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Physiological tolerance of marine calanoid copepod eggs to sulphide

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ABSTRACT: The impact of anoxia and anoxia-sulphide (8 different numeric sulphide concentrations, ranging from 10 µmol l^{-1} to 10 mmol l^{-1}) on hatching of subitaneous eggs of the planktonic copepod *Acartia tonsa* Dana was evaluated. The experiments were consequently conducted at pH 8.1. Subitaneous eggs, spawned by laboratory-reared specimens, were exposed to anoxia and anoxia-sulphide for different periods (3, 7, 14, 30, 60, 120, 180 and 240 d) and then transferred to normoxic conditions. Short-term (3 to 60 d) exposure to anoxia or anoxia-sulphide did not significantly affect the hatching success of the eggs, but hatching generally declined with increasing length of exposure. After 60 d of exposure there were significant differences between the effects caused by anoxia and anoxia-sulphide (sulphide concentrations ≥250 µmol l^{-1}). After 240 d of exposure there were significant differences in hatching between eggs treated with anoxia and those with anoxia-sulphide (all sulphide concentrations). A short-term experiment, where subitaneous eggs were simultaneously exposed to oxygen and different sulphide concentrations, indicated that sulphide is capable of crossing the eggshell. The metabolic rate of eggs exposed to normoxic conditions was 1.86 ± 0.57 µJ h^{-1}. Eggs exposed to anoxia had a metabolic rate of 0.08 ± 0.02 µJ h^{-1}, whereas eggs exposed to anoxia and 14.7 mmol l^{-1} sulphide had a metabolic rate of 0.25 ± 0.001 µJ h^{-1}. Based on the metabolism experiments and the fact that internal egg pH was ~6, an unknown sulphide defence mechanism is suggested to be present in *A. tonsa* eggs.

KEY WORDS: *Acartia tonsa* · Copepoda · Subitaneous eggs · Anoxia · Sulphide · Survival · Metabolism · Defence mechanism

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INTRODUCTION

Calanoid copepods are prominent members of the zooplankton communities in estuarine and costal regions in terms of numbers, biomass and productivity. Typical boreal estuarine and costal genera include *Acartia, Centropages* and *Labidocera* (Marcus & Lutz 1994). A great majority of marine calanoid copepods spawn their eggs freely into the water column. The eggs are denser than the surrounding seawater and they begin to sink. The eggs may be subitaneous, which normally hatch within a few days, or diapause, which undergo a refractory phase (Grice & Marcus 1981). In shallow waters, subitaneous eggs may sink to the bottom before hatching if limited vertical turbulence occurs. Once at the seabed, a quiescent state might be induced in the eggs, probably due to low oxygen concentrations (<0.15 ml l^{-1}), which inhibits hatching (reviewed by Grice & Marcus 1981). For the duration of the time that eggs remain buried in the sediment they are likely to be exposed to anoxia. Therefore, viability and development of the benthic resting eggs are greatly influenced by the environmental conditions in the sediment (Uye & Fleminger 1976). The greatest numbers (e.g. 10^6 m^{-2}) of buried eggs from planktonic copepods occur in estuarine muddy sediment at shallow water depths (Marcus 1996).

In marine environments, the disappearance of oxygen is often correlated with the formation of toxic hydrogen sulphide. When oxygen is absent, first nitrates and then sulphates serve as electron acceptors in the bacterial mineralisation of organic matter. The reduction of sulphates leads to the formation of sulphide and H_2S. In particular, soft seabeds, which often are poorly

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aerated, are characterised by periods of substantial sulphide production and H_2S (Vopel et al. 1998). Marine sediments consist of an upper oxic and deeper anoxic sulphide-containing layer, in which considerable amounts of sulphide (up to 10 mmol l^{-1}) may be found at a depth of a few mm or cm (Fenchel & Riedel 1970). Under conditions of reduced oxygen concentrations in the bottom water layer, sulphide may diffuse into the sediment-water interface and cause the overlaying water to become anoxic and sulphidic, thus affecting fauna in the top sediment (Nilsson & Rosenberg 1994). In aqueous solutions, the equilibrium concentration of the different sulphide species (i.e. H_2S, HS^- and S^{2-}) varies with pH. In natural open seawater, the pH ranges from 7.9 to 8.3 (Hinga 1992, 2002) and the dominant sulphide species is the hydrosulphide anion (HS^-). In porewater, where pH can be as low as 6.0 to 6.5, the dominant species is the natural molecular gas H_2S (Vismann 1996a). H_2S is capable of crossing biological cell membranes freely, whereas the HS^- anion may be electrically excluded (Bagarinao 1992). The total sulphide toxicity towards marine benthic invertebrates is pH dependent and increases with decreasing pH (Vismann 1996a).

According to Diaz (2001), hypoxic, anoxic and sulphidic conditions are occurring more frequently in sediments and bottom waters due to increased eutrophication. The survival of copepod eggs exposed to anoxia has been studied by Uye & Fleminger (1976), Lutz et al. (1992) and Marcus et al. (1994). These studies all indicate that copepod eggs can survive anoxia for days to weeks and, in some cases, years. However, in addition to low oxygen concentrations, copepod eggs are also exposed to hydrogen sulphide in natural anoxic environments. The impact of sulphide on egg viability of planktonic copepods under laboratory conditions has only been studied by Marcus et al. (1997) and Invidia et al. (2004). They both concluded that there was no significant difference in viability between eggs exposed to anoxia-sulphide (at pH 8.2) and those exposed to anoxia alone. Additionally, at pH 6.5, the exposure of eggs to anoxia-sulphide was more detrimental than when exposed to anoxia alone (Invidia et al. 2004).

The brine shrimp *Artemia franciscana* is frequently used as a model organism in metabolic studies because the embryos exhibit an astonishing long term tolerance to anoxia. In a number of direct calorimetric experiments, the brine shrimp embryo was shown to be capable of reducing the energy flow to 2.4% of the aerobic level. For longer periods of anaerobiosis the metabolic depression can extend down to 0.5% of the aerobic level (Kemp 1999). Whether embryos of *Acartia tonsa* are equally capable of metabolism depression is not known. Likewise, it is not known whether metabolic depression also occurs under sulphidic conditions.

