

## Biochemistry of Anhydrobiosis in *Beddingia siricidicola*, a Biological Control Agent of *Sirex noctilio*

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**Abstract:** Proto-anhydrobiosis of the nematode, *Beddingia siricidicola*, was achieved by incubation in polyethylene glycol or various concentrations up to 4 M of glycerol. The associated changes in the levels of glycerol, unbound proline, trehalose, lipids, and glycogen were determined by alkylation strategies, followed by gas chromatography or gas chromatography/mass spectrometry. The level of glycerol reached 8.9% of dry weight, proline 2.4% of dry weight, and trehalose 8.0% of dry weight within *B. siricidicola* that were incubated in 1.5 M glycerol over 6 d, while glycerol reached 17.9% of dry weight after incubation for the same period in 4 M glycerol. Movement was thereby reduced but the nematodes from 1.5 M glycerol revived after a few minutes upon rehydrating and they were able to avoid osmotic damage by rapidly excreting the glycerol, much of it being expelled within the first hour. The potential for storage and transport of this nematode for the biological control of the pine-killing wasp, *Sirex noctilio*, was greatly improved when nematode suspensions were maintained in 1.5 M glycerol under refrigeration.

**Key words:** anhydrobiosis, *Beddingia siricidicola*, biochemistry, derivatization, gas chromatography, glycerol, osmosis, proline, protectants, *Sirex noctilio*, trehalose.

The remarkable ability of nematodes to survive adverse environmental conditions has been recently reviewed (Perry and Wharton, 2011). Some nematode species such as *Tylenchus polyhynchus*, *Anguina tritici*, and *Ditylenchus dipsaci* can become completely anhydrobiotic so that they contain no water, neither osmotically active nor inactive (Holmstrup, 2014); have no perceptible respiration (Crowe et al., 1992; Danks, 2000) and can survive for as long as 39 yr in this state (Steiner and Albin, 1946; Fielding, 1951; Limber, 1973; Perry, 1977; Norton, 1978). The dauer larvae of *Caenorhabditis elegans* can also survive extreme desiccation (Erkut et al., 2011; Erkut et al., 2013). These nematodes require an initial slow desiccation at high relative humidity (e.g., 97%, water activity [Aw] 0.97) before complete drying and then, eventually, slow rehydration to recover optimally from the anhydrobiotic state.

A feature of the biochemistry of anhydrobiotic nematodes is the production of increased levels of trehalose and glycerol during the slow desiccation or induction phase (Madin and Crowe, 1975; Womersley and Smith, 1981), but several other biochemical changes are also involved, including the formation of stress (heat shock) proteins and late embryogenesis abundant (LEA) proteins (Solomon et al., 2000; Browne et al., 2002; Goyal et al., 2003). The stage following synthesis of increased levels of trehalose and glycerol has been described as proto-anhydrobiosis (Tsai and van Gundy, 1989) and this can occur in many nematodes that cannot then proceed to full anhydrobiosis (Saeed et al., 1988; Tsai and van Gundy, 1989; Womersley and Ching, 1989; Grewal et al., 2011). In these cases, proto-anhydrobiosis gives them increased protection against

various adverse conditions. Proto-anhydrobiosis can be induced as a result of various forms of desiccation including osmotic stress (Lamitina et al., 2006; Perry and Wharton, 2011) and is exemplified by incubation in glycerol solutions (Popiel and Vasquez, 1991; Curran et al., 1992; Nugent et al., 1996; Qiu and Bedding, 2002; Chen and Glazer, 2004). Qiu et al. (2000a) have shown, using glycerol-1,1,2,3,3-<sup>2</sup>H<sub>5</sub>, not only that glycerol can readily permeate *Steinernema carpocapsae* but that permeated glycerol is incorporated into trehalose by a gluconeogenesis pathway as a consequence of osmotic stress.

The nematode, *Beddingia (Deladenus) siricidicola*, is the most important control agent of the pine-killing woodwasp, *Sirex noctilio*. The woodwasp, originally from Europe, has invaded New Zealand, Australia, South America, South Africa and, very recently, North America and is a severe pest once established (Slippers et al., 2012). The nematode has a parasitic cycle that usually results in complete sterilization of female *S. noctilio*. It also has free-living cycles that feed on the woodwasp's symbiotic fungus, *Amylostereum areolatum*, as the fungus grows throughout the killed tree (Bedding, 1967, 1972, 2009). Many *S. noctilio* females can attack and oviposit (and inject fungus) into each tree, resulting in larvae boring throughout the tree while feeding on the fungus (Gaut, 1969). When parasitized *S. noctilio* oviposit in such a tree, they deposit eggs filled with nematodes that migrate through the tree's tracheids, feeding on the fungus, reproducing and eventually changing form to parasitize any available larvae. Because the nematodes can breed throughout the tree, nearly 100% of *S. noctilio* from a nematode-infected tree may be parasitized and these woodwasps can now emerge and infect other trees.

Although *S. noctilio* females are responsible for much of the spreading of nematodes from one tree to others, it requires human intervention to optimize the spread of the nematode and its introduction into new plantations as they become susceptible to the woodwasp. This is accomplished by establishing trap trees (Haugen

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et al., 1990; Bedding, 2009; Iede et al., 2012; Carnegie and Bashford, 2012), which are then manually inoculated with about 100,000 nematodes per tree (Bedding and Akhurst, 1974). The nematodes are produced by adding monoxenic cultures on fungus aseptically to flasks of sterilized wheat/rice where they breed up and are harvested in water after about 6 wk (Bedding and Akhurst, 1974; Bedding, 2009). The resulting nematodes are then sent in aqueous suspension to forestry managers so that they can be used to inoculate trap trees throughout various pine plantations.

