High resistance to oxidative damage in the Antarctic midge *Belgica antarctica*, and developmentally linked expression of genes encoding superoxide dismutase, catalase and heat shock proteins

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A B S T R A C T

Intense ultraviolet radiation, coupled with frequent bouts of freezing–thawing and anoxia, have the potential to generate high levels of oxidative stress in Antarctic organisms. In this study, we examined mechanisms used by the Antarctic midge, *Belgica antarctica*, to counter oxidative stress. We cloned genes encoding two key antioxidant enzymes, superoxide dismutase (SOD) and catalase (Cat), and showed that SOD mRNA was expressed continuously and at very high levels in larvae, but not in adults, while Cat mRNA was expressed in both larvae and adults but at a somewhat reduced level. SOD mRNA was expressed at even higher levels in larvae that were exposed to direct sunlight. Catalase, a small heat shock protein, Hsp70 and Hsp90 mRNAs were also strongly upregulated in response to sunlight. Total antioxidant capacity of the adults was higher than that of the larvae, but levels in both stages of the midge were much higher than observed in a freeze-tolerant, temperate zone insect, the gall fly *Eurosta solidaginis*. Assays to measure oxidative damage (lipid peroxidation TBARS and carbonyl proteins) demonstrated that the Antarctic midge is highly resistant to oxidative stress.

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1. Introduction

In addition to the conspicuous challenges of low temperature and desiccation, organisms living in Antarctica are bombarded with especially high levels of ultraviolet radiation during the summer, an effect that has been exacerbated recently due to openings in the ozone layer (Solomon, 1990; Liao and Frederick, 2005; Weatherhead and Andersen, 2006). Adding to the oxidative stress caused by ultraviolet radiation is the potential for oxygen radical generation by frequent freeze–thaw and anoxia cycles (Joanisse and Storey, 1998; Hermes-Lima and Zenteno-Savin, 2002).

Oxygen radicals and non-radicals, like hydrogen peroxide, are known collectively as reactive oxygen species (ROS). ROS can cause lipid peroxidation which disrupts membrane fluidity, and the degradation products can initiate apoptosis in the mitochondria (Halliwell and Gutteridge, 1999; Green and Reed, 1998). Oxidative damage to proteins can range from specific amino acid modifications and fragmentation of the peptide chain to total enzyme inactivation by superoxide anions (Stadtman, 1986). ROS can also lead to DNA deletions, mutations, base degradation, single-strand breakage and cross-linkage of proteins (Imlay and Linn, 1988; Imlay, 2003). Superoxide radicals generated by oxidative stress act as oxidants or reductants that lead to the production of hydroxyl radicals (Fridovich, 1995). The hydroxyl radicals, though short-lived, are highly reactive and readily damage DNA by denaturing nucleic acids (Lesser, 2006). Two of the enzymes most crucial for inactivating these potentially damaging oxygen agents are superoxide dismutase (SOD) and catalase (Orr and Sohal, 1994). SOD converts superoxide into oxygen and hydrogen peroxide, while catalase then converts hydrogen peroxide into oxygen and water.

In this study, we examine the response of an Antarctic insect, the midge *Belgica antarctica* Jacobs (Diptera, Chironomidae), to oxidative stress. This insect, which is endemic to maritime Antarctic, has a patchy distribution on the Antarctic Peninsula and its nearby islands (Gressitt, 1967; Convey and Block, 1996). The midge is freeze-tolerant and spends much of its 2-yr life cycle as a larva encased in a matrix of ice and substrate, but during the brief (approximately 2 months) austral summer the larvae feed on algae and bacteria located in the substrate near penguin rookeries (Sugg et al., 1983). The wingless adults emerge late December to early January. They can be found in aggregations on the surfaces of rocks located near the larvae, but they never stray far from the

moist habitats that are essential for their survival (Benoit et al., 2007a).

A previous study (Grubor-Lajsic et al., 1996) reported high antioxidant enzyme activity in the midge larvae. We extend that work by cloning the genes that encode SOD and catalase in *B. antarctica* and by examining the expression patterns of those genes in the larvae and adults. Because Antarctic organisms are particularly vulnerable to ultraviolet radiation (Frederick and Lubin 1994; Weiler and Penhale, 1994; Weatherhead and Andersen, 2006), we monitored the expression of the genes encoding SOD and catalase, as well as the genes encoding three heat shock proteins in response to ultraviolet radiation. We also assessed total soluble antioxidant capacity, trehalose concentrations, and measures of oxidative damage, lipid peroxidation (TBARS) and protein damage (carbonyls). For a point of reference, we compared the antioxidant capacity of *B. antarctica* to that of a temperate zone species, *Eurosta solidaginis*, a gall fly that is also freeze-tolerant (Baust and Lee, 1982).

2. Materials and methods

2.1. Insects

B. antarctica Jacobs larvae and adults were collected in January and February, 2006 and 2007, on Norsel Point, and Torgersen and Cormorant Islands near the United States Antarctic Program's research station, Palmer Station (Anvers Island, Antarctica). Larvae were collected with the substrate, while the adults were collected using aspirators. Prior to use in our experiments, the larvae were sorted from the substrate and placed, as groups of 10, in 1.5 ml microcentrifuge tubes containing 0.5 ml of water and held at 4 °C until used (less than 24 h). Adults were kept in Petri dishes in groups of 50 at 4 °C until used.

2.2. Clones

The clones were obtained by two methods. The clone of SOD was obtained by suppressive subtractive hybridization (SSH), in which an RNA pool from hydrated larvae held at 4 °C was subtracted from an RNA pool of larvae held at 4 °C that had been desiccated for 12 h at 98.2% RH to remove roughly 10% of their body water. The cDNAs were synthesized using the BD SMART^{TM} PCR cDNA Synthesis kit and subtracted using a PCR-Select^{TM} cDNA Subtraction Kit (Clontech, Mountain View, CA). cDNAs encoding SOD, a small heat shock protein and a control gene, 28S, were among the cDNAs obtained by this method, although none of the cDNAs proved to be unique to either of the original RNA pools. Full-length sequence for SOD was obtained by rapid amplification of cDNA ends using a 3′-Race kit (Invitrogen, Carlsbad, CA).