With the ultimate purpose of discussing the fate of eggs from boreal neritic calanoid copepods that potentially sink before hatching and reach anoxic/sulphidic marine sediments, we performed a series of laboratory experiments. We mimicked a situation where eggs produced by free spawning calanoids during fall are buried in marine sediments, and re-enter the pelagic environment the proceeding spring-summer or even later. We studied the viability of the eggs when exposed to anoxia, and to anoxia plus different sulphide concentrations, for different time periods. The aim of the present study was to assess hatching and viability of *Acartia tonsa* eggs exposed to anoxia and different sulphide concentrations for different periods of time, and to substantiate the results by measuring the metabolic activity of *A. tonsa* eggs exposed to similar conditions. The present study addressed the following questions: (1) Does sulphide affect the viability of *A. tonsa* eggs? (2) Do *A. tonsa* eggs exhibit a minimum tolerance level to sulphide, and does time exposure matter? (3) Does sulphide actually pass through the eggshell? (4) Is metabolism affected when *A. tonsa* eggs are exposed to anoxia and to anoxia-sulphide? (5) Do *A. tonsa* eggs have a defence mechanism against or detoxification system for sulphide?

MATERIALS AND METHODS

Long-term experiment. ***Sampling and maintenance of cultures:*** *Acartia tonsa* eggs used in the experiments came from laboratory cultures that were raised over the last 25 yr under the same conditions (Støttrup et al. 1986). The copepods were cultivated in our laboratory in 70 l tanks filled with seawater (~30 psu) and covered with opaque plastic. The water temperature was 17 ± 1°C and atmospheric air was gently supplied to ensure that algal food remained in suspension. Copepod cultures were fed daily with 1000 ml of a suspension of the chryptophyte *Rhodomonas salina* (approx. 8 µm equivalent spherical diameter) of approximately 10^6 cells ml^{-1}. This gave a mean cell density of approx 15×10^3 ml^{-1}, above half-saturation according to Berggreen et al. (1988) and Hansen et al. (1997).

Alga was continuously cultivated in round bottom culture flasks (2 to 5 l) filled with pasteurised natural seawater (30 psu) with added artificial B1-media (Hansen 1989) in a 17 ± 1°C walk-in climate room. The cultures were maintained in logarithmic growth phase, and received approximately 125 µE m^{-2} s^{-1} of photosynthetic active radiation (PAR) continuously (measured by a Li-Cor LI-1000 Data Logger equipped with a Li-Cor model quantum Serial No. Q30253 sensor).

Experimental procedure: A 0.1 M $NaHCO_3$-buffer solution was prepared in 0.2 µm GF/F filtered sea-

water. The buffer was deoxygenated by bubbling vigorously for 1 h with nitrogen gas, and then by bubbling gently over night. The pH was adjusted to 8.10 with diluted HCl. The salinity of the buffer-solution was determined to 35.5 psu.

Eggs produced within 24 h were collected from the bottom of the copepod culture tanks with a siphon. After collection, eggs were cleaned through meshes of 3 different sizes (100, 90 and 70 µm). Cleaned eggs were finally transferred to screw-capped vials (20 ml, Kimble) by sub-sampling. The sub-sampling was performed with a bottle containing the cleaned eggs and a known volume of seawater, and a 10 ml 'Kip-automat' (NS 29.2/32, Witeg) — a glass device constructed to dose an accurate volume of liquid from a bottle. The sub-sample (10 ml) of the egg-suspension was sieved onto a 45 µm mesh that retained the eggs. The mesh and eggs were gently transferred to screw-capped vials. The anoxic vials were filled with anoxic $NaHCO_3$-buffer, creating a surface meniscus. A small piece of household film (Vita Wrap) was gently placed on top to avoid air-bubbles, and the lid was firmly closed. The number of eggs in each vial, regardless of treatment, was 108.9 ± 5.5.

A pilot project (data not shown) verified that the $NaHCO_3$-buffer did not affect hatching success of the eggs (*t*-test, $p > 0.05$). A control experiment to monitor the viability of the actual egg batch used throughout the long term experiment was conducted in 10 Petri dishes. The total number of eggs in each Petri dish was counted before they were left to hatch for 4 d at normoxic conditions at 17°C. Every day, a few drops of *Rhodomonas salina* suspension were added to feed the newly hatched nauplii, and after 4 d the number of eggs and nauplii was counted under a dissecting microscope.

Sulphide stock solution (~0.1 M) was prepared from anhydrous Na_2S and 0.2 µm GF/F filtered seawater, which had been bubbled with nitrogen gas over night. The actual sulphide concentration of the stock solution was determined to be 0.112 M using the Cline method (Cline 1969). The sulphide samples were prepared by adding 15 ml of anoxic $NaHCO_3$-buffer to the vials containing the eggs, and then a suitable aliquot of sulphide stock solution was added to the vials to obtain the numeric sulphide concentrations. The vials were filled and closed the same way as described for the anoxic samples. Before the addition of the required amount of sulphide, only 10 ml of anoxic $NaHCO_3$-buffer was added to vials with sulphide concentrations of 10 mmol l^{-1}, and finally pH was adjusted to approximately 8 (via addition of diluted HCl). The sulphide concentrations in the vials were only numeric and the actual sulphide concentration in each vial was first determined at the end of the exposure.

Vials were kept in an insulated box filled with water at 17°C. This temperature was chosen because copepods were cultivated at this temperature and, according to the literature, many experiments on *Acartia tonsa* were conducted at this temperature. After exposure, oxygen concentrations were measured with an OX50 oxygen microsensor (Unisense). In vials with 10 mmol l^{-1} sulphide, the oxygen-electrode was poisoned by the sulphide so that the signal indicated $-\infty$. The actual sulphide concentrations in the vials were thus determined using the Cline method (Cline 1969) and the pH measured with a combined pH-electrode (Radiometer).

Copepod eggs were washed with 0.2 µm filtered seawater and counted under a dissecting microscope. Normally no or only a few eggs hatched during the incubation. The eggs were left in Petri dishes with fresh seawater for 4 d at 17°C. After 4 d of normoxic conditions, the number of eggs and nauplii was counted again and hatching success calculated.

The stage of the nauplii after 180 and 240 d of exposure was determined and categorised as 'stage NIII or higher' or as nauplii at a lower development stage. Eggs and nauplii were kept in antibiotic seawater (Penicillin-Streptomyci: 10 000 units ml^{-1} Penicillin and 10 mg ml^{-1} Streptomycin in 0.2 µm GF/F filtered seawater) at normoxic conditions at 17°C. The hatching success was monitored after 4 d and then at least every fourth day for 16 and 11 d, respectively. A control experiment was conducted, where the effects of antibiotics on hatching success were examined, and no significant differences were apparent between the hatching success of eggs kept in antibiotic seawater and those in normal seawater (data not shown).

