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EFFECT OF ANAEROBIOSIS ON CYSTEINE PROTEASE REGULATION DURING THE EMBRYONIC–LARVAL TRANSITION IN *ARTEMIA FRANCISCANA*

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Summary

Hydrated encysted embryos of the brine shrimp *Artemia franciscana* have the ability to withstand years in anaerobic sea water using metabolic strategies that enable them to inactivate all cell metabolic activities and then to resume development when placed in aerobic sea water. However, this unique characteristic of *Artemia franciscana* embryos is lost during a very short period, at the embryonic–larval transition period of development, coincident with the appearance of prenauplius larvae. Thus, while encysted embryos show complete inhibition of proteolysis over at least 4 years under anoxia, control of this activity, together with resistance to anoxia, is lost in newly hatched nauplius larvae after only a few days in anaerobic sea water. In contrast to encysted embryos, young larvae in anaerobic sea water produce large amounts of lactic acid, which reaches a concentration of nearly 50 mmol l^{-1} within 12 h of incubation. The accumulated lactic acid is believed to reduce the intracellular pH (pHi) to considerably less than 6.3, the value found in encysted embryos after 5 months in anaerobic sea water. We find that larvae, in contrast to embryos, lose cytoplasmic proteins at the rate of 4–5 ng h^{-1} $larva^{-1}$ upon transfer to anaerobic sea water, while yolk proteins are not degraded in either embryos or larvae under anoxic conditions. The decline in cytoplasmic protein levels in anaerobic larvae may be due to activation of an endogenous cysteine protease (CP) as the pHi becomes acidic. Contributing to the apparent uncontrolled CP activity is a decrease in the level of cysteine protease inhibitor (CPI) activity during the embryonic–larval transition period, resulting in an increase in the CP/CPI ratio, from approximately 0.5 in embryos to greater than 1.0 in newly hatched larvae. Finally, data are presented to suggest that loss of the 26 kDa stress protein from embryos during the embryonic–larval transition may also contribute to the loss in resistance of young nauplius larvae of *A. franciscana* to anaerobic conditions.

Key words: *Artemia franciscana*, brine shrimp, anoxia, anaerobiosis, cysteine protease, cysteine protease inhibitor, intracellular pH, 26 kDa protein, stress protein.

Introduction

Many Metazoa, especially aquatic invertebrates, have acquired the ability to survive during relatively long periods under hypoxic or anaerobic conditions (Hochachka and Guppy, 1987; Storey and Storey, 1990; Bryant, 1991; Hochachka *et al.* 1993; Pörtner and Grieshaber, 1993; Grieshaber *et al.* 1994; Guppy *et al.* 1994; Hand and Hardewig, 1996). Most organisms well-adapted to anaerobic conditions have survival times (LT_{50}) of days or weeks, only to resume normal physiological functions when normal environmental oxygen levels are restored. However, in one member of this group of organisms, the brine shrimp *Artemia franciscana*, larvae and adults have limited resistance to anoxia, but their encysted embryos have an LT_{50} well in excess of 4 years (Clegg, 1994, 1997).

Adaptation to anaerobic conditions appears to have been achieved through metabolic rate depression to levels that are commonly only 5–10 % of corresponding metabolic activity levels in aerobic environments (Storey and Storey, 1990; de Zwaan *et al.* 1991; Hochachka *et al.* 1993; Hand and Hardewig, 1996). In addition, there is evidence that some organisms resistant to anaerobiosis have acquired the ability to stabilize membrane functions (Hochachka, 1986; Hand and Hardewig, 1996). Thus, numerous metabolic arrest strategies have evolved, including phosphorylation/dephosphorylation mechanisms (Whitwam and Storey, 1991), proteolytic activity regulation (Anchordoguy and Hand, 1994, 1995), transition from aerobic to anaerobic metabolism (Ip *et al.* 1993), production of stress-related proteins (Brooks and Storey, 1993; Clegg *et al.* 1994, 1995; Jackson and Clegg, 1996) and perhaps protein–protein interactions which alter the free/bound forms of key metabolic enzymes (Lazou *et al.* 1994). In the case of *A. franciscana*, encysted embryos show the most extreme depression of metabolism in response to anoxia of any known organism, becoming reversibly ametabolic under anaerobic