Catalase was obtained by designing primers using sequences from *Anopheles gambiae*, *Aedes aegypti* and *Drosophila melanogaster* (forward primer 5′-CCGTCAAGTTCTACACTGAGGA-3′ and reverse primer 5′-CAGAACATGTCCGGATCCTT-3′). The full coding sequence of catalase was obtained using the 3′-Race kit (Invitrogen) and the Genome Walker Universal kit (Clontech).

The heat shock protein clones (*hsp70* and *hsp90*) were previously isolated from *B. antarctica* by our group with designed primers based on homology (Rinehart et al., 2006).

2.3. Northern blot hybridization

RNA used for northern blot hybridizations was extracted from the treatment groups of 75 larvae or 50 adults described below, using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Northern blot hybridization was performed by running 4 μg of RNA in a 1.4% agarose, 0.41 M formaldehyde gel. Based on signal strength, 1 μg of RNA was used for the 28S control to verify equal loading. The RNA was transferred to a positively charged nylon membrane (Hybond-N+, Amersham Biosciences, Piscataway, NJ) using the Turboblotter rapid downward transfer system (Schleicher & Schuell Inc.). DNA clones for SOD, catalase, a small heat shock protein (smHsp), Hsp70, Hsp90 and 28S were labeled using the DIG-High Prime labeling kit and hybridization was perform using the DNA Labeling and Detection Starter Kit II (both from Roche Diagnostics, Switzerland). The membranes were exposed using Blue Lite Autorad Film (ISC BioExpress) for 5–30 min depending on DIG probe strength. The membranes were stripped using the DIG kit guidelines and re-hybridized with the control gene (28S). All northern blots were performed in triplicate.

2.4. Stress treatments

To evaluate gene expression and to assess total antioxidant capacity and damage due to oxidative stress, larvae were subjected to three stresses that commonly occur in their habitat: heat shock, freezing and anoxia. To administer heat shock and freezing stresses groups of 10 larvae were placed in 1.5 ml microcentrifuge tubes with 100 μl of distilled water and exposed to 1 h heat shock at 30 °C or freezing at −5 °C for 2 d, with and without a 2 h recovery. For exposure to anoxia, groups of 10 larvae were placed in 15 ml glass vials with distilled water, and a stream of nitrogen was bubbled into the container for 2 min; larvae were then maintained in the sealed vials at 4 °C for 2 d. Even though the adults are not likely to experience anoxic conditions in their short life, we wanted to record any possible SOD and catalase upregulation following anoxia therefore we exposed adults to anoxic conditions in the same manner but without water. Controls consisted of larvae and adults that were maintained at their normal summer habitat temperature, 4 °C. RNA was extracted, as described above, immediately after the stresses were administered.

Larvae were also stressed by exposure to unfiltered ultraviolet radiation from direct Antarctic sunlight. For this experiment, larvae were held in water (0.5 cm in depth) in 10 cm i.d. glass Petri dishes that were maintained at 4–5 °C and monitored with a thermometer hourly. Groups of larvae ($N = 75$) were exposed to direct sunlight for 3, 6, and 9 h on 25 January 2007. During the entire period, the test site remained in the sunlight, with the exception of two 3–5 min periods of cloud cover. Immediately after exposure, RNA was extracted from the samples. The experiment was independently replicated three times.

2.5. Total antioxidant capacity

Larvae were collected and held in distilled water overnight (12–16 h) to allow clearance of the gut prior to assessment. For comparison, the antioxidant capacity of the freeze-tolerant, over-wintering larvae of a temperate zone species, the goldenrod gall fly was also assessed. *E. solidaginis* (Fitch) (Diptera, Tephritidae) larvae were collected near Miami University in Oxford, OH, in late November 2006 and maintained within the intact galls at 4 °C for 4 months prior to measurement of antioxidant capacity.

Groups of 25 larvae or 40 adults of *B. antarctica* were homogenized in ice-cold Coast's solution (Coast 1988), and sonicated for 30 s to disrupt cells. A 25 μl aliquot of the crude homogenate was stored at −80 °C and reserved for the TBARS assay. The remainder of the homogenate was then centrifuged at 4 °C for 10 min at 2000 × g to separate cellular fragments.

The supernatant was collected and stored at −80 °C until assayed. Individual larvae of *E. solidaginis* were similarly prepared and stored.

The trolox equivalent antioxidant capacity (TEAC) was measured using an ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation decolorization assay (Re et al., 1999). The ABTS radical cation (ABTS$^+$) was produced by reacting an ABTS stock solution (7.0 mM) with potassium persulfate (2.45 mM final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h. The radical cation was stable in the dark at room temperature for 48 h. For use in the assay, the ABTS$^+$ was diluted with phosphate-buffered saline (PBS) to an absorbance of 0.7 at 734 nm. The antioxidant capacity was assessed by monitoring the decrease in absorbance at 734 nm exactly 10 min after the addition of 1 ml of diluted ABTS$^+$ to 10 μl of biological sample. Samples were compared to Trolox standards (0–15 μmol/ml; final concentration) and expressed as Trolox equivalents.

2.6. Lipid peroxidation

The concentration of thiobarbituric acid reactive substances (TBARS) in larvae of *B. antarctica* was determined using a commercially available assay kit (OXItek; ZeptoMetrix Corp., Buffalo, NY). The assay was conducted on crude tissue homogenates by combining 25 μl of homogenate with 25 μl of the supplied SDS solution and 625 μl of TBA reagent in a 2 ml microcentrifuge tube. The mixture was then incubated at 95 °C for 1 h. After cooling to room temperature, 675 μl of butanol was added to each sample and the tubes were then centrifuged 5 min at 1000g. Two 220 μl aliquots of the butanol layer were transferred to a 96-well plate and the absorbance ($\lambda = 532$ nm) read on a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA). The TBARS concentration (nmol/ml) was determined by averaging duplicate samples calculated from a standard curve prepared from various concentrations (0–50 nmol/ml) of malondialdehyde. TBARS concentration was expressed per mg of soluble protein in the crude homogenate. Protein concentrations in the prepared tissue homogenates were determined by the Bradford method (Bio-Rad, Hercules, and CA) using bovine serum albumin (0–1 mg/ml) as a standard.