Short-term experiment. In a series of experiments, *Acartia tonsa* eggs produced within 24 h were exposed to different sulphide concentrations in the presence of oxygen and at pH ~8.2 (pH ~8 in the long-term experiment), in order to study the possible diffusion of hydrogen sulphide (H_2S) across the egg membrane. If the hatching success of eggs exposed to sulphide decreased with increasing sulphide concentrations or exposure time, this would indicate that hydrogen sulphide was capable of crossing the egg membrane. In all experiments, the initial water volume (2 l) was bubbled with atmospheric air for at least 30 min. The experimental computer protocols were controlled by a set-up that simultaneously recorded and controlled pH and total sulphide (for details, see Vismann 1996b). The eggs were cleaned as earlier described and, via the sub-sampling method described above, transferred to a small cylindrical container (25 ml) equipped with 45 µm mesh at both ends, so that they were easy to find after exposure. Exposure time ranged from 2 to 5 d. At the end of experiment, eggs were transferred to Petri dishes

where the number of eggs and nauplii was counted. Normally no or only a few eggs hatched. After 4 d of exposure to normoxic conditions at 17°C, the eggs and nauplii were counted again and hatching success calculated. For every experiment and batch of eggs, a control experiment was conducted in Petri dishes to test hatching success under normoxic conditions.

Internal pH and buffer capacity measurements. pH measurements were conducted on 2 different days on a homogenate of approximately 300 000 eggs that were 1 and 5 d-old (cold stored, *sensu* Drillet et al. 2006). Eggs were cleaned as described earlier, collected on a 70 µm mesh, and tissues were pressed from below towards the mesh to remove the seawater. The eggs were then transferred to an Eppendorf tube and exposed to liquid nitrogen for 10 min and defrosted; this procedure was completed 5 times in a row to ensure that all of the eggs were ruptured. The pH-measurements of egg fluids were carried out directly in the Eppendorph tubes with a small pH-electrode (diameter 0.5 mm) from Radiometer, and were measured 3 times consecutively for each batch of eggs. The pH of water from the copepod culture tanks was also measured.

The buffer capacity towards acid and base were conducted separately on 2 batches of eggs: towards acid was measured using a batch of 128 000 eggs via titration with 0.01 M HCl, and towards base was measured using a batch of 100 000 eggs via titration with 0.0095 M NaOH. A microburette (200 µl) was used to add the titrant. The amount of titrant varied from several µl to several ml, depending on the variability of pH. The pH-electrode was used to mix the egg fluids with the titrant.

Metabolism experiments. ***Experimental procedure:*** Copepod eggs produced within 24 h were cleaned as previously described and transferred to a sub-sampling bottle. A known volume of 0.2 µm filtered seawater was added, and a control experiment was conducted in Petri dishes to determine hatching success of the actual batch of eggs as well as to provide an estimate of the total amount of eggs incubated. Sub-sampling gave 4 equally sized portions, and the eggs were accumulated on a 70 µm mesh. The mesh was pressed from below with tissues in order to remove the seawater (previously described) and afterwards washed with anoxic antibiotic buffer (Penicillin-Streptomycin: 10 000 units ml^{-1} Penicillin and 10 mg ml^{-1} Streptomycin in GF/F filtered $NaHCO_3$-buffer) for approximately 5 min, and again pressed with tissues.

Isothermal microcalorimetry: Calorimetry is a nonspecific technique for the direct measurement of metabolic activity, and is used for the direct determination of heat quantities and of heat production rates or thermal powers. All calorimetric measurements were conducted on type 2277 thermal activity monitors (TAM) from Thermometric. The sample was loaded into a 3 cm^{-3} stainless steel calorimetric vessel. For samples exposed to oxygen or sulphide, the calorimetric vessel was mounted on a type 2250 perfusion calorimetry module (Thermometric). Throughout the thermal equilibration and data acquisition periods, the sample was stirred by a turbine stirrer at 60 rpm, to ensure a constant supply of oxygen or equal distribution of sulphide to the eggs. To thermally equilibrate the sample (17°C), it was gradually lowered into the calorimeter over a period of 60 to 120 min. At this stage, the data collection was initiated and monitored for 15 to 38 h. The experiments conducted in the TAM were kept at 17°C and the baseline value for each channel was measured (data not shown). The baselines represented the amount of heat produced in the channel when there were no contributions from any biological or chemical processes involved. Negative baselines were added to measured signals from the experiments, whereas positive baselines were subtracted from measured signals in order to take baseline levels into account. After the TAM measurements were conducted, oxygen concentrations in the TAM vessels were determined by the Unisense oxygen microsensor (OX50). Oxygen concentration was determined to be 0.002 ± 0.004 ml O_2 l^{-1} for anoxic experiments, and 3.67 ± 0.25 ml O_2 l^{-1} for normoxic experiments, ensuring no oxygen limitation during measurement.

For the anoxic experiments, eggs were transferred to the TAM vessel, 2 ml of the anoxic Penicillin/Streptomycin solution was added, and the egg suspension was bubbled with nitrogen gas for 5 to 10 min during which time the vessel was covered with Parafilm. Afterwards, a small chink between the vessel and the lid was left open and a gentle stream of argon gas was supplied for 1 to 2 min in order to remove oxygen from the gas phase above the sample.

Experiments on anoxia-sulphide followed the same procedure as above, except that 300 µl of a sulphide stock solution was added so that actual sulphide concentration in the TAM vessel was 14.7 mmol l^{-1}. A high sulphide concentration ensured that the sulphide concentration was sufficient to have an influence on the metabolism of the eggs.

The aerobic experiments followed the same procedure, with the exception that egg suspensions were bubbled with atmospheric air instead of nitrogen for 5 to 10 min and then the TAM vessels were closed.

All TAM vessels were sealed with an O-ring and cleaned with ethanol before they were lowered into the TAM, and thermostated before measurements were initiated.

Data analysis. Each vial was considered to be a replicate for data analysis of egg hatching. The final hatching percentage for each vial was arc-sin transformed,

because data are expressed as percentages. Means and SD were calculated from the transformed values for each incubation period and for each collection date.

RESULTS

Long-term experiment

The oxygen concentration in vials ranged from 0.0 to 0.76 ml O_2 l^{-1} throughout all treatments and over time. From 30 d onwards, oxygen concentrations slightly decreased to between 0.0 and 0.09 ml O_2 l^{-1}. The final oxygen concentration in most of the 296 vials was less than 0.02 and approached 0.0 ml O_2 l^{-1}.

