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Biomarkers and energy reserves in the isopod *Porcellionides pruinosus*: The effects of long-term exposure to dimethoate

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HIGHLIGHTS

- Dimethoate field application dose induced low to moderate toxicity to isopods.
- Dimethoate also generates oxidative stress leading to high lipid peroxidation rates.
- Terrestrial isopods increase dimethoate's degradation rates on soils.

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ABSTRACT

Terrestrial isopods from the species *Porcellionides pruinosus* were exposed to the recommended field dose application (0.4 mg/kg soil) and a sublethal concentration (10 mg/kg soil) of dimethoate at two temperatures that can be generally found in several countries (20 °C and 25 °C) and are commonly used as reference temperatures. The organisms were exposed for 28 days and sampled at the following time points: 24 h, 48 h, 96 h, 7 days, 14 days, 21 days, 28 days; organisms were then changed to clean soil for a recovery period of 14 days during which organisms were sampled on day 35 and 42. For each sampling time, the enzyme activities of acetylcholinesterase (AChE), glutathione-*S*-transferases (GST), catalase (CAT), lactate dehydrogenase (LDH) were determined as well as the following: total lipid, carbohydrate and protein content; energy available (Ea); energy consumption (Ec); cellular energy allocation (CEA) and lipid peroxidation rate (LPO). The integrated biomarker response (IBR) was calculated for each sampling time and for each of the above parameters. Mortality was also recorded during the study.

The results obtained showed that dimethoate causes toxicity by several mechanisms. This study found evidence for the inhibition of the acetylcholinesterase enzyme, which has been previously reported, and also evidence of oxidative stress, which altered the levels of GST, CAT or LPO. In addition, the study showed that the two concentrations used of dimethoate caused the activation of different general detoxification mechanisms, and also that the same concentration at different temperatures induced different toxicity responses.

1. Introduction

The potential impact of a stressor in ecosystems requires the observation of effects at different levels of biological organization, starting at the molecular level and ending at the population/community level (Moore et al., 2004). At a molecular level, several biomarkers have been used as efficient tools due to their sensitivity, quickness and accurate relationship between toxicant exposure and respective biological response (Morgan et al., 1999). However, results from molecular approaches may have limited information if they are not related to higher and more complex biological levels of organization. Indeed, the effects at lower organizational levels may not necessarily be observed or be meaningful at superior biological levels whenever they consist on the acquisition of a new homeostasis status (Morgan et al., 1999), or they just do not represent any major life-changing effects. Nevertheless, they can provide crucial information on stressors' modes of action, which improve the knowledge on their related effects. Depletion of energy reserves and energy metabolic costs can be used as another parameter to evaluate chemical exposure effects. Due to the stress induced by xenobiotics, metabolic changes can induce the depletion of energy reserves especially under long time exposures, negatively affecting individuals' growth or reproduction, and finally impairing population dynamics and structures (de Coen and Janssen, 2003).

In the soil compartment, organisms play an important role on decomposition and fragmentation processes, and their exposure to xenobiotics may change overall soil functions, causing a decrease in soil quality and soil services (MEA, 2005). There is a wide range of xenobiotics that can appear in the terrestrial compartment. Organophosphorous compounds (OP) are one of the most extensively used pesticides

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in agriculture practices. One of the most commonly applied is dimethoate, which successfully combines a selective toxicity to insects through a systemic action. By acting on the enzyme AChE, this pesticide inhibits the degradation of acetylcholine thereby producing extensive cholinergic stimulation and neurotoxicity (de Coen and Janssen, 2003). When looking at xenobiotics' exposures, the overall conditions of exposure can also bring additional stress or induce changes in physiological responses of organisms when they are dealing with chemical exposure.

Terrestrial isopods are macrodecomposers that play an important role in the decomposition processes, vegetal litter fragmentation and re-cycling process of nutrients (Ferreira et al., 2010; Loureiro et al., 2006; Zimmer, 2002; Zimmer et al., 2003). The terrestrial isopod species *Porcellionides pruinosus* has been described as a good test-organism to evaluate soil contamination or other environmental changes in their habitat (Jansch et al., 2005; Loureiro et al., 2005, 2009; Takeda, 1980; Vink et al., 1995). Several individual parameters have been chosen as indicators of isopod health status but also as parameters tightly related to their function in soils. Feeding activities, including excretion rates, reproduction, growth and behaviour are amongst the most used parameters in isopods ecotoxicological tests. Along with these, the use of neurotoxicological (acetylcholinesterase), detoxification (glutathione *S*-transferases), oxidative stress (catalase, glutathione peroxidase, and lipid peroxidation), energy related (lactate dehydrogenase) biomarkers' basal activities and energy reserves (total lipid, carbohydrate and protein content) can be used as good evaluation tools that will provide useful information and a connection between these two ecological levels. In previous studies, these biomarkers have been used to determine the basal levels on organisms from well-established lab cultures (Ferreira et al., 2010) and they have also shown to respond to the short term exposure of pesticides (e.g. Jemec et al., 2009, 2012).

Time of exposure is one of the key factors to improve ecological relevance. In isopod bioassays, tests are usually carried out for 48 h, when considering the avoidance behaviour test (Loureiro et al., 2002), 14 or 28 days when regarding feeding inhibition tests (Loureiro et al., 2006), 14 days for survival (Calhôa et al., 2012; Santos et al., 2010), 21 days for bioaccumulation tests (Sousa et al., 2000), or more than one month when reproduction is being evaluated (Calhôa et al., 2012). Long term exposure tests are advisable when a more comprehensive approach is required in order to integrate chemical fate and changes in bioavailability with time, but also to consider the ability of organisms to recover when exposure ends.

Therefore, the main goal of this study was to evaluate and understand the long-term effects of dimethoate using several enzymatic biomarkers and energy reserves in the terrestrial isopod *Porcellionides pruinosus*. Organisms were exposed to two dimethoate concentrations (a recommended field dose application and a concentration below EC50 level) and two different exposure temperatures (20 °C and 25 °C) during a 28 day exposure period followed by a 14 day recovery period. The results were then combined using the integrated biomarker response index (IBR).

The mode of action (MoA) analysis of dimethoate to the terrestrial isopod *P. pruinosus* was determined in several ways: 1) the toxicity and inherent effects of two dimethoate concentrations; 2) the toxicity effect of dimethoate combined with temperature; 3) response patterns at the different times of exposure and 4) differences between the exposure and recovery period. Within this approach, dimethoate degradation in soils was also integrated in the results in the presence or absence of terrestrial isopods.

2. Materials and methods

2.1. Test organism and culture procedure

The organisms used in this study belong to the species *Porcellionides pruinosus* Brandt (1833), and were previously collected from a horse manure heap and maintained for several generations in laboratory cultures. In culture, isopods were fed *ad libitum* with alder leaves (*Alnus glutinosa*) and maintained at two different temperatures (20 ± 1 °C and 25 ± 1 °C), with a 16:8 h (light:dark) photoperiod. Twice a week cultures were sprayed with water and food provided. Only adult organisms (15–25 mg wet weight) were used in the experiments and no distinction between sexes was made, although pregnant females were excluded.

2.2. Soil spiking

LUFA 2.2 soil (Speyer, Germany) was spiked with two different concentrations of dimethoate (0.4 and 10 mg dimethoate/kg soil), with a final moisture content equivalent to 50% of the soil water holding capacity. The concentration of 0.4 mg dimethoate/kg soil represents the recommended field dose for dimethoate application (TitanAG, 2010) and the 10 mg dimethoate/kg soil was used based on the study by Fischer et al. (1997) that found in LUFA 2.2 soil for the isopod *Porcellio scaber*, a EC50 value of 17.5 mg dimethoate/kg soil for growth; 16.8 mg dimethoate/kg soil for mancae/surviving females and 15.4 mg dimethoate/kg soil for pregnant/surviving females. Therefore, the concentration of 10 mg dimethoate/kg soil was chosen as a sublethal value.

