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ORIGINAL ARTICLE

Stress responses of the sea cucumber *Holothuria forskali* during aquaculture handling and transportation

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ABSTRACT

Animal welfare during handling and transportation to aquaculture facilities or public aquaria is commonly estimated by addressing injury and mortality levels. Although these procedures have been optimized for different species, data on individual species' cellular capabilities to tolerate stress are still scarce. In the present study, several biomarkers related with oxidative stress and energy metabolism were assessed in *Holothuria forskali* during animal acclimation, pre-transport, transport and quarantine. Combined analyses confirmed that sea cucumbers experienced high oxidative stress during transport, but had the capability to deal with it using a complex of cellular defence mechanisms, which enabled recovery from oxidative stress without permanent damage. Through a better understanding of individual species and the development of optimal parameters, this approach has the potential to improve animal wellbeing during and after acclimation, transportation and recovery processes.

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Introduction

Public aquaria have been increasingly concerned with animal welfare, public engagement and the conservation of species. Opportunities for public aquaria to improve the sustainability of the aquatic animal trade have been discussed in Tlusty et al. (2013). For the purpose of species protection, public education and research, organisms of interest are carefully collected from their natural environment and transported to public aquaria worldwide, by air or road, on a daily basis (Chandurvelan et al. 2013; Dhanasiri et al. 2013; Manuel et al. 2014; Boerrigter et al. 2015). Also, despite attracting less public attention, improving animal welfare during organism transport to commercial aquaculture facilities will probably increase organism fitness to better suit business needs. Standard techniques and equipment to optimize animal welfare, and minimize injuries and mortality rates during long-distance transport, have been established for several vertebrate species, including the guppy (Teo et al. 1989), ratfish and tiger rockfish (Correia 2001), scalloped hammerhead shark (Young et al. 2002), devil-ray, meagre and ocean sunfish (Correia et al. 2008), among others. Stress during transport has also been largely studied on commercially important invertebrate species, such as the crab *Cancer pagurus* Linnaeus, 1758 (Barrento et al. 2010), the lobster *Homarus americanus* H. Milne Edwards, 1837 (Lorenzon et al. 2007) or different jellyfish (Pierce 2009). However, stress reduction is not only vital during the transportation itself but should also be regarded during the preceding animal collection and acclimation. To ensure long-term survival of different animals, optimizations have been performed with regard to oxygen saturation, ammonia minimization, temperature and pH stabilization (Correia et al. 2011, 2008). As those studies have primarily been focused on endpoints such as injury and mortality levels, information regarding stress levels and physiological responses at the cellular level is scarce, but of paramount importance.

Stress typically leads to elevated oxygen consumption and subsequent production of reactive oxygen species (ROS), potentially leading to a reduction in fitness that may ultimately lead to death. Key antioxidant enzymes that protect the cells against ROS include superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR). Nevertheless, if the cellular antioxidant system fails to lower ROS levels, oxidative damage can occur in tissues and cellular macromolecules in the form of, for instance, lipid peroxidation (LPO) and DNA strand breaks (Alves et al. 2016; Silva et al. 2016). Since the mechanisms to cope with stress and maintain internal homeostasis are highly

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energy-consuming, the use of biomarkers involved in energy metabolism can also give valuable information about the organisms' stress levels. Lactate dehydrogenase (LDH) and isocitrate dehydrogenase (IDH) are examples of enzymatic biomarkers related to anaerobic and aerobic metabolisms, respectively, and their activity measurements can give an indication of the metabolic costs of stress responses. To address the global energy budget of organisms, the cellular energy allocation (CEA) indicator can also be applied. CEA is defined as the ratio of total available energy (E_A, i.e. sum of proteins, lipids and carbohydrates) over energy consumption (E_C), which is estimated by measuring the electron transport system (ETS) activity – indicative of the organisms' cellular respiration rate (De Coen et al. 2001; De Coen & Janssen 2003; Verslycke et al. 2004a, 2004b).

The black sea cucumber *Holothuria forskali* Delle Chiaje, 1823 is an echinoderm widely distributed in the Mediterranean Sea and the north-east Atlantic Ocean (Mercier & Hamel 2013). Along the coasts of the West Region of Portugal, *H. forskali* is the prevalent species of holothurian, commonly occurring in shallow waters (0–50 m of depth) around hard-bottom areas, especially on vertical surfaces (Southward & Campbell 2006). Because of its great abundance, accessibility and popularity for public aquaria and aquaculture, *H. forskali* is a suitable species to investigate the biochemical stress responses during a transport simulation, including preceding acclimation and subsequent quarantine periods. Biomarkers related to the antioxidant system and energetic status were measured in two target tissues: the respiratory tree and the longitudinal muscle. The respiratory tree is exclusively present in holothurians (Dolmatov & Ginanova 2009) and serves as the primary respiratory system through which holothurians extract most of their oxygen (Newell & Courtney 1965).

