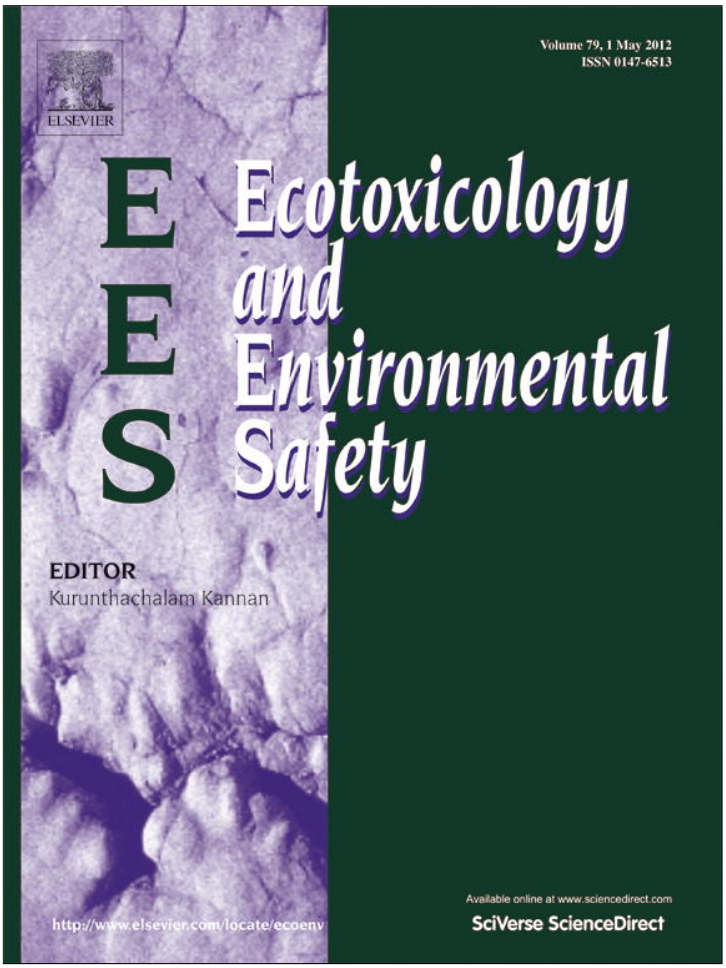

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Highlighted Article

The link between antioxidant enzymes catalase and glutathione S-transferase and physiological condition of a control population of terrestrial isopod (*Porcellio scaber*)

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ABSTRACT

The aim of this work was to investigate if the activities of catalase and glutathione S-transferase in a control population of terrestrial isopods (*Porcellio scaber*) are correlated with the physiological condition of the isopods. For this purpose, the activities of these enzymes were analysed in isopods from a stock population and in parallel, the physiological condition of the same specimens was assessed using a histological approach based on epithelial thickness and lipid droplets. We found a correlation between antioxidant enzymes and the physiological condition of the isopods. This implies that these enzymes could be used as predictive indicators of the physiological condition in a stock population before comprehensive toxicological studies are conducted and also in control group after the experiment. When a control group is found to be very heterogeneous in terms of physiological condition, the experiment should be repeated with a larger number of experimental animals. The findings of this study will contribute to more accurate experimental design of toxicity tests when using biomarkers. This should encourage other researchers to increase their effort to know the physiological state of their test organisms.

1. Introduction

The approach using antioxidant enzymes to assess the effects of chemicals on organisms is now well established (Jemec et al., 2010). Interpretation of data on antioxidant enzymes in chemically stressed organisms relies on comparison with unexposed animals as controls and it is therefore of vital importance that the antioxidant enzymes in control animals be well-defined. This paper was motivated by our observation that the activities of antioxidant enzymes catalase (CAT) and glutathione S-transferase (GST) in a stock population of terrestrial isopods (*Porcellio scaber*) vary despite prevalence of constant abiotic conditions during cultivation. We have already determined that gender and age of isopods are not the sources of variability (Jemec et al., 2008) and this paper was aimed at investigation of whether the activities of CAT and GST in a control population are correlated with the physiological condition of isopods.