Presently, there are problems of survival of *B. siricidicola* during storage and transport, requiring that nematodes be refrigerated and used within a week of receipt. This is often inconvenient. By way of comparison, Bedding et al. (2003) have used nonfibrous microcellulose at Aw *c.* 0.97 to induce proto-anhydrobiosis and thus successfully store and transport various species of the entomopathogenic nematodes, *Steinernema* and *Heterorhabditis* spp. (Bedding, 2006). When *B. siricidicola* larvae and adults are gradually acclimatized in 50% glycerol, they can be stored in liquid nitrogen and have been recovered up to 24 yr later (Bedding, 1993). Liquid nitrogen storage of *B. siricidicola* has been essential to prevent strain deterioration but is impractical for storing the nematodes for field use.

The aims of this study are threefold: (i) to examine the biochemical response of *B. siricidicola* to controlled dehydration as well as to the impregnation of these nematodes with various levels of glycerol, (ii) to attempt to induce proto-anhydrobiosis artificially to facilitate storage and transport, and (iii) to test a simple and robust means of evaluating specific biochemical constituents that are relevant to proto-anhydrobiosis.

#### MATERIALS AND METHODS

*Treatment of B. siricidicola*: The *B. siricidicola* nematodes were obtained from Ecogrow Pty Ltd, Queanbeyan, NSW, and were extracted in tap water from approximately 6 wk-old monoxenic cultures feeding upon the fungus *A. areolatum* on wheat/rice in 500-ml flasks (Bedding and Akhurst, 1974). The extracted nematodes were of various larval stages but < 1% were adult. After sedimentation, the nematodes were evenly distributed on double layers of milk filter, which lined sieves just

touching the surface of water (5 cm depth) in bowls. Nematodes that had migrated through the tissues were harvested from the water beneath the sieves after 24 hr and washed by sedimentation before counting.

Counting of nematodes followed appropriate serial dilution of thoroughly bubbled stock suspension. The final dilution, based on estimates of the numbers involved, was made so that 1-ml aliquots from it contained from 100 to 200 nematodes. These were counted under a dissecting microscope with transmitted sub-stage illumination, after adding and spreading the 1-ml aliquots in the center of a counting tray having 6-mm reticulations. A transfer pipet with a 1-ml tip cut at the end so that nematodes could not cause a blockage was used for sampling. For each stock suspension, serial dilution was repeated three times and three counts were made of each final dilution. The average of the nine counts was made and this was multiplied by the dilution factors. The concentration of nematodes in the original stock solution was then adjusted as required.

Fresh nematodes were obtained from separate flasks for the initial studies of Table 1 but, for all subsequent experiments (Tables 2-6), fresh nematodes were obtained by amalgamation of the contents of several flasks. Nematodes were added to various concentrations of glycerol or polyethylene glycol (PEG, average molecular mass 6,000) at 100,000/ml and bubbled within separating funnels at room temperature (RT) at a rate just sufficient to maintain them in suspension. When the molarities for glycerol treatments were higher than 1.5 M, the nematodes were initially acclimatized in 1.5 M glycerol for 12 hr before gradual adjustment to higher concentrations. The Aws of the solutions were measured using an AquaLab instrument (CX2; Decagon Devices, Washington, DC). Aw is the relative humidity, expressed as a decimal, evaluated after equilibration in the surrounding air within a sealed container. Aw may be the most satisfactory means of describing and comparing the various types of desiccation, such as air, substrate, or osmotic drying, within different environments.

Where nematodes were stored for long-term survival experiments (Tables 5 and 6), a suspension of nematodes (10 ml containing 1 million) previously bubbled in 1.5 M glycerol (Aw 0.97) for 3 d at RT was added to each 60-ml Falcon tube stored flat under refrigeration

TABLE 1. Analyses of *Beddingia siricidicola* after treatment with polyethylene glycol (PEG) or glycerol.

Dry weight (mg)	Treatment <sup>a</sup>	Glycerol <sup>b</sup>	Trehalose <sup>b</sup>	Total lipid <sup>b,c</sup>
9.04 ± 0.17	Fresh	0.16 ± 0.03%	2.81 ± 0.21%	22.19 ± 1.72%
7.53 ± 0.22	4 d, PEG	8.93 ± 0.48%	7.41 ± 1.10%	11.89 ± 0.50%
10.68 ± 0.43	Fresh	1.30 ± 0.10%	1.84 ± 0.08%	22.38 ± 0.84%
10.86 ± 0.18	4 d, 1.5 M Glycerol	12.65 ± 0.56%	4.67 ± 0.42%	8.85 ± 0.62%

<sup>a</sup> The nematodes for the PEG and glycerol experiments were obtained from separate flasks.

<sup>b</sup> Percentage dry weight; mean ± SD (*N* = 3) of 0.5 million nematodes per treatment.

<sup>c</sup> Total lipid was estimated as the sum of the ethyl esters of palmitate (hexadecanoate), palmitoleate ((9*Z*)-hexadec-9-enoate), stearate (octadecanoate), oleate ((9*Z*)-octadec-9-enoate), vaccenate ((11*E*)-octadec-11-enoate) and linoleate ((9*Z*,12*Z*)-octadeca-9,12-dienoate).

TABLE 2. Analyses of *Beddingia siricidicola* for glycerol, proline, and trehalose with increasing concentrations of exogenous glycerol.

