Available online at www.sciencedirect.com
SCIENCE DIRECT
Cryobiology 52 (2006) 74–82
CRYOBIOLOGY
www.elsevier.com/locate/ycryo

Oxidative DNA damage and antioxidant defenses in the European common lizard (*Lacerta vivipara*) in supercooled and frozen states ☆

Yann Voituron [a,*,1], Stéphane Servais [a,1], Caroline Romestaing [a], Thierry Douki [b], Hervé Barré [a]

[a] *Physiologie Intégrative Cellulaire et Moléculaires (UMR 5123), Campus La Doua, Bât 404, 4° Etage, 43 bd du 11 novembre 1918, F-69622 Villeurbanne Cedex, France*

[b] *Laboratoire "Lesions des Acides Nucleiques", Service de Chimie Inorganique et Biologique, UMR 5046, CEA/DSM/Département de Recherche Fondamentale sur la Matière Condensée, CEA-Grenoble, 38054 Grenoble Cedex 9, France*

Received 3 September 2004; accepted 30 September 2005
Available online 29 November 2005

Abstract

The European common lizard (*Lacerta vivipara*) tolerates long periods at sub-zero temperatures, either in the supercooled or the frozen state. Both physiological conditions limit oxygen availability to tissues, compelling lizards to cope with potential oxidative stress during the transition from ischemic/anoxic conditions to reperfusion with aerated blood during recovery. To determine whether antioxidant defenses are implicated in the survival of lizards when facing sub-zero temperatures, we monitored the activities of antioxidant enzymes and oxidative stress either during supercooling or during freezing exposures (20 h at −2.5 °C) and 24 h after thawing in two organs of lizards—muscle and liver. Supercooling induced a significant increase in the total SOD and GPx activity in muscle (by 67 and 157%, respectively), but freezing had almost no effect on enzyme activity, either in muscle or in liver. By contrast, thawed lizards exhibited higher GPx activity in both organs (a 133% increase in muscle and 59% increase in liver) and a significant decrease in liver catalase activity (a 47% difference between control and thawed lizards). These data show that supercooling (but not freezing) triggers activation of the antioxidant system and this may be in anticipation of the overgeneration of oxyradicals when the temperature increases (while thawing or at the end of supercooling). Oxidative stress was assessed from the content of 8-oxodGuo and the different DNA adducts resulting from lipid peroxidation, but it was unaltered whatever the physiological state of the lizards, thus demonstrating the efficiency of the antioxidant system that has been developed by this species. Overall, antioxidant defenses appear to be part of the adaptive machinery for reptilian tolerance to sub-zero temperatures.

Keywords: Freeze tolerance; Supercooling; Reptile; Superoxide dismutase; Catalase; Glutathione peroxidase; Lipid peroxidation induced DNA adducts; 8-oxodGuo

☆ This work was supported by a research grant from the CNRS, France.
* Corresponding author. Fax: +33 4 72 43 11 72.
E-mail address: yann.voituron@univ-lyon1.fr (Y. Voituron).
[1] These authors contributed equally to this work.

0011-2240/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.cryobiol.2005.09.006

Several species of cold-blooded vertebrate that hibernate on land are commonly exposed to sub-zero temperatures. To ensure their survival during winter, these animals have developed cold hardiness strategies that are commonly divided into two main groups: freeze avoidance via an extensive supercooling capacity and freeze tolerance. The freeze avoidance strategy is characterized by various metabolic adaptations involving the release or masking of potent ice nucleators, the accumulation of low molecular weight carbohydrates and antifreeze proteins that depress the freezing point [39]. By contrast, the freeze tolerance strategy implies that animals can endure the conversion of a fraction of body water into ice. This second strategy is characterized by mechanisms such as the production of ice nucleators which allow the initiation of freezing at high sub-zero temperatures and the production of cryoprotectant substances, which allow controlled propagation of ice within the body [32]. Among the several species of Holarctic vertebrates, winter survival is mainly based on supercooling and the principal mechanisms developed involve the removal of active nucleating agents from body fluids and modification of the integument to provide a highly efficient barrier to the penetration of ice from frozen soil into the body compartments [26]. On the other hand, freeze tolerant vertebrates principally accumulate high concentrations of carbohydrates, such as glucose and/or glycerol [32]. In the supercooled state, the heart rate is known to be extremely low [3] which reduces tissue perfusion and causes accumulation of lactate within the body [16]. Freezing also induces ischemia and anoxia because when the extracellular body fluids freeze the circulation stops and the cells rely on internal fermentation of fuel reserves for survival. Increase in temperature and thawing then reintroduce oxygen into the tissues and organs, thereby restoring aerobic metabolism. However, reperfusion also potentially creates the conditions for oxidative stress.