Catalase and GST usually change as a response presumably, to reactive oxygen species (ROS) (Livingstone, 2001). Catalase detoxifies hydrogen peroxide (Halliwell and Gutteridge, 2007), while GST is involved in the cellular detoxification of xenobiotic substances and the endogenous products formed during lipid peroxidation. In chemically stressed organisms, an increase of antioxidant enzyme activities resulting from enhancement in ROS levels in comparison to controls is expected (Razinger et al., 2008). At higher concentrations of exogenous chemicals, the activities of antioxidant enzymes can be lower due to inhibition either directly, by generated ROS, or indirectly, as a consequence of general cellular dysfunction (Escobar et al., 1996).

The terrestrial isopod (*Porcellio scaber*) Latreille 1804 (Isopoda, Crustacea) is commonly used as a test organism in ecotoxicity studies, and its physiology is well known (Drobne et al., 1999). The physiological condition of isopods has been assessed previously by histological appearance of their digestive gland (hepatopancreas) (Köhler et al., 1996; Žnidaršič et al., 2003; Lešer et al., 2008) which is the isopod's major digestive organ with intestinal, hepatic, and pancreatic functions (Hames and Hopkin, 1989; Drobne, 1997; Jemec et al., 2008). The hepatopancreatic epithelium contains two cell types, the large B cells that project into the lumen of the hepatopancreas and wedge-shaped small S cells. The B cells are secretory and absorptive, and usually contain many lipid droplets, glycogen, and metal ions stored in granules. The S cells accumulate metals, calcium, and urate (Wägele, 1992). Thinning of hepatopancreatic epithelium and depletion of lipid droplets in B cells occurs upon chemical and physiological stress (e.g. starvation) (Ribeiro et al., 2001; Odendaal and Reinecke, 2003; Žnidaršič et al., 2003; Lapanje et al., 2008; Lešer et al., 2008).

In this work, hepatopancreatic CAT and GST activities in a stock population of isopods were analysed and in parallel, the physiological condition of the same specimen was assessed using the histological

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approach based on epithelial thickness and lipid droplets. The aim was an assessment of whether antioxidant enzyme activities and physiological condition of isopods are correlated. This would mean that the physiological condition of isopods could be determined based on the activities of CAT and GST.

2. Materials and methods

2.1. Chemicals

The following chemicals were purchased from Sigma (Germany): eosin Y (dye content 90%), glacial acetic acid, dibasic and monobasic potassium phosphate, 30% hydrogen peroxide, 1-chloro-2,4-dinitrobenzene, and L-glutathione (reduced form). BCA protein assay reagents A and B were purchased from Pierce (U.S.A.). Xylene, ethanol, and chloroform were purchased from Merck (Germany); isopropanol from Fluka (Germany), and Canada balsam from Kemika (Croatia). Paraplast Plus was purchased from Sherwood Medical (U.S.A). All chemicals were of the highest commercially available grade, typically 99% or higher.

2.2. Test organisms

Terrestrial isopods (*Porcellio scaber*, Latreille 1804) were collected under the litter layer in an uncontaminated location near Ljubljana. In the laboratory, the animals were kept in a terrarium (20 × 35 × 20 cm) filled with a 2–5 cm layer of moistened soil and a thick layer of partly decomposed hazelnut tree leaves (*Corylus avellana*). The substratum in the terrarium was heated to 80 °C for several hours to destroy predators, in particular the spiders, before the introduction of the isopods. The culture was kept at a controlled room temperature (21 ± 1 °C), 16:8 h

