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SHORT COMMUNICATION

POST-ANOXIC VIABILITY AND DEVELOPMENTAL RATE OF *ARTEMIA FRANCISCANA* ENCYSTED EMBRYOS

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Free-living animals differ widely in their tolerance to the absence of molecular oxygen (anoxia): most die within hours or days under these conditions, and even well-adapted forms such as intertidal invertebrates do not survive more than a month or so of continuous anoxia (Hochachka, 1980; Hochachka and Guppy, 1987; Bryant, 1991). A striking exception is the brine shrimp, *Artemia franciscana*, whose encysted embryos (cysts) have previously been reported to survive 4 months of continuous anoxia with no decrease in viability (Dutrieu and Chrestia-Blanchine, 1966) and 7 months with only a 25% reduction (Stocco *et al.* 1972). Both studies were terminated at the times given so that anoxic survival limits of this remarkable organism remain to be determined. In view of these observations, and the interest in cyst metabolism during anoxia (see Hand and Gnaiger, 1988; Clegg and Jackson, 1989*a*; Hand, 1990; Hofmann and Hand, 1990), further study of anoxic survival seemed worthwhile.

Activated cysts in 'vacuum-packed' cans (San Francisco Bay Brand, Hayward, California) were processed and stored at −20°C (Clegg, 1986). These cysts are 'activated' in the sense that their hydration under permissive conditions (aerobic sea water at 25°C) results in the resumption of metabolism and development. Activated cysts contrast with the diapause cysts released from maternal females: *diapause* is a state of obligatory dormancy that is terminated by desiccation, among other treatments, resulting in activated cysts (Drinkwater and Clegg, 1991). Both diapause and activated cysts experience hypoxia/anoxia in nature (see Drinkwater and Clegg, 1991).

Dried cysts (about 100 mg) were hydrated under anoxic conditions (Clegg and Jackson, 1989*a*) by the addition of 7 ml of anoxic sterile sea water (deoxygenated with 100% N_2 for 4 h) containing penicillin (1.3 mg ml^{-1}) and streptomycin (1.2 mg ml^{-1}) in an 8 ml screw-cap glass vial (N_2 in the 1 ml gas phase). The caps were wrapped with several layers of Parafilm to prevent loosening, and the vials were stored on their sides under ambient conditions of light and temperature (21–24°C). After anoxia, the cysts from each vial were collected by filtration and some were immediately assayed for *emergence* of embryos from their surrounding

Key words: anoxia, *Artemia franciscana*, encysted embryos.

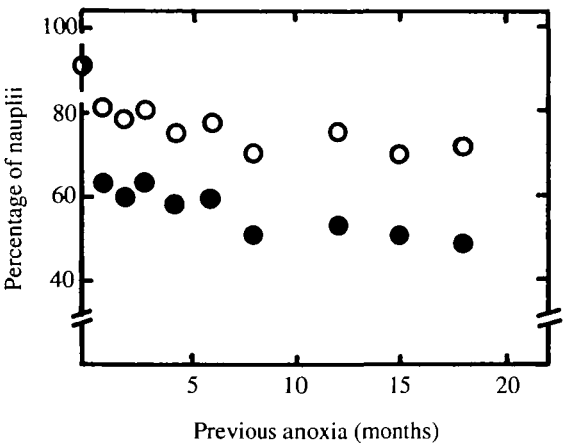

Fig. 1. Larval hatching (percentage of nauplii) from cysts previously exposed to continuous anoxia. Filled circles are anoxic cysts transferred directly to aerobic sea water; open circles are anoxic cysts desiccated for 24 h and then incubated in aerobic sea water. Each point represents at least 200 cysts; the bar on the zero time axis give the standard deviation for four replicates of at least 200 cysts.

shells and subsequent *hatching* of swimming larvae. The remaining cysts from each vial were dried in air for 24 h and then treated as above. Hatching assays were carried out at 21–24 °C in sealed plastic depression plates for 5 days in sea water (SW). Each plate contained 20 wells (0.6 ml) to which were added 0.3 ml of SW and at least 10 cysts; thus, at least 200 cysts were assayed for each data point. All vials were examined after anoxia to verify that no emergence had occurred during the anoxic exposure.

