

OXIDATIVE STRESS AND ANTIOXIDANTS IN OVERWINTERING LARVAE OF COLD-HARDY GOLDENROD GALL INSECTS

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Summary

Antioxidant and pro-oxidant systems were studied in overwintering larvae of two cold-hardy gall insect species, the freeze-tolerant fly *Eurosta solidaginis* and the freeze-avoiding moth *Epiblema scudderiana*. An increase in the levels of the oxidized form of glutathione suggested slight oxidative stress in both species during the winter. Freeze-tolerant *Eurosta solidaginis* larvae generally had decreased activities of antioxidant enzymes in the winter, indicating that these larvae do not face increased challenge from oxidative stress during the numerous freeze–thaw events they experience. Instead, existing defences must be sufficient to prevent any damage. By contrast, increased winter activities of antioxidant enzymes in freeze-avoiding *Epiblema scudderiana* suggest that these larvae must

defend against the formation of reactive oxygen species. This may result from the oxidative nature of winter metabolism in these larvae, as well as a dependence on lipid oxidation as their fuel over this season. Xanthine dehydrogenase activity decreased dramatically in both species during the autumn, reducing the potential for the formation of the pro-oxidant xanthine oxidase. Indeed, xanthine oxidase activity fell to undetectable levels by winter in *Epiblema scudderiana* and was not detectable at any time in *Eurosta solidaginis*.

Key words: *Eurosta solidaginis*, *Epiblema scudderiana*, insect cold-hardiness, reactive oxygen species, antioxidant.

Introduction

The discoveries of superoxide (O_2^-) by Gerschman *et al.* (1954) and superoxide dismutase by McCord and Fridovich (1969) opened the way for what is now a broad and far-reaching field of study involving the detection, characterization and analysis of the role of reactive oxygen species (ROS) in both normal and pathological processes of cellular metabolism. The formation of ROS *in vivo* under normal conditions is now well established (Halliwell and Gutteridge, 1989). Superoxide is relatively stable under physiological conditions of pH and ionic strength but, since it is even more so in organic solvents, it has been postulated that the diffusion of this nucleophile across or into membranes may spread damage beyond the original site of radical formation (Fuller *et al.* 1988; Valentine *et al.* 1984). Hydrogen peroxide is itself a weak oxidizing agent but in the presence of transition metals (such as iron or copper) it can react with superoxide to form the hydroxyl radical (OH^\bullet), which is extremely reactive and damaging to biological systems (Halliwell and Gutteridge, 1986). Damage resulting from oxidative stress, defined as any condition where the rate of ROS production surpasses the ability of antioxidant systems

to buffer them, has been demonstrated under numerous conditions (notably ischaemia-reperfusion, iron-overload and increased oxidative metabolism such as during exhaustive exercise). All cellular components are susceptible to attack by ROS. Damage to proteins, DNA and lipids (more particularly to polyunsaturated fatty acids) may result in loss of function, conformational changes and the formation of cytotoxic low molecular mass breakdown products (Sies, 1986; Davies, 1987; Fuller *et al.* 1988; Gutteridge and Halliwell, 1990).

To deal with the potential dangers of ROS, a number of antioxidant defences have arisen. Their function is to maintain low steady-state levels of ROS and other radicals in the cell, a process involving precise regulation of their location and amount. The enzymes superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), selenium-dependent glutathione peroxidase (GPox), selenium-independent glutathione peroxidase (GSTPox) and the glutathione-S-transferases (GST) are recognized as a key interacting line of defence against ROS and their products of attack (Halliwell and Gutteridge, 1989). SOD dismutates superoxide anions directly (McCord and Fridovich, 1969), but in so doing

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produces potentially toxic hydrogen peroxide, H₂O₂. CAT and GPox (the selenium-dependent glutathione peroxidase) act to remove peroxides. GSTPox activity is attributed to some multifunctional isoenzymes of GST and has been shown to catalyze the reduction of a wide range of organic hydroperoxides, but not of H₂O₂ (Mannervik, 1985; Flohé, 1982; Flohé and Gunzler, 1984). GST itself will conjugate reduced glutathione to, and thus help remove, nucleophilic xenobiotics or damaged cellular components from the organism. GR, an NADPH-dependent reductase, serves to replenish substrate for GPox, GSTPox and GST by converting oxidized glutathione (GSSG) to its reduced form (GSH). The tripeptide glutathione (GSH) is the principal non-protein sulphhydryl compound in tissues and, in addition to its role as a substrate for GPox, GSTPox and GST, it is a scavenger of hydroxyl and singlet oxygen, can reactivate some enzymes inhibited under oxidizing conditions, and has also been shown to be involved in vitamin E regeneration (Halliwell and Gutteridge, 1989; Reddy *et al.* 1982). An increase in the level of GSSG at the expense of GSH is generally viewed as an indication of oxidative stress.