The actual sulphide concentrations in the 10, 25 and 50 µmol l^{-1} sulphide experiments were very close to one another (Table 1); in reality, the difference between the 3 sulphide concentrations was less than 5 µmol l^{-1}. Therefore, hatching success under these 3 sulphide concentrations was pooled (Fig. 1). In general, the actual sulphide concentrations were lower than the intended concentrations. The actual sulphide concentrations showed a decrease with increasing incubation time (Table 1).

Mean pH in vials changed from 8.14 to 8.46, but a significant difference was only observed between vials exposed for 7 d and 240 d (ANOVA, $p < 0.00000$).

Hatching success

The batch of copepod eggs used had a hatching success of 75% when they were kept at 17°C for 4 d under normoxic conditions (control). No hatching occurred during either anoxia or sulphide treatments, irrespec-

Table 1. *Acartia tonsa*. Actual mean sulphide concentrations ± SD in vials at different exposure times reported under numeric sulphide concentrations. No. of replicates = 5, except for Days 3 and 180 where no. of replicates = 1

Exposure (d)	Actual sulphide concentration (µmol l^{-1}) 10 l^{-1}	25 l^{-1}	50 l^{-1}	100 l^{-1}	250 l^{-1}	500 l^{-1}	1000 l^{-1}	10000 l^{-1}
3	15	17	20	130	235	535	586[a]	2670[a]
7	2 ± 1	5 ± 5	9 ± 7	57 ± 24	155 ± 120	856 ± 73	1592 ± 78	629 ± 112
14	3 ± 3	5 ± 4	7 ± 4	60 ± 24	296 ± 235	622 ± 182	1029 ± 69	2187 ± 116
30	4 ± 2	8 ± 6	7 ± 4	24 ± 8	186 ± 15	456 ± 272	1326 ± 109	4119 ± 124
60	2 ± 1	4 ± 4	5 ± 7	35 ± 15	122 ± 65	283 ± 212	999 ± 71	4448 ± 116
120	3 ± 1	2 ± 1	4 ± 4	45 ± 53	95 ± 67	381 ± 124	696 ± 193	6897 ± 457
180	0.4	2	2	7	11	46	1656	1922
240	2 ± 1	14 ± 17	5 ± 4	4 ± 3	26 ± 39	132 ± 137	302 ± 251	1854 ± 221

[a]Samples were not sufficiently diluted; therefore not all sulphide required was bound in the methylene blue-complex, which led to an under-estimation of sulphide concentrations

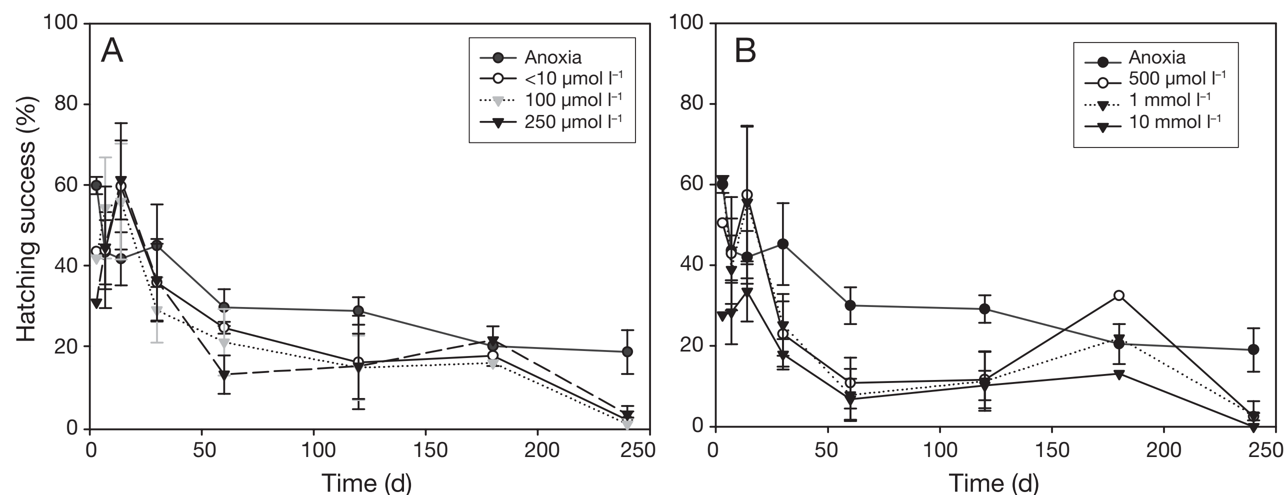

Fig. 1. *Acartia tonsa*. Long-term experiment: mean ± SD hatching success of subitaneous eggs exposed to anoxia and different sulphide concentrations. (A) Anoxia and numeric sulphide concentrations from 10 to 250 µmol l^{-1} (NB: hatching success in numeric sulphide concentrations from 10 to 50 µmol l^{-1} was pooled). (B) Anoxia and numeric sulphide concentrations from 500 µmol l^{-1} to 10 mmol l^{-1}

tive of exposure time. The eggs resumed hatching only after transfer to normoxic seawater.

Egg viability was variable in the anoxia and sulphide vials, but generally declined with increasing length of exposure (Fig. 1). Under anoxic conditions, hatching success decreased from 60% after 3 d to 19% after 240 d of incubation, and from 27.6% after 3 d to 0% after 240 d of incubation when exposed to the highest sulphide concentration.

There was no significant difference between the hatching success of eggs exposed to anoxic conditions and that of eggs exposed to all sulphide concentrations when exposure was shorter than 60 d. However, at 60 d there was a significant difference between the hatching success of eggs exposed to anoxia and those exposed to sulphide concentrations ≥250 µmol l^{-1} (ANOVA, p = 0.05). After 120 d, there was a significant difference between hatching success of eggs exposed to anoxia and of those exposed to sulphide concentrations ≥500 µmol l^{-1}. After 240 d of incubation, the hatching success of all eggs exposed to all sulphide concentrations reached a level below 5%, and sulphide concentrations of 10 µmol l^{-1} none of the eggs hatched. There were significant differences between hatching rates of eggs exposed to all sulphide concentrations and of those exposed to anoxia (ANOVA, p = 0.05). A 2-factor ANOVA showed that the effect of sulphide ($p \leq 0.000000$), time ($p \leq 0.000000$), and the interactions between sulphide and time was very strong ($p \leq 0.000002$). Hence, increasing sulphide concentrations had a significant effect on egg hatching success, and this effect increased over time.