Overall, the goal of this study was to investigate stress responses of *H. forskali* at the biochemical level during collection, acclimation, transport and quarantine, with the use of cellular biomarkers indicative of oxidative stress and changes in the energy metabolism, providing tools to address an organism's fitness. The results of this study will prove invaluable to inform future handling and transportation practices.

Materials and methods

Collection of organisms (Holothuria forskali)

Organisms of similar sizes (18 ± 2 cm; contracted state) were collected at Carreiro de Joanes (39°21′14.3″N, 9°23′43.6″W), Peninsula of Peniche, central Portugal, by scuba diving. During collection, the animals were temporarily maintained in 50 l transport containers with regularly renewed seawater to ensure adequate water quality. The organisms were transferred to the aquaculture facilities, within the hour, where six organisms were immediately dissected, processed and placed at −80°C until further analysis (0 d) (see procedure section). The remaining organisms were kept in a recirculating 600 l water tank with a 12 h:12 h (light:dark) photoperiod, temperature (T) at 17 ± 0.5°C, oxygen saturation (DO) at 90 ± 10%, salinity at 32 ± 1‰, pH at 7.7 ± 0.1 and ammonia concentration (cNH_3) under 0.006 mg/l. Water conditions were monitored on a daily basis using a multiparametric probe, YSI Professional Plus (Yellow Springs, Ohio, USA), and Tropic Marin® AMMONIA-/AMMONIUM-TEST (Niklausen, Luzern, Switzerland). The temperature and salinity conditions in the tanks were chosen to mimic the natural conditions of the collection site and were maintained throughout the entire experiment.

Experimental set-up

Acclimation

During the acclimation period (8 days), the organisms were kept in 600 l tanks filled with seawater and connected to a TMC 5000 l filtration system (Tropical Marine Centre, London, UK). For daily feeding, a 5 l mixture of microalgae (1.5×10^5 cell/ml) was prepared consisting in equal parts of the following species: *Rhodomonas lens* Pascher & Ruttner, *Isochrysis galbana* Parke, *Phaeodactylum tricornutum* Bohlin and *Chlorella* sp. At each sampling day throughout the acclimation period, seven organisms were randomly sampled, processed, and kept at −80°C until further analysis. Sampling occurred on days 1, 2, 4 and 8.

Pre-transport and transport

Two days prior to the transport (i.e. the pre-transport period, days 8–10), the organisms were fasted to decrease the amount of nitrogenous waste released during transport. After sampling on day 10, a 16 h transport was simulated according to previous studies (e.g. Correia et al. 2011), transferring the organisms into 7 l plastic bags (five animals per bag). All bags were filled with approximately 1/3 of water from the acclimation system and 2/3 of medicinal oxygen (Gasin, Portugal), sealed and stored inside a styrofoam box, identical to the ones used in actual transports (Berka 1986). Water parameters (pH, dissolved oxygen and temperature) were monitored using Hannah Instruments Oxy-Check HI9147 (oxygen and temperature; Nuşfalău, Romania) and VWR Symphony SP70P (pH;

Singapore) at 0 h and 8 h of transport. Sampling of five individuals occurred at 8 h and 16 h (see tissue preparation section) and oxygen was renewed after the 8 h-sampling.

Quarantine

Subsequent to the 16 h-transport sampling, the bags containing the remaining organisms were opened and carefully placed in a recirculating water tank as described for the acclimation period, corresponding to the quarantine period in a public aquarium. For 24 h, the water inside the bags was regularly renewed with tank water to ensure adequate quality. After 24 h, the animals were entirely relocated into the 'quarantine' tanks. Sampling occurred at 24 h, 48 h and 96 h during the quarantine period.

Tissue preparation

Upon sampling, organisms were cold shock sacrificed, and each organism was dissected for tissue samples of the respiratory tree and longitudinal muscle, which were homogenized using an Ystral homogenizer (D-79282, Ballrechten-Dottingen, Germany). Homogenized samples were split into several fractions, depending on the biomarkers to be analysed in each tissue, in 2 ml microtubes and stored at −80°C until further analyses.