In this study, we examine the role of oxidative stress and antioxidants in two species that have been extensively studied as models for insect cold-hardiness. Freeze-tolerant larvae of *Eurosta solidaginis* (Fitch) (Diptera, Tephritidae) undergo numerous freeze-thaw cycles during the winter, with up to 65% of total body water being converted into extracellular ice (Storey and Storey, 1992). In these freeze-tolerant animals, the anoxic conditions brought about by ice formation in extracellular spaces (Storey *et al.* 1981; Storey and Storey, 1985) and the return to aerobic conditions parallel exactly ischaemia-reperfusion, a condition well known to lead to ROS formation and damage in a number of systems during cold or warm ischaemia-reperfusion (Halliwell and Gutteridge, 1989; Fuller *et al.* 1988; Ruuge *et al.* 1991). However, since the freeze-tolerant larvae survive this stress in nature, they must possess defence mechanisms sufficient to deal with this insult. Although the exact mechanisms of ROS formation in reperfusion are largely unknown, one commonly accepted source is the conversion of xanthine dehydrogenase (XDH) to the oxidase form by proteolytic cleavage following protease activation under anoxic conditions (McCord, 1987). Upon reoxygenation, xanthine oxidase (XO) generates superoxide as its reduction product, unlike the NADH produced by xanthine dehydrogenase. In contrast to *Eurosta solidaginis* larvae, those of freeze-avoiding *Epiblema scudderiana* (Clemens) (Lepidoptera, Olethreutidae) survive the winter cold by utilizing a freeze-avoidance strategy, where body fluids are in the liquid state throughout the season. Although freeze-avoiding organisms are not faced with the problem of oxygen reperfusion following ischaemia or anoxia, and are therefore not expected to suffer as great an oxidative stress as their freeze-tolerant counterparts, peroxidative damage is well known to occur in cold-stored tissues, even down to -80 °C (Whiteley *et al.* 1992a,b). In addition, the low steady oxidative metabolism that these larvae maintain during the winter will

undoubtedly lead to a low steady state of ROS formation. In both cold-hardiness model systems, there is therefore the potential for ROS formation and oxidative damage during overwintering.

Materials and methods

Chemicals

2-Vinylpyridine was obtained from Aldrich Chemical Co., Milwaukee, WI, USA. All other biochemicals were obtained from Sigma Chemical Co., St Louis, MO, USA, or Boehringer Mannheim Corp., Montréal, Québec, Canada. Distilled deionized water was used in the preparation of all aqueous solutions.

Animals

Galls containing larvae of *Eurosta solidaginis* and *Epiblema scudderiana* were collected from goldenrod plants in fields around Ottawa during September 1990 and kept outdoors in lightly coloured cloth sacks hanging above the snowline. At each sampling date, a few sacks containing galls were brought indoors and placed in incubators set to the outdoor temperature for that day. Galls were removed from the sacks, quickly opened, and larvae were removed. Larvae assessed to be alive by visual inspection were quickly dropped into a container of liquid nitrogen. Frozen larvae were then transferred to plastic scintillation vials for storage at -80 °C until use.

Preparation of extracts

Larvae were homogenized 1:10 (w/v) in 1.5 ml Eppendorf tubes using a glass, hand-held homogenizer. One of two homogenization buffers was used, determined by which provided the best recovery of enzyme activity. Buffer A (1 mmol l⁻¹ EDTA in 50 mmol l⁻¹ potassium phosphate, pH 7.5) was used for SOD and total XDH+XO, whereas buffer B (buffer A + 10 mmol l⁻¹ dithiothreitol) was used for GST, GR and CAT. All homogenizations were carried out in the presence of a few crystals of phenylmethylsulphonyl fluoride (approximately 0.1 mmol l⁻¹). Homogenates were centrifuged at 15 000g for 20 min at 4 °C, and the supernatant was removed and desalted by centrifugation through a Sephadex G-25 spun column (0.5 cm × 6 cm; pre-equilibrated with the appropriate buffer). The clear eluate was used as the source of all enzymes. Samples for GSTPox were homogenized 1:5 (w/v) in buffer A and were sonicated with a Kontes micro-ultrasonic cell disrupter for 15 s before centrifugation at full speed at 4 °C in a Brinkmann model 5412 centrifuge, and were then desalted through a spun column.

Antioxidant enzyme assays

All assays were conducted on a Pye-Unicam SP8-100 or a Gilford 250 recording spectrophotometer at 22 °C. All data are reported as enzyme units mg⁻¹ soluble protein.

Total SOD activity (Cu,Zn-SOD and Mn-SOD) was measured by the method of Paoletti *et al.* (1986). The assay was run in 50 mmol l⁻¹ potassium phosphate (pH 7.4) containing 2.5 mmol l⁻¹ MnCl₂, 5 mmol l⁻¹ EDTA, 3.9 mmol l⁻¹

2-mercaptoethanol (MeSH) and 0.27 mmol l^{-1} NADH. Superoxide, formed from a chemical reaction between MnCl_2 , EDTA, MeSH and oxygen, oxidizes NADH and decreases its absorbance (340 nm) at a rate dependent on superoxide concentration. In the presence of increasing SOD activity, the rate of NADH oxidation slows as a result of decreasing O_2^- concentrations. 1 unit of SOD was defined as the activity of enzyme that inhibited the oxidation of NADH by 50% and was determined from an inhibition curve (rate of NADH oxidation *versus* supernatant volume).