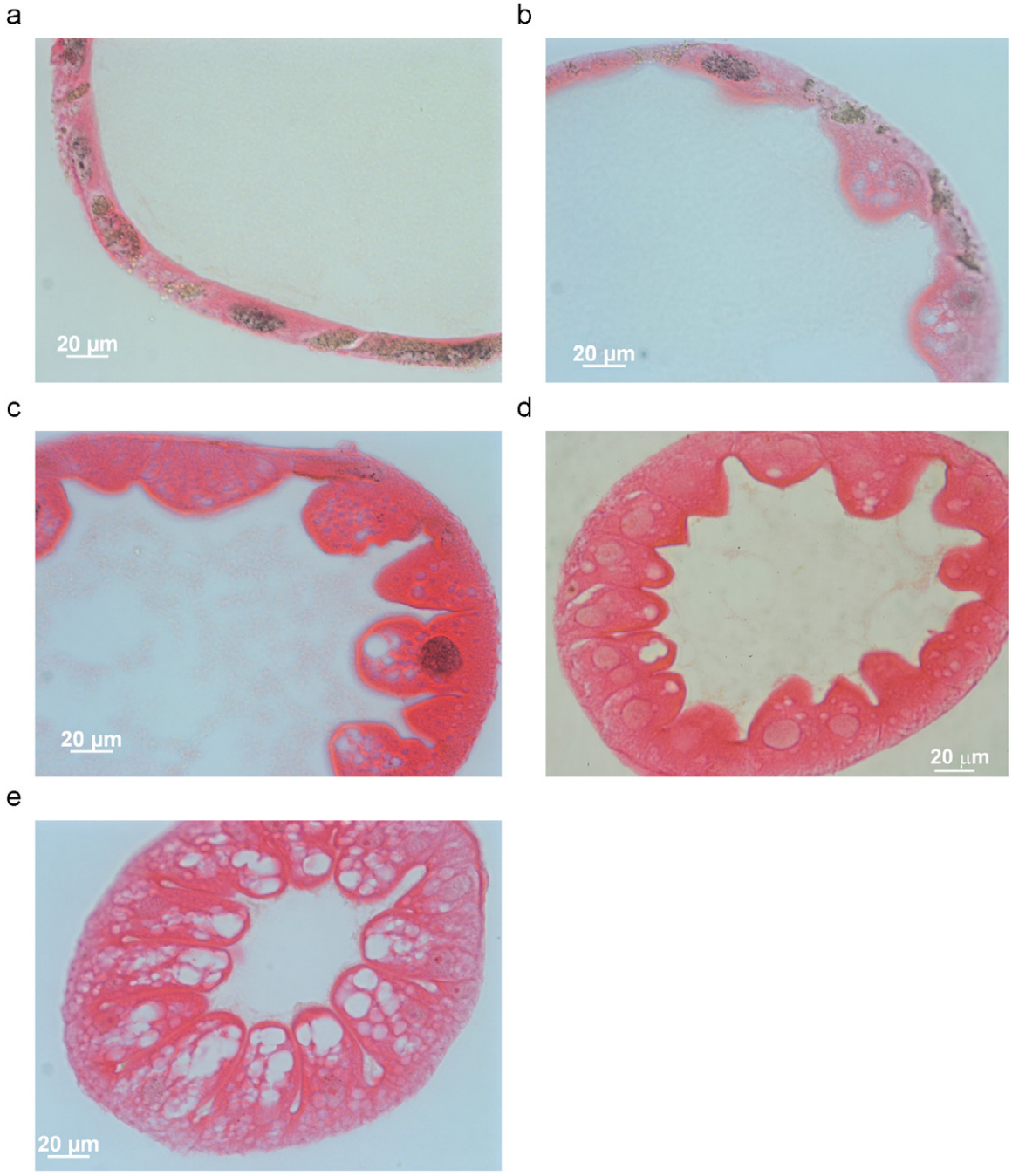

Fig. 1. Examples of different shapes and sizes of epithelial B cells, which were used to determine the epithelial thickness of hepatopancreas of terrestrial isopod *P. scaber*. The epithelium is classified into 5 classes on the basis of the size and the shape of B cells from class 1 (Fig. 1a) totally flattened B cells to class 5 (Fig. 1e) large, dome shaped B cells. Lipids are seen as white empty droplets.

light/dark regime and high humidity. The adults of both sexes (intermoult and early premoult stages; 25–80 mg fresh body) were used, because it had been shown previously that gender and age do not affect either of the investigated parameters in isopods (Jemec et al. 2008; Lešer et al., 2008).

2.3. Experimental

Epithelial thickness, lipid droplet abundance, and antioxidant enzyme activities of digestive glands were investigated in a total of 83 specimens. All four parameters were measured in the same digestive glands of the same organism. The digestive glands consist of four blind-ending tubes. In this study, one tube was used for histological examination and the other three were prepared for enzyme analysis. Epithelial thickness and lipid content did not vary between the tubes (Lešer et al., 2008).