Indeed, oxygen metabolism is known to generate reactive oxygen species (ROS), as by-products [6,12] and these can initiate free radical chain reactions that may damage cellular components such as DNA, proteins, and lipids. Both superoxide radicals ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) can lead to the formation of the hydroxyl radical ($OH^{\cdot}$) which is the most reactive form of ROS. While the superoxide radicals are produced by the "leaky" mitochondrial respiratory chain, or from the activity of oxidases such as xanthine oxidase, aldehyde oxidase and NADPH oxidase, hydrogen peroxide (H_2O_2) is mainly formed by dismutation of $O_2^{\cdot -}$ [5,14]. To neutralize the oxidative effect of ROS, all aerobic organisms have developed biochemical defenses, including low molecular weight free radical scavengers and enzymatic antioxidant systems [38]. When the generation of ROS exceeds the capacity of the antioxidant defense systems, oxidative stress occurs in the cell. Many organisms are likely to experience wide variations in oxygen availability to their tissues when facing natural conditions like birth, exercise, hibernation, and overwintering [4,7,18,23,29]. To endure these situations, animals have developed different degrees of tolerance to ischemia and anoxia, including reliable antioxidant systems.

Questions concerning antioxidant defenses at sub-zero temperatures have received some attention lately, but only in two species of insect [23], a frog [22], and a reptile [20]. In spite of these three studies, no data are available to make direct comparisons between the biochemical adaptations associated with freeze tolerance and supercooling by examinating these processes in the same species. Recent studies have shown that the European common lizard (*Lacerta vivipara*) tolerates both the supercooled state and the frozen state in its natural environment. Laboratory experiments have shown that this lizard can remain for at least 3 weeks in the supercooled state and for 3 days in the frozen state [9]. The maximum ice content (about 50% of the body water converted into ice) is attained after 5 h freezing and then remains constant [37]. Since these two physiological states potentially induce prolonged hypoxia, the present work aims to monitor the activities of the main antioxidant enzymes [superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT)] during either supercooling or freezing induced by exposure to −2.5 °C for 20 h, and 24 h after thawing at 5 °C. Two organs were studied—muscle and liver. To assess the disturbance of the balance of prooxidant/antioxidant, we measured the oxidative damage to DNA in cells: that is extensively used as an indicator of the occurrence of oxidative stress [8]. We focused our interest on two types of DNA lesion: (i) 8-oxo-7,8-dihydro-2′-deoxy guanosine (8-oxodGuo), which is a marker for direct DNA oxidation [17] and (ii) lipid peroxidation DNA adducts that come from the release of aldehydic products during the breakdown of lipid peroxides. The latter metabolites include malondialdehyde (MDA) and simple aldehydes such as α,β-unsaturated aldehydes like acrolein

and 4-hydroxy-2-nonenal (4-HNE). Most of these aldehydes have been found to react with DNA bases, giving rise to adducts. MDA adds to 2′-deoxyguanosine to yield a pyrimidopurinone exocyclic adduct (M1dGuo). 4-HNE also converts guanine into an exocyclic propano adduct. In addition, the epoxidation product of 4-HNE has been shown to give rise to ethenobases from adenine and guanine [11].

Materials and methods

Animals

Lacerta vivipara inhabits mainly damp habitats such as meadows, peatbogs, and heathlands, and hibernates in a shallow terrestrial hibernaculum. *L. vivipara* specimens (2.94 ± 1.25 g) were collected from a highland population in the Cévennes mountains in France (latitude, 44.30°N; longitude, 3.45 °E; altitude, 1450 m) in late September. They were held in boxes with sand and wet moss and cold acclimated at 4 °C for 6–7 weeks in the dark without feeding before use. The present investigation was carried out according to the ethical principles laid down by the French (Ministère de l′Agriculture) and European Convention for the protection of Vertebrate Animals used for Experimental and Scientific purposes (Council of Europe No. 123, Strasbourg, 1985).

Preparation of supercooled, frozen, and thawed animals

Freezing exposures were performed as previously reported [36,37]. Briefly, lizards were placed on a pad of absorbent wet paper, itself placed inside a 50 ml Falcon tube. Animals were then progressively cooled at a constant rate of 0.2 °C min^{-1} in a Low Temperature Incubator (Model 815 from Precision) from an initial temperature of 4 °C down to the crystallization temperature (T_c). We considered that the freezing exposure of each lizard began immediately after its exotherm and ended when this individual was removed from its tube 20 h later. During the freezing period all the individual tubes were placed in an incubation chamber set to −2.5 °C (±0.1 °C). A 20 h freezing period was chosen because this induces an ice content of about 50% ice and recovery of the frozen lizards strongly decreased with longer periods of freezing [37]. Lizards were thawed in an incubator at +3 °C for 24 h.

For supercooling exposure, the lizards were placed on a pad of absorbent dry paper and progressively cooled at a constant rate of 0.2 °C min^{-1} down to −2.5 °C. They were then maintained at this temperature for 20 h.