2.7. Carbonyl proteins

Carbonyl proteins were measured from whole body homogenates of larvae by reacting samples with 2,4-dinitrophenylhydrazine (DNPH) to generate dinitrophenyl-hydrazones (Reznick and Packer, 1994). For the assay, a 200 μl aliquot of sample supernatant was added to each of two 2 ml microcentrifuge tubes; 800 μl of a DNPH solution (10 mmol DNPH in 2 M HCl) was added to one tube, and 800 μl of 2 M HCl to the other. The sample mixtures were then incubated at room temperature in darkness for 1 h. Proteins were precipitated with the addition of 1 ml 20% TCA and centrifuged at 10,000g for 10 min. The resulting pellet was washed 3x with 1 ml volumes of an ethanol:ethyl acetate (1:1) solution and similarly centrifuged between washes to remove excess DNPH. The final pellet was solubilized in 500 μl of 6 M guanidine hydrochloride. Carbonyl content was determined from the difference between the average absorbance ($\lambda = 370$ nm) of duplicate samples of DNPH-reacted and unreacted HCl samples. Carbonyl proteins were expressed per milligram of protein in the sample as described above.

2.8. Trehalose concentration

Trehalose content was determined as described by Chen et al. (2002). Five groups of 25 larvae were weighed and immediately frozen at −80 °C. Larvae were homogenized in 0.25 ml of 0.25 M Na$_2$CO$_3$ and incubated at 95 °C for 2 h to denature proteins. Following the denaturing of proteins, 0.15 ml of 1 M acetic acid and 0.6 ml of 0.2 M sodium acetate were added, and the mixture was centrifuged at room temperature for 10 min at 12,000g. Aliquots (200 μl) of the supernatant were placed into two tubes; one was used as background, and the other was digested by trehalase (0.05 units/ml) overnight at 37 °C. Glucose concentration was then measured using the glucose (GO) assay kit (Sigma, St. Louis, MO, USA).

2.9. Statistical analyses

Means were compared with analysis of variance (ANOVA) and Bonferroni–Dunn tests (Statview from SAS Institute, Cary, NC, USA). Data are presented as mean ± S.E.M. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Superoxide dismutase

The full-length clone of *SOD* that was produced was identified, by nucleotide and amino acid sequence comparisons, as SOD2 (Fig. 1). The identification was confirmed by the presence of two highly conserved domains for iron/manganese superoxide dismutases. The N-terminus contains the long alpha antiparallel hairpin and the C-terminal ends in the characteristic mixed alpha/beta fold. An amino acid comparison of the 217 amino acid clone shows that *SOD2* (*SOD-Mn*) from the Antarctic midge (GenBank accession no. DQ507228) shares a 67% and 68% amino acid identity to *D. melanogaster* and *A. aegypti*, respectively.

Strong upregulation of transcripts encoding SOD2 was found in all samples from the midge larvae, i.e., the untreated controls as well as those exposed to heat shock, freezing or anoxia (Fig. 2). By contrast, the gene was not expressed in control or anoxia-treated adults. Thus, in the larvae, *SOD2* was strongly and continuously expressed, and expression levels were not further elevated by any of the environmental stresses that we measured. By contrast northern blots did not reveal the transcript in the adults, regardless of the stress applied. The transcript of the control gene, *28S* (GenBank accession no. DQ459549), was present in all treatments and replications, including the adults.

SOD2 was also continuously expressed in larvae exposed to direct sunlight (Fig. 3). A modest upregulation occurred after a 6 and 9 h exposure to direct sunlight.

3.2. Catalase

The *catalase* gene clone (GenBank accession no. EU344974) shares an amino acid identity of 93% and 89% with *D. melanogaster* and *A. aegypti*, respectively (Fig. 4). The 506 amino acid coding sequence obtained contains the heme-binding pocket characteristic of heme-catalases (Fig. 4).

Although the transcript encoding *catalase* was not as strongly expressed as the *SOD* transcript, the *catalase* transcript was present in both larvae and adults. Expression at 4 °C was higher in larvae than in adults (Fig. 2). Freezing and anoxia both lowered the level of expression in larvae, but expression was elevated by heat shock. In the adults, anoxia elevated expression.

Catalase was strongly upregulated in larvae in response to natural sunlight (Fig. 3). A strong upregulation was detected when the larvae were exposed to direct sunlight for 3 h but declined somewhat after 6 and 9 h.

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Belgica       1  MMSLCRKVVPASRGLASLTSVRTKVTLPELGYDYAALEPIISREIMEIHHSKHHQAYVTN
Aedes         1  MIALRSAVLVSTRNVAAVLGCRNKHTLPDLPYDFGALEPVICREIMEVHHQKHHNAYVTN
Drosophila    1  ------MFVARKISQTASLAVRGKHTLPKLPYDYAALEPIICREIMELHHQKHHQTYVNN

Belgica      61  YNAAKDQLDEAVAKGDTNKIISLQGALRFNGGGHLNHSIFWKNLSSTNSSPSDALQKAII
Aedes        61  LNAAEEQLAEAVAKKDTSKIIQLGGALKFNGGGHINHSIFWKNLSPERSDPSAELKKLLE
Drosophila   55  LNAAEEQLEEAKSKSDTTKLIQLAPALRFNGGGHINHTIFWQNLSPNKTQPSDDLKKAIE

Belgica     121  TNFKSLDNLKTEMKTAAVGVQGSGWAWLGLNKKTGHLQVVQCANQDPLQATTGLTPLFGI
Aedes       121  RDFHGLENFKKEMKAAAVAVQGSGWAWLGYNQKTKALQVAACPNQDPLQATTGLVPLLGI
Drosophila  115  SQWKSLEEFKKELTTLTVAVQGSGWGWLGFNKKSGKLQLAALPNQDPLEASTGLIPLFGI

