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Energy reserves and cellular energy allocation studies: Should food supply be provided?

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ABSTRACT

Energy budget studies offer important information in terms of organisms' fitness and this has become an increasingly common stress marker. The method is well developed but the issue of food supply (addition or not) during exposure is not consensual. Moreover, chemicals such as cadmium (Cd) are known to affect food uptake, e.g. via feeding inhibition, hence energy budget results could be because of decreased uptake and not direct toxicity. On the other hand, food deprivation can be a stressor itself and bias results. In the present study we compared exposure with (F) and without food (NF) along time (2, 4 and 8 days). The Cellular Energy Allocation (CEA) method was used to study the effects of Cd in the soil standard species *Enchytraeus albidus* (Oligochaeta). In control conditions (un-spiked soil), carbohydrate and lipid budgets were mobilized in NF. When testing Cd, variations in carbohydrates and lipid budgets depend on the presence of food and time of exposure. The main hypothesized mechanisms triggered by Cd exposure were similar (i.e. induction of protein synthesis and increase in energy consumption). Differences between F and NF over time indicate that the process of mobilization/preservation of energy reserves depends on the food/energy intake (e.g. in Cd exposed organisms, mobilization of proteins occurred within 2 days when food was present, while in the absence of food carbohydrate and protein budgets were mobilized from 2 to 4 days). Comparisons between F-NF studies should not be done directly. Moreover, we recommend exposure without food because it allows a better discrimination of effects (in particular within periods of exposure).

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1. Introduction

Energy budget is an indication of the organisms' overall condition (Calow, 1991). The use of Cellular Energy Allocation (CEA) (De Coen and Janssen, 2003, 1997) to assess the energy budget of an organism in response to stressors is well established, being used in several invertebrate species, notably in enchytraeids (Gomes et al., 2015a, 2015b; Novais and Amorim, 2013; Novais et al., 2013). The rationale is that stress can cause organisms to mobilize their energy reserves (e.g. for detoxification processes) with costs for biological processes such as growth or reproduction. Testing procedures for exposure of organisms vary regarding the supply or not of food. There is no consensus in the current practices, while several studies are conducted with food (e.g. (De Coen and Janssen, 2003, 1997; Novais and Amorim, 2013; Novais et al., 2013; Soetaert et al., 2007)) other do not supply food, to avoid the interference of chemicals' effects on feeding behaviour and energy intake (Verslycke and Janssen, 2002) or to avoid disturbing soil metal availability (Beaumelle et al., 2014). On the other hand, if no food is added, starvation could act as an additional stress, depending on the test organism or exposure duration. In the present study we compared the response of energy reserves to exposure with food (F) and without food (No Food: NF) using the standard soil species *Enchytraeus albidus* (Oligochateta) (OECD, 2004; ISO, 2005). Cadmium (Cd) was selected because it is known to inhibit food consumption/ingestion rate in various organisms (Pestana et al., 2007; Russell et al., 1981; Wicklum and Davies, 1996), hence it is relevant for the present study. The objectives were to investigate how the presence and absence of food affect the energy budget of *E. albidus* i) in control conditions (un-spiked soil), and ii) in Cd exposure, in terms of CEA (including Energy available and consumed). The factor time of exposure (from 0 to 2 days, 2 to 4 days and 4 to 8 days) was included to investigate the short-term temporal variations of CEA.

2. Materials and methods

2.1. Test species

The test species *Enchytraeus albidus*, Henle, 1837 was used. Organisms were maintained in laboratory cultures for several years and under controlled conditions in a mixture of LUFA 2.2 natural soil (Speyer, Germany) and Organization for Economic Cooperation and Development (OECD) artificial soil (3:1). Cultures were kept at a temperature of

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18 °C (±1) with a photoperiod 16:8-h light:dark, and the organisms were fed twice a week with finely ground and autoclaved rolled oats.

2.2. Test soil, chemical and spiking procedure

The natural standard soil LUFA 2.2 (Speyer, Germany) was used. The main characteristics can be described as follows: pH (0.01 M $CaCl_2$) = 5.5, organic matter = 1.77%, CEC (cation exchange capacity) = 10.1 meq/100 g, WHC (water holding capacity) = 41.8%, and grain size distribution of 7.3% clay, 13.8% silt, and 78.9% sand.

Cadmium (Cd) was purchased from Fluka as $CdCl_2$ (99%).

Spiking was performed by adding Cd as aqueous solution to the pre-moistened soil to reach the final concentration of 150 mg Cd/kg of soil (dry weight). The spiked soil was left to equilibrate for three days after which water was added up to 50% of the soil WHC.

The selected concentration of Cd corresponds to the reproduction EC_{90} (i.e. 90% reduction in reproductive output) and LC_{50} (i.e. 50% mortality) (Novais et al., 2011).