2.3. Experimental procedure

Toxicity tests were performed in plastic boxes (26 length × 18 width × 7.5 height cm), containing approx. 2 cm height of natural LUFA 2.2 soil (Speyer, Germany) and 40 isopods (per box). Test organisms were collect from culture boxes, weighted (15–25 mg) and placed in each test-box. Organisms with abnormalities, moulting characteristics or pregnant females were excluded from trial. Although food was provided *ad libitum* in the form of alder leaf disks (Ø 10 mm), it was made available considering also that a soil coverage by leaves could influence isopods' exposure to the spiked soil. Therefore, food was added in small quantities but continuously reintroduced throughout the test period. Organisms were exposed to 0.4 and 10 mg dimethoate/kg soil in a 16:8 h (light:dark) photoperiod, at two different temperatures: 20 °C and 25 °C. Both temperatures are relevant temperatures for Mediterranean countries, and can be found elsewhere during a year time and are widely used in ecotoxicological assays with several species of terrestrial isopods (Calhôa et al., 2012; Dailey et al., 2009; Loureiro et al., 2006; Morgado et al., 2013; Ribeiro et al., 1999; Santos et al., 2011).

A total of five replicates were performed for each concentration and temperature. Four organisms from each box/replicate were collected at the following time points: 0 h, 24 h, 48 h, 96 h, 7 days, 14 days, 21 days, 28 days (exposure period) and 35 days, 42 days (recovery period). In the results section, the 35 and 42 days of test duration will be denominated as 7 and 14 days of post-exposure.

The enzymatic biomarkers glutathione *S*-transferases (GST), glutathione peroxidase (GPx), catalase (CAT) and lipid peroxidation (LPO) were measured using a pool of two full-body organisms per replicate. Another organism was divided into head and body to analyse acetylcholinesterase (AChE) and lactate dehydrogenase (LDH) activity, respectively, each part corresponding also to a replicate. All chemicals used in these experiments were obtained from Sigma-Aldrich Europe, except the Bradford reagent, which was purchased from Bio-Rad (Germany), and hydrogen peroxide from Fluka.

For the energy reserves (total lipid, carbohydrate and protein content) and electron transport system, only one organism was used as a replicate.

At each sampling time, the number of dead organisms were recorded and removed from the test boxes.

2.4. Measured parameters and IBR

The protocol used to process samples was previously described by Ferreira et al. (2010) and is extensively described in the supplementary

data. The lipid peroxidation (LPO) assay was adapted from the methods described by Bird and Draper (1984) and Ohkawa et al. (1979) to a microplate format. The glutathione *S*-transferases (GST) and glutathione peroxidase (GPx) activities were determined as described by Habig et al. (1974) and Mohandas et al. (1984), respectively. Catalase (CAT) activity was determined based on the method described by Clairborne (1985) and adapted to a microplate format. Lactate dehydrogenase (LDH) activities were measured using the method described by Vassault (1983), adapted to microplate format by Diamantino et al. (2001) and the acetylcholinesterase (AChE) activity according to the Ellman method (Ellman et al., 1961), adapted to microplate format described by Guilhermino et al. (1996). For all biomarkers, protein concentration was determined according to the Bradford method (Bradford, 1976), adapted from BioRad's Bradford micro-assay set up in a 96 well flat bottom plate, using bovine γ-globuline as a standard.

Considering energy parameters, to determine total protein, carbohydrate and lipid contents, oxygen consumption rate in the electron transport system (energy consumption - Ec), energy available (Ea) and cellular energy allocation (CEA) protocols were adapted from de Coen and Janssen (1997). The Ea, Ec and CEA value were calculated as described by Verslycke et al. (2004):

$$\text{Ea(available energy)} = \text{carbohydrates} + \text{lipids} + \text{proteins(mJ/mg org.)}$$
$$\text{Ec(energy consumption)} = \text{ETS activity(mJ/mg org./h)}$$
$$\text{CEA(cellular energy allocation)} = \text{Ea/Ec(/h)}$$

To integrate results from the different biomarkers and understand global/general responses, the integrated biomarker response (IBR) was calculated according to Beliaeff and Burgeot (2002); details can also be found in the supplementary data.

2.5. Chemical analysis

Determination of total dimethoate concentration per kilogram of soil was performed by the Marchwood Scientific Services Ltd. The lower detection limit for dimethoate was 0.4 μg/kg soil. The method used to analyse soil spiked with dimethoate involved air drying and grinding the samples. Then 0.5 gram of sample was used for extraction

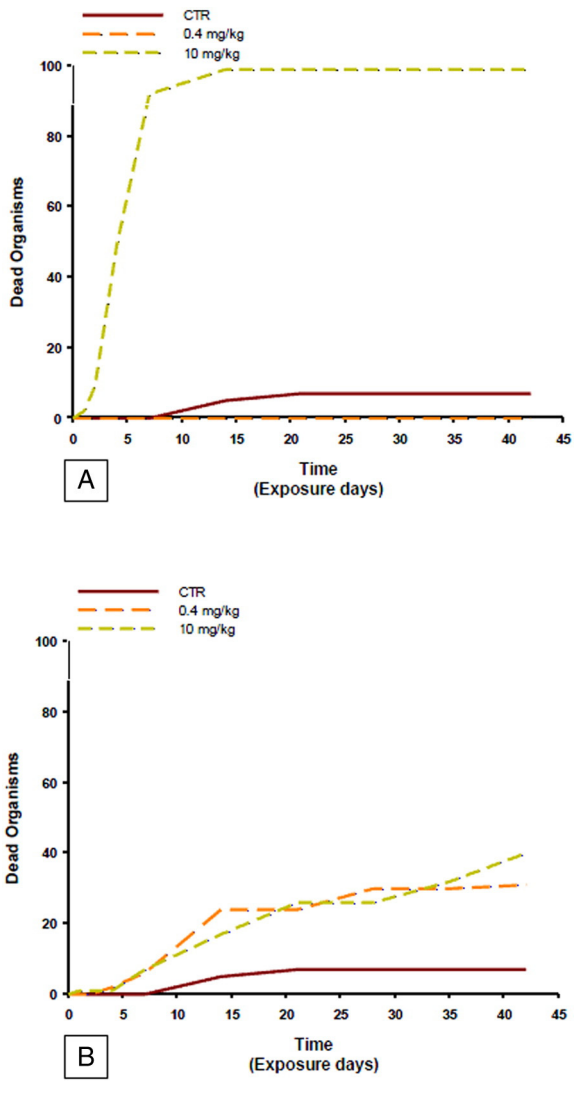

Fig. 1. Cumulative total number of dead organism from the species *Porcellionides pruinosus* during the exposure and recovery period in the control and exposed to 0.4 mg dimethoate/kg soil and 10 mg dimethoate/kg soil. A total of 200 organisms were exposed per treatment. A – organisms exposed to 20 °C, B – organisms exposed to 25 °C.

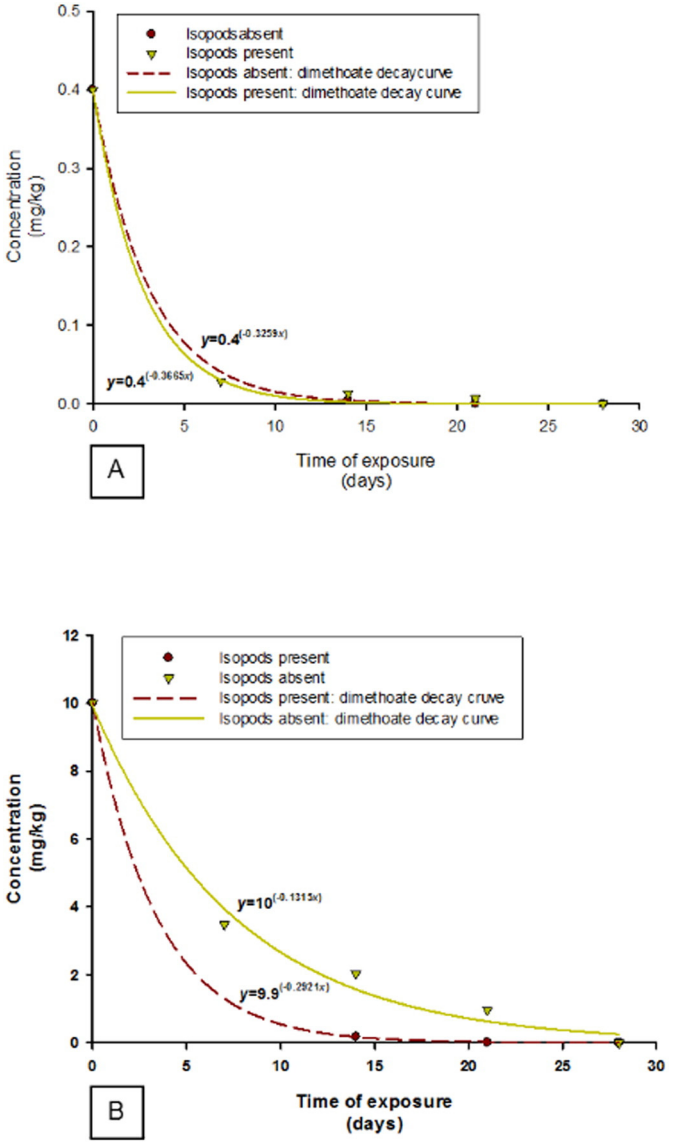

Fig. 2. Dimethoate decay curve for soil spiked with 0.4 mg dimethoate/kg soil (A) and 10 mg dimethoate/kg soil (B) at 25 °C exposure in the presence and absence of terrestrial isopods.

with acidified acetonitrile. The sample was then filtered and the filtrate used for analysis by Liquid Chromatography-Tandem Mass Spectrometry following a pre-treatment buffering stage. The instrument used for the analysis was an Agilent 6410 Triple quad LCMS-MS. Standards were prepared in solvents at 7 levels with recoveries in the range of 80–120%. The water sample analysis passed for all the samples except the drying and grinding stages.