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conditions (Hontario *et al.* 1993; Hand, 1995; Clegg, 1997). Although significant clues about the mechanism(s) responsible for 'switching off' the major metabolic processes in these anaerobic embryos have been put forward, a complete picture has not yet emerged (see, for example, Busa *et al.* 1982; Hand and Gnaiger, 1988; Clegg and Jackson, 1989*a*,*b*; Clegg, 1994; Anchordoguy and Hand, 1994, 1995; Hofmann and Hand, 1994; Kwast *et al*. 1995; Kwast and Hand, 1996*a*,*b*; Hand and Hardewig, 1996).

In the study reported here, we show that the dramatic and stringent metabolic controls operative in anaerobic embryos of *A. franciscana* are altered during the embryonic–larval transition period of development, resulting in a marked decrease in the LT_{50} from greater than 4 years in anaerobic embryos (Clegg, 1997) to 1 or 2 days in anaerobic larvae. The importance of lactic acid production, consideration of intracellular pH (pHi) regulation and the control of cysteine protease activity in anaerobic embryos and larvae provide the main focus of this paper.

Materials and methods

Preparation of samples

Encysted gastrula embryos of *Artemia franciscana* (Kellogg) were obtained from the *Artemia* Reference Centre (Ghent, Belgium) and Sanders (Ogden, UT) and stored dry at −20 °C. When needed, encysted embryos were hydrated in sea water for at least 5 h at 0–4 °C, then collected on a cloth filter, rinsed with distilled water, blotted to remove excess water and weighed. Each milligram contained 105±5 hydrated cysts (mean ± S.E.M., N=10). Hydrated encysted embryos (0 h embryos) were cultured in aerobic or anaerobic sea water in the presence of antibiotics using established culture conditions (Clegg, 1993). Embryos undergoing aerobic development were collected after 10–12 h of incubation at 25–27 °C, while young swimming larvae (N-1) were collected after 15–18 h of incubation at this temperature. For the anoxia experiments, embryos or larvae were concentrated on a cloth filter, washed with nitrogen-purged Millipore-filtered (pore size 0.45 μm) sea water, and 10 000–15 000 organisms were added to 8 ml scintillation vials and sealed with a cap and Parafilm wrappings. (Note that larvae placed in anaerobic sea water become immotile within 90 min at room temperature.) Control larvae were maintained in aerobic sea water under otherwise identical conditions to larvae in anaerobic sea water. At the desired times of incubation, the organisms were collected on small cloth filters and either used immediately or frozen in liquid nitrogen and stored at −70 °C.

Preparation of embryo and larval extracts

Embryos or larvae were homogenized in a buffer containing 150 mmol l^{-1} sorbitol, 70 mmol l^{-1} potassium gluconate, 5 mmol l^{-1} monobasic potassium phosphate and 35 mmol l^{-1} Hepes and adjusted to either pH 6.8 or 7.4. The homogenate was centrifuged at 1630 ***g*** for 10 min to obtain the supernatant and pellet (nuclei and yolk platelets) fractions. The pellet was washed once with homogenization buffer and, after centrifugation at 1630 ***g***, the washing buffer was added to the original supernatant. These fractions were used for protein analysis where the 1630 ***g*** supernatant represents mainly cytoplasmic proteins, while the 1630 ***g*** pellet represents proteins in the yolk platelets/nuclei fraction of embryos and larvae (Clegg *et al.* 1994, 1995). For cysteine protease and cysteine protease inhibitor analyses, the 1630 ***g*** supernatant was centrifuged at 15 000 ***g*** for 30 min (4 °C) to obtain the mitochondria plus lysosome (M+L) fraction and a supernatant (PMF) that we call the *aqueous cytoplasm* throughout this paper.