Belgica     181  DVWEHAYYLQYKNLRPSYVDAIWDIVNWKDVSERFSA------
Aedes       181  DVWEHAYYLQYKNLRPNYVDAIWDVVNWKDVSERLAKAQ----
Drosophila  175  DVWEHAYYLQYKNVRPSYVEAIWDIANWDDISCRFQEAKKLGC
```

Fig. 1. Amino acid homology comparison of superoxide dismutase 2 (Mn) from *B. antarctica*, *A. aegypti* and *D. melanogaster*. The percentage identity shared is 68% and 67%, respectively. The solid line (—) shows the conserved N-terminal long alpha antiparallel hairpin. The dashed line (---) shows the conserved C-terminal mixed alpha/beta fold. Consensus matches are shown in black, partial matches in gray and no matches in white.
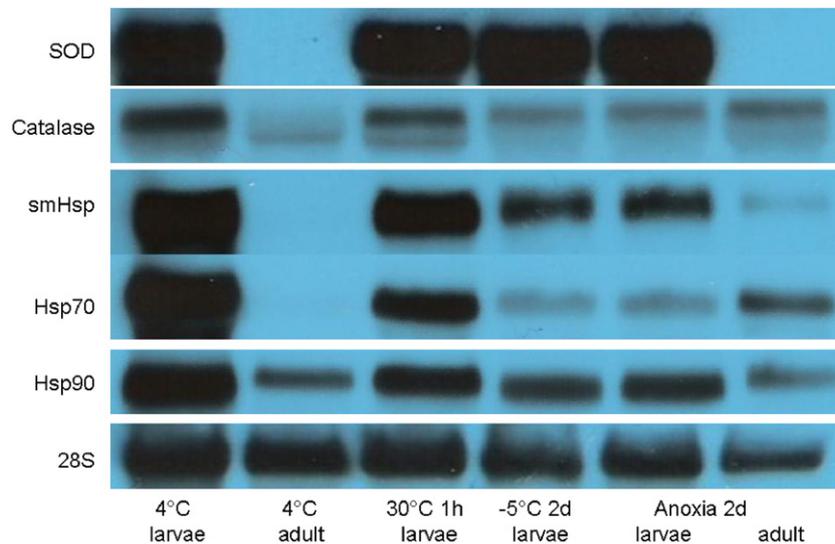

Fig. 2. Northern blot hybridizations showing mRNA expression of *SOD*, *catalase*, a *smHsp*, *Hsp70* and *Hsp90* in response to a variety of environmental stresses. *28S* was used as a control. The treatments were: larvae at 4 °C (summer habitat temperature), adults at 4 °C, larvae heat shocked at 30 °C for 1 h, larvae frozen at −5 °C for 2 d, larvae under anoxic conditions for 2 d, and adults under anoxic conditions for 2 d.

3.3. Small Hsp

A small heat shock protein (GenBank accession no. DQ459548) was equally expressed in both control larvae and those that were heat shocked (Fig. 2). Expression in response to freezing and anoxia was not as strong as observed following heat shock. The adults did not express this gene under control conditions, but a mild upregulation was evident following anoxia (Fig. 2). Larval expression of this gene was reduced compared to that of the controls, after 3 h of direct sunlight exposure (Fig. 3). However, strong upregulation occurred by 6 h and persisted to 9 h.

3.4. Hsp70

Heat shock protein 70 (GenBank accession no. DQ459546) was constitutively expressed in larvae, but a decline in expression was evident in response to freezing and anoxia (Fig. 2). *Hsp70* was not expressed in control adults but was upregulated in adults exposed to anoxia. Larvae exposed to direct sunlight for 3 h (Fig. 3) expressed the gene at a level similar to the controls, but a 6 h exposure to direct sunlight elicited strong upregulation that persisted for at least 9 h.

3.5. Hsp90

Heat shock protein 90 (GenBank accession no. DQ459547) was expressed at nearly constant levels in all larval treatments (Fig. 2). *HSP90* was also present in the adult controls and following anoxia. A mild upregulation was seen in larvae exposed to direct sunlight for 6 or 9 h (Fig. 3).

3.6. Total antioxidant capacity

B. antarctica appears to be well equipped to defend against ROS, as they possessed relatively high antioxidant capacity (Fig. 5). Relative to another freeze-tolerant insect, the larvae of
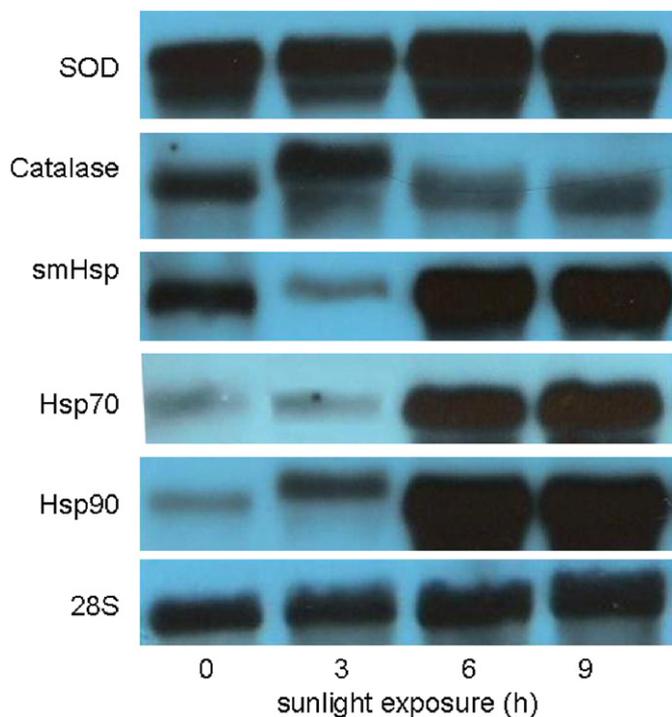
Fig. 3. Northern blot hybridizations showing the mRNA expression patterns of *SOD*, *catalase*, a *smHsp*, *Hsp70* and *Hsp90* in response to direct Antarctic sunlight. *28S* was used as a control. The larvae were exposed to sunlight for 0, 3, 6 or 9 h. Water temperature within the Petri dishes was maintained at 4 °C (summer habitat temperature).

the goldenrod gall fly *E. solidaginis*, the antioxidant capacity of *B. antarctica* larvae was nearly five times higher. Surprisingly, the short-lived midge adults possessed higher ($>1.5\times$) antioxidant defense than the larvae (Fig. 5).

Relative to control larvae (maintained at 4 °C), no significant differences in total antioxidant capacity were observed following larval freezing, anoxia or heat shock (Fig. 6). All measurements were taken after a 2-h recovery at 4 °C; however, these values did not differ significantly from measurements taken 30 min following removal from the stresses (data not shown).

3.7. Trehalose content

Compared to the control ($23.21 \pm 0.41\,\mu g/mg$ DM; $n = 5$) no significant increase in trehalose concentration of the larvae was observed following exposure to anoxia or heat shock (Fig. 7). Trehalose concentration was significantly ($P < 0.05$) higher ($\sim 1.3\times$) following freezing; however, this increase did not correspond to an elevation of the total antioxidant capacity of larvae following freezing.

3.8. Oxidative damage

Markers of oxidative damage were assessed following exposure to the aforementioned environmental stressors. Relative to controls, no significant differences in either TBARS or carbonyl proteins were observed following freezing, anoxia, or heat shock (Fig. 8A and B). These results suggest that the high antioxidant capacity of the larvae is sufficient to prevent oxidative damage induced by ROS, and no further elevation of these markers for oxidative damage occurs when the larvae are subjected to stress.

4. Discussion