Soil samples analysed consisted of a pool of 5 soil replicates for each concentration used to expose isopods. Similarly, soil spiked with the same concentrations of dimethoate but with no isopods was also sampled. Samples were taken at the beginning of the exposure, and at days 7, 14, 21 and 28.

2.6. Data analysis

A one-way analysis of variance (ANOVA) or a Student test (*t* test) was performed to compare differences between treatments at each sampling time and Dunnett's comparison test was carried out to discriminate statistically different treatments from the control (SPSS, 1999). When possible, data transformation was used to achieve normality. When data did not show a normal distribution or homoscedasticity, the non-parametric test Kruskal-Wallis One Way Analysis of Variance on Ranks was used.

Data values that were higher or lower than the mean value, plus or minus two times the standard deviation, were considered outliers, and withdrawn from analysis (Rousseeuw and Croux, 1993). Whenever there was enough data ($n > 3$, due to high mortality rates), a two-way analysis of variance (two-way ANOVA) was performed to check for interactions between time and concentration. The two-way ANOVA was performed separately for the exposure and the recovery period. The one-way ANOVA and two-way ANOVA with significance of $\alpha = 0.05$. Due to the mortality observed within each temperature, under 20 °C a two-way ANOVA could only be performed for the exposure period and for the 25 °C exposure for the recovery period.

Dimethoate decay on time was calculated using non-linear regression curves at the two different temperatures, with and without the presence of isopods, and calculated as an exponential single decay curve with 2 parameters ($C_t = C_0 e^{-K_0 t}$), where C_t is the dimethoate concentration in soil (mg/kg soil), C_0 is the initial concentration of dimethoate in soil, K_0 the decay rate of dimethoate in soil (/day), and t the time (days).

3. Results

Organisms exposed to dimethoate under the two different temperature regimes (20 °C and 25 °C) showed different mortality patterns. In Fig. 1 it is presented the number of dead organisms and the cumulative number at a given sampling time. In the 20 °C exposure regime, due to the mortality observed at the highest concentration, on the 14th day of recovery it was not possible to analyse the energy related parameters (proteins, lipids, carbohydrates, Ec, Ea and CEA). Similarly in the 25 °C exposure regime, the high mortality observed for the highest concentration did not allow the analysis of the energy related parameters (proteins, lipids, carbohydrates, Ec, Ea and CEA) for the 14th day of exposure and all parameters forward.

The decay rate for the lower and higher concentrations of dimethoate in soil at 20 °C with isopods present was respectively 0.32/d ($y = 0.4e^{-0.3216x}$; $r^2 = 0.9994$) and 0.13/d ($y = 9.9e^{-0.1277x}$). For the exposure at 20 °C where no isopods were present, no data is available due to analytical constraints.

The decay rates of dimethoate in soil at 25 °C are presented in Fig. 2. At the lower concentration, the decay rates for soil with and without isopods were respectively 0.37/day ($y = 0.4e^{-0.3665x}$ $r^2 = 0.9987$) and 0.33/day ($y = 0.4e^{-0.3259x}$; $r^2 = 1.0000$). For the higher concentration, those rates were 0.29/day ($y = 10e^{-0.2921x}$; $r^2 = 1.0000$) and 0.13/day ($y = 9.9e^{-0.1315x}$; $r^2 = 0.9903$), for soil with and without isopods.

3.1. Biomarkers activity and energy reserve content

The activity of the biomarkers and energy reserves of organisms exposed at 20 °C and 25 °C during the exposure and the recovery period is presented in Figs. 1SD and 2SD (suppplentarry data) and in Table 1. Details relative to the significant differences found between treatments and control are presented as supplementary data (Table 2SD).

The main target enzyme of the pesticide dimethoate (AChE) showed significant differences mainly at the higher concentration (10 mg dimethoate/kg soil) for both temperatures. The lower exposure concentration only showed significant differences at 20 °C after 48 h of exposure. Regarding the oxidative stress related biomarkers (LPO, GST, CAT and GPx), significant differences were mainly observed at 25 °C exposure and after 48 h/96 h of exposure or during the recovery period (more evident at the higher exposure concentration). Finally

Table 1
Significant differences found for biomarkers and energy reserves between control organisms from the species *Porcellionides pruinosus* and those exposed to dimethoate at 20 °C and 25 °C. Data refers to a 28 day exposure period followed by a 14 day recovery period. Red boxes denote deleterious effects, green boxes denote positive effects and grey boxes represent parameters that could not be measure due to the lack of organisms. ↗ denotes significant increase, ↘ denotes significant decrease; one-way ANOVA, ANOVA on ranks or Student's *t*-test, $p \leq 0.05$.

			AChE		GST		LPO		CAT		LDH		Lipids		Carbohydr.		Proteins		Ea		Ec		CEA	
			20°C	25°C	20°C	25°C	20°C	25°C	20°C	25°C	20°C	25°C	20°C	25°C	20°C	25°C	20°C	25°C	20°C	25°C	20°C	25°C	20°C	25°C
Exposure	24h	0.4mg/kg									↘													↗
		10mg/kg	↘	↘							↘												↗	
	48h	0.4mg/kg	↘			↗					↗											↗		
		10mg/kg	↘	↘		↗				↘	↗				↘									
	96h	0.4mg/kg										↗				↗		↗		↗				
		10mg/kg	↘	↘			↘			↗	↗				↘	↗				↗		↗	↗	
	7d	0.4mg/kg																				↗		
		10mg/kg	↘	↘				↗			↘	↗						↘						
	14d	0.4mg/kg									↗													
		10mg/kg	↘	↘																				
	21d	0.4mg/kg			↗	↘							↘	↗					↘				↘	↗
		10mg/kg	↘										↘						↘		↗		↘	
	28d	0.4mg/kg												↘				↘						
		10mg/kg	↘																					
Post-exposure	7d	0.4mg/kg							↘		↗	↗												
		10mg/kg	↘														↘							
	14d	0.4mg/kg				↘		↗																
		10mg/kg							↘															