After supercooling, freezing, and thawing the lizards were killed by decapitation and organs were quickly dissected out and frozen by immersion in liquid nitrogen. Organ samples were then stored at −80 °C until use.

Antioxidant enzyme activities

Frozen tissue samples were rapidly weighed and homogenized using a Potter–Elvehjem homogenizer at 4 °C, in a buffer containing KH_2PO_4 (100 mM), DTT (1 mM), and EDTA (2 mM), pH 7.4. After centrifugation (3000*g*, for 5 min), the supernatant was used for the enzymatic assays.

Superoxide dismutase

Superoxide dismutase (SOD) activity was assayed spectrophotometrically (at 550 nm) by monitoring the rate of acetylated cytochrome *c* reduction by superoxide radicals generated by the xanthine–xanthine oxidase system at 25 °C in reaction buffer (xanthine 0.5 mM, cytochrome *c* 0.2 mM, KH_2PO_4 50 mM, and EDTA 0.1 mM, pH 7,8) [13]. One activity unit of SOD is defined as the amount of enzyme which inhibits the rate of acetylated cytochrome *c* reduction by 50%. To distinguish mangano-SOD (MnSOD), which is exclusively located in mitochondrial matrix, from cuprozinc-SOD (CuZnSOD), which is primarily located in the cytosol, SOD activity was determined after incubation with NaCN (1 mM). At this concentration, cyanide inhibits the CuZn isoform of the enzyme, but does not affect the MnSOD isoform [13]. SOD activity was expressed as unit per milligram of total protein.

Glutathione peroxidase

The total activity of glutathione peroxidase (GPx) activity was assayed with cumene hydroperoxide (1 mg ml^{-1}) as substrate by measuring spectrophotometrically the reduction of NADPH ($\varepsilon = 6.22 \times 10^3$ L mol^{-1} cm^{-1}) at 340 nm (Tappel, 1978). Measurement was carried out at 37 °C in reaction buffer (GSH 0.25 mM, NADPH 0.12 mM, GR 1 U ml^{-1}, and NaCN 10 mM).

GPx activity was expressed as milliunit per milligram of total protein.

Catalase

The activity of catalase (CAT) was determined by the method of Aebi [1]. Each supernatant (200 μl) was incubated 30 min at 0 °C with ethanol (95%, 2 μl). After adding Triton (1%, 2 μl) the sample was centrifuged at 5000*g* for 5 min. The supernatant was used for measurement of CAT activity by using the first-order rate constant of the decomposition of H_2O_2 by tissue CAT at 20 °C in buffer (pH 7.4) containing KH_2PO_4 (40 mM) and HNa_2PO_4 (60 mM). One unit of catalase activity was calculated by using $k = (2.3/dt)(\log A_1/A_2)$, where k is CAT activity, dt is change in time, A_1 is initial absorbance, and A_2 is final absorbance. CAT activity was expressed in millikelvin per milligram of total protein.

Nuclear DNA oxidative damage content

As previously described [27] determination of 8-oxodGuo needs some precautions to limit artefactual oxidation during DNA extraction.

Isolation of nuclei

Samples were homogenized with a Potter glass homogenizer in buffer (320 mM sucrose, 10 mM Tris, 5 mM $MgCl_2$, 0.1 mM desferroxamine mesylate, and 1% Triton 100, pH 7.5). After homogenization, the sample was centrifuged at 1500*g* for 5 min at 4 °C. The supernatant was discarded and the pellet was washed before centrifugation at 1500*g*.

DNA isolation

Nuclear pellets were resuspended in 600 μl extraction buffer (10 mM Tris, 5 mM EDTA, and 0.15 mM desferroxamine mesylate, pH 8) with addition of SDS (10%). RNase A (3 μL, 100 mg/ml) and RNase T1 (8 U) were then added and the samples were incubated for 15 min at 50 °C. The samples were then incubated with Qiagen proteinase (20 mg/ml) for 60 min at 37 °C. Isolation of nucleic acids was achieved by adding a NaI solution (7.6 M NaI, 40 mM Tris, and 20 mM EDTA, pH 8), and isopropanol (100%). The samples were centrifuged for 15 min at 5000*g* at room temperature. The nucleic acids pellets were rinsed. Following the last centrifugation (5000*g*, 10 min), the DNA pellet was solubilized in deionized water containing 0.1 mM desferroxamine mesylate.

Digestion

Phosphodiesterase II (Ph II, 1 μl, 0.004 U; Sigma, St. Louis, MO) and 5 U of nuclease P1 (5 μl in 300 mM of 10 μM $ZnSO_4$, pH 5, Sigma, St. Louis, MO) were added to the DNA solution (100 μl) together with 5 μl MN/SPD buffer (200 mM succinic acid, 100 mM $CaCl_2$, pH 6). The resulting solution was incubated for 2 h at 37 °C. Then, 12 μl PA buffer (500 mM Tris, 1 mM EDTA, pH 8) was added together with 0.003 U phosphodiesterase I (Ph I, 0.25 μl, Sigma, St. Louis, MO) and 4 U alkaline phosphatase (0.2 μl, Roche Molecular Biochemicals). The sample was incubated at 37 °C for 2 h. HCl (8 μl, 0.1 N) and chloroform (30 μl) were added. The sample was vortexed. The resulting solution was centrifuged (5000*g*) and subsequently transferred into HPLC injection vials.