The continuous and strong expression of the mRNA encoding superoxide dismutase, in the absence of any measurable stress, indicates that the midge larvae have evolved a mechanism to prevent oxidative stress by the massive production of antioxidant agents. Rather than responding to stress by initiating transcription of the *SOD* gene, the midge larvae are already maximally expressing this gene, and none of the environmental stressors we examined caused the gene to be further upregulated. This is much akin to the continuous upregulation of heat shock proteins observed in these larvae (Rinehart et al., 2006). The continuous expression of *SOD* suggests that the larvae have adapted to produce the enzyme in anticipation of the wide range of environmental stressors that they confront in the Antarctic environment, and the lack of increased oxidative damage we report is consistent with that. None of the three stresses we tested (heat shock, freezing, or anoxia) generated an increase in ROS damage to lipids or proteins beyond what was observed in the control. This result is consistent with a previous report showing significantly higher levels of SOD activity in *B. antarctica* compared to temperate zone insects (Grubor-Lajsic et al., 1996). As pointed out by Hochachka and Somero (2002), an organism living in a low temperature environment may compensate for low enzymatic activity by producing more enzyme, but that may not be the case here. The high levels of SOD activity recorded by Grubor-Lajsic et al. (1996) were observed at 4–5 °C. *B. antarctica* thus appears to both highly express the SOD gene as well as show an elevated level of enzyme activity.

In addition to the freeze–thaw cycles that invertebrates experience in Antarctica, up to 140/yr (Block, 1997), ultraviolet radiation (UVR) may be a driving force contributing to the persistent SOD expression pattern we observed. The active, summer larvae of *B. antarctica* are normally found within the substrate, in close association with algae, grass, moss, soil and animal detritus (Peckham, 1971). This habitat is likely to provide considerable protection against UVR, yet periods of melting snow, driving summer rainstorms and ocean spray can readily expose the larvae, and it is in these situations that the larvae are particularly vulnerable to UVR. Freshly fallen snow also contains a high content of hydrogen peroxide (Abele et al., 1999), thus the larvae may be challenged during both summer and winter. Ultraviolet radiation is classified into three major forms based on wavelength (UVA, UVB, UVC), but UVA and UVB present a greater challenge than UVC, which is absorbed in the upper atmosphere (Karentz, 1994; Cockell, 2001). UVA, radiation in the 320–400 nm range, is not attenuated by the ozone layer and leads to the production of ROS. This is due to photooxidation reactions by endogenous photosensitizers since nucleic acid bases absorb UV radiation weakly above 320 nm (Ravanat et al., 2001; Cadet et al., 2005). UVB, radiation in the 280–320 nm range, damages DNA by the formation of cyclobutane pyrimidine dimers at adjacent thymine residues and generates ROS with high efficiency in an aqueous medium (Tevini, 1993).

In the austral summer, the surface temperature of the midge larval habitat can reach 25–27 °C, an extremely high temperature for this insect (Rinehart et al., 2006). The larvae may avoid these high temperatures by moving further down into the substrate to find cooler locations (Peckham, 1971). However, melting snow and summer rainstorms commonly displace larvae from their micro-habitats, exposing them directly to ultraviolet radiation.

Even though the hole in the ozone layer allows UVB radiation to reach the surface of the Antarctic Peninsula, peak UVB activity in this region occurs in November (Karentz and Bosch, 2001), and during this time the larvae are still well protected from UV radiation by a layer of snow. The hole in the ozone layer appears to

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Belgica        1  -------DGPPKFTPLMRCRKSLINFRVT-TLGHGAPVAVKTASLTVGQRGPILLQDVNF
Aedes          1  RIGINMS-RNPAENQLNLFKESQKDKSVA-TTGNGAPLGTKTATATVGERGPVVLQDVHF
Anopheles      1  ----------------RIGINLQDKVTA-TMSHGAPVGTKTASETAGPRGPVLLQDVHL
Drosophila     1  RIGINMAGRDAASNQLIDYKNSQTVSPGAITTGNGAPIGIKDASQTVGPRGPILLQDVNF

Belgica       53  VDEMAHFDRERIPERVVHAKGAGAFGLFEVTHDISKYTSAKIFEKIFDPSQRETYELCFH
Aedes         59  LDEMSHFDRERIPERVVHAKGAGAFGYFEVTHDITQYCAAKVFEKV----GKKTPLAVRF
Anopheles     43  IDELAHFDRERIPERVVHAKGAGAFGYFEVTHDITQYCAAKLFEKV----GKKTPLAVRF
Drosophila    61  LDEMSHFDRERIPERVVHAKGAGAFGYFEVTHDITQYCAAKIFDKV----KKRTPLAVRF

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Belgica      113  FILGGESGSADAARDPRGFAMKFYTDEGIWDLVGNNTPIFFIRDPILFPSFIHTQKRNPQ
Aedes        115  STVGGESGSADTARDPRGFAVKFYTDDGVWDLVGNNTPIFFIRDPILFPSFIHTQKRNPA
Anopheles     99  STVGGESGSADTVRDPRGFAVKFYTDDGVWDMVGNNTPIFFIRDPVLFPSFIHTQKRNPA
Drosophila   117  STVGGESGSADTARDPRGFAVKFYTEDGVWDLVGNNTPVFFIRDPILFPSFIHTQKRNPQ