Dry weight (mg)	Treatment	Glycerol <sup>a</sup>	Proline <sup>a</sup>	Trehalose <sup>a</sup>
13.82 ± 0.27	Fresh	1.33 ± 0.18%	0.43 ± 0.01%	1.76 ± 0.11%
10.66 ± 0.53	6 d, water	1.40 ± 0.12%	0.17 ± 0.07%	3.64 ± 0.07%
13.18 ± 0.27	6 d, 1.5 M	8.94 ± 0.14%	2.42 ± 0.03%	7.95 ± 0.55%
20.07 ± 0.44	6 d, 3 M	16.38 ± 0.83%	1.01 ± 0.12%	8.61 ± 0.63%
17.42 ± 0.31	6 d, 4 M	17.87 ± 0.81%	0.83 ± 0.12%	9.86 ± 0.22%

<sup>a</sup> Percentage dry weight; mean ± SD (*N* = 2); 0.5 million nematodes per treatment.

at 5 ± 0.5°C, with caps slightly loosened to permit aeration. With a limited number of samples, nematodes in 1.5 M glycerol were added directly to Falcon tubes without first bubbling. Polyethylene glycol (40% in water) was used to achieve *Aw* 0.97, it having such a high molecular mass that, unlike glycerol, it is unable to penetrate the nematodes. The 3 M glycerol solutions had an *Aw* of 0.93 and 4 M glycerol solutions had an *Aw* of 0.91. These measurements of *Aw* are relative values and a correction factor is necessary to obtain absolute values (Marcolli and Peter, 2005).

**Filtration of *B. siricidicola*:** When it became evident that glycerol was rapidly excreted during washing with water, processing was performed as quickly as possible. Thus, the treated nematodes were recovered by gentle centrifugation (1,000 rpm) and decantation, with successive suspensions of the nematodes in aqueous ethanol of the same molarity as the treatment. The nematode suspension was then rapidly filtered through a glass fiber filter paper (Whatman GF/C, 1.2 µm pore size, 24 mm diameter) (Whatman, Little Chalfont, Buckinghamshire, UK) using a ceramic Buchner funnel and water pump evacuation. Before use, the individual glass fiber filter papers were dried at 100°C and weighed to the nearest 10 µg (Ohaus AP 250D electronic balance). The nematodes were washed five times on the filter paper with aqueous ethanol of the same molarity as the treatment. The nematodes were killed on the filter using the brief application of a hair dryer (*c.* 70°C) before evacuation at 5 Pa with a rotary pump and storage in a vacuum desiccator over silica gel for 6 d at RT to preserve the structural integrities of the target biomolecules. The filter papers were then reweighed to determine the amount of the nematode sample. For each treatment and control there were 0.5 million nematodes per sample, unless otherwise indicated.

**Water content of *B. siricidicola*:** To determine the amount of water in fresh *B. siricidicola* and those from incubation

in 1.5 M glycerol for 6 d, replicate suspensions of 0.5 million nematodes were filtered on glass fiber papers as above and washed thoroughly with water or 1.5 M ethanol, respectively. For each sample, the nematodes were carefully scraped from the glass fiber onto a cellulose filter paper where the nematode clump was rolled and gently pressed to remove the residual surface water (similar to Wharton, 1996). The nematode clump was weighed, vacuum dried over silica gel at RT as described above, and reweighed to evaluate the average amount of water for the fresh and incubated samples.

**Excretion from *B. siricidicola*:** Excretion experiments were carried out on replicate samples of 2 million nematodes that had been incubated in 1.5 M glycerol for 6 d at RT. Saline (0.75 M) was used to wash the filtered nematodes because it was suspected that 1.5 M ethanol might narcotize the nematodes and thereby perturb the excretion profile. However, saline was not as efficient as 1.5 M ethanol for removing superficial glycerol from the nematodes and small quantities of glycerol were detected in the surrounding water during the first 15 min that elapsed. Therefore, the initial period for assessing the rate of excretion was taken as 0.25 to 0.5 hr. Excretion of glycerol for the successive elapsed times was assessed after gentle centrifugation (1,000 rpm) and decantation. Following addition of the appropriate internal standard, the aqueous solutions were centrifuged briefly at 3,000 rpm to ensure that there had been no inadvertent transfer of buoyant nematodes or other insoluble material. Each aqueous solution was concentrated to low volume in a rotary evaporator at 50°C bath temperature, taken up in 20% aqueous ethanol and evaporated to dryness at RT under a nitrogen stream. The mean dry weight after glycerol incubation and before rehydration was estimated by extrapolation from that of 0.5 million nematodes subsequent to 6 d in 1.5 M glycerol.

TABLE 3. Analyses of *Beddingia siricidicola* for lipid content of the same samples listed in Table 2.

Treatment	Palmitate <sup>a</sup>	Palmitoleate <sup>a</sup>	Stearate <sup>a</sup>	Oleate <sup>a</sup>	Vaccenate <sup>a</sup>	Linoleate <sup>a</sup>	Linoleate/Oleate
Fresh	1.91 ± 0.37%	0.94 ± 0.42%	2.78 ± 0.54%	11.10 ± 0.09%	13.26 ± 0.38%	8.64 ± 0.02%	0.78
6 d, water	2.06 ± 1.17%	1.70 ± 0.43%	1.85 ± 0.97%	8.40 ± 1.04%	8.78 ± 1.60%	8.17 ± 0.25%	0.97
6 d, 1.5 M	0.77 ± 0.28%	0.60 ± 0.05%	1.21 ± 0.13%	4.82 ± 0.42%	5.47 ± 0.51%	4.73 ± 0.20%	0.98
6 d, 3 M	0.29 ± 0.01%	0.70 ± 0.02%	0.68 ± 0.16%	3.23 ± 0.13%	4.56 ± 0.26%	3.79 ± 0.02%	1.17
6 d, 4 M	0.20 ± 0.04%	0.35 ± 0.12%	0.50 ± 0.16%	1.71 ± 0.45%	2.29 ± 0.83%	2.56 ± 0.14%	1.5

<sup>a</sup> Percentage dry weight; mean ± SD (*N* = 2); 0.5 million nematodes per treatment.

