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Diapause termination and development of encysted *Artemia* embryos: roles for nitric oxide and hydrogen peroxide

Heather M. Robbins[1], Gilbert Van Stappen[2], Patrick Sorgeloos[3], Yeong Yik Sung[3,4], Thomas H. MacRae[1,*] and Peter Bossier[2]

[1]Department of Biology, Dalhousie University, Halifax, NS, Canada, B3H 4J1, [2]Lab Aquaculture & Artemia Reference Center, Faculty of Bioscience Engineering, Ghent University, Rozier 44, 9000 Gent, Belgium, [3]Department of Fisheries and Aquaculture, Faculty of Agrotechnology and Food Science, University Malaysia Terengganu, 21030, Kuala Terengganu, Malaysia and [4]Institute of Tropical Aquaculture (AQUATROP), University Malaysia Terengganu, 21030, Kuala Terengganu, Malaysia

*Author for correspondence (tmacrae@dal.ca)

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SUMMARY

Encysted embryos (cysts) of the brine shrimp *Artemia* undergo diapause, a state of profound dormancy and enhanced stress tolerance. Upon exposure to the appropriate physical stimulus diapause terminates and embryos resume development. The regulation of diapause termination and post-diapause development is poorly understood at the molecular level, prompting this study on the capacity of hydrogen peroxide (H_2O_2) and nitric oxide (NO) to control these processes. Exposure to H_2O_2 and NO, the latter generated by the use of three NO generators, promoted cyst development, emergence and hatching, effects nullified by catalase and the NO scavenger 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide (PTIO). The maximal effect of NO and H_2O_2 on cyst development was achieved by 4 h of exposure to either chemical. NO was effective at a lower concentration than H_2O_2 but more cysts developed in response to H_2O_2. Promotion of development varied with incubation conditions, indicating for the first time a population of *Artemia* cysts potentially arrested in post-diapause and whose development was activated by either H_2O_2 or NO. A second cyst sub-population, refractory to hatching after prolonged incubation, was considered to be in diapause, a condition broken by H_2O_2 but not NO. These observations provide clues to the molecular mechanisms of diapause termination and development in *Artemia*, while enhancing the organism's value in aquaculture by affording a greater understanding of its growth and physiology.

Key words: diapause, nitric oxide, hydrogen peroxide, *Artemia*.

INTRODUCTION

The brine shrimp *Artemia* avoids predation and competition by residing in high salinity habitats where they frequently experience drying, anoxia, food depletion and temperature fluctuation. To survive environmental stress these crustaceans undergo two different developmental pathways, with females consequently releasing either swimming larvae (nauplii) or encysted gastrulae (cysts) (MacRae, 2003). The larvae undergo several moults to reach adulthood and then reproduce, but cysts enter diapause, a physiological condition where development stops, metabolism is greatly reduced and stress tolerance is high (Drinkwater and Clegg, 1991; Clegg, 1997; MacRae, 2003; MacRae, 2005). Resistance to stress depends on the rigid, semi-permeable cyst shell (Anderson et al., 1970; Morris and Afzelius, 1967; Van Stappen, 1996), trehalose (Clegg and Jackson, 1998) and the accumulation of molecular chaperones such as p26, ArHsp21, ArHsp22 and artemin which prevent irreversible protein denaturation and inhibit apoptosis (Liang and MacRae, 1999; Villeneuve et al., 2006; Sun et al., 2006; Chen et al., 2007; Qiu and MacRae, 2008a; Qiu and MacRae, 2008b).

The structure and stress tolerance of *Artemia* cysts are relatively well characterized, with both contributing to diapause maintenance, but information on diapause induction and termination has been slower to emerge. *Artemia* embryos presumably enter diapause in response to a cue from the female but the signal and its origin are unknown. Several up-regulated genes have been identified in diapause-destined embryos at 2 days post-fertilization, and one of these encodes a homologue of the mammalian transcription cofactor p8, a protein with the potential to regulate cell growth, development, apoptosis and stress tolerance (Qiu et al., 2007; Qiu and MacRae, 2007). Diapause termination in *Artemia* has yet to be examined systematically at the molecular level although the proteome of *A. sinica* diapause cysts has been investigated (Zhou et al., 2008), as have changes in the proteome of post-diapause *A. franciscana* cysts (Wang et al., 2007). Exposure to specific environmental stimuli such as light, desiccation and cold promotes resumption of cyst development and metabolism, and these conditions are habitat specific with variation among cyst populations (Drinkwater and Crowe, 1987; Van Der Linden et al., 1988; Drinkwater and Clegg, 1991; Nambu et al., 2008). For example, *A. franciscana* from the San Francisco Bay, a highly variable environment, terminate diapause in response to several cues including either cold or drying whereas *A. franciscana* from the Great Salt Lake required both drying and cold, although there is contrary evidence regarding this latter point (Nambu et al., 2008). *Artemia monica,* found in Mono Lake, a large Alpine lake, terminates diapause only after a long cold period but not in response to drying. Additionally, several empirically developed techniques which are strain/batch dependent and of varying effectiveness have been employed to terminate *Artemia* cyst diapause, including dehydration/rehydration, freezing, cold storage, decapsulation and exposure to hydrogen peroxide (H_2O_2) (Van Stappen, 1996; Van Stappen et al., 1998). In this report the effect of nitric oxide (NO) and H_2O_2 on the development of *Artemia* embryos was investigated, confirming that H_2O_2 ends diapause and showing for the first time that both H_2O_2 and NO modulate cyst development. The results suggest cell/molecular mechanisms that control diapause termination and promote post-diapause development, poorly understood processes in many animal species. Furthermore, the work has implications in aquaculture because *Artemia* is used extensively in the diets of crustaceans and fish (Sorgeloos, 1980), as well as in forestry and agriculture, where the ability to undergo diapause increases the destructive potential of pest insects.

MATERIALS AND METHODS

Artemia cysts

Encysted embryos (cysts) from a parthenogenetic strain of *Artemia* were harvested in Bolshoye Yarovoy, Russia, during 2005–2006 [Artemia Reference Center (ARC) code number BY 1706] (Baitchorov and Nagorskaja, 1999; Van Stappen et al., 2009). These cysts, which were received dry (water content 10.1%) and stored vacuum packed at 4°C, were used in this study as an alternative to the more commonly studied *A. franciscana* because they were readily available, slow to break dormancy during storage and yielded only 25% hatching when incubated in non-supplemented sea water in capped tubes. The tendency to remain in dormancy provided a constant supply of uniform cysts over a long time period and a broad range over which to examine breakage of dormancy because hatching of control cyst populations was low. Cysts, stored in the dark before use, were incubated at 26–28°C with constant illumination in 25 ml of sea water under varying experimental conditions as detailed below. Cyst development was the same with Instant Ocean® (Belcopet, Brugge, Belgium) artificial sea water and with 0.22 μm filtered sea water from the Northwest Arm, Halifax, NS, Canada, both at 32 g l^{-1} salinity, and with mixing by rotation or on a reciprocating shaker (P>0.9).