For each individual, 0.4 g (wet weight (ww)) of respiratory tree was homogenized in 2 ml 0.1 M potassium-phosphate buffer (pH 7.4) and different fractions were separated to measure LPO, DNA damage and ETS. The rest of the homogenate was centrifuged at 10,000 g for 20 min (4°C) to obtain the post-mitochondrial supernatant (PMS) for the measurement of CAT, SOD and GR activities.

Similarly, 0.8 g (ww) of muscle was homogenized in 4 ml ultrapure water and different fractions were separated to measure LPO, DNA damage and CEA. The rest of the homogenate was centrifuged at 10,000 g for 20 min (4°C) to obtain PMS, to which an equal volume of 0.2 M potassium-phosphate buffer (pH 7.4) was added (final molarity of 0.1 M), for the measurement of CAT, SOD and GR activities. To measure LDH and IDH activities, the remaining homogenate was centrifuged at 3000 g for 5 min (4°C) and the supernatant, with an equal volume of 0.2 M potassium-phosphate buffer (pH 7.4), was used for the enzyme activity measurements.

Biomarker analysis

In all enzymatic assays and measurements, either potassium-phosphate buffer (0.1 M, pH 7.4) or ultrapure water was used as blank, depending on the medium present in the samples. Spectrophotometric measurements were done in triplicate, at 25°C, in a Synergy H1 Hybrid Multi-Mode microplate reader (Biotek® Instrument, Vermont, USA).

Protein quantification for the normalizations was performed using bovine γ-globulin (BGG, Sigma-Aldrich, USA) as standard protein, following the Bradford method (Bradford 1976), using 96-well flat-bottom plates. Absorbance was read at 600 nm and results expressed in the mg of protein/ml.

Oxidative stress-related biomarkers

Lipid peroxidation (LPO). LPO was measured in the form of thiobarbituric acid reactive species (TBARS), following the method of Ohkawa et al. (1979) and Bird & Draper (1984), with adaptations published by Filho et al. (2001) and Torres et al. (2002). The samples were deproteinized with 12% trichloroacetic acid following the addition of 0.73% 2-thiobarbituric acid (TBA), and the mixture was kept at 100°C for 1 h. After centrifuging the samples at 11,500 g for 5 min, the supernatant was used to measure absorbance at 535 nm. LPO levels were calculated and expressed in nmol TBARS per g of ww using the TBA molar extinction coefficient at 535 nm of 1.56×10^5 M/cm.

DNA damage. The degree of DNA damage was assessed by measuring DNA strand breaks using the DNA alkaline precipitation assay of Olive (1988) with de Lafontaine et al. (2000) adaptations. Tissue homogenates (50 µl) were incubated with 500 µl of 2% SDS solution containing 50 mM NaOH, 10 mM Tris, 10 mM EDTA and 500 µl of 0.12 M KCl at 60°C for 10 minutes. Samples were placed on ice for 15 minutes to induce the precipitation of SDS associated nucleoproteins and genomic DNA, and were finally centrifuged at 8000 g (4°C) for 5 min to enhance precipitation. To quantify levels of damaged DNA molecules, the supernatant was mixed with Hoesch dye (1 µg/ml bis-benzimide, Sigma-Aldrich, USA) in a 96-well microplate. Fluorescence was measured using an excitation/emission wavelength of 360/450 nm. Results were expressed as mg of DNA per g of ww, using calf thymus DNA (Sigma-Aldrich, USA) as standard, using a curve with DNA concentrations between 0 and 20 µg/ml.

Catalase (CAT) activity. CAT activity was measured following the method described by Claiborne (1985). After adding 0.03 M H_2O_2 to the PMS, CAT activity was measured following the decrease in absorbance at 240 nm for 3 min. CAT activity was expressed in

µmol/min/mg of protein, using the H_2O_2 molar extinction coefficient at 240 nm of 40 M/cm.

Superoxide dismutase (SOD) activity. SOD activity was measured following the method described by McCord & Fridovich (1969). After adding 0.05 M K-phosphate buffer (pH 7.4), 0.14 mM xanthine, 0.06 M cytochrome C and 0.01 U/ml xanthine oxidase to the PMS, SOD activity was measured following the decrease in absorbance at 550 nm for 5 min. SOD activity was expressed in U/mg of protein using a SOD standard of 1.5 U/ml, where 1 U represents the amount of enzyme in the sample that causes 50% inhibition of cytochrome C reduction.

Glutathione reductase (GR) activity. GR activity was measured following the method described by Cribb et al. (1989). After adding 0.05 M K-phosphate buffer (pH 7.4) (containing 0.2 mM NADPH, 1 mM GSSG and 0.5 mM DTPA) to the PMS, GR activity was measured following the decrease in absorbance at 340 nm for 2 min. The enzymatic activity was calculated using the NADPH molar extinction coefficient at 340 nm of 6.2×10^3 M/cm and expressed in nmol/min/mg of protein.