Catalase (CAT) activity was measured by the method of Aebi (1984) in the presence of 7.5 mmol l^{-1} H_2O_2 in 50 mmol l^{-1} potassium phosphate (pH 7.0). The removal of peroxide was measured at 240 nm ($\epsilon=0.03941 \text{ mmol}^{-1}$). 1 unit was defined as the activity of enzyme that catalysed the reduction of 1 μmole of peroxide per minute.

Selenium-independent glutathione peroxidase activity (GSTPox) was assayed in 50 mmol l^{-1} potassium phosphate (pH 7.0) in the presence of 1 mmol l^{-1} EDTA, 0.2 mmol l^{-1} NADPH, 2 mmol l^{-1} NaN_3 , 1 i.u. of GR, 5 mmol l^{-1} GSH and 1.2 mmol l^{-1} cumene hydroperoxide (Ahmad and Pardini, 1988). A blank without homogenate was used as a control for the non-enzymatic oxidation of NADPH upon addition of cumene hydroperoxide. 1 unit was defined as the activity of enzyme that catalyzed the oxidation of 1 μmole of NADPH per minute, monitored at 340 nm. Selenium-dependent glutathione peroxidase (GPox), measured with H_2O_2 in place of cumene hydroperoxide, was not detected in the larvae at any time.

GST was assayed in 50 mmol l^{-1} potassium phosphate (pH 6.5) containing 1 mmol l^{-1} EDTA, 6 mmol l^{-1} GSH and 1 mmol l^{-1} 1-chloro-2,4-dinitrobenzoic acid (CDNB) (Habig and Jakoby, 1981). The reaction was initiated by the addition of CDNB after initial velocities had been recorded to account for non-enzymatic conjugation of GSH and CDNB. 1 unit was defined as the activity of enzyme that catalysed the formation of 1 μmole of the conjugate *S*-2,4-dinitrophenylglutathione per minute, monitored at 340 nm ($\epsilon=9.61 \text{ mmol}^{-1}$).

GR was assayed by following NADPH oxidation at 340 nm in the presence of 5 mmol l^{-1} GSSG, 0.2 mmol l^{-1} NADPH and 1 mmol l^{-1} EDTA in 50 mmol l^{-1} potassium phosphate buffer, pH 7.4 (Dillio *et al.* 1983). GR activity was corrected for spontaneous reaction by measuring velocities prior to the addition of enzyme. 1 unit was defined as the activity of enzyme that reduced 1 μmole of NADPH per minute.

Xanthine dehydrogenase and oxidase assays

Total xanthine oxidase plus xanthine dehydrogenase was measured spectrophotometrically ($\epsilon_{295}=12.591 \text{ mmol}^{-1}$ for urate) in the presence of 1 mmol l^{-1} (*Epiblema scudderiana*) or 0.1 mmol l^{-1} (*Eurosta solidaginis*) xanthine and 6 mmol l^{-1} NAD in 50 mmol l^{-1} potassium phosphate, pH 7.8 (Stirpe and Della Corte, 1969). 1 unit of activity was defined as the activity of enzyme that catalyzed the formation of 1 nmole of urate per minute.

Xanthine dehydrogenase and oxidase activities were also measured by the fluorometric assay of Beckman *et al.* (1989).

Single preweighed larvae were homogenized 1:5 (w/v) with a hand-held glass homogenizer in Eppendorf tubes in ice-cold 50 mmol l^{-1} potassium phosphate (pH 7.0) containing 0.5 mmol l^{-1} EDTA. Homogenates were centrifuged for 10 min at $15000g$ in a Biolabs 13 microcentrifuge at 5°C . Supernatants were desalted by passage through a Sephadex G-25 spun column ($0.5 \text{ cm} \times 6 \text{ cm}$). Pterin ($40 \mu\text{mol l}^{-1}$ final assay concentration) was added to the desalted supernatant in 50 mmol l^{-1} potassium phosphate buffer (pH 7.0) for the determination of xanthine oxidase activity. Methylene Blue was then added ($20 \mu\text{mol l}^{-1}$ final concentration) to the same cuvette for the determination of total XDH+XO activity. Allopurinol, an inhibitor of XO and XDH, was then added to a final concentration of $40 \mu\text{mol l}^{-1}$ (*Eurosta solidaginis*) or $120 \mu\text{mol l}^{-1}$ (*Epiblema scudderiana*) to ensure the absence of non-specific activity. Finally an internal standard of isoxanthopterin (approximately 0.1 nmol per cuvette) was added to each assay to calibrate the intensity readings. The exact concentration of the isoxanthopterin standard was determined spectrophotometrically at 336 nm ($\epsilon=13.01 \text{ mmol}^{-1}$) and the final concentration added to the assay cuvette was calculated. 1 unit was defined as the activity of enzyme that catalyzed the formation of 1 nmole of isoxanthopterin per minute.