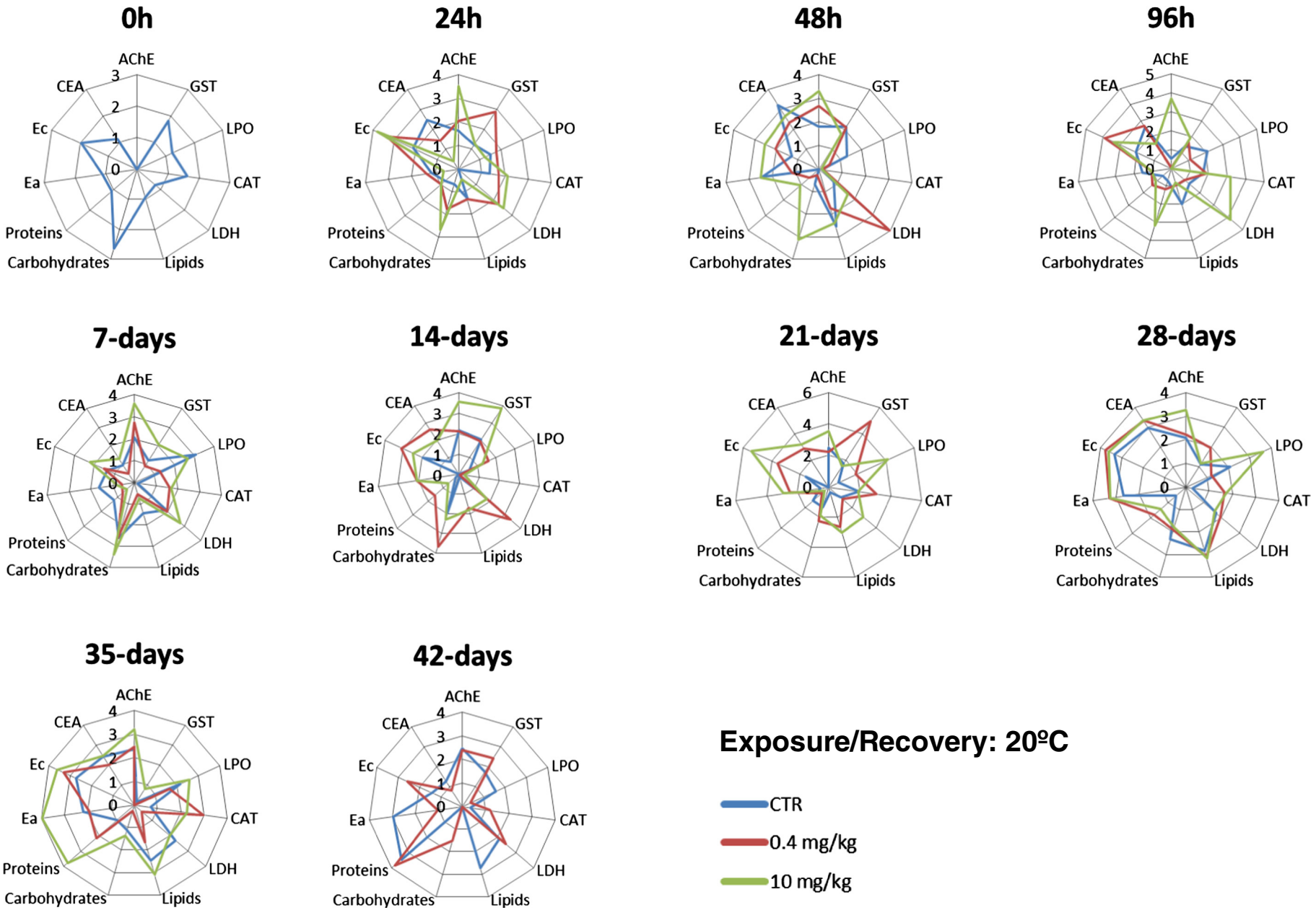

Fig. 3. Star plots for each sampling time of *Porcellionides pruinosus* exposed to 20 °C (exposure period: 0 h, 24 h, 48 h, 96 h, 7 days, 14 days, 21 days, 28 days; recovery period: 35 days, 42 days). AChE = acetylcholinesterase, GST = glutathione *S*-transferases, LPO = lipid peroxidation, CAT = catalase, LDH = lactate dehydrogenase, Ea = available energy, Ec = energy consumption, CEA = cellular energy allocation.

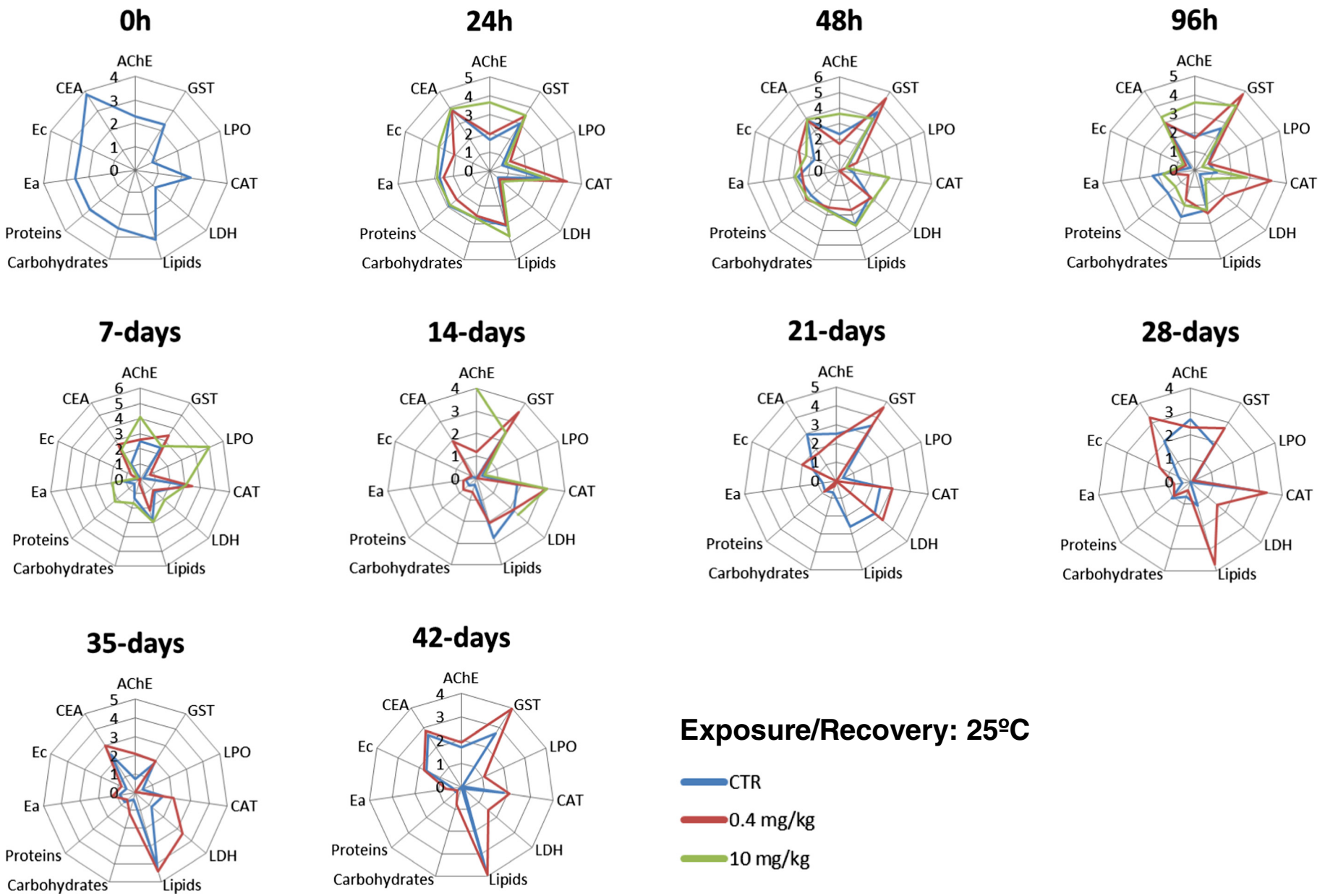

Fig. 4. Star plots for each sampling time of *Porcellionides pruinosus* exposed to 25 °C (exposure period: 0 h, 24 h, 48 h, 96 h, 7 days, 14 days, 21 days, 28 days; recovery period: 35 days, 42 days). AChE = acetylcholinesterase, GST = glutatione *S*-transferases, LPO = lipid peroxidation, CAT = catalase, LDH = lactate dehydrogenase, Ea = available energy, Ec = energy consumption, CEA = cellular energy allocation.