Energy metabolism-related biomarkers

Lactate dehydrogenase (LDH) activity. LDH activity was measured following the method described by Vassault (1983) with the adaptations of Diamantino et al. (2001). After adding NADH and pyruvate to the sample, LDH activity was measured following the decrease in absorbance at 340 nm for 5 min that corresponds to the oxidation of NADH when pyruvate is being converted to lactate. Results were expressed as nmol/min/mg protein, using the NADH molar extinction coefficient at 340 nm of 6.3×10^3 M/cm.

Isocitrate dehydrogenase (IDH) activity. IDH activity was measured following the method described by Ellis & Goldberg (1971) with the microplate adaptations of Lima et al. (2007). After adding DL-isocitric acid and $NADP^+$ to the sample, IDH activity was measured following the increase in NADPH formation at 340 nm for 3 min. Results were calculated according to a molar extinction coefficient of NADPH at 340 nm of 6.22×10^3 M/cm and expressed as nmol/min/mg protein.

Cellular energy allocation (CEA)

CEA was determined by comparing available energy (E_A) and consumed energy (E_C) in the muscle tissue. E_A was measured by summing the total content of proteins, carbohydrates and lipids using spectrophotometry measurements according to De Coen & Janssen (2003). Briefly, protein content was measured via Bradford's method (1976) following the absorbance at 600 nm using bovine serum albumin (BSA, Sigma-Aldrich, USA) as standard. Carbohydrate content was measured with 5% phenol and 95% H_2SO_4 following the absorbance at 490 nm and using glucose (Sigma-Aldrich, USA) as standard (De Coen & Janssen 2003). Total lipids were extracted according to Bligh & Dyer (1959) and measured by following the absorbance at 400 nm using tripalmitine (Sigma-Aldrich, USA) as standard. The results of protein, carbohydrate and lipid fractions were then transformed into energetic equivalents using enthalpy combustion (24 kJ/g proteins, 17.5 kJ/g carbohydrates and 39.5 kJ/g lipids), as described by De Coen & Janssen (2003). E_C was calculated based on the measurement of ETS activity (King & Packard 1975), by adding a solution of NADPH and INT (p-iodo-nitro-tetrazolium, Sigma-Aldrich, USA), following the absorbance at 490 nm for 3 min. The oxygen consumption rate was calculated based on stoichiometry (for each 2 mmol of formazan formed, 1 mmol of O_2 is consumed). The quantity of oxygen consumed was then transformed into caloric values using oxyenthalpic equivalents of 484 kJ/mol O_2 for an average lipid, protein and carbohydrate mixture (Gnaiger 1983; De Coen et al. 2001). After caloric transformation, CEA was calculated as the ratio of E_A over E_C and expressed in mJ per mg of muscle ww (Verslycke et al. 2004a, 2004b).

Statistical analysis

All statistical analyses were performed using the statistical package *SigmaPlot* version 11.0 (1997). To assess differences within the first acclimation periods (days 0, 1, 2, 4 and 8), as well as within time points during transport (day 10: 0, 8 and 16 hours), and within time points during the quarantine period (days 10–14: 0, 24, 48 and 96 hours), a one-way analysis of variance (ANOVA) was performed for each tissue. Prior to each analysis, data were examined for normality (Kolmogorov–Smirnov test) and variance homogeneity (Levene's test). In order to improve normal distribution and homogeneity of variance, the dataset was log10 or square root-transformed. In case of significant outcomes, the post-hoc Dunnett's method was applied for comparisons with the respective starting point. To compare the two days of pre-transport (days 8 and 10), a Student's *t*-test was applied. Results are presented as mean with the respective

standard error. For all statistical tests, the significance level was set at $P < 0.05$.

Results

Acclimation

Upon collection of the organisms (0 d), LPO levels were elevated but showed a significant decrease already at 1 d of acclimation (Figure 1a). On the contrary, DNA damage gradually increased from 0 d up to 8 d in both tissues (Figure 1b). Regarding the antioxidant enzymes, responses differed between tissues: in the respiratory tree, only an increase in CAT was observed in the first 2 d of acclimation but the values returned to baseline after 4 d, while in muscle CAT activity was significantly reduced at 8 d and SOD activity was inhibited since 1 d (Figure 1c,d). No significant changes occurred regarding GR activity (Figure 1e). Concerning the endpoints related to energy metabolism, there was a significant increase in the ETS activity in the muscle at 8 d (Figure 2a) along with a reduced lipid content (Figure 2b) and decreased LDH activity during the acclimation period (Figure 2d).