Viability measurements

The resistance of embryos and larvae to anaerobic stress, or measurement of survival time in anaerobic sea water, was determined by transferring organisms to aerobic sea water (at room temperature, 20–23 °C) after various times in anaerobic sea water, and counting the number of embryos that hatched into swimming larvae or larvae that recovered full swimming activity by 12–18 h in aerobic sea water. Only larvae that recovered full swimming activity were considered to be fully viable and resistant to anaerobic conditions.

Protein and lactate analyses

Protein analyses were carried out on the 1630 ***g*** supernatant and pellet fractions in two ways. For the quantification of proteins, the supernatant and pellet (mainly yolk platelets) were solubilized in 100 mmol l^{-1} NaOH, diluted 10-fold with water then analyzed using the bicinchoninic (BCA) reagent with bovine serum albumin as the standard according to the manufacturer's instructions (Pierce, Rockford, IL, USA). For qualitative analysis, sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) was carried out on 7 % to 18 % gradient gels according to the procedure of Laemmli (1970) on samples taken from the 1630 ***g*** supernatant and pellet derived from embryos and larvae and diluted with loading buffer to the desired protein concentration.

Lactic acid content of larvae was measured in the 6 % perchloric-acid-soluble fraction of total homogenates of larvae and Millipore-filtered incubation medium using a lactate-dehydrogenase-based commercial kit for the determination of lactate (Sigma Chemical Co., St Louis, MO, USA) as described previously (Clegg and Jackson, 1988).

Analysis of cysteine protease and cysteine protease inhibitor activity

The cysteine protease (CP) and cysteine protease inhibitor activity (CPI) in embryos and larvae were measured after fractionation of the aqueous cytoplasm (PMF) on a Sephadex G-150 (SF) column (1 cm×77 cm). The PMF (obtained by centrifugation of the 1630 ***g*** supernatant at 15 000 ***g***) was passed through a Sephadex G-25 column to remove the low molecular mass components, then concentrated to approximately 1 ml using a Centricon 3 filter (Amicon, Oakville, Ontario, Canada). The concentrate representing the aqueous cytoplasm from 30 000–40 000 organisms was applied

to the Sephadex G-150 column and the protein eluted with a buffer containing 15 mmol l^{-1} potassium phosphate, 25 mmol l^{-1} potassium chloride and 10 % glycerol, pH 6.8. The CP and CPI activities eluted from the column were assayed using the trinitrobenzene sulfonic acid (TNBS) reagent described previously (Warner and Shridhar, 1985; Warner and Sonnenfeld-Karcz, 1992). The CP used in the CPI assays was purified to homogeneity in one of our laboratories (Windsor) by Andrea Aiton using a modification of a published procedure (Warner and Shridhar, 1985). One milliunit of CP activity (mEU) is defined as the release of 1 nmol min^{-1} of amino peptide from the substrate (protamine sulfate) at pH 5 and 30 °C. One milliunit of CPI activity (mIU) is the amount of protein that completely inhibits 1 mEU of CP activity under the conditions used above. Protease activity in the aqueous cytoplasm of embryos and larvae was also visualized by SDS–PAGE on 7 % to 18 % gradient gels.

Analysis of cysteine protease and 26 kDa protein on western blots

Various embryo and larval cytoplasmic fractions from the Sephadex G-150 column were analyzed for the major cysteine protease and 26 kDa stress protein by SDS–PAGE and western blotting using a horseradish peroxidase reporter system, as described recently (Clegg *et al.* 1994, 1995; Warner *et al.* 1995). The cysteine protease antibodies were obtained from rabbits and affinity-purified as described previously (Warner *et al.* 1995), while the 26 kDa protein and anti-26 kDa protein antibodies were generous gifts from Dr Tom MacRae of Dalhousie University (Halifax, Nova Scotia, Canada).