Samples were first analyzed by HPLC with electrochemical detection (HPLC-EC) to quantify 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) as described previously [28].

DNA samples were then analyzed for their content of lipid peroxidation adducts by HPLC–MS/MS as described previously [28]. For this purpose, the vials used for the HPLC-EC analysis were freeze-dried. DNA samples were then analyzed for their content of lipid peroxidation adducts by HPLC–MS/MS. For this purpose, the vials used for the HPLC-EC analysis were freeze-dried. The resulting residue was solubilized in 30 μl water containing 200 fmol of the [$^{15}N_5$]-labeled derivatives of 1,N^2-εdAdo, 1,N^2-εdGuo, and 6-(1-hydroxyhexanyl)-8-hydroxy-1,N^2-propano-2′-deoxyguanosine HNE-dGuo propano adduct. The latter compounds were used as internal standard for isotopic dilution calibration. M1dGuo was quantified by external calibration. The samples were then analyzed by reverse phase HPLC and an API 3000 mass spectrometer (Perkin-Elmer/SCIEX, Thornhill, Canada) This was used in the multiple reaction monitoring mode. 1,N^6-Etheno-2′-deoxyadenosine (ethdA), 1,N^6-etheno-2′-deoxyguanosine (ethdGuo), pyrimidopurinone malondialdehyde adduct to 2′-deoxyguanosine (M1dGuo), and 1,N^2-propano adduct between dGuo and 4-hydroxy-2-nonenal (HNE-dGuo) were quantified by high performance liquid chromatography associated with tandem mass spectrometry (HPLC–MS/MS) in the positive mode. In each sample, the amount of DNA injected was inferred from the area of the peak corresponding to 2′-deoxyguanosine monitored in a UV spectrophotometer.

Protein measurement and statistics

Protein concentration was measured by the Lowry method [24].

Data are presented as means ± SE. Statistical analysis was performed with Statview computer statistical package. Data were analyzed by the non-parametric Kruskall–Wallis test and the Mann–Whitney test to compare two means. We used non-parametric tests because of violation of the parametric assumption of homogeneity of variance. A 5% ($p < 0.05$) level of significance was used in all tests.

Results

Antioxidant enzyme activities

Figs. 1–3 show the activities of enzymes involved in the antioxidant defenses of *L. vivipara*, in two tissues and in the following groups: control (4 °C); frozen (20 h at −2.5 °C); supercooled (20 h at −2.5 °C);

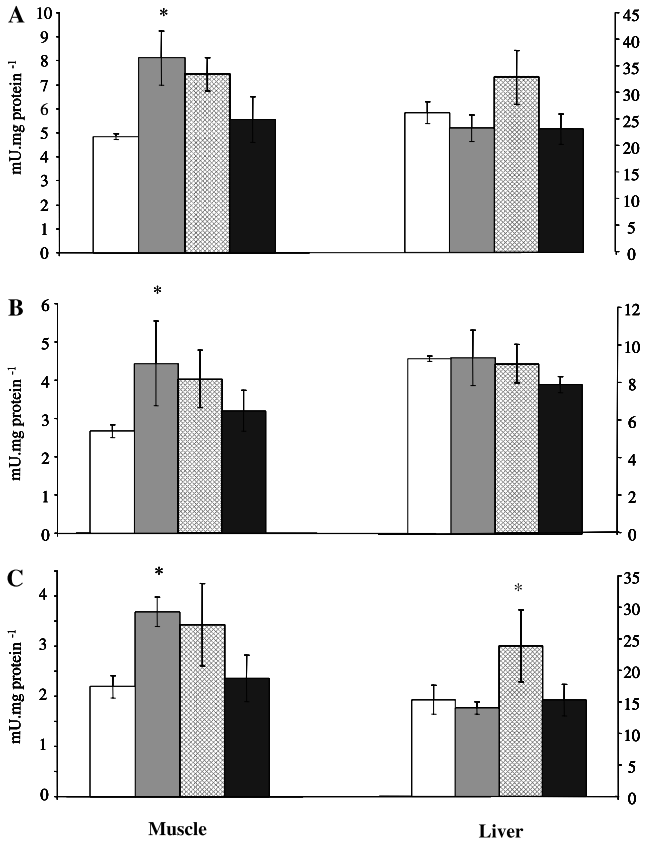

Fig. 1. (A) Total superoxide dismutase (SOD), (B) MnSOD, and (C) CuZnSOD activity in tissues of *L. vivipara*. Open bars, control lizards (4 °C, $n = 5$); grey bars, supercooled (20 h at −2.5 °C, $n = 4$) lizards; crosshatched bars, frozen (20 h at −2.5 °C, $n = 5$) lizards, black bars, thawed (24 °C at 5 °C, $n = 5$) lizards. Data are means ± SE. Values were considered statically significant at $p < 0.05$. (∗) Significantly different from corresponding control value.