Additionally, a population of *A. franciscana* Kellogg 1906 cysts approximately 85% in diapause was obtained from the Great Salt Lake in UT, USA, as a gift from Dr Brad Marden, and used to test the effects of H_2O_2 and NO on diapause termination (see below).

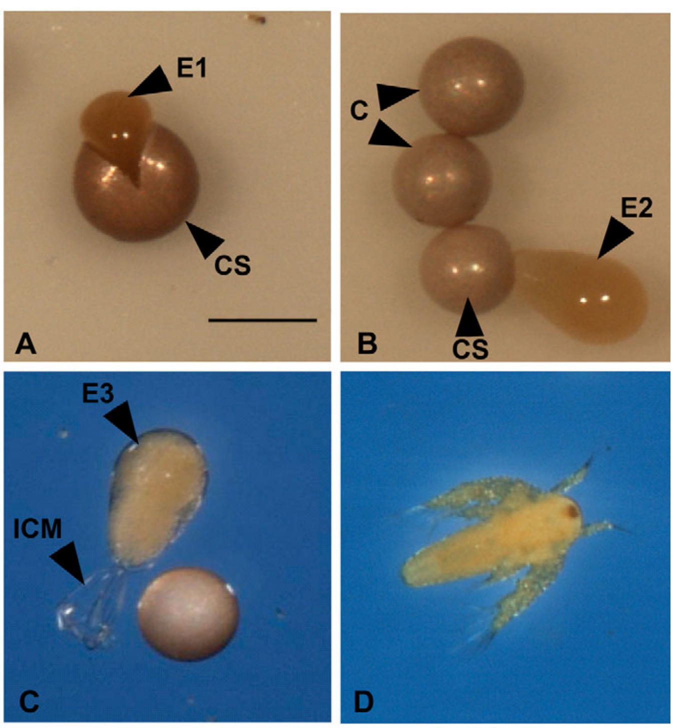

Fig. 1. *Artemia* life history stages used for developmental quantification. *Artemia* were fixed in Lugol's solution and photographed with a Nikon AZ 100 microscope. (A) An E1 larva emerging through the crack in a cyst shell; (B) a completely emerged E2 larva enclosed in a hatching membrane and attached to a cyst shell; (C) an E3 larva released from a cyst but enclosed in a hatching membrane with the inner cuticular membrane attached; (D) hatched (swimming) larva shortly after rupture of the hatching membrane. C, cyst; CS, cyst shell; ICM, inner cuticular membrane. The bar in A represents 260 μm and all figures are the same magnification.

Promotion of cyst development by NO and H_2O_2

Tightly capped 50 ml plastic tubes containing 95.0±0.5 mg of *Artemia* cysts in Instant Ocean® artificial sea water supplemented separately with the NO donors 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine (Papa NONOate; half-life 15 min), *N*-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]-1,3-propanediamine (Spermine NONOate; half-life 39 min) and 3-morpholine syndnonimine (Sin-1 chloride; half-life 20 h), or with H_2O_2 (Sigma-Aldrich, St Louis, MO, USA) were incubated with rotation. To quantify larval emergence and hatching (Fig. 1) (Go et al., 1990; Rafiee et al., 1986), which served as measures of cyst development, 6 samples of 250 μl from each of three 50 ml tubes were mixed individually with 250 μl of sea water and 2 drops of Lugol's solution (Van Stappen, 1996) prior to counting with the aid of a dissecting microscope. Two drops of NaOCl [14% (technical) active chlorine] and NaOH (32% w/v) were then added to dissolve cyst shells, revealing non-hatched embryos for counting. The extent of development was determined as the percentage of developed cysts, which was calculated as either the number of hatched larvae or the number of hatched and emerged larvae, obtained from 100 cysts (represented by the sum of hatched larvae, emerged larvae and undeveloped cysts containing embryos).

To determine whether development was dependent on NO generation three capped 50 ml plastic tubes containing 95.0±0.5 mg of cysts in Instant Ocean® artificial sea water supplemented individually with 0.5 μmol l^{-1} Papa NONOate, 6.0 μmol l^{-1} Spermine NONOate and 6.0 μmol l^{-1} Sin-1 chloride were incubated with rotation for 48 h after addition of the NO scavenger 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) (Sigma-Aldrich) to 300 μmol l^{-1}. In related experiments, a volume of 0.88 mol l^{-1} H_2O_2 sufficient to give a final concentration of 0.18 mmol l^{-1} upon addition to culture tubes was exposed to bovine liver catalase (Sigma-Aldrich) at 0.0024 mg ml^{-1} in 0.05 mol l^{-1} potassium phosphate buffer, pH 7.0, for 60 min at 25°C. The H_2O_2–catalase mixture was then put in four capped 50 ml plastic tubes containing 95.0±0.5 mg of cysts in Northwest Arm sea water and these were incubated on a reciprocating shaker for 48 h. The percentage of developed cysts was calculated as described above.

To ascertain the duration of NO and H_2O_2 exposure required to achieve maximal development four capped 50 ml plastic tubes containing 95.0±0.5 mg of cysts in Northwest Arm sea water supplemented with either 0.5 μmol l^{-1} Papa NONOate or 0.18 mmol l^{-1} H_2O_2 were incubated on a reciprocating shaker for varying times. The cysts were then washed three times with sea water and incubated in non-supplemented sea water such that the combined incubation time in the presence and absence of either NO or H_2O_2 was 24 h. The percentage of developed cysts was calculated as described above.

Acquisition of diapause cysts and termination of dormancy

In addition to experiments done in capped tubes, four Petri plates (8.5 cm diameter) containing 0.10±0.05 mg of cysts in non-supplemented Northwest Arm sea water or in sea water containing either 0.5 μmol l^{-1} Papa NONOate or 0.18 mmol l^{-1} H_2O_2 were incubated concurrently in the absence of agitation for 60 h with removal of hatched larvae every hour. The percentage of developed cysts was calculated by determining the absolute number of larvae that hatched from embryo-containing cysts in each plate. To obtain diapause cysts, Petri plates containing non-supplemented Northwest Arm sea water and 0.10±0.05 mg of *Artemia* cysts were incubated for 7 days with hatched larvae removed periodically. Cysts remaining at the end of 7 days were harvested and incubated in stationary Petri plates containing Northwest Arm sea water supplemented with either Papa NONOate or H_2O_2 in varying concentrations. The percentage of developed cysts was calculated as described above.