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Belgica      173  THLKDPDMFWDFISLRPETTHQVMFLFGDRGTPDGNRFMNGYGSHTFKLTTEKGEAYYCK
Aedes        175  THLKDADMFWDFISLRPESTHQVMFLFADRGTPDGYRFMNGYGSHTFKLINAQGKPVYCK
Anopheles    159  THLKDPDMFWDFISLRPETTHQIMFLFSDRGTPDGYRFMNGYGSHTYKLVNADGKPVYCK
Drosophila   177  THLKDPDMFWDFLTLRPESAHQVCILFSDRGTPDGYCHMNGYGSHTFKLINAKGEPIYAK

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Belgica      233  FHFKSAQGVKNLSTETADKLAGSDPDYSTRDLFNAIQKGDFPSWNMHIQIMTFEQAETYK
Aedes        235  FHFKSNQGIKNLEARRADELAGSDPDYSIRDLYNAIAKGECPSWNLKIQVMTFEQAEQHS
Anopheles    219  FHFKTDQGIKNLDPARANELTATDPDYSIRDLYNAIAKKDFPSWLKVQVMTFEQAEKVP
Drosophila   237  FHFKTDQGIKNLDVKTADQLASTDPDYSIRDLYNRIKTCKFPSWTMYIQVMTYEQAKKFK

                  -----------------------------------------------------------
Belgica      293  YNPFDVTKVWSQKDFPLIPVGKFTLDRNPNNYFAEVEQIAFSPSHLVPGIEPSPDKMLQG
Aedes        295  FNPFDVTKVWPQNEFPLIPVGRMVLDRNPSNYFAEVEQIAFAPSHLVPGIEASPDKMLQG
Anopheles    279  YNPFDLTKVWPQNEFPLIPVGRMVLDRNPSNYFAEVEQAAFAPSHLVPGIEPSPDKMLQA
Drosophila   297  YNPFDVTKVWSQKEYPLIPVGKMVLDRNPKNYFAEVEQIAFSPAHLVPGVEPSPDKMLHG

                  -----------------------------------------------------------
Belgica      353  RLFSYADTQRHRLGANYLQLPVNCPYRVSVKNYQRDGPMTFNDNQAGAPNYYPNSFEGPE
Aedes        355  RLFSYADTHRHRLGANYLQLPVNCPYRVSMKNYQRDGPMNVTDNQGGAPNYYPNSFGGPE
Anopheles    339  RLFAYADTHRHRVGANYLMLPVNCPYRVATRNFQRDGPMNCTDNQGGAPNYFPNSFSGPQ
Drosophila   357  RLFSYSDTHRHRLGPNYLQIPVNCPYKVKIENFQRDGAMNVTDNQDGAPNYFPNSFNGPQ

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Belgica      413  PTQRARTLQQP-YKLSGEVHRFDSGD-EDNFSQATIFWNTVLDGAARKRLVDNMAGHIVN
Aedes        415  PCGFAHKLQNSKFNVSGDVNRFESGETEDNFAQPGIFYRRVLDEAARERMITNMVNHMSA
Anopheles    399  TCPRAHKLQNTPLKLSGDVNRYETGD-EDNFSQATVFYRRVLDDAGRQRLINNIVGHLKD
Drosophila   417  ECPRARALSSC-PVTGDVYRYSSGDTEDNFGQVTDFWVHVLDKCAKKRLVQNIAGHLSN

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Belgica      471  AQGFIQERAIGNFAKVSADFGKMLVGAINLKKTAKM
Aedes        475  ASPFIQERAVQNFSQVDADFGRRLTEGLKLRRSAKM
Anopheles    458  ASPFLQERAVKNFAMVDADFGRHLSEGLKLRRTANL
Drosophila   476  ASQFLQERAVKNFTQVHADFGRMLTEELNLAKSSKF
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Fig. 4. Amino acid homology comparison of catalase from *B. antarctica*, *A. aegypti* and *D. melanogaster*. The percentage identity shared for the cloned gene fragment is 71% and 69%, respectively. The dashed line (---) area marks the heme-binding pocket characteristically found in heme-catalases. Consensus matches are shown in black, partial matches in gray and no matches in white.

have formed only within the past 25 yr (Solomon, 1990), thus it is not likely that this midge, with its 2-yr life cycle, has already adapted to that type of ultraviolet radiation. UVA radiation, however, is at its highest in the months of December and January (Liao and Frederick, 2005), a period when the larvae are actively feeding and are most likely to be vulnerable to exposure. It is during this late December/early January period that adults are present and can be seen crawling over the rocks surfaces, where they are clearly vulnerable to UVA radiation.

We thus suspect that the constant high levels of *SOD* in the larvae serve as a preventive measure against UVA exposure. And, the lack of further upregulation *SOD* in response to Antarctic sunlight supports this conjecture. We also suspect that the numerous freeze–thaw cycles in Antarctica render invertebrates from this continent especially susceptible to the production of free radicals. The larvae become anoxic during freezing and are subjected to oxygen reperfusion and free radical generation upon thawing (Joanisse and Storey, 1998). We thus anticipate that SOD provides protection against the oxygen reperfusion following such bouts of anoxia.

The lack of *SOD* expression in adults suggests that the short-lived adults (7–10 d) do not degrade superoxide anions. This is surprising given that the adults spend most of their time walking on the surfaces of rocks or plant material where they are vulnerable to sunlight and the heated substrate. Even though adults are less likely than larvae to experience bouts of freezing or anoxia, the lack of *SOD* expression after an anoxic period is surprising and suggests the possibility that adults may lack the ability to produce this antioxidant enzyme. Due to a shortage of adults available during our field seasons in Antarctica, we were unable to directly test this hypothesis.

We suspect the difference in *SOD* expression between larvae and adults of *B. antarctica* reflects the contrasting durations of these two life stages. The brief period of adult life would not likely
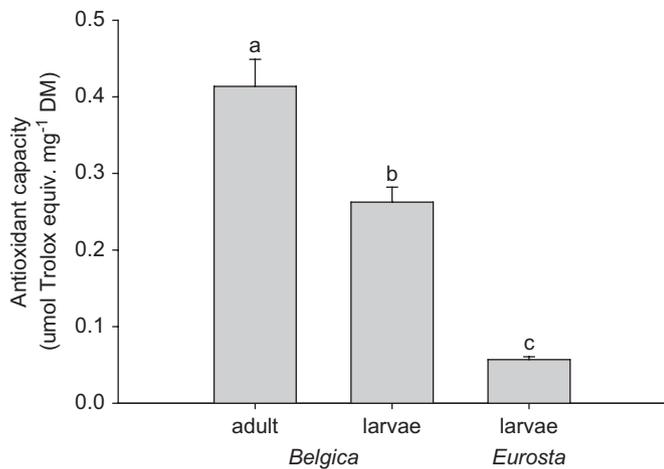
Fig. 5. Total soluble antioxidant capacity of *B. antarctica* adults and larvae, and freeze-tolerant larvae of the goldenrod gall fly, *E. solidaginis*. Values are mean± ⊖ S.E.M. (*n* = 5). Different letters represent significant differences (*P*<0.05) between groups.