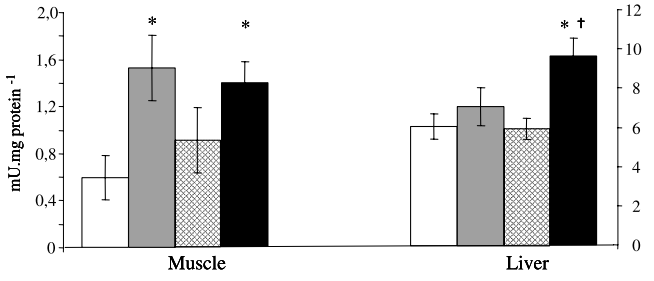

Fig. 2. Glutathione peroxidase (GPx) activity in tissues of *L. vivipara*. Details as in Fig. 1. (∗) Significantly different from corresponding control value; (†) significantly different from corresponding frozen value.

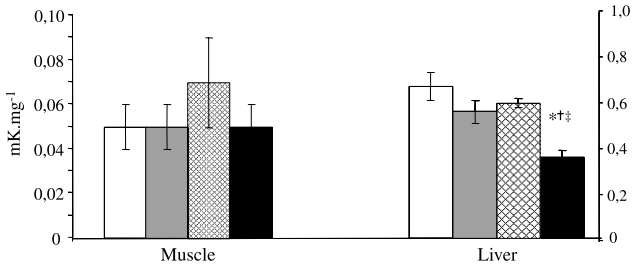

Fig. 3. Catalase (CAT) activity in tissues of *L. vivipara*. Details as in Fig. 1. (∗) Significantly different from corresponding control value; (†) significantly different from corresponding frozen value; (‡) significantly different from corresponding supercooled value.

Table 1
Liver DNA oxidative damage in *L. vivipara* after supercooling, freezing, and thawing

	Lacerta vivipara				*P* values
	Control	Supercooled	Frozen	Thawed	
8-OxodG	2.43 ± 0.21	2.33 ± 0.37	2.20 ± 0.45	1.88 ± 0.16	0.34
EthdA	4.43 ± 0.59	2.31 ± 0.61	3.72 ± 0.30	3.53 ± 0.64	0.18
EthdGuo	5.57 ± 0.21	3.70 ± 0.93	5.18 ± 0.49	5.19 ± 0.99	0.42
M1dGuo	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.03 ± 0.01	0.81
HNE-dGuo	0.03 ± 0.02	0.07 ± 0.04	0.06 ± 0.03	0.03 ± 0.02	0.86

Data are lesions per 10^6 unmodified bases, means ± SE with $n = 5$ for frozen and thawed groups and $n = 4$ for control and supercooled groups.

thawed (24 h at 4 °C). Overall, the activities of antioxidant enzymes were always greater in liver than in muscle (between four- and sevenfold according to the enzyme) and they differed markedly between the different physiological states of *L. vivipara*.

The total SOD activity increased significantly during supercooling in muscle tissue (by up to 67% of control values—Fig. 1A). Such an increase was detected for both cytosolic and mitochondrial SOD (68 and 66%, respectively—Figs. 1B and C). On the other hand, total SOD activity in liver was unaffected by freezing and supercooling (Fig. 1A) although a significant increase in cytosolic SOD after freezing was observed (about 56% in comparison with controls—Fig. 1C). Mitochondrial SOD activity was equivalent irrespective the physiological state (Fig. 1B).

Muscle GPx activity was significantly higher with supercooling and 24 h after thawing (about 157 and 133%, respectively, Fig. 2). GPx activity in liver followed the same patterns but with a lesser effect of supercooling.

Hepatic catalase activity was not altered during freezing and supercooling but showed a significant decrease 24 h after thawing and that applied to all other groups (for instance, a 47% decrease in thawed lizards with respect to control. By contrast, the experimental treatment had no effect on catalase activity in muscles (Fig. 3).

Oxidative stress status by nuclear DNA oxidative damages measurement

Levels of modified bases within DNA were undetectable in muscle but this was explainable by the low DNA contents of these samples. However, with the liver, 8-oxodGuo and DNA adducts from lipid peroxidation were detectable but showed little or no oxidative stress induced by supercooling, freezing or thawing in *L. vivipara*. The five modified DNA bases assessed in this study did not show significant variations between the different physiological states (see Table 1).