TABLE 4. Analyses of glycerol excreted in incubation water after successive intervals from rehydrating *Beddingia siricidicola* following 6 d in 1.5 M glycerol.<sup>a</sup>

Duration	Glycerol excreted <sup>b</sup>	Glycerol excreted <sup>c</sup>	Excretion rate <sup>d</sup>
0.25–0.5 hr	0.05 ± 0.02%	0.03 ± 0.02 ng/nem	1.8 ± 1.1 pg/min/nem
0.5–1 hr	1.99 ± 0.90%	0.4 ± 0.21 ng/nem	15.6 ± 7.0 pg/min/nem
1–2 hr	2.62 ± 0.57%	0.61 ± 0.13 ng/nem	10.2 ± 2.2 pg/min/nem
2–3 hr	1.82 ± 0.39%	0.43 ± 0.09 ng/nem	7.1 ± 1.6 pg/min/nem
3–4 hr	1.54 ± 0.19%	0.36 ± 0.04 ng/nem	6.0 ± 0.7 pg/min/nem

<sup>a</sup> Mean ± SD ( $N = 3$ ); 2 million nematodes per treatment.

<sup>b</sup> Percentage dry weight excreted.

<sup>c</sup> Mean weight excreted per nematode.

<sup>d</sup> Mean excretion rate per nematode.

*Prolonged storage of B. siricidicola*: For *B. siricidicola* that had been stored under refrigeration at ~5°C for 6 mon in 1.5 M glycerol, replicate samples of 1 million nematodes were centrifuged at 1,000 rpm and, after decantation of the supernatant solution, the suspension was filtered and washed eight times with 1.5 M ethanol. The dried nematode-impregnated glass fiber filters were then processed as indicated below. Further replicate samples of 1 million nematodes that had been stored for 6 mon were retrieved by centrifugation at 1,000 rpm and the suspensions were washed three times with water. The nematodes were then left to revive in tap water for 6 hr. Each suspension was recovered by gentle centrifugation (1,000 rpm), filtered, washed eight times with water and the dried nematode-impregnated glass fiber filters were processed as indicated below.

*Internal standards for analyses of target metabolites*: The internal standards used were sucrose (for trehalose), 1,2,4-butanetriol (for glycerol), valine (for unbound proline), ethyl n-heptadecanoate or n-heptadecanoic acid (for trans-esterified lipids), and mannose for hydrolyzed glycogen.

*Biochemical extractions and analyses by Method A*: Method A is analogous to that described previously for the extraction and analyses of glycerol, trehalose and lipids in individual termites (Lacey et al., 2010) and was applied to the samples recorded in Table 1. Briefly, the lipids were extracted from the washed and dried nematodes on the filter papers with absolute ethanol at 100°C for 1 hr under nitrogen and then transformed to ethyl esters with acidified ethanol under the same experimental conditions, followed by gas chromatographic analysis. Glycerol and trehalose were extracted with water at 100°C for 0.5 hr and the dried extracts were derivatized as described below in Method B, followed by gas chromatographic analysis.

*Biochemical extractions and analyses by Method B*: The improved Method B was developed for subsequent samples (Tables 2–6) when it was discovered that the lipids could be trans-esterified directly by alkylation at RT and, moreover, that unbound proline could be rapidly derivatized by *N,O*-dialkylation. In addition, it was found that the target metabolites, other than glycogen, could be recovered from the dried glass fiber filters by sequential solvent extractions at RT. These improvements circumvented the vulnerability of unsaturated lipids to aerial oxidation and saccharides to possible degradation at the elevated temperatures described in Method A. Moreover, cellulose filter paper is quite unsuitable for recovering and processing the metabolites because it adsorbs the polar ones such as glycerol and trehalose tenaciously.

The dried nematode-impregnated filter paper was folded and placed into the barrel of a shortened Pasteur pipet (barrel 2.5 cm, stem 0.5 cm). The internal standards (800 µg 1,2,4-butanetriol, 200 µg valine, 450 µg sucrose) in 20% aqueous ethanol (0.1 ml) were applied to the paper and the solvent was evaporated. The filter paper was wetted with n-heptadecanoic acid (800 µg) in 0.2 ml dichloromethane/methanol (2:1) and the pipet was left in a closed 5-ml screw-capped glass tube (Alltech, Sydney, Australia) at RT for 1 hr. The solvent extract was centrifuged (2,000 rpm) into a screw-capped glass tube (2 ml) and the filter paper was washed thoroughly with the same solvent mixture (3 × 0.1 ml, each wetted 3 min). The combined extract was divided into two portions in screw-capped glass tubes (2 ml) and each was evaporated to dryness at RT under a nitrogen stream.

The first sample of the dried dichloromethane/methanol extract was dissolved in dimethylsulfoxide (DMSO, 0.3 ml) and excess iodoethane (C<sub>2</sub>H<sub>5</sub>I, 50 µl)

TABLE 5. Analyses of *Beddingia siricidicola* for glycerol, proline, trehalose, and glycogen after 6-mon storage in 1.5 M glycerol and subsequent revival in water for 6 hr.

Dry weight (mg)	Treatment	Glycerol <sup>a</sup>	Proline <sup>a</sup>	Trehalose <sup>a</sup>	Glycogen <sup>a</sup>
21.01 ± 0.15	6 mon	9.98 ± 1.72%	0.26 ± 0.03%	4.71 ± 0.29%	1.18 ± 0.16%
19.22 ± 0.56	6-hr revival	2.99 ± 0.36%	0.04 ± 0.01%	3.17 ± 0.48%	0.98 ± 0.15%

<sup>a</sup> Percentage dry weight; mean ± SD ( $N = 3$ ); 1 million nematodes per treatment.

TABLE 6. Analyses of *Beddingia siricidicola* for lipid content of the same samples listed in Table 4.