Data analysis

The data in Fig. 2 were analysed by a non-linear regression used for the scrutiny of bell shaped concentration–response curves and which employed Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). Data are expressed as the mean ± standard error (s.e.) of three replicates and if smaller than the symbol s.e. is not shown. All curves were computer generated.

Fulfillment of the assumptions of single classification analysis of variance (ANOVA) for Figs 3–6 was verified prior to statistical analysis. Normality was tested using the D'Agostino–Pearson test and the homogeneity of variance was assessed with Bartlett's test. Data were reported as the mean percentage of developed cysts + s.e. for each experiment and were compared using single classification ANOVA (P<0.05) followed by Tukey's *post-hoc* test of multiple comparisons when a difference between groups was indicated. If the experimental data did not meet the assumption of normality, a Kruskal–Wallis non-parametric test of variance was completed (P<0.05). When differences between groups were demonstrated Dunn's *post-hoc* test was performed. All statistical analyses were executed with SYSTAT 10.0 (Statistical Product and Service Solutions, Chicago, IL, USA).

RESULTS

NO and H_2O_2 promote the development of *Artemia* cysts

Incubation for 48 h with any of the three NO generators used in this study yielded almost equal amounts of hatched larvae with few emerged larvae remaining. However, Papa NONOate promoted development most effectively at 0.63 μmol l^{-1} whereas 4.0 μmol l^{-1} Spermine NONOate and Sin-1 chloride were required for maximum development of cysts into larvae (Fig. 2A–C). NO generators at concentrations of 40 or 60 μmol l^{-1} tended to reduce the overall extent of development achieved after 48 h, an effect most prominent with Papa NONOate (Fig. 2A–C). Moreover, after 24 h, Papa NONOate inhibited hatching almost completely at 40 and 60 μmol l^{-1}, an effect overcome by 48 h, whereas the effects of Spermine NONOate and Sin-1 chloride at these concentrations were less dramatic, but obvious (Table 1). These were the only significant differences noted when the extent of hatching was compared at 24 and 48 h (P>0.1). By comparison, after 48 h of incubation, cyst development was promoted most effectively by 0.18 mmol l^{-1} H_2O_2, whereas emergence and hatching were reduced at higher concentrations (Fig. 2D, Table 1). As shown for Papa NONOate, high concentrations of H_2O_2 inhibited hatching after 24 h, but unlike the situation with Papa NONOate, this was not reversed at 48 h. Maximum cyst development obtained with NO generators was approximately 64%, compared with 82% for H_2O_2. Addition of 300 μmol l^{-1} PTIO to tubes containing sea water supplemented individually with 0.5 μmol l^{-1} Papa NONOate, 6.0 μmol l^{-1} Spermine NONOate and 6.0 μmol l^{-1} Sin-1 chloride inhibited cyst development, as did incubation of H_2O_2 with 10 units of catalase prior to use (Fig. 3). Compared with controls the respective inhibition of NO generators and H_2O_2 imposed by PTIO and catalase was complete (P<0.001).

Incubation of cysts for 1 h in sea water containing 0.5 μmol l^{-1} Papa NONOate followed by washing and incubation in non-supplemented sea water for 23 h promoted development compared

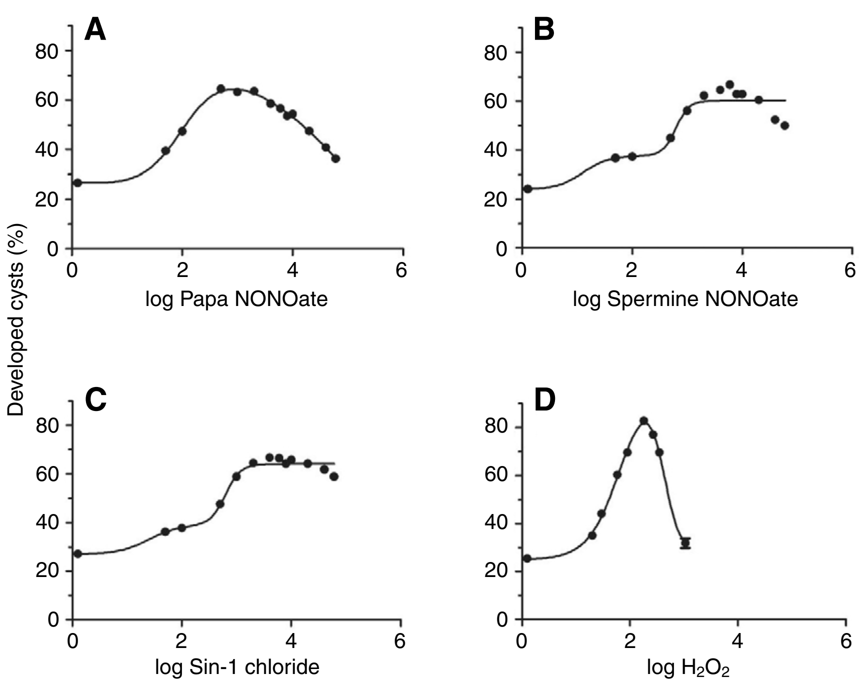

Fig. 2. NO and H_2O_2 promote *Artemia* cyst development. Capped plastic tubes containing 95.0±0.5 mg of cysts in Instant Ocean® artificial sea water supplemented with Papa NONOate (A), Spermine NONOate (B), Sin-1 chloride (C) and H_2O_2 (D) were incubated with rotation for 48 h. All tubes contained 25 ml of head space and were opened after 24 h for sampling; thus hypoxic conditions were avoided. For this and all other experiments the percentage of developed cysts was calculated as described in Materials and methods. *x*-axis values for A–C are in nmol l^{-1}, and those for D are in μmol l^{-1}. Results are presented as the mean values of three replicates with error bars representing s.e., and the *x*-axis is $\log_{10}$. If error bars are not shown the error is smaller than the symbol.