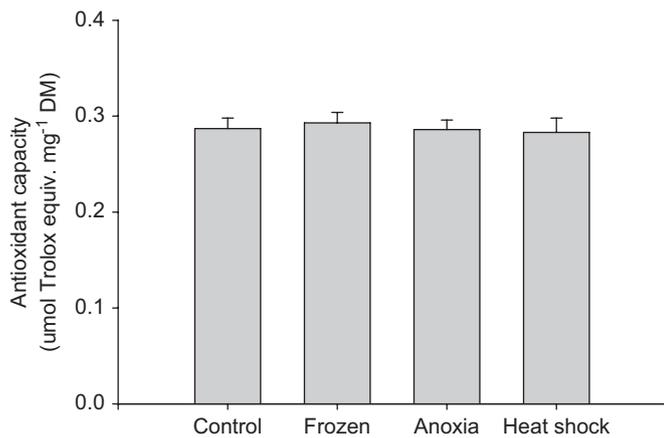

Fig. 7. Trehalose concentration in *B. antarctica* larvae following exposure to a variety of environmental stressors. Larvae were frozen (−5 °C/24 h), held under anoxia (48 h at 4 °C), or heat shocked (30 °C/1 h) and allowed to recover 2 h at 4 °C prior to measurement of trehalose concentration. Control larvae were maintained in water at 4 °C. Values are mean±S.E.M. (*n* = 5). Different letters represent significant differences (*P*<0.05) between treatments.

Fig. 6. Total soluble antioxidant capacity of larvae of *B. antarctica* following exposure to a variety of environmental stressors. Larvae were frozen (−5 °C/24 h), held under anoxia (48 h at 4 °C), or heat shocked (30 °C/1 h) and allowed to recover 2 h at 4 °C prior to assessment of antioxidant capacity. Control larvae were maintained in water at 4 °C. Values are mean±S.E.M. (*n* = 5).

Fig. 8. Indices of oxidative damage, TBARS (A) and carbonyl proteins (B), in *B. antarctica* larvae following exposure to several environmental stressors that may be expected to result in production of reactive oxygen species. Larvae were frozen (−5 °C/24 h), held under anoxia (48 h at 4 °C), or heat shocked (30 °C/1 h) and allowed to recover 2 h at 4 °C prior to measurement of oxidative damage. Control larvae were maintained in water at 4 °C. Values are mean±S.E.M. (*n* = 5).

necessitate an elaborate protection mechanism against oxidative stress, while the larvae are much more susceptible to such injury during the onslaught of environmental stresses they encounter during their 2-yr developmental period. Such a dichotomy is also evident in the heat shock response (Rinehart et al., 2006): the larvae continuously mount a heat shock response, i.e., express their heat shock proteins, while the adults do not. But, unlike the heat shock response, the adults did not upregulate *SOD* following exposure to anoxia. Likewise, larvae are extremely drought-tolerant (Hayward et al., 2007; Benoit et al., 2007b), while adults are not (Benoit et al., 2007a).

Unlike *SOD* mRNA, *catalase* mRNA was present in both larvae and adults. Catalase reduces the amount of hydrogen peroxide, and its expression is known to extend the life span of *D. melanogaster* (Orr and Sohal, 1994), c.f. the house fly *Musca domestica* (Bayne and Sohal, 2002). A synergistic effect between SOD and catalase further extends the life span of *D. melanogaster* (Orr and Sohal, 1994). Catalase mimetics also protect against oxidative stress and increase life span in *Caenorhabditis elegans* (Melov et al., 2000; Sampayo et al., 2003). Catalase is an unusual
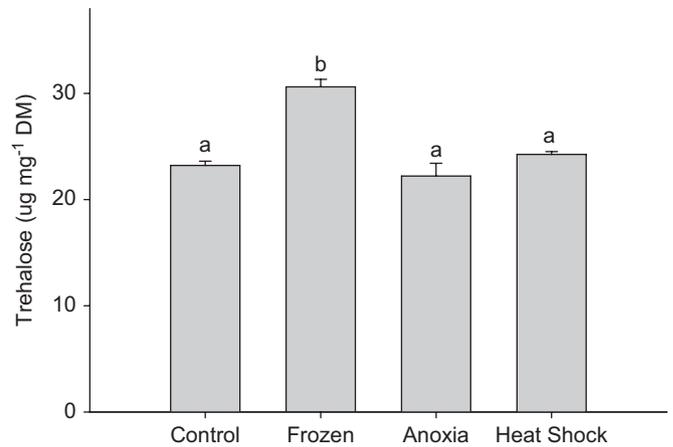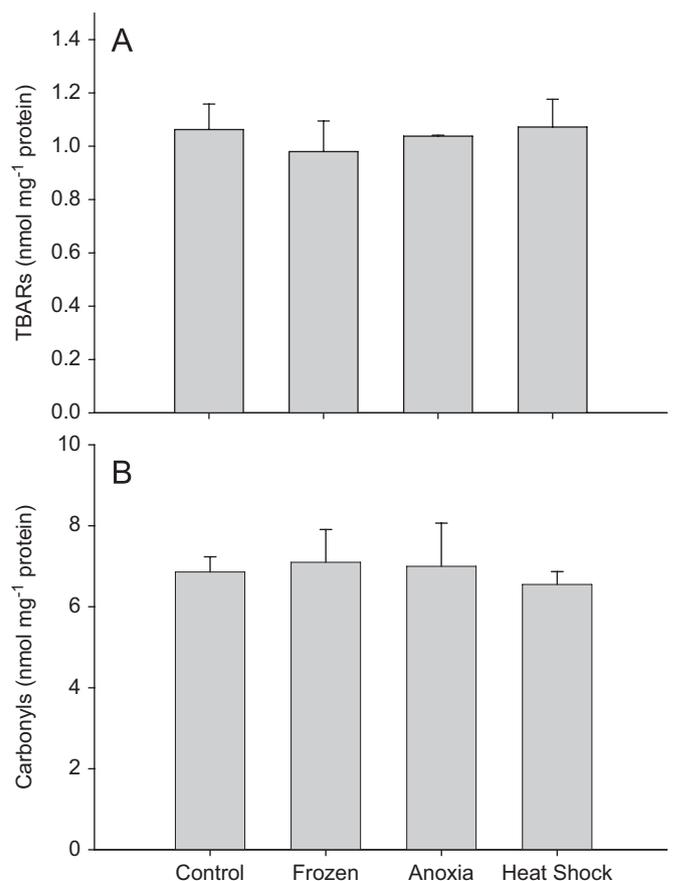
antioxidant enzyme in that it is sensitive to light and has a rapid turnover (Lesser, 2006). The expression levels we observed for *catalase* were less strong than those observed for *SOD*, suggesting

the possibility that catalase may be present at lower levels than *SOD*. The low expression level may also reflect the possibility that alternate enzymes, such as peroxidases, that also catalyze the breakdown of hydrogen peroxide, may be upregulated at this time. However, strong upregulation of *catalase* was seen in response to sunlight, suggesting a possible increase of ROS production due to UVA radiation and/or rapid turnover of the protein in the presence of light.

The *smHsp* and *Hsp70* were continually expressed in larvae, but not adults, as previously reported (Rinehart et al., 2006). It is interesting to note that *Hsp70* was upregulated by anoxia to a greater extent in adults than in larvae, while the *smHsp* was barely expressed. Certainly, larvae experience anoxia more frequently than adults, and quite possibly the adults never experience it. The larvae appear capable of handling anoxia without further upregulation of the *Hsps* while adults possibly rely on the *Hsps*. Expression of *Hsp90* persisted at a constant level in both larvae and adults, and no responsiveness to stress was apparent, but we cannot discount a role for *Hsp90* in other stresses, e.g., desiccation.

In response to Antarctic sunlight, all three *Hsps* were strongly upregulated in larvae within 6 h, and that upregulation persisted for at least 9 h. We carefully controlled temperature during this time, thus we do not think this upregulation can be attributed to heat shock. In addition, the *Hsp* upregulation in response to sunlight was stronger than expression of these three genes following 1 h of heat shock. It is evident that these expression patterns exceed that of the control, suggesting that something else is involved, possibly UVB. UVB is known to elicit *Hsp70* upregulation in sea urchin embryos (Bonaventura et al., 2005; Matranga et al., 2006). The involvement of Hsps in response to ultraviolet radiation treatment suggests that these chaperones are critical for adequate cell function during UV exposure.