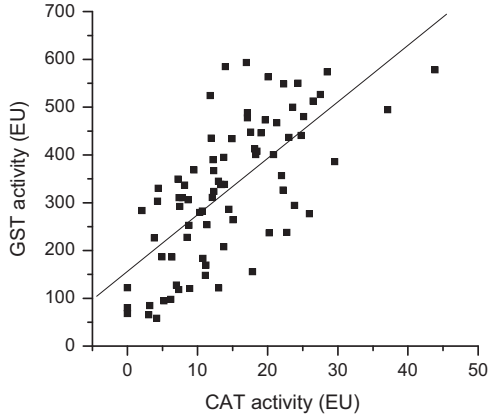

Fig. 2. Interrelation between glutathione S-transferase (GST) and catalase (CAT) activities (83 specimen in total) (EU-enzyme unit: μmol/min/mg protein for CAT, nmol/min/mg protein for GST).

2.4. Analysis of enzyme activities

An enzyme sample was prepared from each animal. The activities of both GST and CAT were measured in each sample in triplicate. Enzyme activities were assessed according to Jemec et al. (2008). Enzyme units were: *n*moles of conjugated reduced glutathione/min/mg protein (extinction coefficient $\varepsilon_{340}=9600\ M^{-1}\ cm^{-1}$) for GST and μmoles of degraded hydrogen peroxide/min/mg protein ($\varepsilon_{240}=43.6\ M^{-1}\ cm^{-1}$) for CAT. Protein concentration was measured using a BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA).

To facilitate the comparison of CAT and GST activities with epithelial thickness and lipid abundance, the activities of both enzymes were ranked in 5 classes. The data for each enzyme were expressed as histograms, the shape of each histogram resembling the normal, Gaussian distribution of values. The width of the bin was considered to be the size of a certain rank. The ranking for CAT activities was performed as follows: rank 1 (0–8 EU), rank 2 (8.1–16 EU), rank 3 (16.1–24 EU), rank 4 (24.1–32 EU), and rank 5 (32.1–40 EU). GST activities were divided into rank 1 (0–120 EU), rank 2 (121–240 EU), rank 3 (241–360 EU), rank 4 (361–480 EU), and rank 5 (481–600 EU).

2.5. Preparation of histological sections

The isolated digestive gland tube was fixed in Carnoy-B fixative (10% glacial acetic acid, 60% ethanol, and 30% chloroform) for 2.5 h at room temperature. The fixative was washed with absolute ethanol for 2 h and the tissue transferred to xylene (three times for 15 min each) and subsequently embedded in melted paraffin wax (Paraplast plus) at 56 °C overnight. The Paraplast was allowed to harden for at least 2 days at room temperature, then 8 μm sections (Reichert-Joung 2040 rotary microtome) of the entire tube were cut. All sections were stained with eosin for 30 min, and then briefly rinsed in 70% alcohol (twice for 2 s each) and in 96% alcohol (twice for 2 s each), dehydrated in isopropanol (twice for 2 min each), and cleared in xylene (twice for 5–10 min each). Slides were mounted using Canada balsam.

2.6. Evaluation of epithelial thickness and lipid droplet abundance in B cells

For evaluation of epithelial thickness and abundance of lipid droplets in B cells, the hepatopancreatic tube was divided into 6 zones each of approximately 1 mm in length. In each of the zones, four equally distant histological sections