Table 1. Recovery of encysted *Artemia* embryos from developmental inhibition imposed by high concentrations of NO and H_2O_2

Inducer	Concentration (μmol l^{-1})	Time (24 h) H	Time (24 h) H+	Time (24 h) E	Time (48 h) H	Time (48 h) H+	Time (48 h) E
Papa NONOate	10	39.1	44.3	5.1	54.3	54.5	0.2
	20	30.6	44.1	13.5	47.4	48.2	0.8
	40	2.9	32.8	29.9	40.8	41.9	1.1
	60	0	29.8	29.8	36.2	37.4	1.2
Spermine NONOate	10	53.1	56.7	3.7	62.9	63.1	0.2
	20	47.9	51.4	3.5	60.5	60.8	0.3
	40	42.3	47.3	5.0	52.4	52.9	0.5
	60	35.9	45.4	9.5	50.0	50.3	0.3
Sin-1 Chloride	10	63.6	66.8	3.1	65.7	66.0	0.3
	20	57.5	61.9	4.4	64.1	64.5	0.4
	40	50.7	54.7	4.0	61.7	61.9	0.2
	60	47.0	52.5	5.4	58.8	58.8	0.1
H_2O_2	180	74.5	77.9	3.4	82.7	83.3	0.6
	270	68.3	80.2	11.8	76.9	81.9	5.1
	350	60.0	80.4	20.4	69.5	81.0	11.5
	1060	15.5	48.8	33.3	31.8	72.6	40.8

H, hatched embryos; H+, hatched + emerged embryos; E, emerged embryos.
Development at 24 h and 48 h is given as a percentage.

with cysts not exposed to Papa NONOate ($P<0.05$) (Fig. 4A). However, to achieve cyst development equivalent to that obtained with continuous exposure to 0.5 μmol l^{-1} Papa NONOate for 24 h required incubation with Papa NONOate for 4 h followed by 20 h in non-supplemented sea water ($P>0.9$) (Fig. 4A). Development was slightly greater for Papa NONOate exposures of 8 h as opposed to 4 h but the difference was not significant ($P>0.6$). After 4 h incubation in sea water containing 0.18 mmol l^{-1} H_2O_2 followed by washing and incubation in non-supplemented sea water for 20 h cyst development was equivalent to that achieved by contact with 0.18 mmol l^{-1} H_2O_2 for 24 h ($P>0.9$) (Fig. 4B). The earliest effect was apparent after a 0.5 h exposure to H_2O_2 followed by 23.5 h in non-supplemented sea water ($P<0.05$) (Fig. 4B). Incubation in H_2O_2 for 8 h before washing and incubation for an additional 16 h in non-supplemented sea water consistently reduced the extent of development compared with 4 or 16 h H_2O_2 exposures followed by incubation in non-supplemented sea water to a combined incubation time of 24 h ($P<0.05$) (Fig. 4B). The decline experienced upon an 8 h H_2O_2 exposure was reversed at 16 h. Regardless of the observed differences, NO and H_2O_2 both initiated developmental processes that continued maximally in the absence of either chemical after 4 h of exposure, although H_2O_2 effects were initiated more quickly.

H_2O_2, but not NO, terminates cyst diapause

Approximately 25% of cysts hatched after 48 h in capped tubes containing non-supplemented sea water (Fig. 2). By comparison, the extent of development in non-supplemented sea water in stationary Petri plates was higher than in capped tubes and very similar to that obtained when NO was present, either in capped tubes or in Petri plates (Figs 2 and 5). Moreover, cysts incubated for 4 h in plates with non-supplemented sea water and then transferred to tubes for an additional 20 h achieved the same level of hatching as cysts experiencing uninterrupted incubation in plates for 24 h. Cyst hatching was, however, greatest in both Petri plates and capped tubes when incubation was in the presence of H_2O_2 (Figs 2 and 5). These results reveal a cyst sub-population that developed upon exposure to H_2O_2 but not when incubated in non-supplemented sea water or with NO. In support of this proposal approximately 50% of cysts failed to develop when maintained at 26–28°C in Petri plates containing non-supplemented sea water for up to 7 days. These cysts, considered to be in diapause, were almost completely refractory to NO in Petri plates (Fig. 6A) ($P>0.1$), whereas approximately 50% hatched when exposed to 0.18 mmol l^{-1} H_2O_2 for 24 h (Fig. 6B). Hatching was reduced at higher H_2O_2 concentrations, although it was not significantly different from the control ($P>0.1$), because development stalled at emergence, as was observed in capped tubes (Table 1). When the experiment was repeated in capped tubes 42.2% of the recovered (diapause) BY 1706 cysts hatched in the presence of H_2O_2 whereas none of the cysts hatched when exposed to NO. To further test the effects of H_2O_2 and NO on diapause termination a population of *A. franciscana* cysts approximately 85% in diapause was employed (see Materials and methods). When incubated with H_2O_2 in capped tubes hatching reached 75–80%, whereas in the

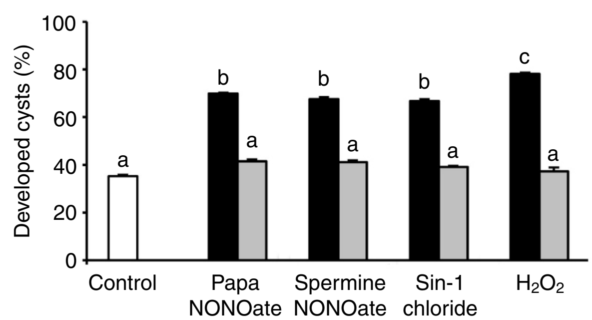

Fig. 3. Promotion of *Artemia* cyst development by NO and H_2O_2 is inhibited by PTIO and catalase. Capped plastic tubes containing 95.0±0.5 mg of cysts in Instant Ocean® artificial sea water supplemented individually with 0.5 μmol l^{-1} Papa NONOate, 6.0 μmol l^{-1} Spermine NONOate and 6.0 μmol l^{-1} Sin-1 chloride were incubated with rotation for 48 h in the presence (grey bars) and absence (black bars) of 300 μmol l^{-1} PTIO. Capped plastic tubes containing 95.0±0.5 mg of cysts in Northwest Arm sea water supplemented with either 0.18 mmol l^{-1} H_2O_2 (black bar) or an equivalent amount of H_2O_2 exposed to catalase before use (grey bar), were incubated for 48 h on a reciprocating shaker. All tubes contained 25 ml of head space and were opened after 24 h for sampling; thus hypoxic conditions were avoided. Control, cysts incubated in the absence of NO generators, H_2O_2 and PTIO (open bar). Results are presented as the mean values of three replicates with error bars representing s.e.; results labelled with the same letter are not significantly different from one another (ANOVA and multiple comparisons, $P<0.001$).