Determination of glutathione levels

Total (GSH+GSSG) and oxidized (GSSG) glutathione levels were measured using a modification of the method of Tietze (1969) by Griffith (1980). This assay monitored the change in absorbance at 412 nm in the presence of 0.6 mmol l^{-1} 5,5'-dithiobis-(2-nitrobenzoic acid), 0.21 mmol l^{-1} NADPH and 0.5 i.u. ml^{-1} glutathione reductase in 125 mmol l^{-1} sodium phosphate buffer containing 6.3 mmol l^{-1} EDTA at a pH of 7.5. Single preweighed larvae were homogenized 1:3 (w/v) in N_2 -bubbled ice-cold sulphosalicylic acid in Eppendorf tubes using a hand-held glass homogenizer. After bubbling with N_2 for approximately 30 s, the homogenates were centrifuged in a Biofuge 15 microcentrifuge at $11000g$ for 5 min at 5°C . The supernatants were divided into two samples for the measurement of total (reduced + oxidized) and oxidized glutathione. Total glutathione was measured directly from the first sample. To measure oxidized glutathione, the second sample was first derivatized by incubation with 2-vinylpyridine (170 mmol l^{-1} final concentration). Extracts neutralized to a pH between 5.5 and 7.0 with 1 mol l^{-1} potassium phosphate (pH 7.3) were assayed for GSSG after 1 h using the same assay as that described for total glutathione. Both total and GSSG values in samples were obtained by comparison with a standard curve of varying GSH concentrations. All assays were corrected for spontaneous reaction in the absence of sample or glutathione reductase.

Protein assay

Soluble protein levels in larval extracts were determined using the Bio-Rad assay kit which is based on the Coomassie Blue dye-binding method (Bradford, 1976) with a standard of bovine serum albumin.

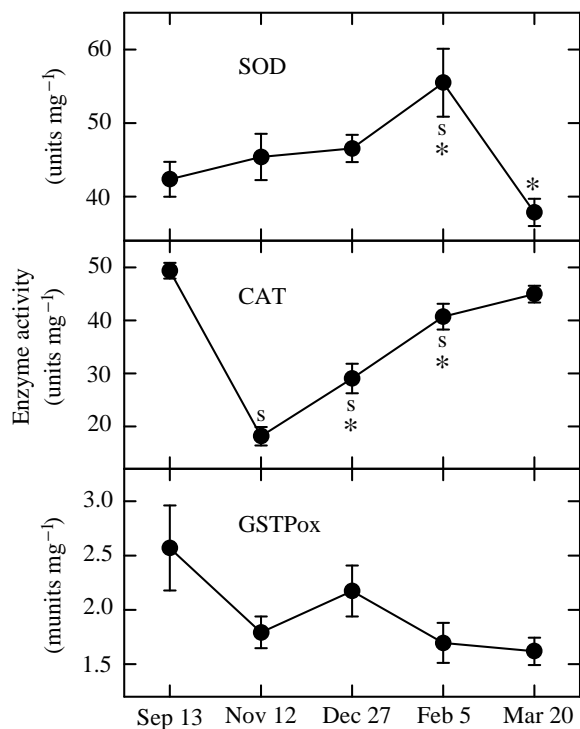


Fig. 1. Activities of superoxide dismutase (SOD), catalase (CAT) and selenium-independent glutathione peroxidase (GSTPox) in overwintering larvae of *Eurosta solidaginis*. Data are means \pm S.E.M. ^sSignificantly different from the September value; *significantly different from the value on the previous sampling date ($P < 0.05$; $N = 4-6$ samples with one larva per sample). Units of enzyme activity are defined in Materials and methods.

Statistical analyses

All enzyme data are reported as means \pm S.E.M. for $N = 4-6$ samples with one larva per sample. Metabolite levels are means \pm S.E.M. for $N = 5$ samples, one larva per sample. Data were analyzed using a one-way analysis of variance (ANOVA). When a significant F ratio was found, a Student-Neuman-Keuls test was performed to examine the difference between means; $P < 0.05$ was considered statistically significant.

Results

The activities of antioxidant enzymes and their winter profiles differed greatly between the two species of larvae. The

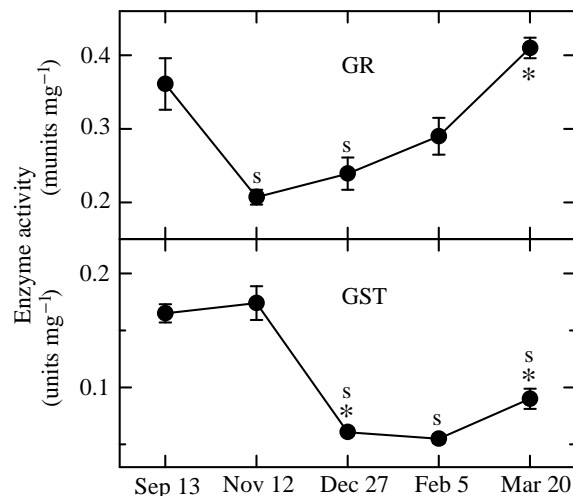


Fig. 2. Activities of glutathione reductase (GR) and glutathione-S-transferase (GST) in overwintering larvae of *Eurosta solidaginis*. Other details as in Fig. 1.

activities of the primary antioxidants, SOD and CAT, and of GST and GR were much higher (two- to fourfold) in larvae of *Epiblema scudderiana* than in those of *Eurosta solidaginis*. Only GSTPox activity was similar in the two larvae. No activity of H_2O_2 -detoxifying GPox was detected in either species.