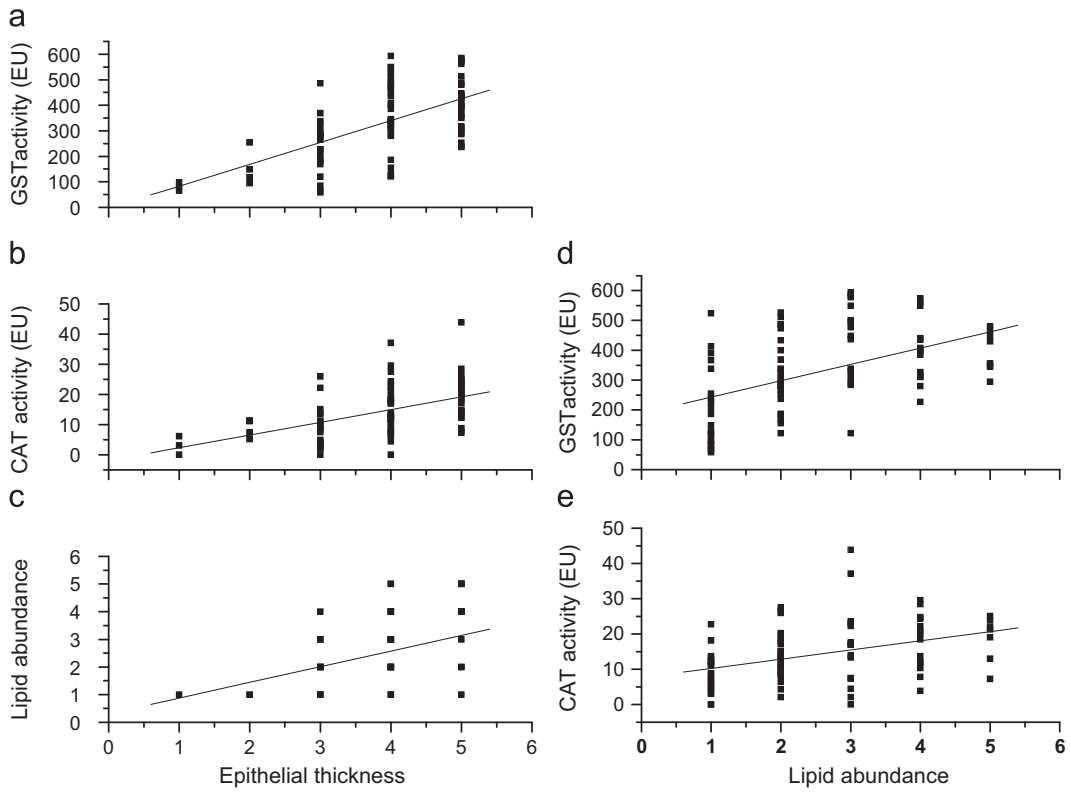

Fig. 3. Correlation between glutathione S-transferase (GST), catalase (CAT), abundance of lipid droplets and epithelial thickness (a,b,c,); and CAT, GST, and abundance of lipid droplets (d,e) (83 specimen in total) (EU-enzyme unit: μmol/min/mg protein for CAT, nmol/min/mg protein for GST).

were selected and morphometrically analysed. Most proximal and distal zones were excluded from further analysis, since the proximal end is often damaged by handling, and the distal end is composed of immature cells and therefore not relevant in terms of morphological characteristics. We analysed a total of 16 histological cross sections per one gland tube. In each animal, only one tube was analysed since it had previously been shown that there are no significant differences among the four tubes of one animal (Lešer et al., 2008).

The epithelial thickness was assessed visually using a light microscope (Axioskop 2 MOT, Carl Zeiss). On the basis of previously developed methodology (Lešer et al., 2008), the epithelia were grouped into five groups (Fig. 1) as follows:

grade 1: totally flat B cells.
grade 2: totally flat B cells with regions of slightly flattened or pyramidally shaped B cells. Degrading cells can be observed occasionally.
grade 3: pyramidal shaped B cells or slightly flat B cells. Occasionally, degrading cells can be observed.
grade 4: large, dome shaped B cells with regions of pyramidally shaped or slightly flat B cells.
grade 5: large, dome shaped B cells.

The abundance of lipid droplets in the B cells of the hepatopancreatic epithelium was classified visually as: (grade 1) sparse, (grade 2) low, (grade 3) moderate, (grade 4) high, or (grade 5) very high as described in Lešer et al. (2008).

2.7. Assessment of physiological condition of specimens

On the basis of epithelial thickness and abundance of lipid droplets in B cells, animals were divided into three groups to each of which a distinct physiological condition was assigned based on previous knowledge (Lešer et al., 2008):

(a) Non-stressed: epithelium grades 4 and 5; abundance of lipid droplets grades 3, 4, and 5.
(b) Moderately stressed: epithelium grades 3, 4, and 5 and abundance of lipid droplets grades 1 and 2, or epithelium grade 3; abundance of lipid droplets grades 3, 4, and 5.
(c) Severely stressed: epithelium grades 1 and 2 and abundance of lipid droplets grades 1–5.