Treatment	Palmitate <sup>a</sup>	Palmitoleate <sup>a</sup>	Stearate <sup>a</sup>	Oleate <sup>a</sup>	Vaccenate <sup>a</sup>	Linoleate <sup>a</sup>	Linoleate/Oleate
6 mon	0.26 ± 0.04%	1.19 ± 0.20%	0.34 ± 0.10%	1.83 ± 0.27%	4.13 ± 0.50%	5.70 ± 0.71%	3.11
6-hr revival	0.29 ± 0.07%	1.38 ± 0.12%	0.35 ± 0.10%	2.11 ± 0.12%	4.42 ± 0.29%	6.74 ± 0.91%	3.19

<sup>a</sup> Percentage dry weight; mean ± SD (*N* = 3); 1 million nematodes per treatment.

by brief vortex mixing. Powdered sodium hydroxide (NaOH) (*c.* 50 mg) was added and the mixture was agitated at RT for 1 hr. Ice-cold water (0.5 ml) and hexane (0.2 ml) were added and the mixture was vortex-mixed for 10 sec to dissolve the excess NaOH. The aqueous layer was discarded and the hexane solution was washed twice with water (2 × 0.3 ml) and dried with magnesium sulfate (MgSO<sub>4</sub>). The ethyl esters of the fatty acid ligands were identified by gas chromatography/mass spectrometry (GC/MS; Varian CP3800 gas chromatograph, Combipal autoinjector, SGE BPX70 column, 30 m length × 0.25 mm i.d. × 0.25 μm phase thickness, Varian 1200 mass spectrometer, 70 eV electron ionization). The mixture of triethylglycerol and the ethyl esters of the C<sub>16</sub>-C<sub>18</sub> fatty acids, together with their derivatized internal standards, were then quantified by gas chromatography using a Varian 450, a programmable temperature volatilizing injector PTV 1079, a flame ionization detector and Varian Galaxie data acquisition and processing. The GC column was a Chrompack CP-Wax column (30 m length × 0.32 mm i.d., phase thickness 0.25 μm, 2 m retention gap) and the temperature program used was 60°C for 1 min; 15°C/min to 150°C; 40°C/min to 235°C; 235°C isothermal for 12 min.

Powdered NaOH (50 mg) was added to the solution of the second sample of the extract in DMSO (0.3 ml) and vortex-mixed for 5 sec to disperse the powder. Ethyl iodide (50 μl) was added and the mixture was agitated at RT for 6 min. Ice-cold water (0.5 ml) and hexane (0.2 ml) were added immediately to quench the reaction and the mixture was vortex-mixed for 10 sec to dissolve the excess NaOH. The aqueous layer was discarded and the hexane solution was washed twice with water (2 × 0.3 ml) and dried (MgSO<sub>4</sub>). The mixture of *N,O*-diethylproline and triethylglycerol, together with their derivatized internal standards, were separated on the same CP-Wax column using the temperature program: 60°C for 1 min; 10°C/min to 200°C; 200°C isothermal for 1 min.

After extraction with dichloromethane/methanol, the filter paper within the Pasteur pipet was dried, wetted with 20% aqueous ethanol (0.2 ml) and left in a closed screw-capped glass tube (5 ml) at RT for 1 hr. The extract was centrifuged (2,000 rpm) into a screw-capped glass tube (2 ml) and the filter paper was washed thoroughly with the same solvent mixture (3 × 0.1 ml, each wetted 3 min). The combined extract was evaporated under a nitrogen stream. The dried extract was dissolved in DMSO (0.3 ml) and C<sub>2</sub>H<sub>5</sub>I (50 μl) by vortex-mixing briefly. Powdered NaOH (50 mg) was added and the

mixture was agitated at RT for 1 hr. Crushed ice (0.5 g) and hexane (0.2 ml) were added and the mixture was vortex-mixed for 15 sec to dissolve the excess NaOH. The aqueous layer was discarded and the hexane solution of derivatized disaccharides was washed twice with water (2 × 0.3 ml) and dried (MgSO<sub>4</sub>). Octaethyltrehalose and octaethylsucrose were analyzed on the same CP-Wax column using the temperature program: 60°C for 1 min; 40°C/min to 235°C; 235°C isothermal for 20 min.

Glycerol and 1,2,4-butanetriol in the dried solutions from the excretion experiments (Table 4) were perethylated as indicated above for 6 min. The signals for any excreted proline were not detectable. Likewise, when portions of the solutions were separately evaporated and perethylated as above for 1 hr, there were no detectable signals for excreted trehalose.

When it became evident that trehalose was not being excreted, a procedure was developed to enable glycogen analyses for the nematodes that had been stored for 6 mon in 1.5 M glycerol and revived thereafter in tap water for 6 hr. After extraction of the other components (glycerol, unbound proline, lipids, and trehalose) from the glass fiber filters, water (0.2 ml) was applied to the paper within the Pasteur pipet, which was then heated within the closed screw-capped glass tube (5 ml) at 100°C for 20 min. The aqueous extract was centrifuged into a sample tube (2 ml) at 2,000 rpm. The filter paper was washed thoroughly with water (3 × 0.1 ml, each wetted 3 min at 100°C) and the combined extract was evaporated to dryness under a nitrogen stream. The residue was dissolved in 5% hydrochloric acid (0.1 ml) and heated in the closed tube at 100°C for 1 hr to hydrolyze the glycogen completely to glucose. The amount of covalently bound protein in glycogen is very small (Calder, 1991) and was therefore ignored in the subsequent calculation. Mannose (400 μg) was added as internal standard and the solution was evaporated to dryness under a nitrogen stream. The residue was extracted with 10% aqueous ethanol (0.1 ml), centrifuged and the supernatant solution of glucose and mannose was evaporated to dryness. The residue was dissolved in DMSO (0.3 ml) and powdered NaOH (50 mg) was added. The mixture was vortex-mixed for 5 sec and, after addition of C<sub>2</sub>H<sub>5</sub>I (50 μl), it was agitated at RT for 30 min. The reaction mixture was quenched with ice-cold water (0.5 ml) and hexane (0.2 ml) and vortex-mixed for 10 sec to dissolve the excess NaOH. The aqueous layer was discarded and the hexane solution was washed twice with water (2 × 0.3 ml) and dried (MgSO<sub>4</sub>). Pentaethylglucose

and pentaethylmannose were analyzed on the same CP-Wax column using the temperature program: 60°C for 1 min; 10°C/min to 235°C; 235°C isothermal for 2 min. The peaks for the pyranose anomers were summed for quantification and the quantity of glycogen was estimated as 90% of that of glucose, since hydrolysis of glycogen increases the mass of the glucosyl units.