Table 2. *Acartia tonsa*. No. of nauplii at stage <NIII or ≥NIII, and % ≥NIII nauplii (of total) after (A) 180 and (B) 240 d of incubation in anoxia and different numeric sulphide concentrations

Sulphide (µmol l^{-1})	Total	<NIII	≥NIII	≥NIII%
(A)				
Anoxia	12	3	9	75
10	10	0	10	100
25	4	3	1	25
50	4	0	4	100
100	16	9	7	44
250	6	3	3	50
500	5	4	1	20
10^3	8	8	0	0
10^4	2	2	0	0
(B)				
Anoxia	15	4	11	73
10	13	3	10	77
25	8	3	5	63
50	13	4	9	69
100	7	5	2	29
250	14	10	4	29
500	12	8	4	33
10^3	12	10	2	17
10^4	0	0	0	0

Table 3. *Acartia tonsa*. Mean pH ± SD, temperature ± SD, sulphide ± SD and sulphide set-point for short-term experiments

Sulphide (µmol l^{-1})	pH	Temperature (°C)	Measured (mV)	Set-point (mV)
55.9	8.2 ± 0.1	17.0 ± 0.1	−549.2 ± 14.0	−532
108.8	8.2 ± 0.1	16.8 ± 0.1	−549.7 ± 5.2	−545
393.4	8.3 ± 0.03	16.8 ± 0.1	−563.4 ± 3.6	−560
1.2×10^3	8.3 ± 0.2	17.1 ± 0.1	−567.8 ± 5.3	−565
6.4×10^3	8.3 ± 0.1	16.8 ± 0.1	−567.1 ± 0.3	−566
12.8×10^3	8.3 ± 0.3	17.1 ± 0.1	−579.7 ± 4.3	−580
22.5×10^3	8.3 ± 0.1	17.0 ± 0.1	−616.0 ± 2.0	−615

Stage development

The viability of eggs incubated under anoxic conditions for 180 and 240 d and then left for 4 d in normoxic conditions had a hatching success of 12.2 and 1.1%, respectively. Hatching successes were calculated again after 16 d (eggs exposed for 180 d) and 11 d (eggs exposed for 240 d) in normoxic conditions, and increased further to 19.3 and 1.8%, respectively. A smaller fraction of nauplii (hatched from eggs incubated under anoxic conditions for 180 and 240 d) reached the NIII stage when eggs were exposed to high sulphide concentrations (Table 2).

Short-term experiment

Mean ± SD pH ranged from 8.2 ± 0.1 to 8.3 ± 0.1 in all experiments. All sulphide measurements (in mV) were very close to the sulphide set-point and exhibited very little variation in SD, which indicated that the eggs had been exposed to constant sulphide concentrations during the entire incubation (Table 3).

Hatching success generally declined with increasing sulphide concentrations and exposure time (Fig. 2). There was a significant difference between hatching success in the control and the anoxic treatment, as well as between the control and all sulphide concentration treatments (ANOVA, p = 0.05). A significant difference in hatching success was observed between anoxia and sulphide concentrations ≥1.2 mmol l^{-1} after 2 d, whereas hatching success of eggs exposed to low sulphide concentrations did not differ from those exposed to anoxia (ANOVA, p = 0.05). Decreasing hatching success with increasing sulphide concentration and exposure time indicated that the hydrogen sulphide was capable of crossing the egg membrane.

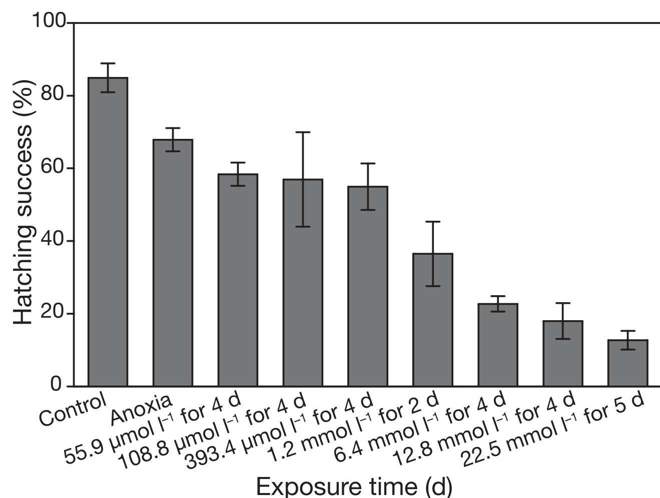

Fig. 2. *Acartia tonsa*. Short-term experiment: mean ± SD hatching success of subitaneous eggs exposed to oxygen and different sulphide concentrations (simultaneously) for different time periods (5 replicates each). After exposure, eggs were maintained under normoxic conditions for 4 d before hatching success was calculated

Internal pH and buffer capacity

The internal mean pH of 1 and 5 d-old *Acartia tonsa* eggs maintained under normoxic conditions was 6.09 and 6.07, respectively, and pH of water from the copepod culture tanks was 7.39 ± 0.13 (n = 8). None of the experiments on buffer capacity indicated that *A. tonsa* eggs had any buffer capacity towards acid or to base (data not shown).

Metabolism experiments

Examples of replicate measurements of the TAM signals of heat production of *Acartia tonsa* eggs under different experimental conditions are shown in Fig. 3. The number of eggs in the 4 experiments were not the same, differing from 12 600 eggs under normoxic conditions, 45 600 eggs each under control and anoxic/sulphidic conditions, and finally 130 000 eggs under anoxic conditions. The control measurement was taken from *A. tonsa* eggs that had been heat-killed at 60°C for 10 to 15 min and served as a biological blind-value.

Heat production (mean ± SD µJ egg^{-1} h^{-1}, where 1 µW = 1 µJ s^{-1}) for a single copepod egg under each treatment was as follows: oxygen: 1.86 ± 0.57 (n = 8); anoxic: 0.08 ± 0.02 (n = 4); anoxic-sulphidic (14.7 mmol l^{-1}): 0.25 ± 0.001 (n = 2); control: 0.03 ± 0.01 (n = 2). *Acartia tonsa* eggs exposed to normoxic conditions had a metabolic activity that was almost 7.5 times higher than that of eggs exposed to sulphide. Eggs exposed to anoxia-sulphide produced more than 3 times as much heat as eggs exposed to anoxia alone.