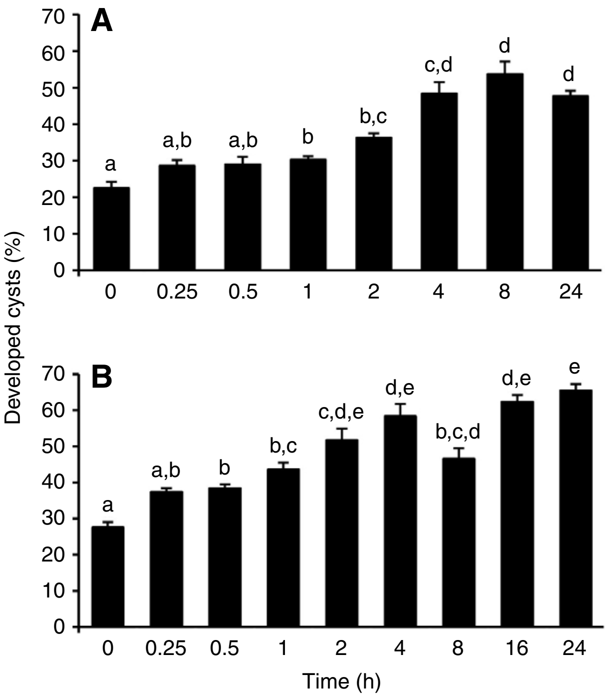

Fig. 4. Time required for maximum promotion of *Artemia* cyst development by NO and H_2O_2. Capped plastic tubes containing 95.0±0.5 mg of cysts in Northwest Arm sea water supplemented with either 0.5 μmol l^{-1} Papa NONOate (A) or 0.18 mmol l^{-1} H_2O_2 (B) were incubated on a reciprocating shaker for the times indicated. The cysts were then washed and incubated in non-supplemented sea water until the combined time in the presence and absence of NO and H_2O_2 was 24 h. Counts at 48 h were not significantly different from those at 24 h and they are not reported. Bars indicate hatched larvae. Results are presented as the mean values of four replicates with error bars representing s.e.; results labelled with the same letter are not significantly different from one another (ANOVA and multiple comparisons, $P<0.05$).

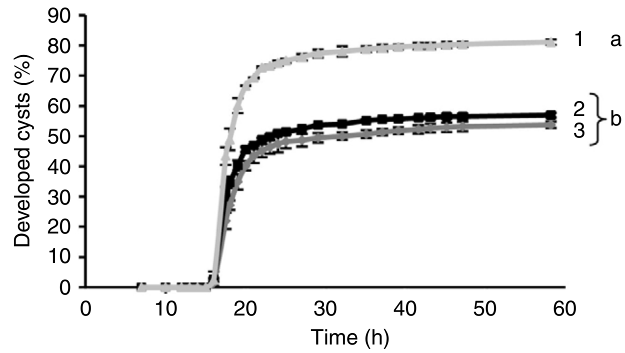

Fig. 5. Promotion of *Artemia* cyst development by H_2O_2 but not NO. Stationary Petri plates containing 0.10±0.05 mg of cysts in Northwest Arm seawater supplemented with 0.18 mmol l^{-1} H_2O_2 (curve 1) and 0.5 μmol l^{-1} Papa NONOate (curve 2), or in non-supplemented seawater (curve 3), were incubated for 60 h with hatched larvae removed every hour. The extent of development is based on the number of hatched larvae. The results are presented as the mean values of four replicates with error bars representing s.e.; results labelled with the same letter are not significantly different from one another at 60 h (ANOVA and multiple comparisons, $P<0.005$).

presence of NO hatching was maximally 11.7%, only marginally higher than in non-supplemented sea water (Table 2).

DISCUSSION

Dehydration/rehydration, cold, freezing/thawing and light, alone or in combination, terminate *Artemia* embryo diapause and promote cyst development (Drinkwater and Crowe, 1987; Van Der Linden et al., 1988; Drinkwater and Clegg, 1991; Nambu et al., 2008), but the intracellular molecular changes driven by these physical factors are unknown. In addition, environmental chemicals modulate diapause termination and post-diapause development; however, access for *Artemia* embryos to most molecules is restricted by the cyst shell, a multi-layered chitinous structure (Anderson et al., 1970; Morris and Afzelius, 1967; Clegg, 1986; Clegg et al., 1996). Nonetheless, water and gases do penetrate the shell, and with this in mind the effect of NO and H_2O_2 on encysted *Artemia* embryos was examined. NO and H_2O_2, whose activities are often integrated (Bright et al., 2006; Bian et al., 2006; Zhang et al., 2007; Neill et al., 2008; Forman et al., 2008), were also chosen because they influence physiological and developmental processes in many organisms (Stone and Yang, 2006; Bright et al., 2006; Giorgio et al., 2007; Zhang et al., 2007; Covarrubias et al., 2008; Zhao and Shi, 2009). As one example, these compounds promote the germination of seeds (Neill et al., 2002a; Neill et al., 2002b; Neill et al., 2003; Bethke et al., 2004; Bethke et al., 2006; Hancock et al., 2006; Sarath et al., 2007; Oracz et al., 2007; Bailly et al., 2008), biological structures that share characteristics with *Artemia* cysts.

As demonstrated by their development in Petri plates, but not in capped tubes, the cysts examined in this study contained a sub-population of individuals in a state of dormancy termed quiescence. These cysts failed to hatch when incubated in tubes, where for an unknown reason development was either interrupted or failed to initiate even though diapause was broken. NO, a versatile gaseous free radical signalling molecule typically converted rapidly into NO_3^- and NO_2^- by nitrogen dioxide (Neill et al., 2003; Forman et al., 2008), promoted post-diapause development of quiescent *Artemia* embryos, a process also enhanced by H_2O_2, but it failed to terminate diapause. NO acted at much lower levels than H_2O_2, perhaps due to more efficient penetration of the cyst shell. For crustaceans NO has been studied mainly for its influence on neural plasticity/function, sensory activity, heart action and bacterial

Table 2. H_2O_2 but not NO terminates diapause in *Artemia franciscana* cysts from the Great Salt Lake

Inducer	Concentration (μmol l^{-1})	Development (%)
Papa NONOate	0	8.9
	0.5	10.9
	2.0	11.7
	6.0	11.5
	10.0	9.4
	20.0	10.5
	40.0	9.7
H_2O_2	0	8.6
	30	62.6
	90	66.3
	180	71.2
	270	73.5
	350	74.0
	1060	70.8

Artemia franciscana cysts were incubated for 48 h in capped tubes containing sea water supplemented with NO and H_2O_2. Development includes both hatched and emerged cysts.