The total antioxidant capacity of *B. antarctica* larvae was quite high when compared to another freeze-tolerant insect originating from the temperate zone, *E. solidaginis*. With a capacity of up to five times that of the gall fly *E. solidaginis*, the Antarctic midge appears exceedingly capable of dealing with oxidative stress and free radical formation. Our results from lipid and protein oxidation also confirm that this is indeed the case. The larvae apparently do not increase their antioxidant defense in response to environmental stress as their antioxidant defense is already quite high. Total antioxidant capacity of adults was even higher than that of larvae. This result was surprising because adults do not express *SOD*, and even *catalase* levels were much lower than those observed in larvae. Still, adults are more susceptible to ROS formation due to UV radiation given the fact that they are surface dwellers and are exposed to over 20 h of daylight daily during the austral summer. In addition, certain antioxidant enzymes and compounds with antioxidant capacity are upregulated during reproduction to protect both the ovaries and eggs from ROS formation. Catalase is one such enzyme that has been found in the mosquito *A. gambiae* to not only be upregulated during reproduction but also to have a rescue effect against the decline of egg laying in aging females (DeJong et al., 2007). Chorion peroxidases have been found in *A. aegypti* and presumably play an important role in ROS defense during egg formation (Li and Li, 2006). Therefore, it seems reasonable that the antioxidant defenses that the adults use to protect sperm and eggs result in a higher total antioxidant capacity. But, our experiments have not identified the factors contributing to the ROS defense present in the adult midge.

Sugar-based cryoprotectants are well known to reduce osmotic outflow from the cell and thus reduce injury due to cell dehydration (Storey and Storey, 1988). Trehalose has been implicated in anoxia tolerance by reducing protein aggregation and maintaining proteins in a partially folded position aiding chaperone refolding, enhancing recovery from anoxia exposure and possibly as a carbon source under extraordinary conditions (Chen et al., 2002). Trehalose has also been demonstrated to prevent damage caused by oxygen radicals when combined with heat shock (Benaroudj et al., 2001; Pereira et al., 2001). Our data suggest that larvae do not rely upon stress-induced increases in trehalose concentration as a means to prevent/limit potential oxidative damage. Other sugar-based compounds such as mannitol and glycerol can reduce damage to proteins caused by ROS (Benaroudj et al., 2001), and we cannot eliminate the possibility that the midge larvae use such compounds. We recorded a strong increase in glycerol levels during desiccation (Benoit et al., 2007b), but we did not measure glycerol changes in response to the present stressors.

In summary, the strong and constant upregulation of *SOD* and the rapid upregulation of *catalase* in response to sunlight in the larvae are a likely defense against oxidative damage due to the accumulation of ROS. This protection was noted under field conditions, as well as in response to experimentally induced heat shock, freezing and anoxia. In contrast, the short-lived adults also have a strong antioxidant defense but mount this defense in the absence of *SOD* expression. Possibly the adults are protected from ROS by peroxidases involved in the protection of sperm and eggs. Clearly, the total antioxidant capacity of adults exceeds that of larvae, which in turn is much higher than observed in *E. solidaginis*, a freezing-tolerant insect from the temperate zone. A *smHsp* and *Hsp70* are upregulated when the adults are exposed to anoxia but not under normal conditions, suggesting that the adults are capable of mobilizing Hsps under stressful conditions. For the three stresses (heat shock, freezing and anoxia) tested, the larvae did not further upregulate the *Hsps* beyond their normal constitutive levels. However, all three *Hsps* were strongly upregulated in the defense that the larvae mounted against prolonged exposure to ultraviolet radiation. This indicates that UVR possibly is more threatening to larval well-being than other naturally occurring stresses on the continent. Thus, the larvae and adults have distinctly different protection strategies against ROS, a distinction that most likely reflects their different microhabitats (underground vs rock surfaces), stage duration (2 yr vs 1–2 wk), and functional differences (non-reproductive vs reproductive).

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