DISCUSSION

Long- and short-term experiments

The copepod eggs used in the experiments were subitaneous, as demonstrated by the generally high hatching success of the controls within 4 d. The eggs that failed to hatch were considered to be non-viable, and not, in fact, diapause eggs as described in previous observations (Drillet et al. 2006). Exposure to anoxia and sulphide induced quiescence in the subitaneous eggs of *Acartia tonsa*, but the eggs were able to resume hatching after recovery under normoxic conditions. The hatching success of eggs incubated for 180 and 240 d only increased slightly when the eggs were maintained under normoxic conditions for >4 d; therefore, the general hatching successes calculated after 4 d were considered to be the final hatching success. However, in all experimental conditions, egg viability first declined, then increased, before slowly decreas-

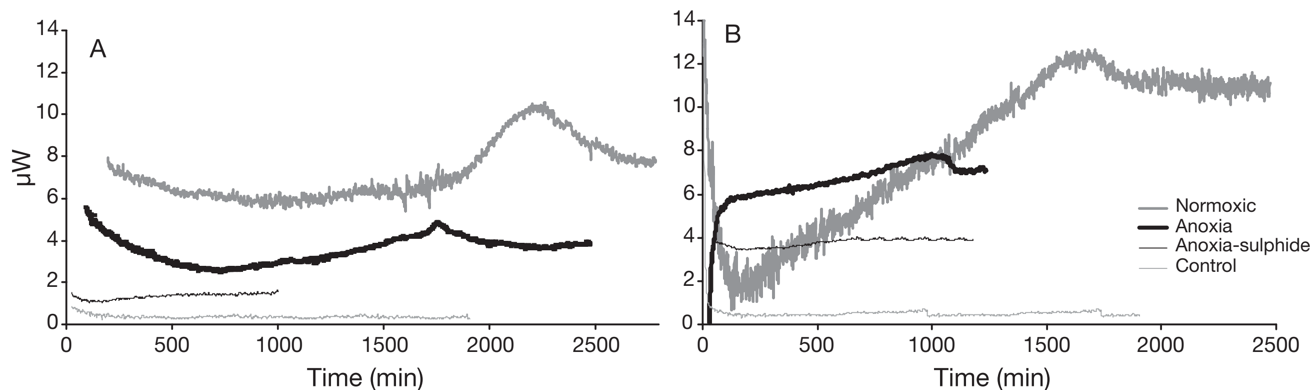

Fig. 3. *Acartia tonsa*. TAM thermograms of heat production of subitaneous eggs exposed to different conditions. (A) and (B) show TAM thermograms for 2 different replicates. No. of eggs in each measurement varies (see text)

ing again with increasing exposure time and increasing sulphide concentrations. The pattern of declining egg viability (after 3 to 7 d of exposure) and then increasing egg viability (after 7 to 14 d of exposure) was an unexpected result, and we have no explanation for this pattern in egg viability. After 60, 120 and 240 d incubation periods, significant declines in hatching success were observed. Marcus & Lutz (1994), Marcus et al. (1997) and Invidia et al. (2004) found that hatching success of *A. tonsa* eggs exposed to anoxia or anoxia-sulphide declined with increasing exposure time, and that after the maximum exposure time of 32 d some eggs were still viable in all experimental conditions. Our hatching results from the first 30 d of incubation were within the range of values reported by the above-cited studies.

Comparison of the effects of anoxia and sulphide on the viability of eggs did not show significant differences when exposure time was <60 d. However, at Day 60 the hatching success of eggs exposed to anoxia was significantly higher than that of eggs exposed to sulphide concentrations ≥250 µmol l^{-1}. As the incubation was prolonged, significant differences between the hatching success of eggs exposed to anoxia and to different sulphide concentrations became even more pronounced, and after 240 d there were significant differences between hatching success after exposure to anoxia and to all sulphide concentrations.

The long-term experiment was supported by results obtained from the short term experiment, where egg viability decreased with increasing sulphide concentrations and exposure time. At low sulphide concentrations ≤393.4 µmol l^{-1} there was no significant difference between the hatching successes of eggs exposed to anoxia or to sulphide, whereas there was a significant difference in hatching success after a 2d-exposure to sulphide concentrations ≥1.2 mmol l^{-1}. Lutz et al. (1994) suggested that 'perhaps prolonged exposure to low oxygen concentrations, as opposite to zero oxygen, is more detrimental to the eggs because in the absence of oxygen, metabolism is shut down completely, whereas under low oxygen conditions some metabolic functions proceed'. Survival under stress depends on the overall reduction in metabolic rate required to conserve substrates and reduce the accumulation of toxic end-products, and is known as 'metabolic rate depression' (Clegg 1997). Our results from exposures <60 d are consistent with this. The addition of sulphide to the vials resulted in complete anoxia from the beginning of the exposure, whereas in 'anoxia' vials we cannot exclude the possibility that there were traces of oxygen present at the beginning of the incubation period. The depression in metabolic rate might have been greater in vials with sulphide than in anoxia vials. Subsequently, cell poisoning was perhaps retarded in the latter treatment because while eggs exposed to sulphide immediately switched to anaerobic metabolism, eggs exposed to anoxia might have begun with aerobic metabolism and then switched to anaerobic metabolism later on. Our results appear to be consistent with those available in the literature: Marcus et al. (1997) reported that there were no significant differences in viability of eggs from 3 calanoid copepod species *Acartia tonsa*, *Labidocera aestiva* and *Centropages hamatus* exposed to anoxia-sulphide and to anoxia alone, and Invidia et al. (2004) reported no significant difference in viability of laboratory-produced *A. tonsa* eggs exposed to anoxia-sulphide (at pH 8.2 and 6.5) and anoxia alone after 15 d. Both studies suggested that eggs exposed to anoxia-sulphide or prolonged exposure to anoxia survived because the eggs had switched to anaerobic metabolism. In our long-term experiment, eggs were incubated for a much longer period than in studies conducted by Marcus et al. (1997) and Invidia et al. (2004). Our results showed that exposure of *A. tonsa* eggs to sulphide over a relatively short time period (<60 d) did not result in significant differences between hatching successes of eggs exposed to sulphide and of those exposed to anoxia alone. For medium exposure times (60 to 180 d), there were significant differences between hatching successes of eggs exposed to anoxia and to those exposed to sulphide concentrations ≥250 µmol l^{-1}, and even low sulphide concentrations had a significant effect on hatching success compared to anoxia when eggs were incubated for 240 d.

Egg viability only appeared to be affected by sulphide when exposure time was ≥60 d, and hatching was almost completely inhibited after 240 d of exposure regardless of the sulphide concentration. Invidia et al. (2004) showed that sulphide exposure (0.78 to 1.12 mmol l^{-1}) for ≤15 d did not appear to be toxic to *Acartia tonsa* eggs, irrespective of pH. Sulphide toxicity became evident only after 32 d of exposure at pH 6.5, but the reduction in hatching success was not significantly different from that of eggs exposed for anoxia alone.