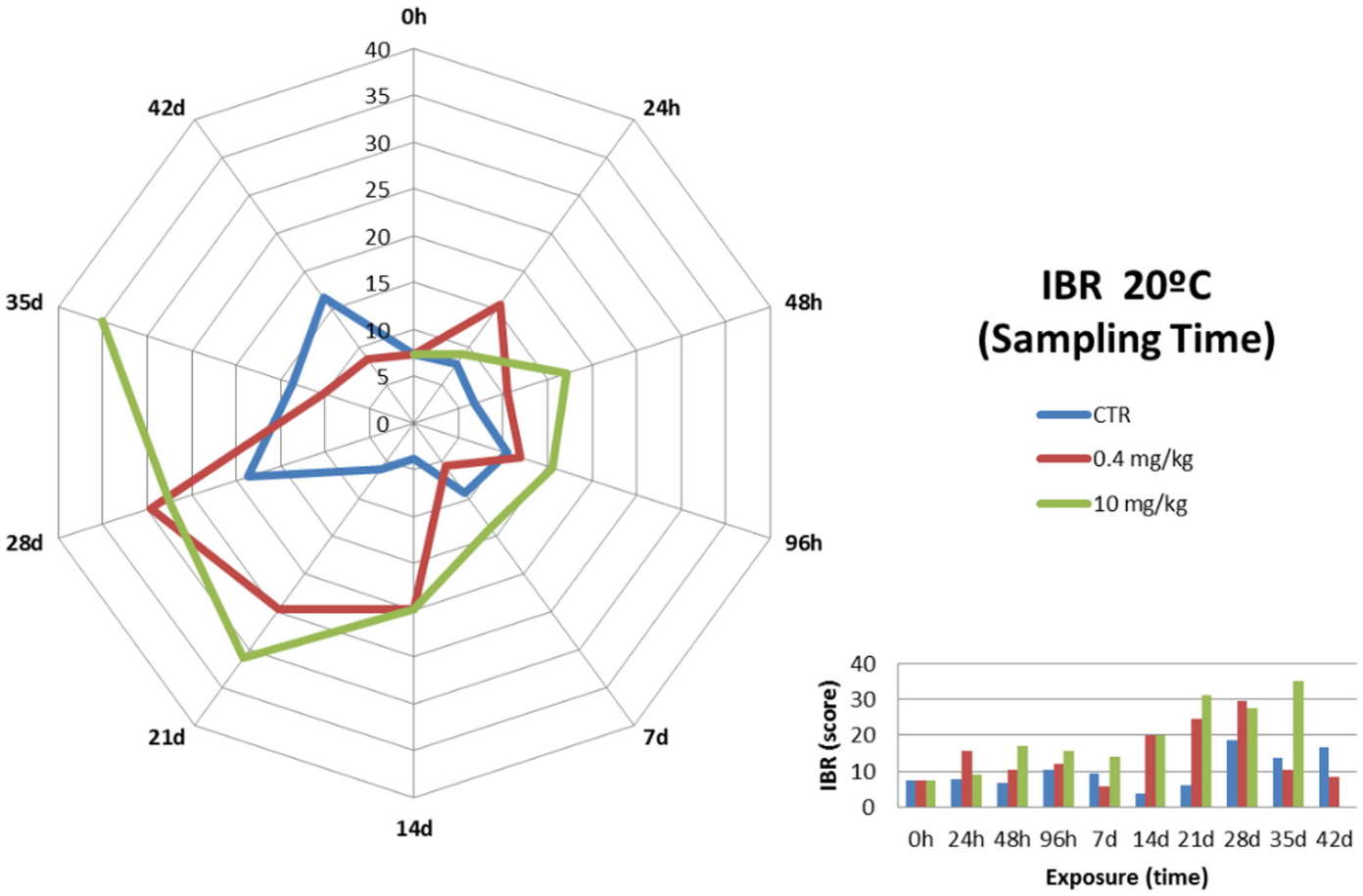

Fig. 5. Integrated biomarker response(IBR) represented by starplot and histogram of *Porcellionides pruinosus* in the control and exposed to dimethoate (0.4 and 10 mg/kg soil) at 20 °C. Exposure period: 0 h, 24 h, 48 h, 96 h, 7 days, 14 days, 21 days, 28 days; recovery period: 35 days, 42 days.

the energy related enzyme LDH presented significant differences at 20 °C for both concentrations during the 7/14 days of exposure.

The significant differences observed at 25 °C on the energy related parameters were mainly found after 96 h of exposure and mostly consisted on positive effects (except for the energy consumption – Ec), whereas for 20 °C negative effects were registered, in particular after 21 days of exposure (except for CEA after 48 h and 96 h of exposure).

At 20 °C, significant interactions between time of exposure and dimethoate concentrations were observed for the energy consumption (Ec), energy available and CEA in the exposure period (two-way ANOVA, ln transformation, $F_{14,75} = 2.967$; $p = 0.001$; two-way ANOVA, ln transformation, $F_{14,79} = 2.355$; $p = 0.009$ and two-way ANOVA, $F_{14,68} = 3.586$; $p < 0.001$, respectively) and for catalase in the recovery period (two-way ANOVA, $F_{4,24} = 5.180$; $p = 0.004$).

At 25 °C, significant interactions between time of exposure and dimethoate concentrations were only observed in the recovery period for LPO, GST, LDH and lipids content (respectively two-way ANOVA, $F_{2,20} = 5.727$; $p = 0.011$; two-way ANOVA, $F_{2,24} = 5.727$; $p = 0.009$; Two Way ANOVA, $F_{2,22} = 9.343$; $p = 0.001$ and two-way ANOVA, $F_{2,21} = 3.959$; $p = 0.035$).

3.2. Integrated biomarker response (IBR)

The IBR starplot is presented in Figs. 3 and 4, and includes the scores of each measured parameter and each sampling time during the exposure and post-exposure period. Better or worse scores (respectively lower and higher values) obtained for each parameter are summarized in Table 1SD (supplementary data).

3.2.1. Exposure 20 °C: IBR and IBR/n analysis

The IBR analysis showed frequently worse scores (higher values) for the highest concentration (10 mg dimethoate/kg soil) than the other treatments (Figs. 5 and 6). When analysing the IBR according to the sampling time (Fig. 5), one can see that only at sampling times 24 h and 28 days of exposure this situation did not occur. Moreover, apart from day 7 and day 28 of exposure, the lower exposure concentration (0.4 mg dimethoate/kg soil) showed always worse scores than the control. When changed to clean soil for recovery this effect disappears and the lower concentration always presented better scores than the control. The absence of an IBR for the highest concentration at day 14 of the recovery phase is due to the aforementioned lack of organisms to measure the complete set of parameters used in the remaining sampling times (high mortality). Organisms in the control treatment always showed better scores except for the 14th day of recovery. A statistical analysis showed that only the highest concentration (10 mg dimethoate/kg soil) is significantly different from the control (one-way ANOVA, $F_{2,26} = 3.678$; $p = 0.039$).

When analysing the parameters individually, organisms in the control treatment always showed better scores, except for LPO and CEA. For the lower concentration the parameters that exhibit the highest toxicity when compared to the control were energy consumption (Ec – 11.3× higher), GST (9.6× higher) and protein content (4.7× higher). For the highest concentration of exposure (10 mg dimethoate/kg soil),