2.8. Statistical analysis

All correlation analysis were done with raw data, expressed with correlation coefficients (r) using a simple regression application in Statgraphics software (Statgraphics Plus for Windows 4.0, Statistical Graphics Corporation). Correlation was the term to describe the relation between an independent and dependent variables (CAT, GST, and abundance of lipid droplets and epithelial thickness), while interrelation was used to describe the relationship between two independent variables (CAT and GST). The enzymes were ranked to allow an easier comparison with epithelium and lipid abundance in different physiological conditions.

3. Results

3.1. Correlation/interrelation between histological parameters and antioxidant enzyme activities

A significant interrelation ($p=0.0001$; $r=0.689$) was observed between the two antioxidant enzyme activities, CAT and GST, in digestive glands (Fig. 2). These are both significantly correlated ($p<0.0001$; $r=0.509$ for CAT, and $p<0.0001$; $r=0.608$ for GST) with the epithelial thickness of the digestive glands and less significantly ($p<0.001$, $r=0.402$ for CAT and $p<0.0001$, $r=0.495$ for GST) to lipid abundance in the epithelial cells of digestive glands (Fig. 3). The abundance of lipids was significantly related to epithelial thickness ($p<0.0001$; $r=0.513$).

3.2. Histological and antioxidant enzymes in animals in different physiological conditions

The results are presented individually for each animal in Fig. 4, and for the whole population in Fig. 5. On the basis of parameters assessed for each individual animal, the animal was categorised as being non-stressed, moderately stressed, or severely stressed.

Inspection of individual specimens showed that the majority of severely stressed animals with very thin epithelia (ranks 1 and 2), and low abundance of lipids also had low CAT and GST activities. Less than 10% of such specimens was found in this control population. The majority of non-stressed animals with thick epithelia had high values of antioxidant enzyme activities and high abundance of lipid droplets. There were some exceptions, in which some animals with very thick epithelia (grades 4 and 5) had low CAT and GST activities and low abundance of lipid droplets (Fig. 4) but, the correlation between the parameters is significant, as can be seen from the correlation factors described above.

When the data of the animals' physiological statistics were grouped (Fig. 5), it was found that non-stressed animals had the investigated parameters with the highest values and severely stressed animals exhibited parameters with the lowest values.