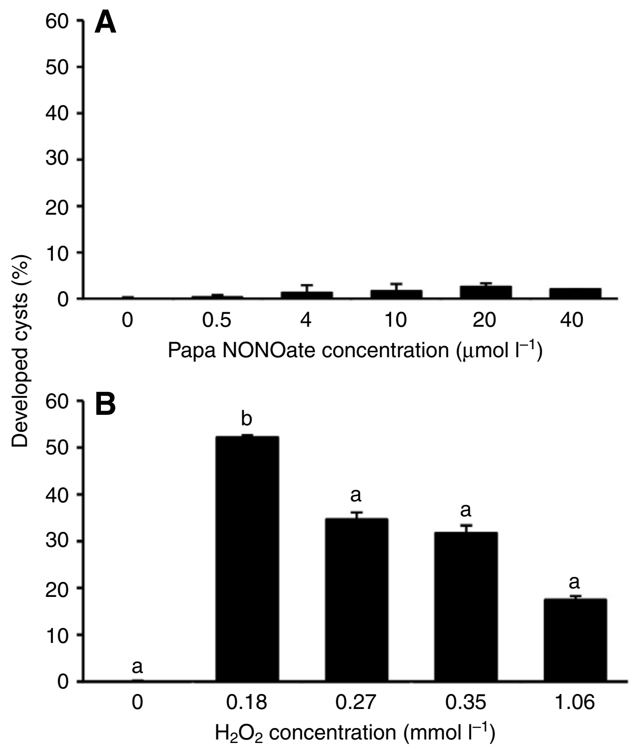

Fig. 6. H_2O_2 but not NO terminates *Artemia* cyst diapause. Diapause cysts, represented by those cysts that failed to hatch after 7 days incubation at 26–28°C in stationary Petri plates containing non-supplemented Northwest Arm sea water, were harvested and then incubated for 24 h in stationary Petri plates containing sea water supplemented with either Papa NONOate (A) or H_2O_2 (B) at the indicated concentrations. Counts at 48 h were not significantly different from those at 24 h and they are not reported. Bars indicate hatched larvae. The results are presented as the mean values of four replicates with error bars representing s.e.; results labelled with the same letter are not significantly different from one another (Kruskal–Wallis and multiple comparisons, $P<0.05$).

resistance (Scholz et al., 2002; Scholz et al., 1998; Christie et al., 2003; Yeh et al., 2006; Ott et al., 2007). This report shows, for the first time to the best of our knowledge, that NO promotes post-diapause development of a crustacean embryo.

NO may advance cyst development by acting as a reactive nitrogen species which drives the formation of NO–metallo linkages in haem-containing proteins (Villalobo, 2006; Forman et al., 2008), the reversible post-translational S-nitrosylation of proteins at cysteine thiol moieties (Bogdan, 2001; Ahern et al., 2002; Villalobo, 2006; Forman et al., 2008) and nitrotyrosine creation by processes either directly or indirectly mediated by H_2O_2 (Bian et al., 2006; Villalobo, 2006; Hancock et al., 2006; Forman et al., 2008). Additionally, NO sparks guanylate cyclase activity, increasing the second messenger cyclic 3′,5′-guanosine monophosphate (cGMP), a regulator of protein kinases, phosphatases and ion channels (Aherm et al., 2002; Kim et al., 2004; Eddy, 2005; Villalobo, 2006). Collectively, NO-driven protein changes may affect cell structure, metabolism and the expression of genes required to enhance post-diapause embryo development (Bogdan, 2001). Employing NO donors, as in this study, short-circuits the need for intracellular NO generation and/or substitutes for external sources of NO in terrestrial and aquatic niches (Bethke et al., 2004; Eddy, 2005).

H_2O_2 is a diffusible, ubiquitously distributed reactive oxygen species (ROS) which modifies intracellular redox potential and is readily formed and destroyed during aerobic metabolism. H_2O_2 was shown in this study to terminate *Artemia* cyst diapause, thus reflecting earlier work (Van Stappen et al., 1998), and to promote development of quiescent cysts. In order to affect development under normal circumstances H_2O_2 may be generated within *Artemia* cysts in response to external cues through the action of peroxidases or NADPH oxidase, the latter susceptible to regulation by Rho-like small G proteins sensitive to environmental signals (Neill et al., 2002b). H_2O_2, which modifies targets directly or by way of intermediate compounds (Winterbourn and Hampton, 2008), oxidizes thiol protein residues and functions as a second messenger *via* enhancement of tyrosine phosphorylation and dephosphorylation (Neill et al., 2002a; Neill et al., 2002b; Hancock et al., 2006; Bian et al., 2006; Forman et al., 2008). Mitogen-activated protein kinases (MAPKs) are stimulated by exogenous H_2O_2 and redox-controlled transcription factors have been identified (Stone and Yang, 2006; Hancock et al., 2006; Giorgio et al., 2007; Covarrubias et al., 2008). Thus, H_2O_2, by its oxidative properties and through its function as a second messenger, may reversibly modify proteins post-translationally. These modifications either activate or inhibit regulatory, metabolic and structural proteins, suggesting how H_2O_2 influences diapause termination and subsequent development in *Artemia* cysts.

When taken together the data indicate that H_2O_2 terminates diapause whereas NO does not, although both compounds promote post-diapause development. NO and H_2O_2 modify proteins post-translationally, perhaps at identical residues (Hancock et al., 2006), and they function as signalling molecules. Knowing this allows interpretation of experimental results and, as elaborated above, the generation of mechanistic models describing *Artemia* diapause termination and post-diapause development. For example, H_2O_2 or another ROS may terminate diapause while promoting NO production during post-diapause development. Such a proposal explains why H_2O_2 and NO, with the latter appearing not to terminate diapause and thus to function downstream of H_2O_2, both promote development of cysts, while clearly portraying H_2O_2 as the key to diapause termination and post-diapause development. Linear relationships between abscisic acid, H_2O_2 and NO, where NO activity depends on H_2O_2, regulate stomatal closure in *Arabidopsis* (Bright et al., 2006) and the activation of antioxidant defence in maize leaves, requiring up-regulation of MAPK and antioxidant enzymes (Zhang et al., 2007). Moreover, as based on their activities in other organisms, the inhibition of *Artemia* hatching by higher concentrations of Papa NONOate and H_2O_2 results from inappropriate post-translational protein modification. A parallel situation is observed in seeds where high levels of an NO generator delay germination and reduce root growth (Bethke et al., 2004; Bailly et al., 2008). Changes to essential proteins could have an immediate effect, hindering cyst development and interrupting emergence, the time when larvae become available to external molecules. Overcoming this inhibition may require the action of intracellular protective mechanisms, reflecting the results shown in Table 1.

To summarize, H_2O_2 terminates *Artemia* diapause whereas NO lacks this activity but was shown for the first time to influence post-diapause development in a crustacean embryo. Speculation concerning NO and H_2O_2 function provides conceptual frameworks upon which to build future investigations of diapause termination, work now in progress. Moreover, a better understanding of diapause termination has practical value because larvae derived from stored *Artemia* cysts are used commercially as feed in aquaculture. Diapause must be terminated prior to hatching and it may be possible to improve this process, an outcome with economic and social significance when the increasing worldwide importance of aquaculture is considered.