Studies of benthic invertebrates (Vismann 1990, Vismann & Hagerman 1996, Hagermann 1998) indicated that exposure to hypoxia/anoxia plus sulphide was more detrimental to the organisms than that of exposure to hypoxia or anoxia alone. Vismann & Hagerman (1996) and Hagerman (1998) suggested that when organisms were exposed to sulphide, the metabolic rate was not dramatically reduced because sulphide detoxification systems were required even under anaerobic conditions. The results from our long-term experiment contradict these studies when exposure time was <60 d, but supported these studies when exposure time was ≥60 d. This suggests that *Acartia*

tonsa eggs have some kind of protection to resist even high sulphide concentrations for a period of 30 to 60 d. Bagarinao (1992) reported that there is no evidence of marine animals being able to exclude sulphide at the body membrane. The fact that the tissue diffusion coefficient of H_2S is very similar to that of O_2 (Powell 1989) led Vopel et al. (1998) to suggest that equilibrium between external and internal sulphide concentrations in small-sized organisms should be reached within a few hours. As suggested by Invidia et al. (2004), the presence of an eggshell capable of blocking entry of sulphide, comparable to that of an adult organism's epithelium, might explain the high tolerance of *A. tonsa* eggs to sulphide. The short term experiment in the present study suggests that sulphide passes through the eggshell, and internal egg sulphide concentrations is expected to increase slowly as a function of exposure time and external molecular H_2S concentration. Only when eggs were exposed for a relatively long time to high H_2S concentrations could the internal sulphide reach a level where toxic effects were observed (Invidia et al. 2004).

pH and buffer capacity

The measured internal pH (6.09 and 6.07) in *Acartia tonsa* eggs was very low compared to that of other organisms; for instance, the internal pH of different invertebrates was determined by Roos & Boron (1981) to range from 7 to 8. The very low internal pH in the eggs might have resulted from peroxisomes that were unintentionally ruptured during exposure to liquid nitrogen: peroxisomes have a pH of 5 to 6 (Alberts 2002) and their rupture would cause a seepage of H^+ that would lower the internal pH of the eggs. On the other hand, an internal pH of 6 might not be an experimental artefact because the yolk, which has a high content of the lipid triacylglycerol (Lee et al. 2006), is hydrolyzed during embryogenesis to fatty acids. Fatty acids have a low pH of approximately 6.5, and their presence might result in an internal pH within the range of 6 to 7.

Why do *Acartia tonsa* eggs have such a low internal pH-value compared to other organisms? An explanation could be that low internal pH in eggs could serve as a defence mechanism towards sulphide. Based upon theoretical considerations, Groenendaal (1981) suggested that lowering the internal pH of an organism could serve as a passive protection mechanism to reduce sulphide influx into the organism's tissues. H_2S dissociates into HS^- and H^+, depending on the pH of the medium. H_2S diffuses across biological membranes much faster than does HS^- (Bagarinao 1992), and penetrates into the organism until an equilibrium with external H_2S concentration is established. Within the organism, the dissociation is shifted towards the H_2S fraction due to lower internal pH. This will reduce the influx of H_2S across the eggshell membrane and will result in a lower internal total sulphide concentration in comparison to the external concentration. If internal pH is lowered enough relative to the external pH, the internal total sulphide concentration might be effectively reduced (Sommer et al. 2000).

Eggs from copepod species that live in estuaries are exposed to the risk of becoming buried in the sediment, where the pH might be as low as 6.0 to 6.5 (Vismann 1996a). An internal pH of approximately 6.1, as measured in *Acartia tonsa* eggs, would reduce the influx of H_2S into the eggs and could thus represent an effective protection against sulphide, or may even be a result of a detoxification mechanism. We hypothesise that low internal pH contributes to the fact that toxic effects were only observed when eggs were exposed to H_2S for a long time. However, this remains to be proven.

Results from the buffer capacity measurements were somewhat uncertain, because the pH-electrode had to be lifted out of the egg matrix fluid to allow room for addition of the titrant. Every time the pH-electrode was separated from the measurement fluid, the pH reading was influenced. Therefore, buffer capacity results must only be considered to be an indication and not as fact. However, these tentative results suggest no buffer capacity in subitaneous *Acartia tonsa* eggs, and should be thoroughly investigated in future studies.

Stage development

A smaller fraction of nauplii that hatched from eggs incubated under anoxic conditions for 180 and 240 d reached the NIII stage when eggs had been exposed to high sulphide concentrations. The nauplii were only categorised as having reached the NIII stage or not. There was no further examination of whether or not they were capable of reaching adulthood. According to Invidia et al. (2004), incubation of eggs at or close to anoxia did not affect the survival of organisms hatching from the exposed eggs. However, exposure to sulphide at pH 6.5 resulted in death at the naupliar stage of the few hatched organisms. After 32 d of exposure at pH 8.2, the internal sulphide concentration might have reached a sublethal level that affected survival and ontogenetic development of nauplii, whereas at pH 6.5 the internal sulphide concentration probably reached a level where hatching success was close to zero. Invidia et al. (2004) suggested that differences in the accumulation of toxic end products during quiescence or sublethal toxic effects of sulphide could account for these observations. In near anoxic conditions, selection was reported to occur at the egg stage. The few eggs

that survived in Invidia et al.'s (2004) study had the highest tolerance and were able to develop into adulthood. In sulphidic conditions, accumulation of toxic end products was lower and reached a sublethal level. They also suggested that a 15 d period might be a threshold time during which adaptation mechanisms required to overcome exposure to near anoxia and sulphide become close to exhausted, owing to their observations that the ability to withstand the cost of quiescence became severely reduced. Our results did not support this hypothesis: our results indicated that this threshold time was not reached until between 120 and 240 d of exposure. It must be taken into account that our study did not examine larval (which reached stage NIII) ability to reach adulthood. The NIII stage is a stage where all nauplii are phagotrophic and no longer dependent on the amount of nutrients provided by the egg yolk (Berggreen et al. 1988, Mauchline 1998).

Metabolism experiments

Results obtained by the TAM were conducted in real time and represented the actual metabolism for specific batches of *Acartia tonsa* eggs during a defined time period. *A. tonsa* eggs that were exposed to normoxic conditions produced the highest amount of heat in the TAM, whereas heat-killed eggs produced the lowest amount. This was as expected, because the heat-killed eggs were considered to be a biological blind-value. However, it was a surprise that *A. tonsa* eggs exposed to anoxia and 14.7 mmol l^{-1} sulphide produced more heat than eggs exposed to anoxia alone. Hence, the depression in metabolic rate observed in *A. tonsa* eggs exposed to anoxia was not sustained when eggs were exposed to 14.7 mmol l^{-1} sulphide. According to the long term experiment, we expected eggs exposed to sulphide to have a lower metabolic activity because we believed that the metabolic rate depression would be greater when eggs were exposed to anoxia-sulphide compared to anoxia alone (Marcus et al. 1997, Invidia et al. 2004). The TAM results indicated that sulphide was capable of diffusing into *A. tonsa* eggs. This was verified by our short term as well as long term experimental data, and in the former caused only a minor reduction in the metabolic rate compared to normoxic conditions. The relatively high heat signal was probably due to sulphide detoxification mechanisms that were needed during anaerobic conditions, as suggested by Vismann & Hagerman (1996) and Hagerman (1998) for crustaceans.