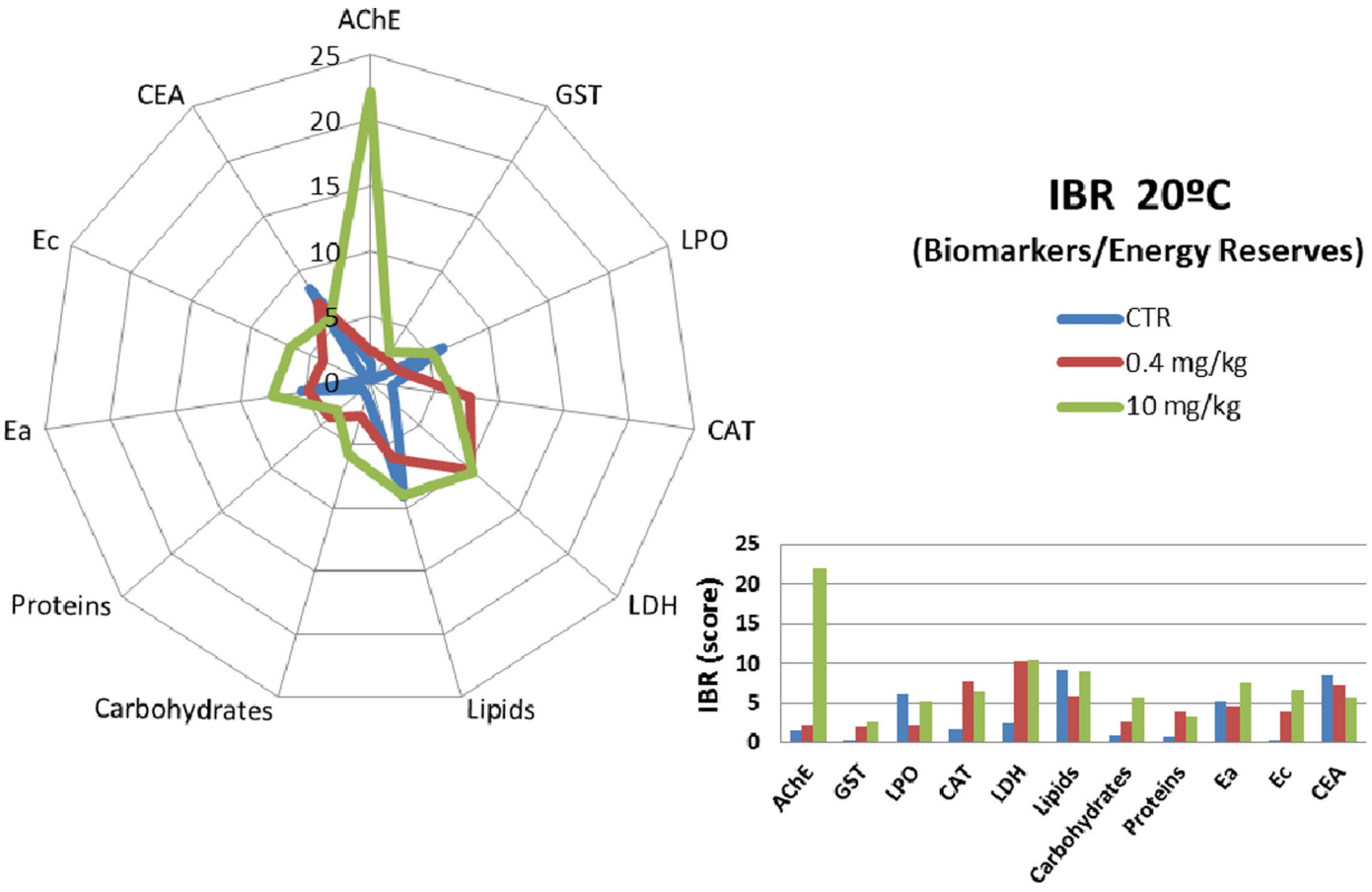

Fig. 6. Integrated biomarker response represented by starplot and histogram of *Porcellionides pruinosus* in control and exposed to dimethoate (0.4 and 10 mg/kg soil) at 20 °C during the exposure and recovery period. AChE = acetylcholinesterase, GST = glutathione *S*-transferases, LPO = lipid peroxidation, CAT = catalase, LDH = lactate dehydrogenase, Ea = available energy, Ec = energy consumption, CEA = cellular energy allocation.

higher toxicity was observed for the energy consumption (Ec – 21.1× higher), AChE (16.7× higher) and GST (13.4× higher).

3.2.2. Exposure 25 °C: IBR and IBR/n

As in the 20 °C exposure, the IBR analysis showed always worse scores for the highest concentration used (10 mg dimethoate/kg soil) than the other treatments (Figs. 7 and 8). Also and except for the 24 h and day 21 of exposure the lower exposure concentration (0.4 mg dimethoate/kg soil) showed always worse scores than the control, showing again a deterioration effect that increases with the exposure concentration (Fig. 7). But, contrary to the 20 °C exposure, the change of organisms to clean soil for recovery did not show any positive effect. As it can be seen in Fig. 7, the IBR for the highest dimethoate concentration could not be assessed for sampling times from day 14 onwards of the exposure phase due to the increased mortality found at 25 °C. The statistical analysis also showed that only the highest concentration (10 mg dimethoate/kg soil) is significantly different from the control (Kruskal-Wallis, $H = 8.348$; d.f. = 2; $p = 0.015$).

For the lower concentration the parameters that exhibit the highest toxicity when compared to the control were CAT (11.0× higher), GST (7.4× higher) and LDH (6.7× higher) (Fig. 8). For the highest concentration of exposure, the parameters that exhibits the highest toxicity were CAT (11.5× higher), AChE (10.8× higher) and LDH (7.6× higher).

4. Discussion

The use of biomarkers to assess toxicity has already been used for a large number of species, scenarios and as a tool to determine which pathways are triggered by stressors, singly applied or as mixtures (e.g. Santos et al., 2010, 2011; Jemec et al., 2009, 2012; Stanek et al., 2006). In these studies, the number of biomarkers used is normally high so the interpretation and integration of all their effects is difficult and further consequences for the organism are hard to determine. This analysis brings several problems in terms of interpretation, since nonexistence of significant differences to the control in one or several biomarkers may still be indicative of stress. In fact, an organism that does not show significant alterations from the control in all biomarkers from a specific pathway may be in a similar state to an organism that shows a significant difference in only one of the biomarkers. The use of IBR for a laboratory exposure and not as a tool to evaluate a specific field scenario has only been reported by Morgado et al. (2013). In the present study, the use of star-plots helped to identify patterns of toxicity that were not so clear when significant differences were analysed. And it proved to be a robust tool, as it could identify significant results between control and exposures in almost all cases, except one (CEA, 24 h, 0.4 mg dimethoate/kg soil, 25 °C) representing 0.30% of total comparisons. An example of this was the AChE activity in organisms exposed to the lowest concentration at 20 °C. When looking only to significant differences between the treatment and control, organisms may be considered in "good conditions", but when analysing the IBR results an inherent toxicity pattern was observed.

In our results, one should highlight that organisms exposed to the highest concentration used (10 mg dimethoate/kg soil) showed a higher mortality at 25 °C. For the 20 °C exposure, mortality was not so high and therefore almost all sampling times could be fulfilled; in addition the observed mortality was very similar to the one found for the lower concentration (0.4 mg dimethoate/kg soil). These differences