Pre-transport

On day 10, corresponding to the pre-transport period (fasting of the organisms occurred between days 8 and 10), LPO levels significantly decreased in the muscle (Figure 1a) as well as the DNA damage levels in both tissues (Figure 1b). CAT activity was significantly induced after this period in both tissues (Figure 1c). At the energetic level, significantly lower ETS and IDH activities were observed in the muscle (Figure 2a,d), as well as reduced carbohydrate levels (Figure 2b).

Transport simulation

Seawater ammonia levels inside the bags during transport simulation were always under 0.024 mol/l. During this period there was a significant increase in CAT activity in both tissues, whereas SOD activity significantly decreased in muscle (Figure 1c,d). Indications of increased oxidative damage were observed at 8 h of transport with elevated LPO levels, which decreased again at 16 h (Figure 1a). With regards to energy-related endpoints, only ETS activity showed a significant increase in the respiratory tree at 8 h of the transport simulation (Figure 2a).

Quarantine

During the quarantine period, no effects on DNA damage were observed but the LPO levels increased through time (Figure 1a,b). CAT activity levels significantly decreased after 24 h in the respiratory tree (Figure 1c). At 48 h, the reduction in ETS levels (muscle) resulted in an increase in global CEA (Figure 2a,c). At 96 h, LDH activity was inhibited while IDH was induced, accompanied by an increase in carbohydrate levels (Figure 2b,d).

Discussion

Available studies concerning animal fitness/stress during transportation and handling have primarily been focused on injury and mortality levels, which can be considered as rough endpoints concerning animal fitness. In the present study, *Holothuria forskali*'s biochemical stress responses during four different periods concerning the transport of these organisms (acclimation, pre-transport, transport simulation and quarantine) were assessed, providing improved tools to address organism wellbeing in these kinds of practices.

Upon collection, test organisms seemed to be able to cope with the change of environment, recover from the initial stress experienced during handling, and acclimatize to aquaculture conditions. Not only did none of the animals die, but there was also evidence for successful acclimation, seen mostly in the biomarkers measured in the muscle, namely: the fast decrease in LPO levels; the decrease in the activity of the antioxidant enzymes SOD and CAT, and the decrease in LDH levels, which indicates that the organisms were reducing their energy requirements (Figure 1). However, when looking at the respiratory tree results, there was an increase in the DNA damage throughout the acclimation period (Figure 1b), indicative of oxidative damage, along with an increase in cellular metabolism (induction in ETS activity; Figure 2a), yet those effects were apparently transient since initial biomarker levels were restored at 10 d. It is likely that the organisms faced an initial imbalance between DNA lesions and repair capacity due to continuous elevated ROS levels, and consequently, rapid accumulation of DNA damage. The decrease of DNA damage from day 8 to day 10, along with the high metabolic rates at 8 d of the acclimation period, seem to indicate that increased DNA repair mechanisms were taking place, ultimately resulting in the lower levels of both biomarkers at 10 d. The process of cellular DNA repair has been studied in circulating immune