Discussion

The European common lizard (*L. vivipara*) is perhaps the most widely distributed of extant lizards. It is one of the four palearctic reptiles inhabiting the subarctic region [30] and thrives in various habitats owing to its physiological plasticity [15]. Different studies have provided conclusive evidence of a well-developed capacity for supercooling and freezing tolerance in this species [9,37]. Its unusual cold hardiness, involving both supercooling and freezing, may promote its winter survival, which is exceptionally high (88–100% at all ages) even in severe winter [2]. Both physiological states induce prolonged ischemia due to the extreme reduction of the heart

pulse rate during supercooling [3] and trapping of cells by ice during freezing [31].

Our results suggest that the activation of antioxidant systems under sub-zero temperatures is a part of the survival strategy of the European common lizard when facing supercooling or freezing (see Figs. 1–3). The data show that SOD and GPx might play a role in this, with a threefold increase in GPx activity, that may be explained by its capacities to detoxify both H_2O_2 and the hydroperoxides resulting from lipid peroxidation [12]. Even if the antioxidant enzyme activity measurements were made far from their normal operating temperature, our results corroborate previous studies demonstrating that antioxidant defenses play key roles in tissue protection during freezing and thawing [20,22,23]. As far as we know, the relations between antioxidant defenses and sub-zero temperatures have so far been studied in only one reptile: the garter snake (*Thamnophis sirtalis*). It has emerged that freezing at −2.5 °C for 5 h resulted in a significant rise in the activity of muscle and lung catalase (by 183 and 63%) and in muscle glutathione peroxidase (by 52%) [20]. This was interpreted as an adaptation to overcome the potentially injurious post-ischemic situation following thawing but the effect of recovery from freezing was not analyzed. The pattern is thus different for *L. vivipara*, which showed no significant difference between control and frozen individuals, whatever the enzyme studied (see Figs. 1–3). Such a discrepancy between the two reptiles may be explained by the different cold hardiness capacities. Indeed, while spontaneous ice nucleation occurs within 1 h of supercooling at high sub-zero temperature (−3.3 °C) in garter snakes [10], supercooled *L. vivipara* can survive for at least 3 weeks at −3.5 °C [9]. That suggests a "global" strategy in *L. vivipara*, whereby sub-zero temperatures induce an increase of the activity of at least two antioxidant enzymes (SOD and GPx) to control possible overgeneration of oxyradicals after supercooling or thawing. In contrast, the garter snake developed specific responses to freezing, the frozen state being the major risk for this species at sub-zero temperatures. To verify such an hypothesis, the activity of antioxidant enzymes should be assessed in animals that have endured significant supercooling, followed by freezing.

From the present data, we can also hypothesize that the liver and muscle cells of *L. vivipara* respond to the stress of sub-zero temperatures by anticipating an overgeneration of oxyradicals during the warming period after thawing or the end of supercooling. Similar responses have already been reported in other animals. For instance, Buzadic et al. [4] showed that the hibernating ground squirrel (*Citellus citellus*) increases its antioxidant defenses during arousal. In other respects, the land snail (*Otala lactea*) shows increased antioxidant defenses in its organs while they are in a hypometabolic (estivating) state as a protective mechanism against oxidative stress during arousal [19]. Since similar patterns can be found in other groups such as fish [25,35], an anticipatory response to the reperfusion of O_2, that can follow estivation, anoxia, supercooling or freezing, may be considered a common trait among animals.

The oxidative stress encountered during sub-zero temperatures was assessed by the contents of 8-oxodGuo and different DNA adducts from lipid peroxidation that are useful biomarkers of oxidative DNA damages [11,17]. Interestingly, to our knowledge, it is the first study that measured 8-oxodGuo and DNA adduct content in ectotherms using HPLC–MS/MS and then ensuring very specific and high sensitive measurement (since less than 1 lesion/10^6 DNA can be detected). Apparently, supercooling, freezing, and thawing do not induce variations of DNA damage, suggesting that a complete freeze–thaw cycle can be accomplished with very little oxidative stress. This lack of damage could be due to the effectiveness of enhanced antioxidant capacities but it is equally valid to suggest that free radical damage does not happen under the experimental conditions used in this experiment. Further investigations focused on longer periods of time and/or lower temperatures of supercooling and freezing are thus needed.

However, it is noteworthy that the values we have obtained in vertebrate ectotherms are superior to those found in endotherms such as the rat (Servais, unpublished data), which suggests higher DNA oxidation and lipid peroxidation. These observations are in complete agreement with other data comparing oxidative stress in the liver of different vertebrates [34]. Indeed, these authors have shown that the TBARS content (lipid peroxidation index) was twice as high in lizard liver than in rat liver. Such higher oxidative damage in ectotherms could result from an unfavorable prooxidant/antioxidant balance induced by a lower cell antioxidant capacity in ectotherms than in endotherms [34]. Previous work also demonstrated different functioning of the respiratory chain in ectotherms, with a lower proton leakage through the inner mitochondrial membrane [33], suggesting

that mitochondrial ROS production in these animals may be relatively high. Thus, it seems to be fundamental for the understanding of this high "basal" oxidative damage, to compare ROS production in ectotherms versus endotherms by focusing studies on the two major respiratory chain complexes implicated in free radical leak (complexes I and III).