Results

Anaerobic survival of embryos and larvae of Artemia franciscana

Fully hydrated encysted gastrula embryos of *A. franciscana* are able to withstand at least 4 years in anaerobic (nitrogen-treated) sea water as determined by their ability to hatch into normal free-swimming larvae upon exposure to aerobic sea water (Clegg, 1997). In contrast, swimming larvae of *A. franciscana* lose viability in less than a day after exposure to anaerobic sea water (Ewing and Clegg, 1969). However, the precise developmental time at which *A. franciscana* larvae become irreversibly damaged by anaerobic exposure was not established in that work. Therefore, to define more closely the developmental stage at which *A. franciscana* larvae become irreversibly affected by anaerobic conditions, we subjected (aerobic) embryos and larvae at different stages of development to anaerobic sea water at 20 °C for varying times and determined their survival as the percentage of embryos hatching or of larvae recovering swimming activity when the organisms were returned to aerobic sea water. The results of this experiment are summarized in Fig. 1. As expected from previous studies, approximately 88 % of 0 h embryos (which were approximately 90 % viable initially) hatched when returned to aerobic conditions after 6 weeks in anaerobic sea water at 20 °C. Embryos incubated in air for 12 h before exposure to anaerobic sea water showed similar stability (88 % survival) after 30 days of anaerobiosis. In striking contrast, prenauplius larvae (E-2 stage) and newly hatched nauplius larvae (N-1 stage) were found to lose viability after only 1–2 days in anaerobic sea water, with the E-2 stage larvae being slightly more resistant than the N-1 stage (nauplius) larvae. It should be noted that after 12 h of aerobic incubation some of the encysted embryos are just ready to begin emergence under conditions which lead to complete hatching of the larvae during the next few hours. It is also noteworthy that both E-2 and N-1 larvae recovered slowly from prolonged anaerobic stress, requiring up to 18 h at room temperature to recover full swimming activity. Alkalization of anaerobic sea water with 40 mmol l^{-1} NH_4Cl improved the rate of recovery and survival profile of E-2 larvae by 15–20 %, but had no such effect on N-1 larvae (data not shown). The implications of this finding are discussed below. Thus, the response of embryos and larvae to anaerobic conditions changes dramatically during a very narrow window of only a few hours during development.

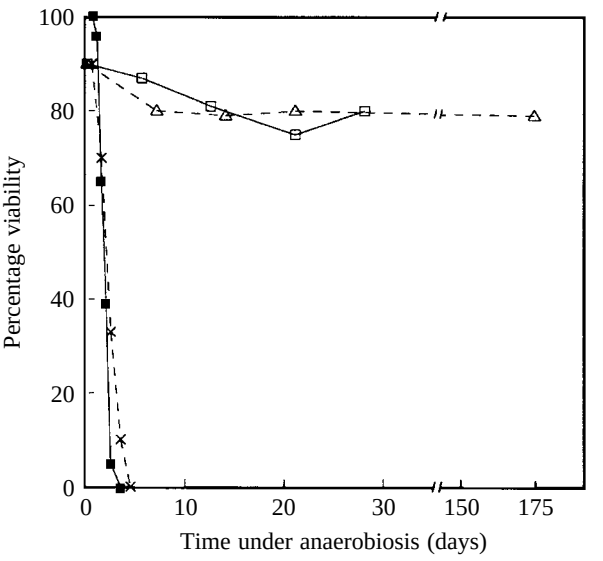

Fig. 1. Survival of embryos and larvae in anaerobic sea water at different stages of development. Embryos after 0 h (△) and 12 h (□) of incubation in aerobic sea water were placed in anaerobic sea water in sealed vials and maintained without shaking at room temperature (20–22 °C) until the times indicated. The animals were then returned to aerobic sea water and the number that hatched into swimming nauplius larvae was recorded. Prenauplius larvae (E-2 stage) (×) and nauplius larvae (N-1 stage) (■) were obtained by incubating embryos in aerobic sea water for 12–15 h. They were then placed in anaerobic sea water and maintained at 20–22 °C. At the times indicated, they were returned to aerobic sea water and the numbers of prenauplius larvae that hatched or larvae that regained full swimming activity were recorded. Three replicates were recorded per print.