If we tentatively extrapolate the calculated per hour heat production from our measurements, and assume that under normoxic conditions 1 *Acartia tonsa* egg produces a constant amount of heat (a proxy for metabolism) during its entire embryogenesis, we obtain a total heat production of 1.86 µJ × 36.7 = 68.2 µJ per egg (development time considered to be 36.7 h at 17°C [Drillet 2003]). If we assume that this heat production represented all the energy stored in the egg yolk, it would take 1 egg maintained under anoxic conditions 37.6 d to metabolise the same amount of energy. For 1 egg exposed to 14.7 mmol l^{-1} sulphide and anoxia it would only require 11.5 d, approximately a one-third reduction compared to eggs exposed to anoxia alone. We question why eggs exposed to sulphide or anoxia alone for <60 d in the present long-term experiment, as well as in the studies conducted by Marcus et al. (1997) and Invidia et al. (2004) (where eggs were exposed for 32 d), did not show any toxic effects, whereas the TAM results showed that eggs exposed to sulphide clearly had a higher metabolism? The fact that the sulphide concentration in the TAM experiment was more than 2 times higher than the highest sulphide concentration measured in the long-term experiment might explain the higher metabolism of eggs exposed to sulphide. However, when taking into account the fact that exposure to sulphide concentrations of only several µM is lethal for aerobic species without special detoxification mechanisms (Fenchel & Finlay 1995), sulphide concentrations higher than 100 µM (and perhaps even lower) in the present long-term experiment should have had a notable effect on the hatching success of *A. tonsa* eggs. Unfortunately, no control experiment (where only sulphide and no eggs were added to the anoxic antibiotic $NaHCO_3$-buffer) was conducted. Nevertheless, the shape of the thermogram in Fig. 3 demonstrated an almost constant heat production over time, which strongly indicates that heat production was constant during the experiment.

If heat production was a result of chemical reactions between sulphide and the $NaHCO_3$-buffer, and not due to biological activity, it would demand that the chemical reactions proceed with constant slow kinetics during the entire experiment, which is not considered to be plausible (P. West pers. comm.). In conclusion, the heat signals of sulphide-exposed eggs are considered to reflect the metabolism of the eggs. Before the measurements were conducted, the vessels were thermostated for 60 to 120 min. During this period, the heat signals of anoxic treatments might have been initially a little higher because the eggs might not have switched to anaerobic metabolism immediately. For the anoxic-sulphidic vessels, the heat signal might also have been higher during this period if the eggs have an acute detoxification system that proceeds at a higher rate at the beginning of exposure. For normoxic vessels, we do not expect higher metabolism at the beginning compared to the rest of the exposure time.

Acartia tonsa is a typical species of eutrophic costal areas (Brylinsky 1981) where the risk of exposure to anoxia and sulphide is pronounced. The present study indicates that subitaneous *A. tonsa* eggs, which sink to the seabed and enter quiescence, can survive weeks to months in sediments but not years. The high tolerance to both anoxia and high sulphide concentrations shown by the eggs could favour their development in the estuaries, compared to those of less tolerant species; however, the population dynamics of *A. tonsa* may subsequently be affected.

Implications for extrapolation of laboratory data

Acartia tonsa reared in the laboratory over the past 25 years was the source of eggs tested in the present study. Therefore, one might be concerned when applying these results to natural populations in the field. An additional concern could be how *Acartia tonsa* eggs from different geographic areas might respond: are consistent results to be expected? Application of results from laboratory-reared organisms to field populations is always questionable. However, we do not consider that our results would differ significantly if experiments were conducted on eggs from a nearby field population or from eggs obtained from other geographical areas, because the present results were in accordance with other studies on eggs produced in the laboratory by organisms obtained from field populations (Marcus et al. 1997, Invidia et al. 2004).

CONCLUSIONS

The present study indicates that early stages of subitaneous eggs of *Acartia tonsa* can survive exposure to anoxia and sulphide for several weeks and months, but not for years. However, increased duration and sulphide concentrations during exposure reduces hatching success. An interesting question is how do eggs survive exposure to anoxia and sulphide? It has been stated that metabolic rate is drastically depressed when eggs are exposed to sulphide, owing to a switch to anaerobiosis (Marcus et al. 1997, Invidia et al. 2004). Invidia et al. (2004) also suggested that the eggshell was capable of blocking the entry of sulphide. But how is this possible when real-time studies (TAM results) demonstrated that eggs exposed to sulphide had a higher metabolism than eggs exposed to anoxia alone, and that eggs become decreasingly viable over time? The inevitable conclusion is that sulphide is capable of crossing the eggshell, and that the eggs have some kind of detoxification system. The costs of activating this detoxification system were responsible for the higher heat production (metabolism) observed when *A. tonsa* eggs were exposed to sulphide. According to simple calculations based on TAM results, *A. tonsa* eggs would require 37.6 d to metabolise the total amount of energy stored in the egg yolk when exposed to anoxia, but only 11.5 d if eggs were exposed to 14.7 mmol l^{-1} sulphide. In the long-term experiment, *A. tonsa* eggs exposed to anoxia were still capable of hatching after an incubation period of 240 d, whereas eggs exposed to sulphide seemed to reach the limit of their tolerance level and could no longer hatch. The eggs survived for a disproportional long time when compared with calculated survival time, which suggests that *A. tonsa* eggs are equipped with an unknown defence mechanism against hydrogen sulphide.

Future studies of *Acartia tonsa* eggs, or copepod eggs in general, should concentrate on real-time observations where eggs are exposed to different sulphide concentrations in order to identify if they have a minimum tolerance level towards sulphide and a threshold exposure period. Measurements of actual internal sulphide concentrations of eggs, in addition to molecular biological studies, could also provide important insights into the mechanistic background and functioning of the suggested sulphide protection or detoxification system. These initiatives could help solve the mystery of how *A. tonsa* eggs cope with long term exposure to anoxia and sulphide.

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