## RESULTS

*Analyses of B. siricidicola after treatment with PEG or glycerol:* The initial experiments using Method A (Table 1) compared dehydration of *B. siricidicola* in 1.5 M glycerol for 4 d at RT (where, presumably, as in *S. carpocapsae* [Qiu et al., 2000a], some glycerol can penetrate) and in PEG 6000 solution at the same  $A_w$  (0.97) and for the same duration (where penetration cannot happen). The nematodes in the glycerol or PEG solution shrank over 6 to 8 hr and ceased movement, but then reinflated and resumed some movement during the next few hours. High levels of glycerol and trehalose accumulated in both cases (Table 1) and the dry weight dropped by 17% after 4 d in PEG, but not in glycerol.

*Analyses of B. siricidicola for glycerol, proline, trehalose, and lipids with increasing concentrations of exogenous glycerol:* Following the improvements in the extraction and derivatization techniques (Method B), the levels of internal glycerol, unbound proline, trehalose, and lipids were evaluated in individual samples dehydrated in glycerol solutions up to 4 M for 6 d at RT (Tables 2 and 3). When the concentration of the external glycerol was increased from 1.5 M to 4 M, the dried nematodes weighed 32% more and the internal glycerol content rose from 8.9% to 17.9% of dry weight (Table 2). The level of unbound proline increased following incubation in glycerol solutions, to a maximum of 2.4% of dry weight. Trehalose levels also responded to the increase in molarity of the external glycerol solutions (Table 2) while the total lipid content diminished (Table 3).

The measured water content of replicate samples (mean  $\pm$  SD) was  $76.8 \pm 2.6\%$  ( $N=5$ ) for fresh *B. siricidicola* and  $79.0 \pm 3.7\%$  ( $N=4$ ) for those incubated in 1.5 M glycerol for 6 d.

Thus, the estimated concentrations of potential osmolytes in the water content of *B. siricidicola* after the latter treatment (Table 2) were 0.26 M for glycerol, 0.08 M for proline, and 0.06 M for trehalose. Method B was used to search for additional polyhydric alcohols (ethylene glycol, erythritol, ribitol, mannitol, sorbitol, and *myo*-inositol) that might have served complementary roles as osmolytes but none was evident. It is recognized that potential osmolytes of low molecular mass could evaporate during vacuum drying of the nematodes and therefore would be invisible to the current procedures for analysis.

*Analyses of glycerol excreted in incubation water after successive intervals from rehydrating B. siricidicola following 6 d in 1.5 M glycerol:* When *B. siricidicola* nematodes were

placed in water after incubation for 6 d in 1.5 M glycerol, they did not burst and excess glycerol was excreted from the nematode into the surrounding water, following an induction period of about 0.5 hr (Table 4). We found that after 4 hr in water the level of excess internal glycerol excreted was 8.0% of dry weight. More than half of the internal glycerol (4.7% of dry weight, Table 4) was excreted in the first 2 hr, though neither the excess trehalose nor proline was excreted during 4 hr (data not shown).

*Analyses of B. siricidicola for glycerol, proline, trehalose, and glycogen after 6-mon storage in 1.5 M glycerol and subsequent revival in water for 6 hr:* Samples of *B. siricidicola* that had been stored for 6 mon in 1.5 M glycerol and subsequently revived in water for 6 hr were analyzed (Tables 5 and 6). The revived nematodes excreted, over this period, 7.0% of dry weight of glycerol (Table 5). In addition to the excretion of glycerol, there were reductions in the levels of unbound proline and trehalose but the percentage dry weight of glycogen did not increase (Table 5). It was also evident that the ratio of linoleate to oleate was much higher after 6-mon storage (3.11, Table 6) than after only 6-d storage in 1.5M glycerol (0.98, Table 3).

## DISCUSSION

Most of the previous studies on biochemical changes following osmotic stress were performed on the rhabditid nematodes *Steinernema*, *Heterorhabditis*, and *Caenorhabditis* (Popiel and Vasquez, 1991; Qiu et al., 2000a; Qiu and Bedding, 2002; Chen and Glazer, 2004; Lamitina et al., 2004; Lamitina and Strange, 2005). However, this study concerns the osmotic dehydration of a tylenchid nematode.

The phenomenon of shrinkage and reflation of *B. siricidicola* after dehydration has also been observed in other nematodes (Lamitina et al., 2004; Strange, 2007) and in mammalian cells (Lang et al., 1998), although, for *C. elegans*, shrinkage occurs within 5 min with some reflation occurring from 6 to 12 min afterward (Lamitina et al., 2004; Strange, 2007). Reflation may occur as the internal osmotic pressure increases with the level of organic osmolytes. The relatively slow loss of water from *B. siricidicola* indicates that this species is an innate dehydration strategist (Perry and Moens, 2011), allowing time to enable the various biochemical changes required for long-term survival. Nevertheless, since the usual environment for this species is within pine tracheids, often deep within the wood, desiccation would generally be slow. While *B. siricidicola* do not fully coil as a result of desiccation, they become C shaped during osmotic dehydration and exhibit the contraction of the cuticular annuli resulting in the "concertina" effect described by Rossner and Perry (1975). This is observable in most *B. siricidicola* stages before reflation, even in 1.5 M glycerol, and is quite pronounced at higher molarities.