With the exception of SOD, the activities of antioxidant enzymes in *Eurosta solidaginis* decreased over the winter (Figs 1, 2). CAT activity decreased to 37% of September values by November, and subsequently increased to a March value comparable with the initial September reading. Although not statistically significant, because of the high variance, GSTPox activity did follow a decreasing trend over the winter, the final March reading being 61% of the initial September value. GR activity decreased to 58% of September values by November and, in a pattern mimicking that of CAT, then increased to a value similar to the September value by February and March. GST activity initially remained high in the autumn, then decreased by 65% from November to December; the value remained low, with a small increase in March. SOD activity remained essentially constant over the study period, with only a slight transient increase in February larvae. Overall, these results indicate a decrease in the potential for antioxidant activity in the organism during overwintering.

Total glutathione (GSH+GSSG) levels did not change

Table 1. Glutathione status in overwintering larvae of *Eurosta solidaginis*

	13 Sep	12 Nov	27 Dec	5 Feb	20 Mar
GSH + GSSG ($mmol\ l^{-1}$)	0.89 \pm 0.03	0.91 \pm 0.04	0.91 \pm 0.04	0.80 \pm 0.02	0.81 \pm 0.04
GSSG ($\mu mol\ l^{-1}$)	13.0 \pm 0.3	7.5 \pm 0.6 ^s	24.5 \pm 2.7 ^{s,*}	13.2 \pm 4.5	5.6 \pm 0.7 ^{s,*}
% GSSG of total	2.93 \pm 0.09	1.66 \pm 0.11 ^s	5.38 \pm 0.44 ^{s,*}	3.44 \pm 1.32 [*]	1.38 \pm 0.16 ^{s,*}

Data are means \pm S.E.M.; $N = 5$ samples with one larva per sample.

^sSignificantly different from September value; *significantly different from previous sampling date value.

GSH, reduced glutathione; GSSG, oxidised glutathione.

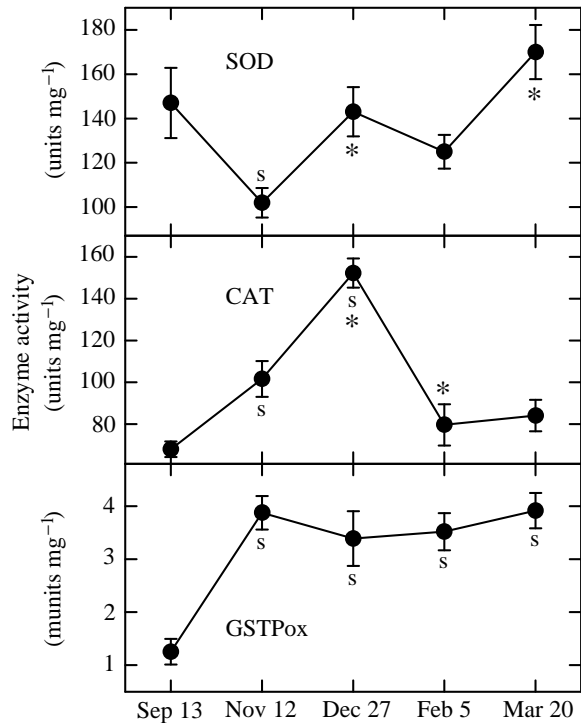


Fig. 3. Activities of superoxide dismutase (SOD), catalase (CAT) and selenium-independent glutathione peroxidase (GSTPox) in overwintering larvae of *Epiblema scudderiana*. Other details as in Fig. 1.

during overwintering in *Eurosta solidaginis* (Table 1). However, the percentage of the total that existed as the oxidized form decreased to 57% of September levels by November, but then increased to 184% of September levels by December. The levels of GSSG then decreased to less than half of the September value by March.

In contrast to *Eurosta solidaginis*, activities of most antioxidant enzymes increased during overwintering in *Epiblema scudderiana* (Figs 3, 4). CAT activities increased by 123% from September to December, returning to September levels by February. GSTPox activity increased by 210% from September to November and remained high for the remainder of the study. GST activity also increased from September to November (by 103%) but, unlike GSTPox, decreased somewhat during the remainder of the study period to a final value 33% greater than the initial September reading. SOD activities remained essentially constant over the study period,

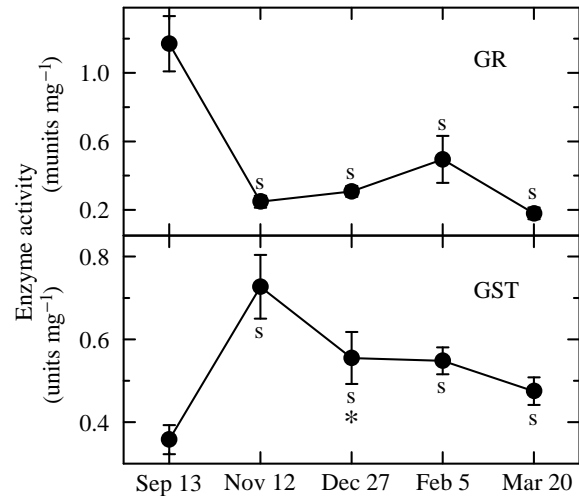


Fig. 4. Activities of glutathione reductase (GR) and glutathione-S-transferases (GST) in overwintering larvae of *Epiblema scudderiana*. Other details as in Fig. 1.

with a slight transient decrease in November and a 36% increase from February to March. Unlike the activities of other antioxidant enzymes in *Epiblema scudderiana*, GR activity decreased by November to 21% of the September value and remained low and relatively constant until March.