The effect of temperature on the potentially toxic effects of anaerobic sea water was also studied (Fig. 2). As expected, loss of viability of larvae in anaerobic sea water was strongly temperature-dependent between 8 °C and 30 °C. Moreover, the loss of viability under anaerobic conditions as a function of

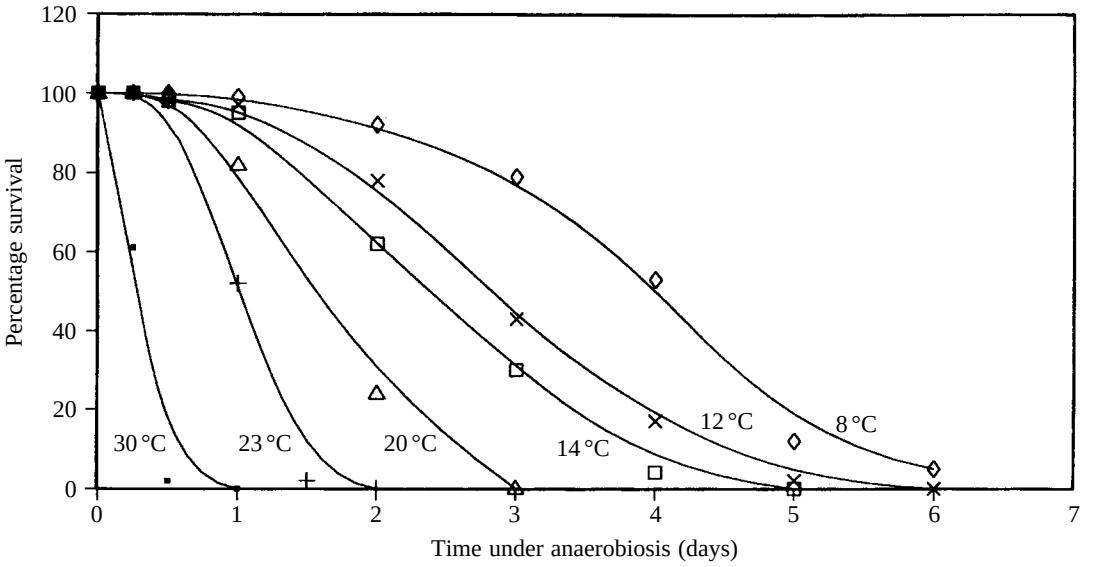

Fig. 2. Effect of temperature on the survival of nauplius larvae (N-1 stage) in anaerobic sea water. Approximately 10000 newly hatched larvae were placed in sealed vials containing anaerobic sea water, and after the larvae had stopped swimming (approximately 90 min, 22–23 °C) they were maintained at the temperatures shown. At various times, samples of 100–200 larvae were returned to aerobic sea water, and the number that regained full swimming activity at room temperature was recorded. Curves were obtained as Best fit lines by Harvard Graphics.

temperature days (days×°C) was relatively constant between 8 °C and 23 °C. In this temperature range, the Q_{10} for larval survival was calculated to be approximately 3.