Fig. 1 shows final hatching levels from previously anoxic cysts. Desiccation increased the hatch level by about 20 %, an observation noted previously in preliminary studies (Drinkwater and Clegg, 1991). Since desiccation terminates diapause in these embryos, we proposed that anoxia may cause some of them (about 20 %) to re-enter diapause. Linear regression of the open circles in Fig. 1 indicates that some of these embryos may survive about 9 years of continuous anoxia (r^2=0.59). While the assumption of linearity remains to be verified, these data demonstrate the exceptional tolerance of these embryos to continuous anoxia.

It should be pointed out that anoxic cysts in SW have been shown to contain the same amount of water as aerobic ones (Clegg and Jackson, 1989*b*). Thus, differential hydration is not a factor in the results observed here.

Stocco *et al.* (1972) found that the onset of emergence was delayed when anoxic cysts were incubated aerobically, and we examined this for the cysts represented in Fig. 1. Fig. 2 shows the time course of emergence and hatching for cysts previously anoxic for 3 months (Fig. 2B,C) compared to controls (Fig. 2A). Similar studies have been made on all the samples in Fig. 1. Emergence is delayed, as Stocco *et al.*

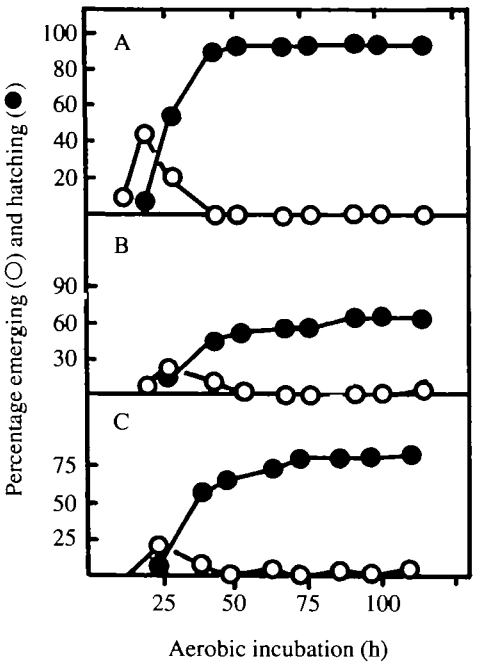

Fig. 2. Emergence (open circles) and hatching (filled circles) of control cysts (A) which had not experienced anoxia, and cysts previously exposed to 3 months of anoxia (B,C). Cysts in B were transferred directly to aerobic sea water, and those in C were first desiccated for 24 h and then assayed as described. At least 200 cysts were used in each of the studies.

(1972) found, but the subsequent hatching of larvae from these embryos proceeds in a similar manner to the controls at the level of resolution of these data. Thus, the time required for half of the 'final' hatching level to be reached ($t_{1/2}$N) can be used as a general measure of the anoxia-induced delay in aerobic development (Fig. 3). Although the precise value of $t_{1/2}$N is uncertain because the total number of cysts that will eventually hatch cannot be predicted, it is clear that the duration of anoxia is related to the delay in post-anoxic development. The metabolic basis for this delay should prove interesting.

Such tidy results as those shown in Fig. 1 were not always obtained (Fig. 4) and the basis of this variability is not known. No obvious relationship was noted between the time of year that anoxia began (or was terminated) and the level of hatching. The static incubation conditions could be a factor since some of the cysts sink, some are suspended and some reside at the liquid–gas phase interface. If anoxia induces diapause, then some of the variability could be due to differences in the extent and 'depth' of diapause induction between samples.