Total glutathione content of *Epiblema scudderiana* larvae decreased slightly over the winter to a final March level of 70% of the September value (Table 2). The percentage of the total present as the oxidized form decreased by 80% from September to November, but then increased to 52% of September levels by December, and the levels remained constant for the remainder of the study period. The changes resulted from both a decrease in GSH+GSSG and a slight increase in the content of GSSG from November onwards.

Total XDH+XO activity decreased sharply from September to late winter in both species. In *Eurosta solidaginis*, the activity decreased by March to 16% of the September value when measured spectrophotometrically and to 10% of the September value when measured fluorometrically (Fig. 5). The activity of XO alone was either not present or below the limit of detection of both assays. In *Epiblema scudderiana* larvae, the activity of total XDH+XO decreased steadily from September to activities below the limit of detection of either assay by March (Fig. 6). XO activity was detectable fluorometrically in September and November, but the activity

Table 2. Glutathione status in overwintering larvae of *Epiblema scudderiana*

	13 Sep	12 Nov	27 Dec	5 Feb	20 Mar
GSH + GSSG (mmol l ⁻¹)	1.2±0.1	0.93±0.06 ^s	0.92±0.03 ^s	0.86±0.08 ^s	0.83±0.05 ^s
GSSG (µmol l ⁻¹)	31.8±2.9	5.9±0.5 ^s	14.6±0.9 ^{s,*}	15.4±2.7 ^s	16.3±2.2 ^s
% GSSG of total	6.2±0.5	1.3±0.2 ^s	3.2±0.2 ^{s,*}	3.5±0.3 ^s	3.9±0.3 ^s

Data are means ± s.e.m.; N=5 samples with one larva per sample.

^sSignificantly different from September value; ^{*}significantly different from previous sampling date value.

GSH, reduced glutathione; GSSG, oxidised glutathione.

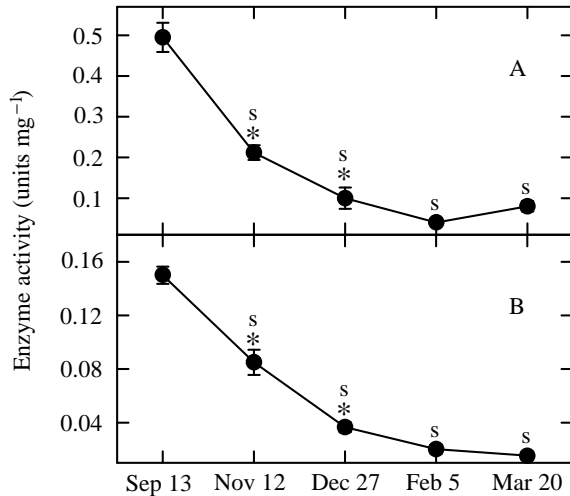


Fig. 5. Activity of total xanthine dehydrogenase + xanthine oxidase measured spectrophotometrically (A) and fluorometrically (B) in overwintering larvae of *Eurosta solidaginis*. Other details as in Fig. 1.

decreased to values below the limit of detection from December onwards.

Soluble protein content did not change significantly from September levels of 95.8 ± 2.3 and 110.4 ± 5.1 mg g⁻¹ wet mass in extracts from *Eurosta solidaginis* and *Epiblema scudderiana* larvae, respectively, over the duration of the overwintering period.

Discussion

Low temperatures alone are not sufficient to prevent ROS formation in organisms. Although Whiteley *et al.* (1992a,b) have shown that the rate of lipid peroxidation of frozen liver homogenates is reduced with decreasing temperatures, the lipid peroxide content measured as malondialdehyde produced is nonetheless increased more than fourfold after 28 days when stored at -20°C and almost twofold in similarly treated homogenates kept at -70°C . This shows that peroxidation occurs readily at low temperatures, and cold-hardy animals, even with a greatly decreased metabolic rate, are still susceptible to ROS generation and damage. They must therefore maintain sufficient antioxidant defences throughout the winter months.

The larvae of the two species showed generally opposite changes in the activities of antioxidant enzymes over the winter. Whereas antioxidant enzyme activities in *Eurosta solidaginis* largely decreased, those in *Epiblema scudderiana* were for the most part increased during the winter months. These results imply that *Eurosta solidaginis* larvae face little challenge by ROS, allowing for the observed decrease in defences, whereas *Epiblema scudderiana* larvae appear to require enhanced protection from these same damaging species.