Conclusions and perspectives

This study provides: (i) an unique comparison of the antioxidant defenses employed during freezing and supercooling in the same species and (ii) the first quantification of cellular oxidative damage, including DNA and lipid damage, by measuring respectively 8-oxo-dGuo and DNA adducts in a vertebrate ectotherm. It also shows that the overall maintenance/enhancement of antioxidant defenses during supercooling/freezing may account for the absence of an increase in oxidative damage in *L. vivipara* tissues. The increase in the activity of antioxidant enzymes observed in organs of supercooled and thawed lizards suggests that antioxidant defenses play a significant part in the adaptive machinery for ectothermal survival under sub-zero temperatures. Importantly, as observed in other cold-hardy animals, *L. vivipara* cells anticipate the reperfusion-linked radical overproduction, that will occur during thawing and at the end of supercooling. Such anticipation seems to be, at least partly, supported by higher activity of SOD and GPx. These findings might provide key information to ameliorate cryopreservation injury in organ transplantation

However, questions remain unanswered and deserve further study. Indeed, the enzymatic antioxidant capacities cannot fully explain the higher oxidative damage reported in ectotherms. The quantification of non-enzymatic antioxidants such as urate, ascorbate, and other enzymes like peroxiredoxins [21] may bring understanding elements.

Acknowledgments

The authors express their thanks to Drs. P.L. Merle (Nice-Sophia Antipolis University, France), J. Costanzo (Miami University, Ohio), and the anonymous referees for the helpful criticism.

References

[1] H. Aebi, Catalase in vitro, Methods Enzymol. 105 (1984) 121–126.
[2] D. Bauwens, Survivorship during hibernation in the European Common Lizard *Lacerta vivipara*, Copeia 3 (1981) 741–744.
[3] G.F. Birchard, G.C. Packard, Cardiac activity in supercooled hatchling painted turtle (*Chrysemys picta*), J. Herpetol. 31 (1997) 166–169.
[4] B. Buzadic, M. Spasic, Z.S. Saicic, R. Radojicic, V.M. Petrovic, B. Halliwell, Antioxidant defenses in the ground squirrel *Citellus citellus*. 2. The effect of hibernation, Free Radic. Biol. Med. 9 (1990) 407–413.
[5] S. Cadenas, C. Rojas, R. Perez-Campo, M. Lopez-Torres, G. Barja, Vitamin E protects guinea pig liver from lipid peroxidation without depressing levels of antioxidants, Int. J. Biochem. Cell. Biol. 27 (1995) 1175–1181.
[6] B. Chance, H. Sies, A. Boveris, Hydroperoxide metabolism in mammalian organs, Physiol. Rev. 59 (1979) 527–605.
[7] L.B. Clerch, D. Massaro, Rat lung antioxidant enzymes: differences in perinatal gene expression and regulation, Am. J. Physiol. 263 (1992) L466–L470.
[8] A.R. Collins, M. Dusinska, C.M. Gedik, R. Stetina, Oxidative damage to DNA: do we have a reliable biomarker? Environ. Health Perspect. 104 (1996) 465–469.
[9] J.P. Costanzo, C. Grenot, R.E. Lee, Supercooling, ice nucleation and freeze tolerance in the european common lizard, *Lacerta vivipara*, J. Comp. Physiol. B 165 (1995) 238–244.
[10] J.P. Costanzo, R.E. Lee, Natural freeze tolerance in a reptile, Cryo-Letters 9 (1988) 380–385.
[11] T. Douki, F. Odin, S. Caillat, A. Favier, J. Cadet, Predominance of the 1,*N*2-propano 2′-deoxyguanosine adduct among 4-hydroxy-2-nonenal-induced DNA lesions, Free Radic. Biol. Med. 37 (2004) 62–70.
[12] W. Droge, Free radicals in the physiological control of cell function, Physiol. Rev. 82 (2002) 47–95.
[13] L. Flohe, F. Otting, Superoxide dismutase assays, Methods Enzymol. 105 (1984) 93–104.
[14] I. Fridovich, Oxygen toxicity: a radical explanation, J. Exp. Biol. 201 (1998) 1203–1209.
[15] C. Grenot, B. Heulin, Sur la plasticité écophysiologique du lézard vivipare, *Lacerta vivipara*, Bulletin de la Société Herpétologique de France 54 (1990) 1–22.
[16] L.M. Hartley, M.J. Packard, G.C. Packard, Accumulation of lactate by supercooled hatchlings of the painted turtle (*Chrysemys picta*): implications for overwinter survival, J. Comp. Physiol. B 170 (2000) 45–50.
[17] H.J. Helbock, K.B. Beckman, B.N. Ames, 8-Hydroxydeoxyguanosine and 8-hydroxyguanine as biomarkers of oxidative DNA damage, Methods Enzymol. 300 (1999) 156–166.
[18] M. Hermes Lima, T. Zenteno Savin, Animal response to drastic changes in oxygen availability and physiological oxidative stress, Comp. Biochem. Physiol. C 133 (2002) 537–556.
[19] M. Hermes-Lima, K.B. Storey, Antioxidant defenses and metabolic depression in a pulmonate land snail, Am. J. Physiol. 268 (1995) R1386–R1393.
[20] M. Hermes-Lima, K.B. Storey, Antioxidant defenses in the tolerance of freezing and anoxia by garter snakes, Am. J. Physiol. 265 (1993) R646–R652.
[21] B. Hofmann, Peroxiredoxins, Biol. Chem. 383 (2002) 347–364.
[22] D.R. Joanisse, K.B. Storey, Oxidative damage and antioxidants in *Rana sylvatica*, the freeze-tolerant wood frog, Am. J. Physiol. 271 (1996) R545–R553.