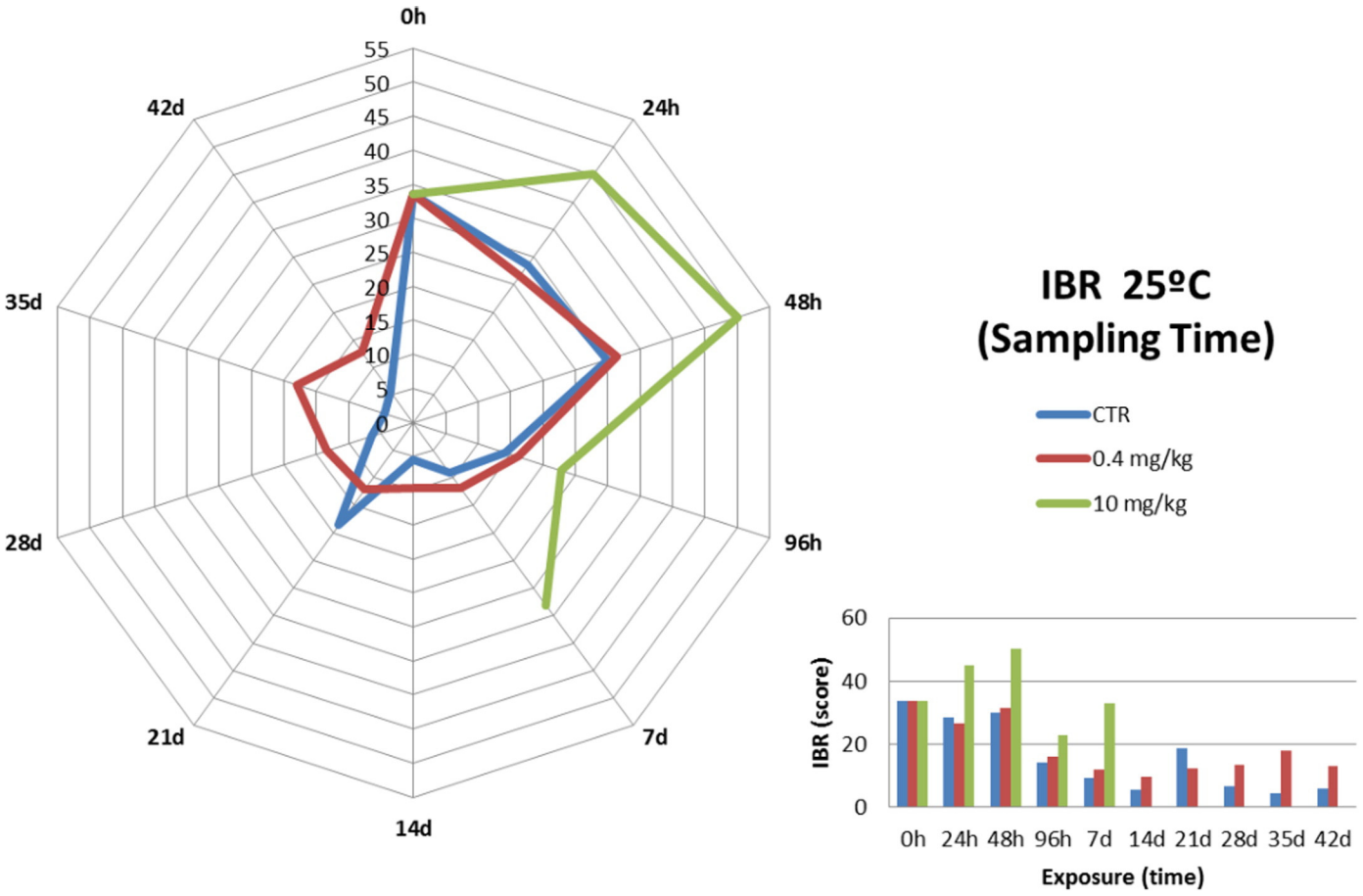

Fig. 7. Integrated biomarker response(IBR) represented by starplot and histogram of *Porcellionides pruinosus* in the control and exposed to dimethoate (0.4 and 10 mg/kg soil) at 25 °C. Exposure period: 0 h, 24 h, 48 h, 96 h, 7 days, 14 days, 21 days, 28 days; recovery period: 35 days, 42 days.

may be explained by faster degradation of dimethoate at 25 °C, which can lead to an increase of metabolites that can be more toxic than dimethoate (Lucier and Menzer, 1970; Martikainen, 1996).

Secondly another highlight from the present study was the strong inhibition of the enzyme acetylcholinesterase in the 10 mg dimethoate/kg exposure. It is widely known that this enzyme is the main target of dimethoate, and its inhibition was expected during the exposure. But an inhibition >90% was observed for the highest concentration for all sampling times at both temperatures. Such inhibition is generally considered to cause severe problems to the organisms and in some cases even death (Guimarães et al., 2007; Lucier and Menzer, 1970). The inhibition of the AChE activity was also observed in a recent work for two other species of earthworms, exposed to dimethoate, where concentrations corresponding to 25% of the field recommended dose inhibited up to 60% of this enzyme activity (Velki and Hackenberger, 2012). In addition, diazinon exposure to isopods via food led to approximately 50% and 90% inhibition, in adults and juveniles, respectively (Stanek et al., 2006). The negative effects of dimethoate in terrestrial isopods based on the locomotion impairment have been reported by Engenheiro et al. (2005), where an AChE inhibition of ~60% was correlated with a shorter path length travelled and more stops per path. Also some previous works using dimethoate and terrestrial isopods showed a higher AChE inhibition and high mortality (Santos et al., 2010, 2011).

This study also highlights the influence of isopods on the increase of dimethoate's degradation rate. This has been reported in a previous work by Loureiro et al. (2002) for the degradation of lindane in soil. The mechanism that underlies this faster rate of degradation of dimethoate in the presence of isopods may be related to their role in ecosystems. Their feeding on decaying vegetal matter and grazing on fungi leads to the release of faecal pellets that are enriched with bacteria present in their gut. This will increase the soil microbiome activity leading in a final step to a possible faster degradation of these organic compounds (Loureiro et al., 2002; Zimmer and Topp, 1999).

Regarding biomarkers, the enzyme GST, which is involved in the detoxification process, did not show any significant differences during the exposure and recovery period at 20 °C and at the lowest dimethoate concentration at 25 °C; on the other hand, the highest dimethoate concentration exposure at 25 °C induced effects on this enzymatic activity. At 25 °C, after 14 days of recovery the decrease on GST activity for the lower concentration cannot be considered an inhibition response, but a basal value according to Ferreira et al. (2010). However, the value for the control showed a significant increase during the test, higher than basal values reported (Ferreira et al., 2010) and higher than values from organisms sampled before the starting of the test for the same temperature. These differences observed for the control are unclear, since a return to basal levels were observed after 14 days of exposure.

An often reported effect of OPs is the induction of oxidative stress, by generating reactive oxygen species (ROS) as well as alterations within the antioxidant and scavenging system (Karami-Mohajeri and Abdollahi, 2011). The study carried out on the biomarkers GST, LPO and CAT showed also some degree of oxidative stress as a result of the exposure to dimethoate. LPO and CAT activity showed small responses to dimethoate exposure, although an increase on LPO was found at the 7 days of exposure at 25 °C (5x higher than control) that could be related to the high mortality observed. So, oxidative stress can be found as a result of dimethoate exposure during an adaptation period of 96 h/7 days and also related to possible effects induced by the higher

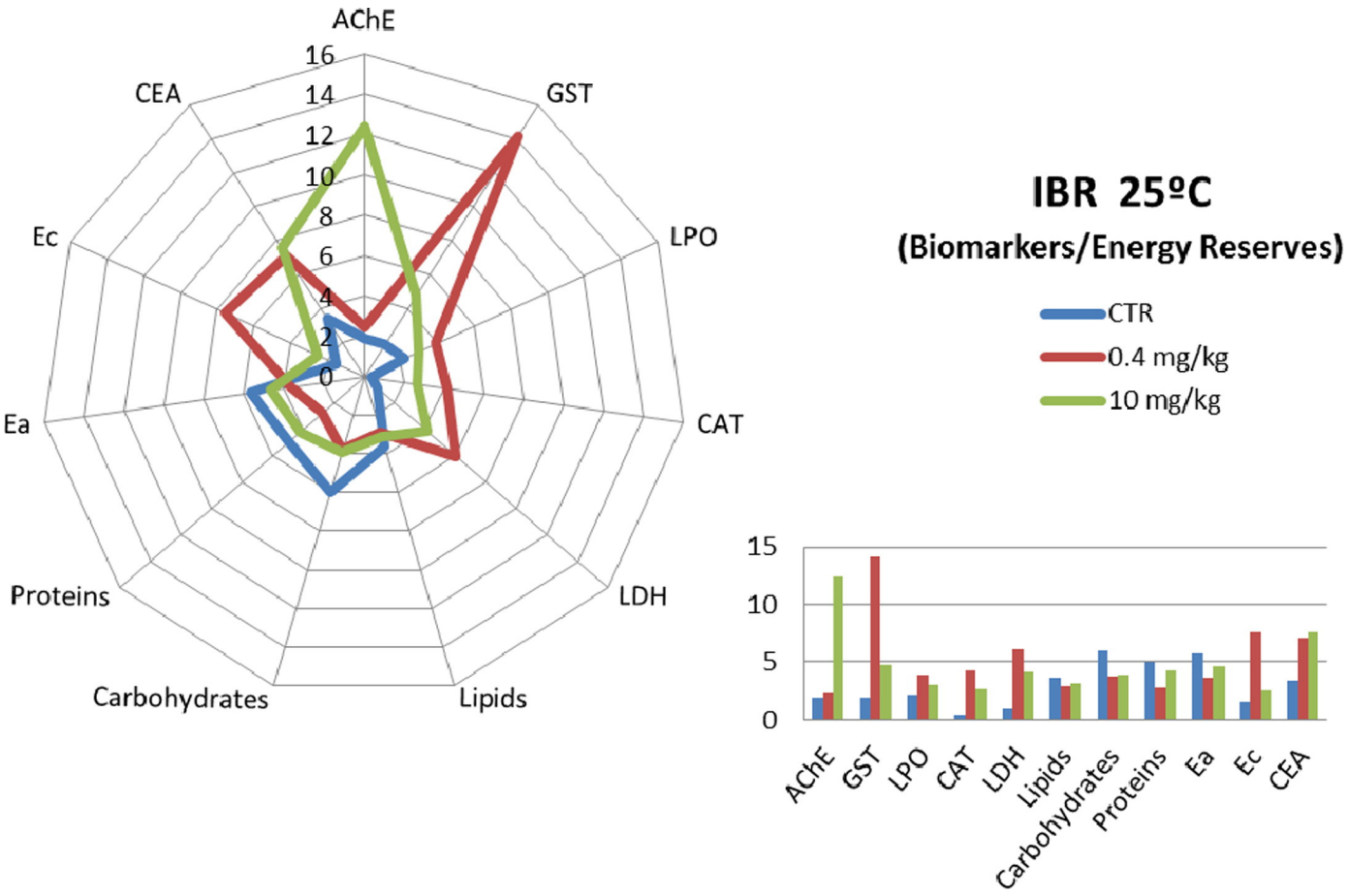

Fig. 8. Integrated biomarker response represented by starplot and histogram of *Porcellionides pruinosus* in control and exposed to dimethoate (0.4 and 10 mg/kg soil) at 25 °C during the exposure and recovery period. AChE = acetylcholinesterase, GST = glutathione *S*-transferases, LPO = lipid peroxidation, CAT = catalase, LDH = lactate dehydrogenase, Ea = available energy, Ec = energy consumption, CEA = cellular energy allocation.

exposure temperature (25 °C). In fact, previous works have shown situations of oxidative stress for exposure to increment temperatures (e.g. Lesser, 2006; Lesser and Kruse, 2004), mainly leading to elevated dehydration (França et al., 2007). In addition CAT activity has been also reported to decrease as a result of the increase of superoxide anions (Velki and Hackenberger, 2012).

