for 21 h into an experimental device (for details see Pilz and Hohberg, 2015), where an atmosphere of 20% CO_2 and accordingly 17% O_2 was adjusted (Table 1). For every respiration measurement ($n = 13$) a single tube was then taken out of the experimental device, lidded immediately to maintain the atmosphere and the contents were instantly pipetted into the measuring cuvette and respiration rates measured as described in Section 2.2 (including control measurements).

2.4. Respiration after major stress (100% CO_2, anoxia)

Thirteen 1.1 ml aliquots per species were set for 21 h into the experimental device (see Section 2.3) with an atmosphere of 100% CO_2 and 0% O_2. Again, aliquots were taken out individually and immediately transferred into the measurement device. Oxygen was added with a gas-tight syringe. Respiration rates ($n = 13$) were determined as quickly as technically possible after oxygen was added, i.e. from minute 7 onwards to avoid disturbance-related overestimations of respiration rates.

2.5. Threshold oxygen concentration

The oxygen concentration, down to which respiration could be maintained, was determined after exposure of nematodes of both

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