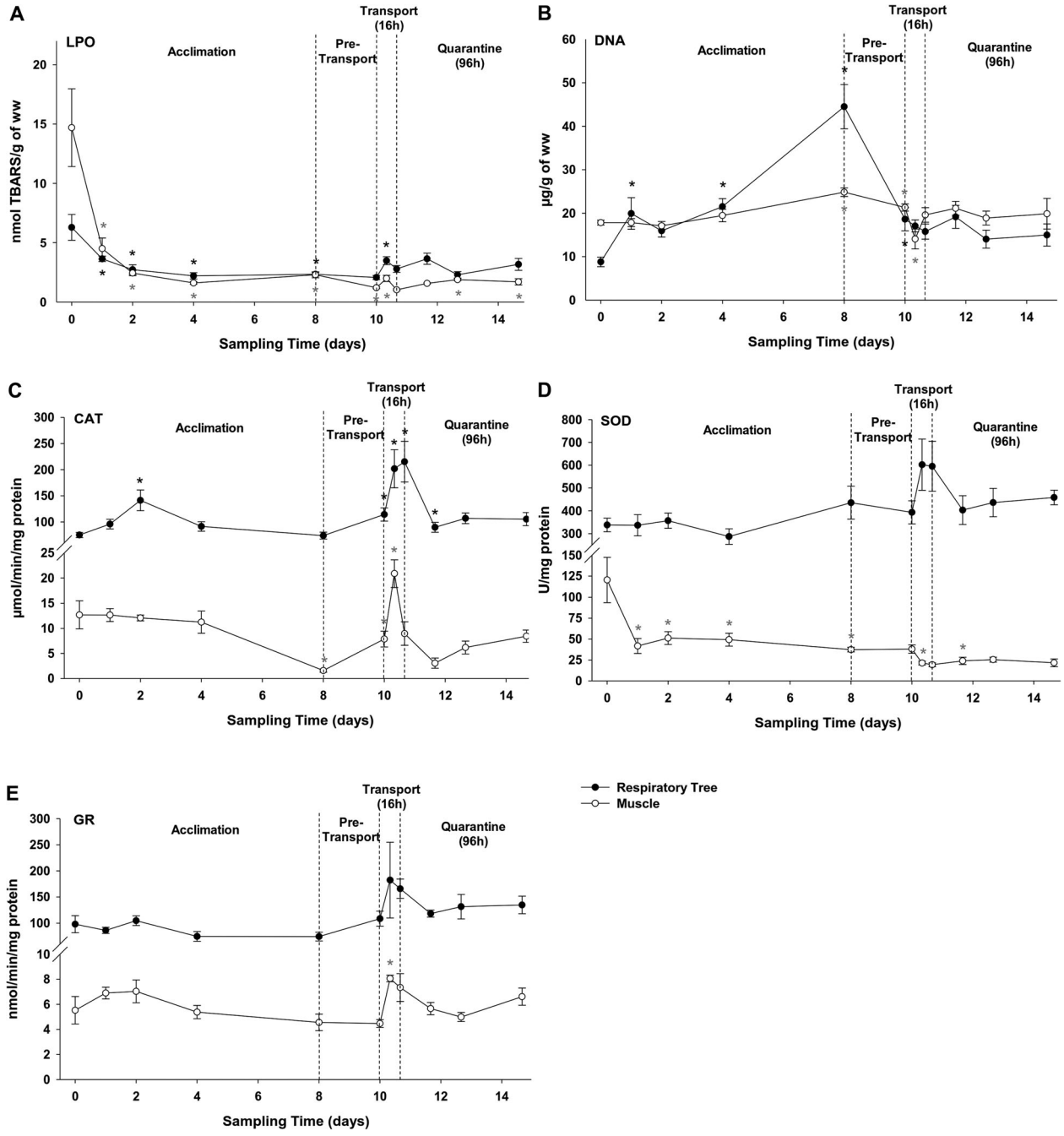

Figure 1. Oxidative stress related biochemical biomarkers in *Holothuria forskali* during acclimation (from 0 d to 8 d), pre-transport (days 8–10), transport (day 10: 0, 8 and 16 h) and quarantine (days 10–14: 0, 24, 48 and 96 h) conditions, in the muscle and respiratory tree: (a) LPO – lipid peroxidation levels; (b) DNA damage levels; (c) CAT – catalase activity; (d) SOD – superoxide dismutase activity; (e) GR – glutathione reductase activity. Graphs show mean values ± standard error. *Represents significant differences relative to the starting point of the respective period (ANOVA, Dunnett's, $P < 0.05$).

cells (coelomocytes) of the sea cucumber *Isostichopus badionotus* (Selenka, 1867) after exposure to H_2O_2 (El-Bibany et al. 2014). It was proposed that environmental stress-induced formation of ROS, particularly by H_2O_2, caused DNA strand breaks (lesions) which were mended via an enzyme complex mechanism – base excision repair. The presence of highly adapted DNA repair mechanisms in sea cucumbers has been claimed with regards to their constant exposure to sediment-associated toxicants (El-Bibany et al. 2014). In our study, the decline in DNA damage from 8 to 10 d might thus be explained by an upregulation of DNA base excision repair in combination with enzymatic ROS defence via CAT and GR. Nevertheless,