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REFERENCES

Ahern, G. P., Klyachko, V. A. and Jackson, M. B. (2002). cGMP and S-nitrosylation: two routes for modulation of neuronal excitability by NO. *Trends Neurosci.* **25**, 510-517.

Anderson, E., Lochhead, J. H., Lochhead, M. S. and Huebner, E. (1970). The origin and structure of the tertiary envelope in thick-shelled eggs of the brine shrimp, *Artemia*. *J. Ultrastruct. Res.* **32**, 497-525.

Bailly, C., El-Maarouf-Bouteau, H. and Corbineau, F. (2008). From intracellular signaling networks to cell death: the dual role of reactive oxygen species in seed physiology. *C. R. Biologies* **331**, 806-814.

Baitchorov, V. M. and Nagorskaja, L. L. (1999). The reproductive characteristics of *Artemia* in habitats of different salinity. *Int. J. Salt Lake Res.* **4**, 287-291.

Bethke, P. C., Gubler, F., Jacobsen, J. V. and Jones, R. L. (2004). Dormancy of *Arabidopsis* seeds and barley grains can be broken by nitric oxide. *Planta* **219**, 847-855.

Bethke, P. C., Libourel, I. G. L. and Jones, R. L. (2006). Nitric oxide reduces seed dormancy in *Arabidopsis*. *J. Exp. Bot.* **57**, 517-526.

Bian, K., Ke, Y., Kamisaki, Y. and Murad, F. (2006). Proteomic modification by nitric oxide. *J. Pharmacol. Sci.* **101**, 271-279.

Bogdan, C. (2001). Nitric oxide and the regulation of gene expression. *Trends Cell Biol.* **11**, 66-75.

Bright, J., Desikan, R., Hancock, J. T., Weir, I. S. and Neill, S. J. (2006). ABA-induced NO generation and stomatal closure in *Arabidopsis* are dependent on H_2O_2 synthesis. *Plant J.* **45**, 113-122.

Chen, T., Villeneuve, T. S., Garant, K. A., Amons, R. and MacRae, T. H. (2007). Functional characterization of artemin, a ferritin homologue, synthesized in *Artemia* embryos undergoing encystment and diapause. *FEBS J.* **274**, 1093-1101.

Christie, A. E., Edwards, J. M., Cherny, E., Clason, T. A. and Graubard, K. (2003). Immunocytochemical evidence for nitric oxide- and carbon monoxide-producing neurons in the stomatogastric nervous system of the crayfish *Cherax quadricarinatus*. *J. Comp. Neur.* **467**, 293-306.

Clegg, J. S. (1986). *Artemia* cysts as a model system for the study of water in biological systems. In *Methods in Enzymology. Biomembranes, Protons and Water* (ed. L. Packer), pp. 230-239. New York: Academic Press.

Clegg, J. S. (1997). Embryos of *Artemia franciscana* survive four years of continuous anoxia: the case for complete metabolic rate depression. *J. Exp. Biol.* **200**, 467-475.

Clegg, J. S. and Jackson, S. A. (1998). The metabolic status of quiescent and diapause embryos of *Artemia franciscana* (Kellogg). *Arch. Hydrobiol. Spec. Issues Adv. Limnol.* **52**, 425-439.

Clegg, J. S., Drinkwater, L. and Sorgeloos, P. (1996). The metabolic status of diapause embryos of *Artemia franciscana* (SEB). *Physiol. Zool.* **69**, 49-66.

Covarrubias, L., Hernández-García, D., Schnabel, D., Salas-Vidal, E. and Castro-Obregón, S. (2008). Function of reactive oxygen species during animal development: Passive or active? *Develop. Biol.* **320**, 1-11.

Drinkwater, L. E. and Crowe, J. H. (1987). Regulation of embryonic diapause in *Artemia*: Environmental and physiological signals. *J. Exp. Zool.* **241**, 297-307.

Drinkwater, L. E. and Clegg, J. S. (1991). Experimental biology of cyst diapause. In *Artemia* Biology (ed. R. A. Browne, P. Sorgeloos and C. N. A. Trotman), pp. 93-117. Florida: CRC Press.

Eddy, F. B. (2005). Role of nitric oxide in larval and juvenile fish. *Comp. Biochem. Physiol.* **142A**, 221-230.

Forman, H. J., Fukuto, J. M., Miller, T., Zhang, H., Rinna, A. and Levy, S. (2008). The chemistry of cell signaling by reactive oxygen and nitrogen species and 4-hydroxynonenal. *Arch. Biochem. Biophys.* **477**, 183-195.

Giorgio, M., Trinei, M., Migliaccio, E. and Pelicci, P. G. (2007). Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals? *Nat. Rev. Mol. Cell Biol.* **8**, 722-728.

Go, E. C., Pandey, A. S. and MacRae, T. H. (1990). Effect of inorganic mercury on the emergence and hatching of the brine shrimp *Artemia franciscana*. *Mar. Biol.* **107**, 93-102.

Hancock, J., Desikan, R., Harrison, J., Bright, J., Hooley, R. and Neill, S. (2006). Doing the unexpected: proteins involved in hydrogen peroxide perception. *J. Exp. Bot.* **57**, 1711-1718.

Kim, H.-W., Batista, L. A., Hoppes, J. L., Lee, K. J. and Mykles, D. L. (2004). A crustacean nitric oxide synthase expressed in nerve ganglia, Y-organ, gill and gonad of the tropical land crab, *Gecarcinus lateralis*. *J. Exp. Biol.* **207**, 2845-2857.

Liang, P. and MacRae, T. H. (1999). The synthesis of a small heat shock/α-crystallin protein in *Artemia* and its relationship to stress tolerance during development. *Dev. Biol.* **207**, 445-456.

MacRae, T. H. (2003). Molecular chaperones, stress resistance and development in *Artemia franciscana*. *Semin. Cell Dev. Biol.* **14**, 251-258.

MacRae, T. H. (2005). Diapause, diverse states of developmental and metabolic arrest. *J. Biol. Res.* **3**, 3-14.

Morris, J. E. and Afzelius, B. A. (1967). The structure of the shell and outer membranes in encysted *Artemia salina* embryos during cryptobiosis and development. *J. Ultrastruct. Res.* **20**, 244-259.

Nambu, Z., Tanaka, S., Nambu, F. and Nakano, M. (2008). Influence of temperature and darkness on embryonic diapause termination in dormant *Artemia* cysts that have never been desiccated. *J. Exp. Zool.* **309**, 17-24.

Neill, S. J., Desikan, R., Clarke, A., Hurst, R. D. and Hancock, J. T. (2002a). Hydrogen peroxide and nitric oxide as signalling molecules in plants. *J. Exp. Bot.* **53**, 1237-1247.