Lang et al. (1998) drew attention to the rapid accumulation of electrolytes  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Cl}^-$  and activation

of the  $\text{Cl}^-/\text{HCO}_3^-$  exchange in mammalian cells exposed to hypertonic conditions and then, because of the toxicity of these inorganic ions, their gradual replacement by organic osmolytes. While this may give some clues as to what happens during the initial shrinkage of nematodes, mammalian cells are only subjected to relatively minor perturbations in external osmolarity. An LEA protein, similar to LEA proteins conferring dehydration in plants, has been found in the anhydrobiotic nematode, *Aphelenchus avenae*, and this responds to dehydration by changing from the unfolded to the folded state (Goyal et al., 2003). It is unknown whether this change could have any effect on the Aw of the nematode but we consider that, while proteins may have some influence (Halling, 1990), there must be other factors at work. Of course, nematode shrinkage, combined with increased osmolyte concentrations at much higher exogenous osmolarities, can ensure equilibration of the internal and external osmotic pressures.

Lamitina et al. (2004) found that, not only does *C. elegans* synthesize glycerol when adapted to and grown on media containing up to 0.5 M NaCl, it reduces glycerol levels by excretion during recovery from hypertonic stress. They postulated that swelling triggers passage of glycerol into the pseudocoelomic fluid and thence to the exterior via the excretory system and/or intestinal epithelium. We have not yet found sites of glycerol excretion in *B. siricidicola* but an additional possibility could be passage by means of aquaporins, proteins that allow transport of small molecules, including glycerol, through membranes (Campbell et al., 2008). Huang et al. (2007) found that two of eight aquaporin genes from *C. elegans* expressed in the oocytes of the frog *Xenopus* enabled transport of both water and glycerol and one transported only glycerol, while Lamitina et al. (2004) showed upregulation of a nematode aquaglyceroporin homolog after hypertonic stress. It has been claimed that osmotic dehydration is different from evaporative dehydration in that, while osmotic stress, freezing, and evaporative dehydration remove freezable water from the system, only evaporative dehydration removes non-freezable water (Hazel and Williams, 1991). Barrett (2011) points out that, as a result of this difference, different solutes can be utilized colligatively to protect against osmotic stress whereas trehalose is the most effective for evaporative dehydration. Moreover, Chen et al. (2006) found two proteins that were upregulated by evaporative dehydration but not by osmotic dehydration. Although these distinctions may well be true for nematodes able to undergo extreme desiccation, it is uncertain whether they also apply at the high Aws promoting proto-anhydrobiosis, particularly for the majority of nematodes that are unable to achieve complete anhydrobiosis. Furthermore, results from osmotic dehydration at high Aws can be more accurate than those from evaporative dehydration because, for the latter, it is difficult to maintain air at precise relative humidities and to dehydrate the nematode evenly.

The loss of dry weight after 4 d in PEG but not in glycerol for the same period and same Aw (Table 1) suggested that proto-anhydrobiosis requires significant energy consumption and this can be provided partly by glycerol permeating from solution. The overall osmolarity may be controlled by the high-osmolarity glycerol (HOG)-signalling pathway (Hohmann, 2002). It was evident from the results in Table 1 that a proportion of the glycerol content of *B. siricidicola* after 4-d incubation in 1.5 M glycerol was derived by biosynthesis, rather than solely by permeation through the cuticle. These results are consistent with those obtained previously with *S. carpocapsae* through studies with isotopically labelled glycerol (Qiu et al., 2000a). The estimated level of total internal glycerol (0.26 M) after 6 d in 1.5 M glycerol (Table 2) indicated that other, as yet unidentified, compounds were involved to enable osmotic equilibrium. Moreover, this internal glycerol concentration is exceeded by the 5 M level accumulating in a wasp larva (Salt, 1961) and the 10 M level in the beetle *Cucujus clavipes* (Bennett et al., 2005).

When *B. siricidicola* were rehydrated after 6 d in 1.5 M glycerol, any possible osmotic damage was obviated by the low permeability of the cuticle and the rapid excretion of glycerol (Table 4). *B. siricidicola* that had been stored for 6 mon in 1.5 M glycerol also excreted excess glycerol during revival over 6 hr (Table 5). However, it was evident that the excess trehalose, instead of lipids (Table 6), was expended during this revival process and was not being anabolized to glycogen (Table 5).

While the total lipids diminished with the increasing molarity of external glycerol, the proportions of the fatty acid ligands did not decline evenly (Table 3). For instance, the increase in the ratio of linoleate to oleate indicated that the more saturated lipids were being consumed at a greater rate than the more unsaturated lipids with the increasing molarities (Table 3). A similar trend was evident in the ratio of palmitoleate to palmitate (Table 3).

For the nematodes stored for 6 mon, the increased ratio of linoleate to oleate (Table 6) indicated that there was a gradual trend toward the more unsaturated fatty acid ligands over the more saturated ligands during the prolonged period, which would be an additional protective mechanism through the maintenance of membrane fluidity (Burnell and Tunnacliffe, 2011).

In addition to their colligative properties, glycerol, unbound proline, and trehalose exhibit non-colligative properties. Glycerol may inhibit Maillard (browning) reactions and oxidations that otherwise would have led to free radicals (Crowe and Crowe, 2008) and it acts as a chemical chaperone that assists in protein refolding (Strange, 2007). Unbound proline can be important in stabilizing membrane phospholipids (Rudolph et al., 1986) and it may have a role as a scavenger of reactive oxygen species, as occurs in plants (Ben Rejeb et al., 2014). Trehalose, a nonreducing disaccharide, is particularly able to interact with and protect macromolecules and membranes during desiccation, thus stabilizing them at

low Aw (Behm, 1997; Crowe et al., 1998; Crowe and Crowe, 2008; Crowe, 2014), and can also inhibit Maillard reactions (Loomis et al., 1979). Trehalose has a low tendency to crystallize and a high ability to replace water and interact favorably with heat-stress proteins (Ring and Danks, 1998; Watanabe, 2006). Under conditions of extreme anhydrobiosis, it is a protectant superior to protein chaperones (Tapia and Koshland, 2014). Nevertheless, nematodes that cannot enter the complete anhydrobiotic state still synthesize increased levels of trehalose and glycerol if exposed to Aw *c.* 0.97 for a limited time and these nematodes can then survive for considerably longer than untreated nematodes (Bedding, 1987; Popiel et al., 1987; Bedding et al., 2003). The *B. siricidicola* nematodes stored in 1.5 M glycerol may be suitable for field use, removing the requirement for forestry personnel to use them within a week of receipt.