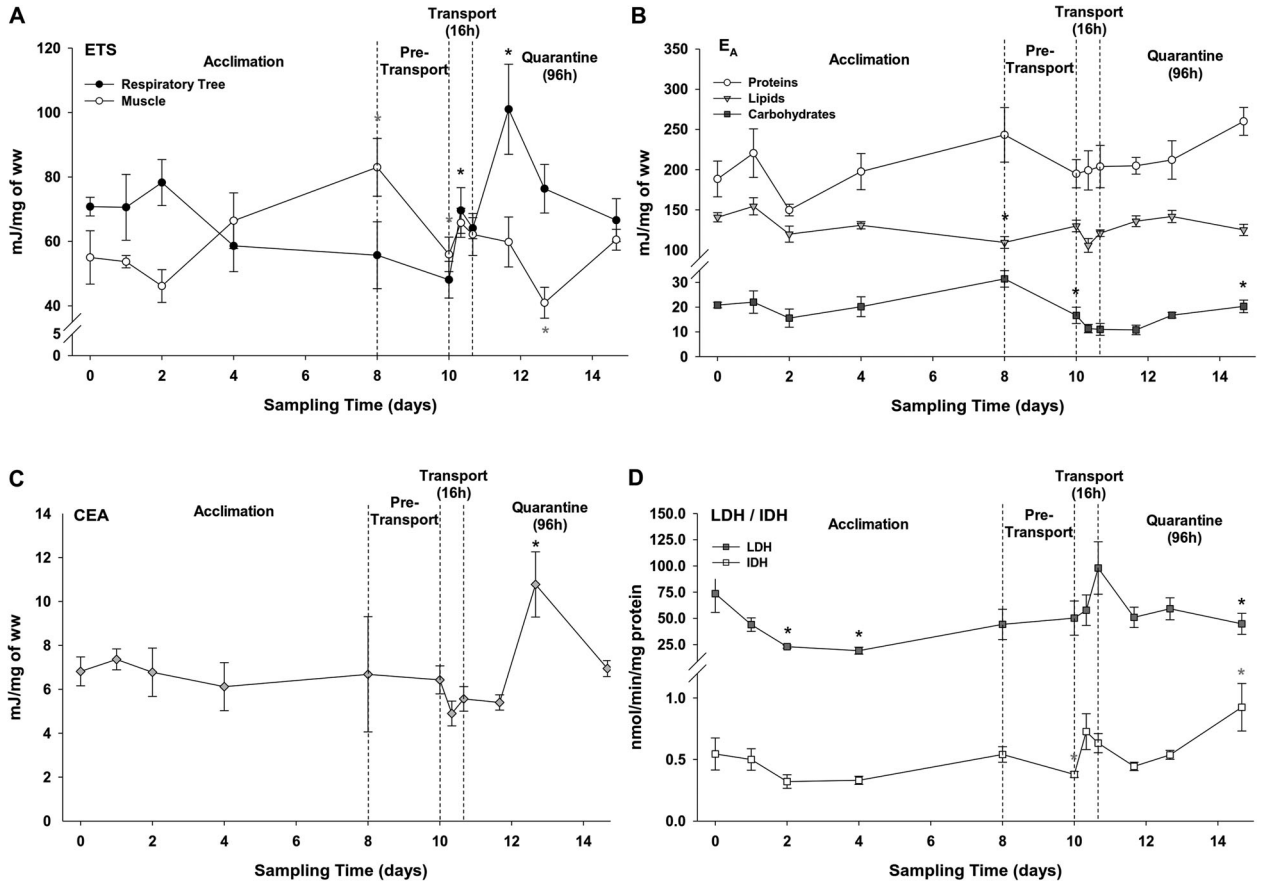

Figure 2. Energy metabolism-related biochemical biomarkers in *Holothuria forskali* during acclimation (from 0 d to 8 d), pre-transport (days 8–10), transport (day 10: 0, 8 and 16 h) and quarantine (days 10–14: 0, 24, 48 and 96 h) conditions. (a) ETS – electron transfer system activity in the muscle and respiratory tree; (b) E_A – energy available in the muscle through total levels of proteins, lipids and carbohydrates; (c) CEA – cellular energy allocation index in the muscle; (d) LDH – lactate dehydrogenase and IDH – isocitrate dehydrogenase activity levels in the muscle tissue. Graphs show mean values ± standard error. *Represents significant differences relative to the starting point of the respective period (ANOVA, Dunnett's, $P < 0.05$).

molecular markers following the gene expression of BER-related enzymes would be necessary to confirm this hypothesis.

Fish and invertebrate species are commonly acclimatized for longer periods (Correia 2001; Young et al. 2002; Lorenzon et al. 2007; Correia et al. 2008, 2011; Pierce 2009; Barrento et al. 2010; Rodrigues et al. 2013; Boerrigter et al. 2015), although such long acclimation periods, prior to international, long-distance transportations by road or air, range from 24 to 72 h. In the present experiment, *H. forskali* was acclimatized for only eight days, yet biomarker analysis confirmed a successful acclimation, and therefore the physiological conditions for transportation were achieved within this comparably shorter acclimation period.