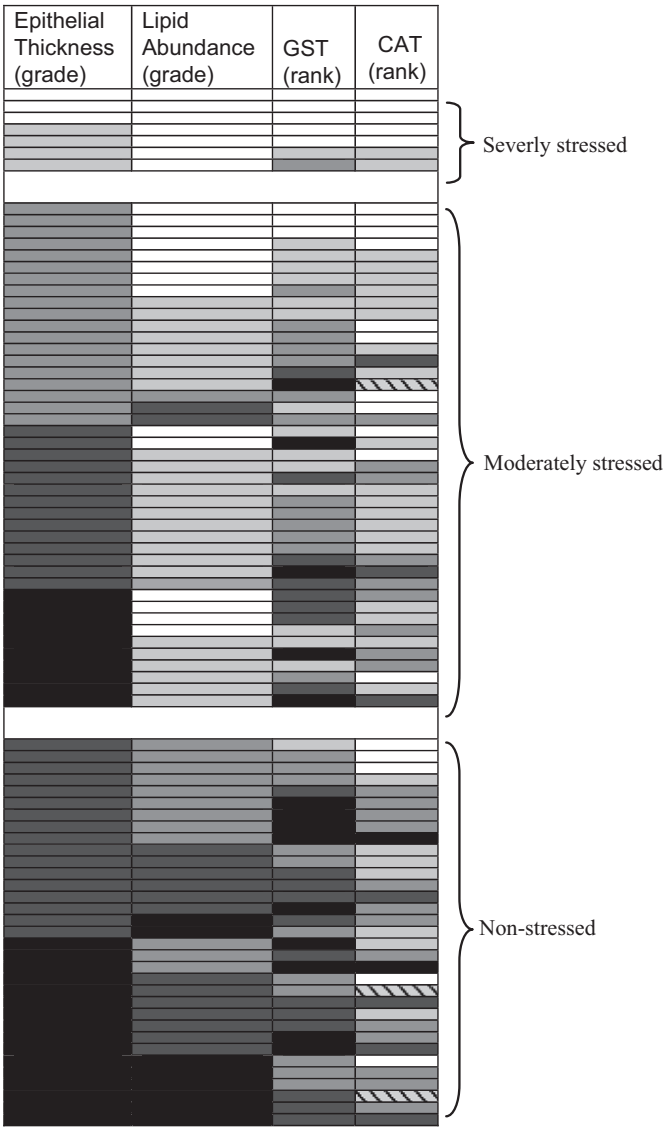

Fig. 4. Histological and biochemical parameters in isopods ($n=80$) of different physiological conditions (severely stressed ($n=7$), moderately stressed ($n=43$) and non-stressed ($n=33$). Each row represents the four parameters evaluated in a single animal. Different grey tones represent different ranks (rank 5: black, rank 4: 75% grey tones, rank 3: 50% grey tones, rank 2: 25% grey tones, and rank 1: white). Where no data were available, the column has diagonal stripes.

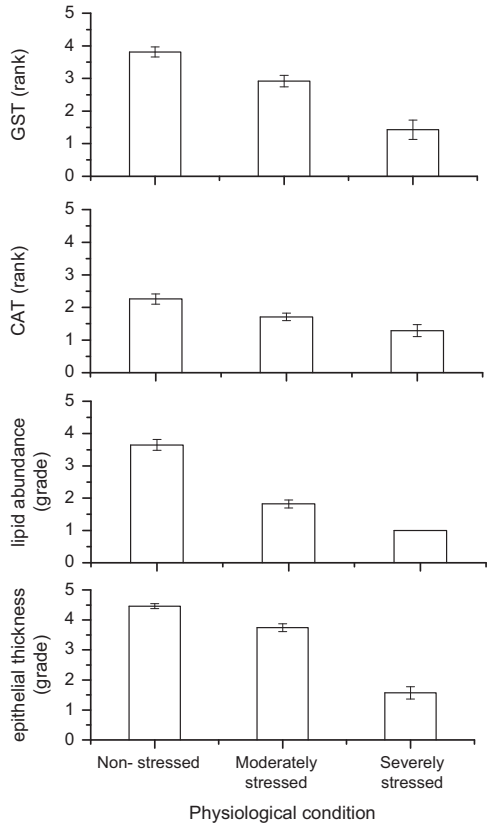

Fig. 5. Average values of glutathione S-transferase (GST), catalase (CAT) activities (expressed in ranks), abundance of lipid droplets, and epithelial thickness (expressed in grades) in severely stressed, moderately stressed, and non-stressed animals (standard errors are shown on bars, total number of animals analysed=83).

The same observation can be made when the results are presented as the portion of animals with a given rank of parameter tested (Fig. 6). It is evident that the proportion of animals with the lowest values for all assessed parameters is the greatest in severely stressed animals, while the proportion of those animals with higher values of tested parameters is high in animals in good physiological state.

4. Discussion

The results of this study show the activities of antioxidant enzymes CAT and GST in control population of isopods (*Porcellio scaber*) to be dependent on the physiological condition of isopods. Non-stressed animals have higher CAT and GST activities than severely stressed organisms. This is different than in chemically stressed organisms, where high activities of antioxidant enzymes usually indicate stress as a response to ROS (Bouskill et al., 2006).

The most probable explanation for the observed correlation between antioxidant enzymes, epithelial thickness, and lipids, is through an animal's metabolic activity. Both histological appearance of tissue (Kallerhof et al., 1996; Dahlhoff et al., 2002; Stöve et al., 2006; Lešer et al., 2008) and antioxidant enzyme levels (Abele et al., 1998, Jones and Obst, 2000; Keller et al., 2004; Perera et al., 2007; Dissanayake et al., 2008) were shown to be related to metabolic activity of an organism. High antioxidant activities are expected at high metabolic rates because of higher ROS levels. In addition, metabolically active organisms have thick epithelia and high lipid content.