2.3. Experimental setup

Testing followed the standard guidelines (ISO, 2005; OECD, 2004) with adaptations following Novais et al. (2013). Fifteen adult worms with well-developed clitellum were introduced in test vessels, each containing 25 g moist soil (50% WHC). The organisms were exposed for 0, 2, 4 or 8 days at 20 °C (±1) and a 16:8 h photoperiod. Five replicates per test condition were performed, each one containing a pool of 15 organisms. The test conditions include Cd treated (150 mg Cd/kg) and control (CT, un-spiked) with (Food: F_50 mg of finely ground and autoclaved rolled oats) and without the addition of food (No Food: NF) at the beginning of the exposure. The test conditions in which food was added will be further referred as CT-F and Cd-F; and the test conditions without the addition of food will be further referred as CT-NF and Cd-NF. At each sampling time, animals of each replicate/test condition were carefully removed from the soil, rinsed in deionized water, weighted, frozen in liquid nitrogen, and stored at −80 °C until further analysis. Organisms from day 0 were sampled directly from cultures and stored following the same procedure.

2.4. Sample preparation

CEA was measured according to the procedure described by Novais and Amorim (2013). Prior the analysis, each replicate (pool of 15 organisms) was homogenized using a sonicator (Branson Sonifier 250) for 10 s, in 1000 μl of ultra-pure water and divided in three sub-samples of 300 μl, i.e. 1) for total protein and carbohydrate content determination, 2) for total lipid content determination and 3) for the electron transport system (ETS) activity.

2.5. Energy available – Ea

Available energy reserves were measured in whole-body homogenates by determining spectrophotometrically the total protein, carbohydrate, and lipid content at each time point, according to the procedures described by De Coen and Janssen (2003, 1997). In short, protein content was determined following the Bradford method (Bradford, 1976) at 600 nm using bovine serum albumin as standard. Total carbohydrate content was determined with phenol 5% and concentrated H_2SO_4 (95–97%) at 490 nm using glucose as standard (De Coen and Janssen, 2003, 1997). Total lipids were extracted according to the method described by Bligh and Dyer (1959) and determined by measuring the absorbance at 400 nm using tripalmitine (Sigma) as standard. All measurements from each replicate were made in triplicate. The available energy reserve measured in 300 μl was then extrapolated for the total volume of 1000 μl and transformed into energetic equivalents using enthalpy combustion (24 kJ/g proteins, 17.5 kJ/g carbohydrates, and 39.5 kJ/g lipids) as described in De Coen and Janssen (2003, 1997).

2.6. Energy consumption – Ec

The energy consumption (oxygen rate consumption) was determined based on measurement of ETS activity (King and Packard, 1975) over the 3 exposure periods (0–2, 2–4, and 4–8 days), following the methodology described in detail by De Coen and Janssen (1997). The ETS activity was measured by adding a solution of the reduced form of nicotinamide adenine dinucleotide phosphate and p-iodo-nitro-tetrazolium (Sigma) and following the increase in absorbance at 490 nm for 3 min. All measurements from each replicate were made in triplicate. The oxygen consumption rate was determined based on the theoretical stoichiometric relationship that for each 2 mmol of formazan formed, 1 mmol of O_2 is consumed in the ETS (De Coen and Janssen, 1997). The quantity of consumed oxygen was then transformed into caloric values using oxyenthalpic equivalents of 484 kJ/mol O_2 for an average lipid, protein, and carbohydrate mixture (De Coen and Janssen, 1997; Gnaiger, 1983).

2.7. Cellular Energy Allocation – CEA

The CEA represents the net cellular energy budget, consisting of the difference between the budget of energy reserves (Ea) and energy consumption (Ec) and was calculated for each time interval, as described in De Coen and Janssen (1997), using the following equation:

$$CEA(\mathrm{mJ/mgOrg}) = \frac{\int_{n-1}^{n} Ea \cdot dt - \int_{n-1}^{n} Ec \cdot dt}{t_n - t_{n-1}}$$

with n being the sampling time (0, 2, 4 or 8 days) and n-1 being the previous time in which measurements were done (e.g. if n is 4 days, then n-1 is 2 days).

The budget of each energy reserve (proteins, carbohydrates, and lipids) was calculated by integrating the changes in energy values over the 3 exposure periods (0–2, 2–4, and 4–8 days). Using energy budgets (integration over time), in addition to the absolute values of energy reserves, has the advantage of describing energy changes over periods of exposure. This should also reflect any required physiological change occurring during the whole exposure period (Smolders et al., 2004). The total Ea value was calculated by summing the integrated energetic values of each energy reserve fraction, corresponding to each of the 3 time intervals. The Ec value was similarly calculated, integrating the change in energy consumption over the same exposure periods.

2.8. Data analysis

Data were tested for normality and homogeneity of variances using Shapiro-Wilk and Levenes's tests, respectively.

Differences in terms of weight of the organisms, Ea, Ec and CEA were tested using three-way analysis of variance (ANOVA) with the factors: food (F and NF), time (0, 2, 4 and 8 days for weight and from 0 to 2, 2 to 4 and 4 to 8 days for Ea, Ec and CEA) and treatment (Cd vs CT); pairwise comparisons were done using the Least Significant Different method with 95% confidence level ($p < 0.05$). The software used was IBM SPSS (version 24).

3. Results

Table 1 summarizes the weight variation of enchytraeids over time.

In CT-F, the weight of the organisms decreased from the 4th to the 8th day, whereas NF organisms lost weight from the 2nd to the 4th day. For Cd-F the organisms' weight was significantly different between 2, 4 and 8 days of exposure (increased from 0 to 2 and from 2 to 4 days

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