Production of metabolites of low molecular masses, such as polyhydric alcohols, unbound amino acids, and disaccharides, is only a part of a nematode's response to dehydration. For example, Erkut et al. (2013) found in dauers of *C. elegans* that 1,833 genes were upregulated while 2,433 genes were downregulated and concluded that these genes are relevant during anhydrobiosis. Ten new proteins were produced and a further 10 upregulated when *S. feltiae* nematodes were incubated in 2.6 M glycerol (Chen et al., 2006). While genomic and proteomic studies of anhydrobiosis are of great interest, it may be that qualitative and quantitative metabolomic investigations of various dehydration regimes will be more immediately informative. For instance, we have found that relative levels of such metabolites in *S. carpocapsae* vary considerably in response to small changes in Aw during induction of proto-anhydrobiosis (unpubl. data).

To enable the derivatizations of the target metabolites in *B. siricidicola* for GC or GC/MS analyses in this study, strategies have been developed through various adaptations of a simple method for methylation (Ciucanu and Kerek, 1984; Ciucanu, 2006). Ciucanu's technique has been used extensively and almost exclusively for the methylation of oligosaccharides, principally for the elucidation of their molecular structures by MS (Dell, 1990; Ciucanu, 2006; Harvey, 2011). We have discovered that, in addition to low-mass saccharides such as trehalose, this technique can permit the derivatization of small quantities of glycerol, unbound proline, and glycogen, transesterification of lipids and esterification of carboxylic acids under mild experimental conditions.

Although we have previously used trimethylsilylation to derivatize metabolites of nematodes (Qiu et al., 2000a, 2000b; Pelleroni et al., 2003) and alpine termites (Lacey et al., 2010), alkylation has a number of advantages over the former technique. The peralkyl derivatives are resistant to hydrolysis and, if necessary, they can be refined by liquid chromatography procedures before GC or GC/MS analysis. They are suitable for autoinjection and they do not contaminate a flame ionization detector

with siliceous residues. The reagents for alkylation are readily available and inexpensive. They need not be strictly anhydrous and the organic solutions can be concentrated for analysis. Moreover, alkylations are readily accomplished at RT. Homologous peralkyl derivatives are easily synthesized by using the appropriate reagent, such as iodomethane, iodoethane, or 1-iodobutane. Such homologs can be used to validate a structural assignment, to confirm a quantitative result or to avoid interference from the chromatographic background.

Furthermore, the modest mass increase for methylation or ethylation of otherwise involatile molecules containing multiple exchangeable hydrogen atoms is a significant advantage for GC/MS analyses using benchtop mass spectrometers. The peralkyl derivatives can be analyzed both on nonpolar and polar GC columns, which can be of particular advantage because isomeric compounds are often differentiated more easily on polar columns. Thus, it was convenient in this study to use the same polar GC phase for the analyses. Finally, many of the nontarget compounds, especially the involatile ones, are readily removed from the peralkyl products through aqueous alkaline washing during the subsequent processing.

An analogous single-step method for the *N,O*-alkylation of unbound amino acids has been previously proposed by Pettitt and Stauffer (1970) but their procedure (reaction with 2-bromopropane and sodium hydride in DMSO for 24 hr) was protracted and sometimes unsuccessful (Blessington and Fiagbe, 1972). The present method for *N,O*-alkylation was simpler and the brief reaction time of 6 min for valine and proline was designed in these particular cases to minimize the formation of tertiary ammonium derivatives (valine) or involatile quaternary ammonium derivatives (proline). The steric hindrance imparted by the use of reagents such as 2-iodopropane can help to curtail such superfluous alkylation, though a more prolonged reaction time would be required for these reagents and for unbound amino acids containing additional functional groups.

Glycerol has been used previously to initiate proto-anhydrobiosis for subsequent storage of nematodes at low temperatures. It is convenient, nontoxic, and is a compatible solute. Several groups (Popiel and Vasquez, 1991; Curran et al., 1992; Bedding, 1993; Galway and Curran, 1995; Nugent et al., 1996) have used it successfully for the liquid nitrogen storage of various species of *Steinernema*, *Heterorhabditis*, and *Pratylenchus* and of *B. siricidicola*. Popiel and Vasquez (1991) were unsure whether glycerol penetrated *S. carpocapsae* infective juveniles during the induction of proto-anhydrobiosis, but Qiu et al. (2000a), using isotopically labelled glycerol, found that much of the glycerol within these nematodes had indeed penetrated from the external solution.

In the natural environment, as pine trees gradually dry, proto-anhydrobiotic induction of *B. siricidicola* nematodes within the tracheids is likely to occur and these nematodes will not be able to feed on fungus or



parasitize *S. noctilio* larvae at the diminished Aws. Nevertheless, in regions of winter rainfall, nematodes may revive and resume feeding, reproduction and parasitism as the wood becomes moister. In regions with limited winter rainfall, such as most of the areas with pine forests in South Africa, nematode parasitism, even in trap trees, is very low indeed (Hurley et al., 2012). Despite the inconvenience involved, it may well be worthwhile collecting billets from *S. noctilio*-infected trees at suitable sites in South Africa early in the season, inoculating them with nematodes and maintaining adequate water levels prior to redistribution of the moist billets in infested areas just before emergence of the woodwasps.

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