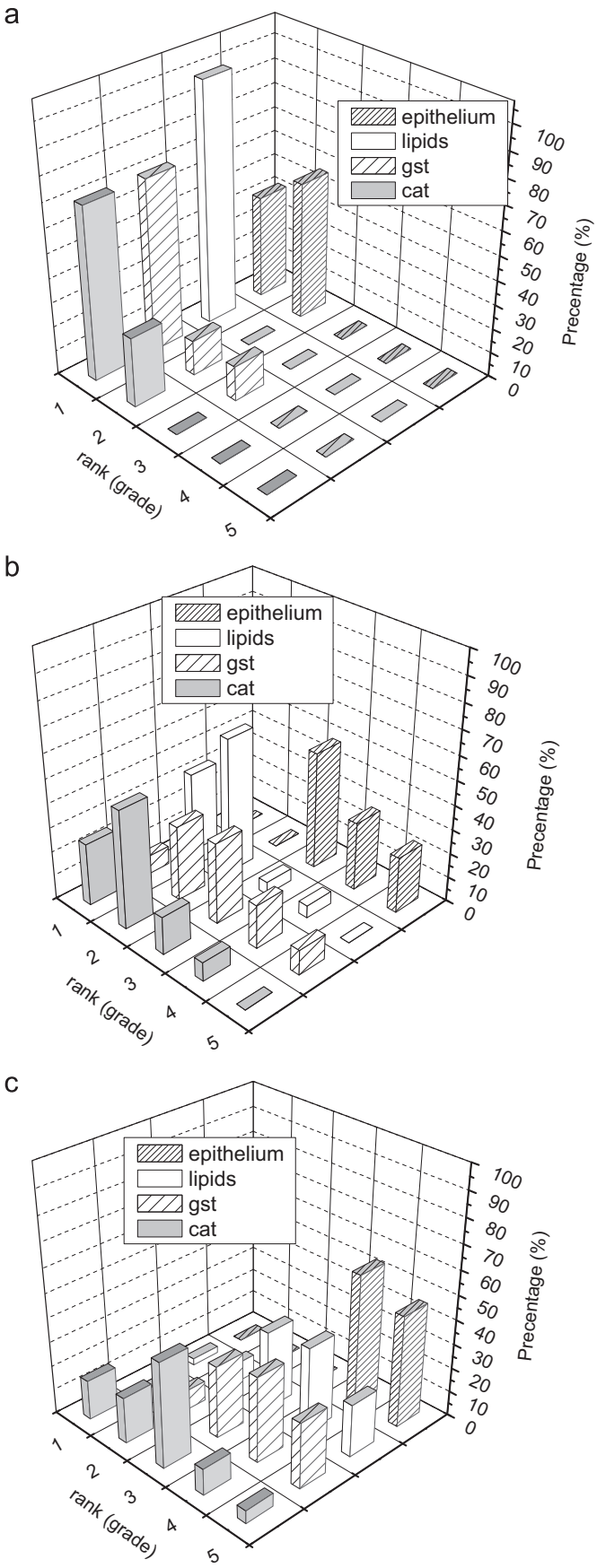

Fig. 6. The percentage of animals of a given rank and in different physiological conditions (severely stressed, moderately stressed, and non-stressed) for each histological and biochemical parameter. (a) Severely stressed organisms. (b) Moderately stressed organisms. (c) Non-stressed organisms.

The correlation between antioxidant enzyme levels and histopathological changes observed in this study was also recorded in the hepatopancreas of the estuarine crab *Chasmagnathus granulatus,* where catalase levels decreased in fasting control animals over 7 days and a parallel change in the histological appearance of hepatopancreas was observed (Pinho et al., 2003). However, in the later study no particular attention was paid to observed correlation.

The identification of stressed animals in a stock culture before conducting experiments is not a trivial task, since they cannot be identified visually. In this paper, we offer evidence that the physiological condition of isopods can be assessed using CAT and GST activities. These enzymes could be used as indicators of the physiological condition of a stock population prior to comprehensive toxicological studies. In addition, the physiological condition of a control population used in a toxicity test could be evaluated after the experiment. When a control group is found to be physiologically very heterogeneous, the experiment should be repeated with a larger number of experimental animals. In some cases, another population of organisms should perhaps be used. The relevance of toxicity data would be increased. This study should encourage other researchers to increase their effort to assess the physiological state of their test organisms in a control culture.

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