Energy response biomarker LDH apparently responded to dimethoate exposure at 20 °C, independently from concentration. After 7 days of exposure and 7 days of recovery this biomarker showed differences at the highest concentration under both temperatures. Although a clear response was not obtained by using this biomarker one can hypothesise that dimethoate may be interfering in the glycogen cycle and some of the effects found for this enzyme might also be associated with decreases in carbohydrates content (Moorthy et al., 1983).

Therefore, one main finding was that there were different responses for each energy reserve within each temperature of exposure. For lipids, an increase was observed in some of the time points and for both temperatures of exposure, but in general dimethoate did not seem to affect the lipid content. The carbohydrates presented a similar pattern of decrease for both temperatures within the first 7 days of exposure, after which the effects of dimethoate seemed to disappear and values tended to the basal levels. As expected, total protein did not present significant changes, as it is known that they are the last energy reserve to change or be used upon stress exposure. As in the previous case, an increase was also observed after 96 h exposure at 25 °C. Lower energy contents were observed mainly at 20 °C and for the lowest concentration. In general, energy reserves presented essential data to understand dimethoate toxicity even indicating a possible interaction within the two different temperatures. Whereas for the 20 °C exposure, almost no increase was found in the energy reserve content for the experimental duration, the 25 °C exposure presented not only a great content increase, but also around the 7/14 days of exposure these reserves reached a plateau, indicating an equilibrium in the organism physiology. In addition, it was also possible to depict small variations after reaching the plateau. The results obtained between the 96 h and 7 days of exposure at 25 °C indicated an increase in carbohydrate, lipid and the protein content leading into a new plateau, which might also indicate some physiological or behavioural changes that the organisms underwent to prevent stress. In this case it seems also plausible to transpose all these results on the interaction between temperature and dimethoate into a disturbance in the moulting cycle. Along with the protein increase, it was also expected an increase in the other energy reserves as observed within our experiment. This impairment in the moulting cycle, which seems to happen when organisms are exposed to 25 °C, may also be a behaviour strategy to increase its feeding rate, so they could endure the stress for a longer time period. Previous works have already reported an increase in the muscle groups located in the anterior and posterior segments, which can be directly related to the protein content, and also that the moult, which is biphasic, and can occur with hours or days (Whiteley and El Haj, 1997). This could be that organisms exposed to 20 °C had a total protein content that did not increase in quantity, but stayed constant, and the increment in the energy reserves resulted from carbohydrate and lipid changes. These results are contradicted in several previous works where a decrease in feeding

behaviour could be observed for terrestrial isopods exposed to stressors (Abdel-Lateif et al., 1998; Donker et al., 1998). Nevertheless one must consider that these studies have been performed with contaminated food, and although small amounts of pesticide must had been adsorbed by the leaves in our study, a possible avoidance was due to the effects on the organisms and not by changes in palatability.

Finally, in this study the available energy as a parameter did not seem to be a good indicator of the organisms' status, since little variation in contents was observed. The same happened with the CEA since differences in Ea values were low, and the organisms' behaviour (represented by the Ec values, where higher consumption ratios relates to organisms' higher activities) were not intense enough to cause an impact. Whenever a decline in CEA was observed, it was indicating either a reduction in available energy or a higher energy expenditure, both resulting in a lower amount of energy available for growth, reproduction or basal activity (de Coen and Janssen, 1997).

Within the two temperatures used, differences were observed in terms of the total available energy, energy consumption and subsequently the CEA. In fact the increase of Ec and decrease in Ea for the exposed organisms at 20 °C is contrary to the patterns shown at the 25 °C exposure, which may suggest that at 20 °C organisms did not slow their metabolism. However, at 20 °C, temperature did not seem to be the dominant factor influencing the energy-related parameters, but dimethoate, as can be seen by the patterns of Ea and Ec, as a result of the detoxification process. Contrary, for the organisms exposed to 25 °C, it did not seem that any of the stressors had a preponderance effect over the other, as organisms decreased significantly their Ec and allocated energy to the detoxification process. At this temperature, the Ea increase might be explained by an increase in isopods' feeding activities. This type of behaviour has not yet been reported and new studies regarding the combined effects of temperature and dimethoate on feeding rates would provide further information.

The recovery period did also provide some important and interesting remarks on organisms previously exposed to dimethoate. From previous works with other non-target organisms, such as earthworms, a slow recovery rate in AChE has also been reported (Aamodt et al., 2007). In the present work at 20 °C and at the highest dimethoate concentration, the AChE inhibition was in accordance with studies that show that it can become an irreversible process (Ranjbar et al., 2005). The organisms exposed to 20 °C followed a similar pattern when regarding oxidative stress biomarkers where a slow recovery was observed, although the LPO rates, that indicate damage from ROS, reached similar levels of those in the control after the end of the test period. For the organisms exposed to 25 °C although a similar pattern can be observed, the LPO rates continued to be significantly higher than the control, which may indicate that this temperature may influence the recovery of the organisms even after 14 days. The fluctuation of the energy related parameters in both the 20 °C and the 25 °C exposure are in accordance with results from biomarkers. In fact it can be hypothesised that the initial fluctuation (96 h/7 days) that could be seen in the exposure period which was more pronounced in the recovery period, was a possible adaptation of organisms to reach homeostasis again.

All this supports the idea that a slow recovery is present, but also suggests an important highlight that these non-target organisms can continuously be affected by dimethoate even when exposed to recommended field doses under single applications. In a broader context the results obtained in this study can be summarized into three major points. First the impact of dimethoate in the main target enzyme AChE, and also in other oxidative stress biomarkers which are necessary to biotransform and handle the reactive oxygen species (ROS) within the organisms' body. Second the impact of temperature for the toxicity of dimethoate that combined with the pesticide may lead to higher toxicity, higher mortality rates and a decline in populations. Finally the sub-lethal effects observed when organisms were exposed to 0.4 mg dimethoate/kg soil, represented by changes in AChE, GST, energy consumption, CEA, can indicate the impairment of key functions essential for the maintenance of these organisms individually, and be transposed to the population level due to a possible effect on their reproduction behaviour/pattern.

5. Conclusions

As previously shown by other studies, dimethoate affected the enzyme AChE but also other biomarkers such as the detoxification enzyme, GST and the damage related biomarker, LPO. The increase of LPO also seemed to be related to the high mortality observed in 25 °C exposure.

The organisms exposed to the lower concentration of 0.4 mg dimethoate/kg soil, simulating a field application dose, presented low to moderate toxicity which was in accordance with the work of Fischer et al. (1997) and therefore was used as baseline for this study. However, in the organisms exposed to the highest concentration (10 mg dimethoate/kg soil), toxicity was mainly due to the inhibition of AChE and the degradation of dimethoate that can possibly lead to the formation of highly toxic metabolites and also reactive oxygen species and oxidative stress that caused high LPO rates.

This study showed an increase in the energy reserve contents within the first 7 days of exposure that could be linked to an impairment of the moult cycle or an increase in the feeding behaviour. This study highlights also a possible strategy of recovery for these organisms with an increase in total lipid content, which was the only energy reserve that returned to baseline levels within the recovery period.

Generally, long term experiments using realistic concentrations should be performed to understand the mechanisms of toxicity of stressors, such as dimethoate, and to develop a baseline for future studies. This will allow better understanding of the specific mechanisms underlying the toxicity process and detoxification pathways. In addition, the results from soil chemical analysis highlight the possibility that isopods can increase the decay rates of dimethoate, as also reported for other pesticides.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2014.08.062.

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