We originally hypothesized that *Eurosta solidaginis* larvae, which undergo numerous freeze-thaw cycles (and the accompanying switch from anaerobic to oxidative metabolism)

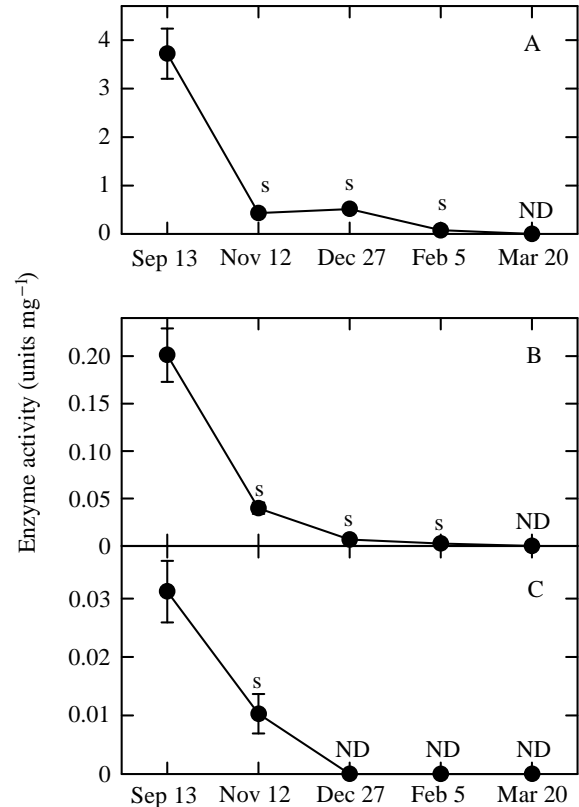


Fig. 6. Activities of total xanthine dehydrogenase + xanthine oxidase measured spectrophotometrically (A) and fluorometrically (B) and of xanthine oxidase measured fluorometrically (C) in overwintering larvae of *Epiblema scudderiana*. ND, not determined; activity below the limit of detection of the assay. Other details as in Fig. 1.

as part of their freeze-tolerance overwintering strategy, may suffer increased ROS formation as the result of oxygen reperfusion after thawing, as has been observed in many higher animal systems undergoing ischaemic episodes (Halliwell and Gutteridge, 1989; Fuller *et al.* 1988; Ruuge *et al.* 1991). However, the observed changes in enzyme activities suggest that this may not be the case. If there was a repeated severe increase in ROS after every thaw of the larvae, antioxidant activities would probably have remained high during the winter. The observed decrease then suggests either that the levels of defences maintained are sufficient to deal with the existing ROS insults or that there is little increase in ROS formation, and perhaps even a decrease in ROS formation. An interesting observation in those systems that undergo reperfusion damage by ROS formation is that there is often both a severe depletion of energy charge and a rapid increase in aerobic metabolic rate (e.g. Blasig *et al.* 1990; Younes and Strubelt, 1988). In *Eurosta solidaginis*, however, winter metabolic rate is reduced and maintained at a low, steady level, and the larvae are well adapted to long-term ischaemia and show little depletion of energy charge under natural winter conditions (Storey and Storey, 1985). These may be key adaptations in preventing ROS formation and the resulting damage in naturally tolerant animals, and they deserve more study.

Another interesting aspect of *Eurosta solidaginis* overwintering is the apparent absence of any xanthine oxidase activity. Xanthine oxidase has been implicated in ROS generation in a number of studies, notably during oxygen reperfusion (Terada *et al.* 1991; Chiricolo *et al.* 1991), since this modified form of XDH uses oxygen as an oxidizing agent which leads to superoxide formation as its reduction product. The absence of such a conversion in the freeze-tolerant larvae could indicate an adaptive strategy to reduce ROS during oxygen reperfusion during thawing. This model of *in vivo* XDH resistance to conversion to XO is not novel and has been demonstrated in a number of euryoxic marine invertebrates, where it is believed to be important for the ability of these animals to tolerate anoxia-reperfusion without cellular damage from ROS (Dykens and Shick, 1988). The detection of xanthine oxidase in the freeze-avoiding *Epiblema scudderiana* indicates that such a mechanism is not operating in this species. In both cold-hardy species, the observed winter decrease in the activity of xanthine dehydrogenase during overwintering (Figs 5, 6) shows that, at the very least, the potential for ROS from this source is greatly reduced. Although the larvae may benefit from this in terms of potential oxidative damage, a reduced risk of ROS formation is not likely to be the cause of these decreased enzyme activities. Instead, the decreasing activity of XDH in the autumn is probably a reflection of the physiological state of the larvae. Since insects generally excrete uric acid, the cessation of food intake in the autumn would lead to a reduced need for enzymes of the uric acid production pathway, including XDH.

Sustained aerobic winter metabolism, even at a reduced rate, appears to be sufficient for the production of ROS at damaging levels in freeze-avoiding *Epiblema scudderiana* larvae, as suggested by the generally increased winter activities of antioxidant enzymes. A decreased effectiveness of antioxidant systems, possibly by temperature (Q_{10}) effects, may be responsible for the observed increase in antioxidant enzyme activities to compensate. The apparent dependence on lipid metabolism during overwintering (Joanisse and Storey, 1996) may also predispose these larvae to ROS damage since fatty acids are particularly susceptible to ROS attack and their metabolism can lead to ROS formation.