Neill, S., Desikan, R. and Hancock, J. (2002b). Hydrogen peroxide signalling. *Curr. Opin. Plant Biol.* **5**, 388-395.

Neill, S. J., Desikan, R. and Hancock, J. T. (2003). Nitric oxide signalling in plants. *New Phytolog.* **159**, 11-35.

Neill, S., Barros, R., Bright, J., Desikan, R., Hancock, J., Harrison, J., Morris, P., Ribeiro, D. and Wilson, I. (2008). Nitric oxide, stomatal closure, and abiotic stress. *J. Exp. Bot.* **59**, 165-176.

Oracz, K., Bouteau, H. E.-M., Farrant, J. M., Cooper, K., Belghazi, M., Job, C., Job, D., Corbineau, F. and Bailly, C. (2007). ROS production and protein oxidation as a novel mechanism for seed dormancy alleviation. *Plant J.* **50**, 452-465.

Ott, S. R., Aonuma, H., Newland, P. L. and Elphick, M. R. (2007). Nitric oxide synthase in crayfish walking leg ganglia: segmental differences in chemo-tactile centers argue against a generic role in sensory integration. *J. Comp. Neur.* **501**, 381-399.

Qiu, Z. and MacRae, T. H. (2007). Developmentally regulated synthesis of p8, a stress associated transcription cofactor, in diapause-destined embryos of *Artemia franciscana*. *Cell Stress Chaperones* **12**, 255-264.

Qiu, Z. and MacRae, T. H. (2008a). ArHsp21, a developmentally regulated small heat-shock protein synthesized in diapausing embryos of *Artemia franciscana*. *Biochem. J.* **411**, 605-611.

Qiu, Z. and MacRae, T. H. (2008b). ArHsp22, a developmentally regulated small heat shock protein produced in diapause-destined *Artemia* embryos, is stress inducible in adults. *FEBS J.* **275**, 3556-3566.

Qiu, T., Tsoi, S. C. M. and MacRae, T. H. (2007). Gene expression in diapause-destined embryos of the crustacean, *Artemia franciscana*. *Mech. Develop.* **124**, 856-867.

Rafiee, P., Matthews, C. O., Bagshaw, J. C. and MacRae, T. H. (1986). Reversible arrest of *Artemia* development by cadmium. *Can. J. Zool.* **64**, 1633-1641.

Sarath, G., Hou, G., Baird, L. M. and Mitchell, R. B. (2007). Reactive oxygen species, ABA and nitric oxide interactions on the germination of warm-season C_4-grasses. *Planta* **226**, 697-708.

Scholz, N. L., Chang, E. S., Graubard, K. and Truman, J. W. (1998). The NO/cGMP pathway and the development of neural networks in postembryonic lobsters. *J. Neurobiol.* **34**, 208-226.

Scholz, N. L., Labenia, J. S., Vente, J. D., Graubard, K. and Goy, M. F. (2002). Expression of nitric oxide synthase and nitric oxide-sensitive guanylate cyclase in the crustacean cardiac ganglion. *J. Comp. Neurol.* **454**, 158-167.

Sorgeloos, P. (1980). The use of the brine shrimp *Artemia* in aquaculture. In *The Brine Shrimp Artemia*: *Vol. 3, Ecology, Culturing, Use in Aquaculture* (ed. G. Persoone, P. Sorgeloos, O. Roels and E. Jaspers), pp. 25-46. Belgium: Universa Press.

Stone, J. R. and Yang, S. (2006). Hydrogen peroxide: A signaling messenger. *Antioxid. Redox Signal.* **8**, 243-270.

Sun, Y., Bojikova-Fournier, S. and MacRae, T. H. (2006). Structural and functional roles for β-strand 7 in the α-crystallin domain of p26, a polydisperse small heat shock protein from *Artemia*. *FEBS J.* **273**, 1020-1034.

Van Der Linden, A., Blust, R., Van Laere, A. J. and DeCleir, W. (1988). Light-induced release of *Artemia* dried embryos from diapause: Analysis of metabolic status. *J. Exp. Zool.* **247**, 131-138.

Van Stappen, G. (1996). Artemia: Use of cysts. In *Manual on the Production and Use of Live Food for Aquaculture. FAO Fisheries Technical Paper No. 361, Food and Agriculture Organization* (ed. P. Lavens and P. Sorgeloos), pp. 107-136. Rome, Italy.

Van Stappen, G., Lavens, P. and Sorgeloos, P. (1998). Effects of hydrogen peroxide treatment in *Artemia* cysts of different geographical origin. *Arch. Hydrobiol. Spec. Issues Advanc. Limnol.* **52**, 281-296.

Van Stappen, G., Litvinenko, L., Litvinenko, A., Boyko, E., Marden, B. and Sorgeloos, P. (2009). A survey of *Artemia* resources of Southwest Siberia (Russian Federation). *Rev. Fish. Sci.* **17**, 1-24.

Villalobo, A. (2006). Nitric oxide and cell proliferation. *FEBS J.* **273**, 2329-2344.

Villeneuve, T. S., Ma, X., Sun, Y., Oulton, M. M., Oliver, A. E. and MacRae, T. H. (2006). Inhibition of apoptosis by p26: implications for small heat shock protein function during *Artemia* development. *Cell Stress Chaperones* **11**, 71-80.

Wang, W., Meng, B., Chen, W., Ge, X., Liu, S. and Yu, J. (2007). A proteomic study on postdiapaused embryonic development of brine shrimp (*Artemia franciscana*). *Proteomics* **7**, 3580-3591.

Winterbourn, C. C. and Hampton, M. B. (2008). Thiol chemistry and specificity in redox signaling. *Free Rad. Biol. Med.* **45**, 549-561.

Yeh, F.-C., Wu, S.-H., Lai, C.-Y. and Lee, C.-Y. (2006). Demonstration of nitric oxide synthase activity in crustacean hemocytes and anti-microbial activity of hemocyte-derived nitric oxide. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* **144**, 11-17.

Zhang, A., Jiang, M., Zhang, J., Ding, H., Xu, S., Hu, X. and Tan, M. (2007). Nitric oxide induced by hydrogen peroxide mediates abscisic acid-induced activation of the mitogen-activated protein kinase cascade involved in antioxidant defense in maize leaves. *New Phytolog.* **175**, 36-50.

Zhao, L. and Shi, L. (2009). Metabolism of hydrogen peroxide in univoltine and polyvoltine strains of silkworm (*Bombyx mori*). *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* **152**, 339-345.

Zhou, Q., Wu, C., Dong, B., Liu, F. and Xiang, J. (2008). The encysted dormant embryo proteome of *Artemia sinica*. *Mar. Biotechnol.* **10**, 438-446.