How do these cysts survive years of continuous anoxia? Many anoxia-tolerant animals reduce their metabolic rates when challenged with anoxic conditions, a phenomenon called 'metabolic rate depression' (MRD) and reviewed recently by

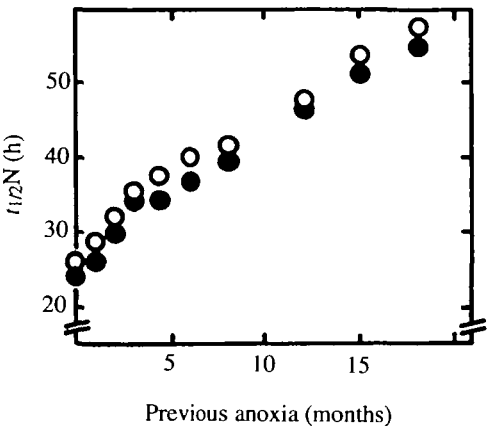

Fig. 3. The time required for half the final hatching level to be reached ($t_{1/2}$N) from cysts previously incubated under anoxic conditions.

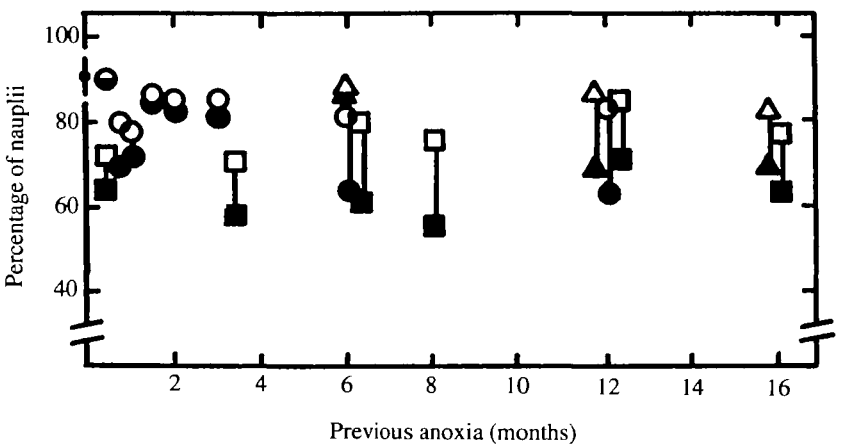

Fig. 4. Larval hatching (percentage of nauplii) from cysts previously exposed to continuous anoxia. Different symbols represent separate experiments, each involving at least 200 cysts. The vertical lines connect data points for cysts from the same experiment: those transferred directly to aerobic sea water after anoxia are filled symbols and those desiccated after anoxia and then incubated in aerobic sea water are open symbols.

Storey and Storey (1990). In general, MRD reduces the metabolic rate to 5–20 % of the aerobic basal rate. The *Artemia franciscana* embryo may represent the lower limit of MRD in which long-term anoxia reduces metabolism to a standstill, 'locking' the cells into a static but stabilized state (Clegg and Jackson, 1989*a*,*b*). In this context, it seemed worthwhile to evaluate the longevity of unfed nauplii from previously anoxic cysts, since even a slow metabolism in anoxic cysts should

Table 1. *Longevity of unfed nauplii hatched from control and previously anoxic cysts*

Anoxia (months)	Life span (days)
0 (controls)	3.7±0.8 (29)
6	3.6±0.9 (36)
15	3.9±0.8 (36)
18	3.9±0.6 (31)

Values are mean±S.D. (*N*).

reduce endogenous stores available to the nauplii. Remarkably (Table 1) no difference in starved life-span was noted for nauplii from cysts experiencing up to 18 months of anoxia compared to those from control cysts. Thus, if an anoxic metabolism exists, it does not seem to utilize anything upon which endogenous larval longevity depends. Of course, it is possible that larval longevity does not depend exclusively on endogenous nutritional stores.

We continue to test the hypothesis that anoxia reduces these embryos, fully hydrated and at ordinary temperatures, to an ametabolic state in which they somehow escape macromolecular denaturation and other damaging processes over periods of years. The mechanisms involved in this stabilization deserve further study.

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