Usually, two days before transportation the organisms are fasted to minimize the release of nitrogen waste products during transportation (e.g. Rodrigues et al. 2013). The fasting period of *H. forskali* during the 2 d pre-transport resulted in the reactivation of CAT and in the decrease of carbohydrate levels, together with a lower cellular metabolism, probably because no energy was being spent with feeding. However, oxidative damage was not observed during this period and the levels of both LPO and DNA damage were even reduced, demonstrating that the organisms were not under oxidative stress. Moreover, although the organisms were fasted and a decrease in energy reserves was observed, the elevated enzyme activities show that the antioxidant enzyme complex had not yet deteriorated, as verified in longer fasting periods (dormancy) for other holothurian species (Klanian 2013).

During the transport simulation, higher cellular respiration rates (ETS), oxidative damage (LPO) and upregulation of antioxidant enzymes (CAT) provided evidence that the animals experienced stress, possibly

caused by increased ammonia excretion, verified after 8 h of transportation. Although the animals had been fasted for 2 d, the high organismal densities might have caused such an increase in ammonia levels, and certainly, for this slow metabolism species, a longer period of fasting before transport would be preferable. However, reducing the number of organisms per bag and renewing high oxygen saturation (>100%) levels halfway through the transport simulation (8 h) was sufficient to counteract ammonia-induced oxidative stress and damage. Furthermore, the energy metabolism of *H. forskali* was barely affected, which might be a result of negligible energy loss due to reduced movement during transport. Nevertheless, lower holothurian densities in the transport bags are advisable to reduce the stress levels, as observed in the present study – a recommendation that would not be possible if only attending to injury status and mortality rates.

During the quarantine period, sea cucumbers were able to recover from the transport-induced stress, as indicated by the decreased activity of antioxidant enzymes, the decrease in cellular respiration, the increase in energy reserves and global cellular energy budget, especially after 48 h. As the animals were slowly acclimatized to the new conditions by keeping them inside their transporting bags within the first 24 h, there was not such a radical change of environment as the one they had experienced after collection from the natural environment. Within the 96 h of quarantine, no injuries or mortality occurred and the physiological/biochemical conditions were stable except for fluctuations in LPO.

Summarizing, *H. forskali* does not require more than one week to acclimate to aquaculture conditions prior to transportation. Ensuring that no major stress occurs during transportation, our data show that 48 h is the minimum quarantine period necessary for the organisms to acclimate to the new conditions. Notwithstanding, if the transportation period needs to be adjusted for this species for longer travel, the quarantine should be adjusted as well.

Oxidative stress parameters together with the energetic biomarkers used were shown to be good indicators of the effects caused by environmental stress during transportation and to be helpful in understanding the organisms' acclimation state. These cellular biomarkers have been successfully applied as stress indicators in other holothurian species, for example to address the stress management of *Apostichopus japonicus* (Selenka, 1867) during starvation and experimental and natural aestivation (Yang et al. 2006; Fangyu et al. 2011; Du et al. 2013). However, although methodological differences in the analysis of both tissues do not allow for direct comparisons, overall enzyme activities were constantly higher in the respiratory tree than in the muscle, indicating that this tissue plays a primary role in the antioxidant system of *H. forskali*. The relations between endpoints and the overall picture of the stress response was more consistent in the muscle, which should therefore be the preferential tissue for such stress effect assessments.

Conclusions

Transport simulation studies with the black sea cucumber (*Holothuria forskali*) have demonstrated that this species exhibits the ability to tolerate transport without considerable and lasting damage. Transporting aquatic animals is necessary in order to conduct scientific research or to provide safe environments for species conservation. Provided that *H. forskali* are given at least four days to acclimate prior to transport, with at least two days fasting immediately before, and two days more to recover in quarantine facilities, our results demonstrated that the cellular mechanisms of these holothurians were able to oppose temporary stress induced by ROS, which cause lipid peroxidation and DNA damage in the cell. Moreover, *H. forskali* was able to maintain global energy allocation during acclimation, fasting, transport simulation and quarantine. During the process of capture and transport, it can be concluded that the biochemical biomarkers used to address stress in the organisms during the different transportation stages provided sensitive and highly informative endpoints. Additionally, these tools are important additions to current monitored endpoints (injuries and mortality rates) and are also early warning endpoints highly related to organism fitness/damage. This approach has shown a potential to improve our understanding of individual species when exposed to stressful conditions, and consequently, support the development of optimal parameters to improve animal wellbeing during acclimation, handling, transportation and recovery.

Disclosure statement

No potential conflict of interest was reported by the authors.

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