There does not appear to be severe oxidative stress in *Eurosta solidaginis* or *Epiblema scudderiana* during the winter, as indicated by the glutathione status of the larvae (Tables 1, 2). Under oxidative stress, there is often an increase in GSSG as a fraction of the total glutathione pool, because reduced GSH is consumed by antioxidant enzymes and as a radical scavenger in its own right. Also, a depletion of the total pool may be associated with long-term oxidative stress. Although there was an increase in the percentage of GSSG of the total pool from November onwards in both species, these values were well within the normal range of other animals (Halliwell and Gutteridge, 1989) and of at least one other insect species, the flesh fly *Sarcophaga peregrina* (Sugiyama and Natori, 1994). In *Eurosta solidaginis*, the December and February increases follow periods of cold sufficient for

repeated freezing and thawing of the larvae (data not shown), which could suggest oxidative stress following reperfusion. The winter increase in GSSG levels in *Epiblema scudderiana*, however, cannot be explained by this mechanism. Instead, the largely aerobic and fat-burning nature of this lepidopteran, which could predispose the larvae to increased risk of ROS generation, may account for why the high GSSG content is maintained well into the spring in this species. The total pool of glutathiones decreased slightly in overwintering *Epiblema scudderiana*, consistent with a greater oxidative stress in this species, as suggested by the enzyme data. In both larvae, it is interesting to note the high September levels of GSSG, especially when compared with November readings. A possible explanation for this is found in the feeding state of the larvae. Phytophagous insects ingest various plant defence compounds, many of which have been identified as pro-oxidants. Studies have shown that insects that feed on plants containing higher levels of these compounds appear to possess greater antioxidant enzyme activities, apparently in order to overcome the plant's natural defence system (Aucoin *et al.* 1991). The goldenrod gall insects, when feeding in the early autumn, are likely to be faced with such compounds. This would lead to increased oxidative stress and may well explain the relatively high GSSG content in both gall species, as well as the higher early autumn antioxidant enzyme activities in *Eurosta solidaginis*. After cessation of feeding, oxidative stress may be reduced, explaining the decreased GSSG content and antioxidant enzyme activities found in *Eurosta solidaginis* by November.

The absence of any GPox activity in the two gall species is consistent with previous work. Unlike vertebrates, where the enzyme is believed to be a key antioxidant enzyme (Simmons and Jamall, 1988; Raes *et al.* 1987), GPox has been found only at very low activities in some insects (Ahmad *et al.* 1989; Aucoin *et al.* 1991). Insects appear to rely instead on elevated activities of catalase and GSTPox for the removal of H_2O_2 and organic hydroperoxides (Ahmad *et al.* 1988a,b, 1989). In many phytophagous insect species, including *Trichoplusia ni*, *Spodoptera eridania* and *Papilio polyxenes*, little or no activity of selenium-dependent GPox was observed (Ahmad *et al.* 1987, 1988a,b; Ahmad and Pardini, 1988; Pristos *et al.* 1988; Aucoin *et al.* 1991). Furthermore, in those cases where minimal activity was seen, the inability to inactivate insect GPox activities by boiling homogenates for 1 h (Ahmad *et al.* 1989) casts some doubt on whether these were truly enzymic in nature. The absence of GPox in phytophagous insect species has been attributed to the fact that plants do not have a requirement for selenium, and therefore it is impossible to acquire this element from dietary sources for the production of the enzyme (Ahmad *et al.* 1989). The hypothesis that phytophagous species have evolved higher levels of catalase to deal with H_2O_2 is supported by the distribution of the enzyme, which is found in all cellular components in insects and is not restricted to peroxisomes as in mammalian tissue (Ahmad *et al.* 1988a,b; Halliwell and Gutteridge, 1989). The high activity of GSTPox in insects would also serve to

compensate for the lack of GPox in removing organic hydroperoxides formed as the result of ROS attack.

The role of other antioxidants during overwintering in the gall insects is as yet unknown. In addition, both species accumulate extremely high levels of low molecular mass cryoprotectants. In *Eurosta solidaginis*, glycerol and sorbitol reach winter levels of 0.6 mol l^{-1} and 0.2 mol l^{-1} , respectively (Morrissey and Baust, 1976; Storey *et al.* 1981; Storey and Storey, 1991). In *Epiblema scudderiana*, glycerol accumulates to 2 mol l^{-1} (Rickards *et al.* 1987; Storey and Storey, 1991). It is possible that these polyols may play a role in preventing oxidative damage.

Overall, the present data show an absence of a general adaptation of antioxidant systems in insects for cold-hardiness (freeze-tolerance and freeze-avoidance). Instead, changes may relate to specific strategies of cold-hardiness. Freeze-tolerant insects, maintaining low aerobic metabolic rates and a high energy charge, and recovering from freezing in a slow, controlled fashion, may not face extensive ROS insult and therefore may require less antioxidant activity. Freeze-avoiding larvae, which maintain aerobic metabolism throughout the winter (albeit at a reduced rate) and may, as in the case of *Epiblema scudderiana*, rely on lipid metabolism for the provision of metabolic energy, could be more susceptible to challenge by ROS and may require increased defences.

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