[23] D.R. Joanisse, K.B. Storey, Oxidative stress and antioxidants in overwintering larvae of cold-hardy goldenrod gall insects, J. Exp. Biol. 199 (1996) 1483–1491.
[24] O.H. Lowry, N.J. Rosbrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[25] V.I. Lushchak, L.P. Lushchak, A.A. Motaa, M. Hermes-Lima, Oxidative stress and antioxidant defenses in goldfish *Carassius auratus* during anoxia and reoxygenation, Am. J. Physiol. 280 (2001) R100–R107.
[26] G.C. Packard, M.J. Packard, To freeze or not to freeze: adaptations for overwintering by hatchlings of the North American painted turtle, J. Exp. Biol. 207 (2004) 2897–2906.
[27] J.L. Ravanat, T. Douki, P. Duez, E. Gremaud, K. Herbert, T. Hofer, L. Lasserre, C. Saint-Pierre, A. Favier, J. Cadet, Cellular background level of 8-oxo-7,8-dihydro-2′-deoxyguanosine: an isotope based method to evaluate artefactual oxidation of DNA during its extraction and subsequent work-up, Carcinogenesis 23 (2002) 1911–1918.
[28] S. Servais, A. Boussouar, A. Molnar, T. Douki, J.M. Pequignot, R. Favier, Age-related sensitivity to lung oxidative stress during ozone exposure, Free Radic. Res. 39 (2005) 305–316.
[29] S. Servais, K. Couturier, H. Koubi, J.L. Rouanet, D. Desplanches, M.H. Sornay-Mayet, B. Sempore, J.M. Lavoie, R. Favier, Effect of voluntary exercise on H_2O_2 release by subsarcolemmal and intermyofibrillar mitochondria, Free Radic. Biol. Med. 35 (2003) 24–32.
[30] I.F. Spelleberg, Adaptations of reptiles to cold, in: A. D'A Bellaires, C.B. Cox (Eds.), Morphology and Biology of Reptile, 1976, pp. 261–285.
[31] K.B. Storey, J.M. Storey, Cellular adaptations for freezing survival by Amphibians and Reptiles, Advances in Low-Temperature Biology, 1993, pp. 101–129.
[32] K.B. Storey, J.M. Storey, Freeze tolerance in animals, Physiol. Rev. 68 (1988) 27–84.
[33] J. St-Pierre, M.D. Brand, R.G. Boutilier, The effect of metabolic depression on proton leak rate in mitochondria from hibernating frogs, J. Exp. Biol. 203 (2000) 1469–1476.
[34] P. Venditti, C.M. Daniele, M. Balestrieri, S. Di Meo, Protection against oxidative stress in liver of four different vertebrates, J. Exp. Zool. 284 (1999) 610–616.
[35] E. Vig, J. Nemcsok, The effect of hypoxia and paraquat on the superoxide dismutase activity in different organs of carp, *Cyprinus carpio* L., J. Fish Biol. 35 (1989) 23–25.
[36] Y. Voituron, B. Heulin, Y. Surget-Groba, Comparison of the cold hardiness capacities of the oviparous and viviparous forms of *Lacerta vivipara*, J. Exp. Zool. A 301 (2004) 367–373.
[37] Y. Voituron, J.M. Storey, C. Grenot, K.B. Storey, Freezing survival, body ice content and blood composition of the freeze tolerant European common lizard, *Lacerta vivipara*, J. Comp. Physiol. B 172 (2002) 71–76.
[38] B.P. Yu, Cellular defenses against damage from reactive oxygen species, Physiol. Rev. 74 (1994) 139–162.
[39] K.E. Zachariassen, Physiology of cold tolerance in insects, Physiol. Rev. 65 (1985) 799–832.