Protein levels in embryos and larvae incubated in aerobic and anaerobic sea water

Encysted embryos of *A. franciscana* become reversibly ametabolic when placed under anaerobic conditions (see Hontario *et al.* 1993; Clegg, 1997; Hand, 1995). Also, rapid suppression of the ubiquitin-mediated proteolytic pathway occurs in *A. franciscana* embryos following transfer to anaerobic sea water, suggesting that protein catabolism is inhibited during anaerobiosis (Anchordoguy and Hand, 1994, 1995; Hand and Hardewig, 1996). To analyze further the effect of aerobic conditions on protein metabolism, we determined the protein levels in embryos and larvae maintained in anaerobic sea water compared with those incubated aerobically. Analysis of the 1630***g*** supernatant and pellet of encysted embryos (i.e. late gastrulae) showed no significant changes in the protein content of these fractions after 4 weeks in anaerobic sea water compared with aerobic control embryos

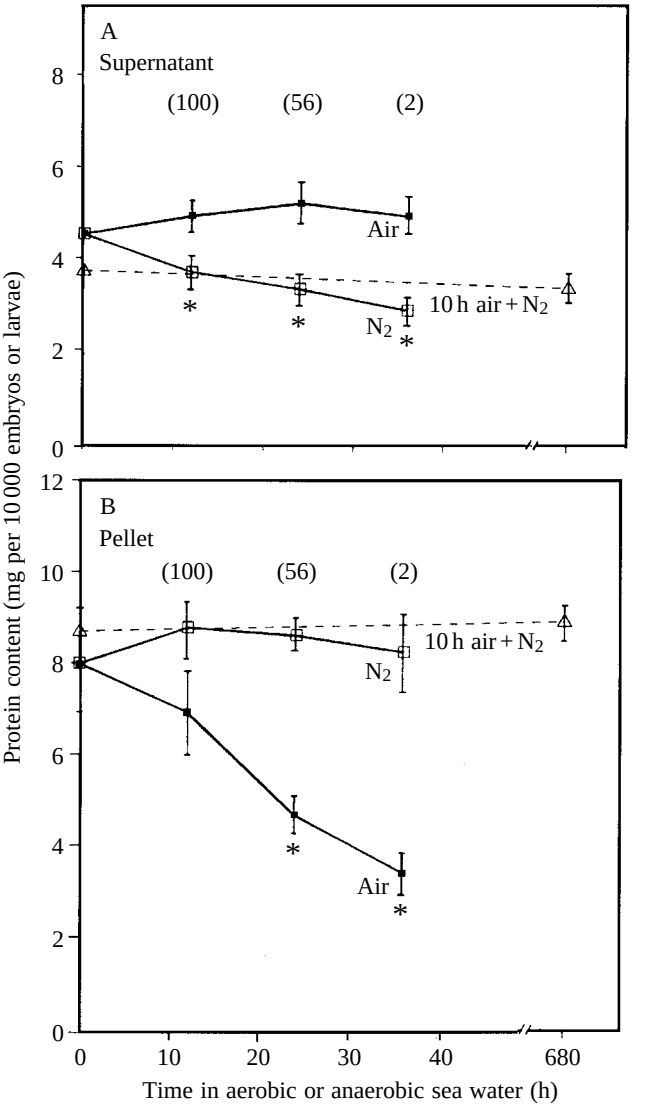

Fig. 3. Protein levels in aerobic and anaerobic embryos and larvae. Newly hatched nauplius larvae (N-1 stage, 10000 per sample) were placed either in aerobic sea water (■) or anaerobic sea water (□) for up to 36 h at 22–23 °C. In addition, embryos incubated for 10 h in aerobic sea water were transferred to anaerobic sea water (△) and maintained at room temperature for an additional 680 h (1 month). At the end of the desired incubation period, embryos and larvae were collected and the protein contents of the 1630***g*** supernatant (A), representing mainly the aqueous cytoplasm, and the 1630***g*** pellet (B), containing mainly yolk platelets and nuclei, were determined. The numbers in parentheses indicate the percentage viability of larvae after the various times in anaerobic sea water. Over 80 % of the encysted embryos (△) were viable after 1 month in anaerobic sea water. An asterisk indicates a value significantly different from that for untreated (control) larvae at P=0.05. Values are means ± S.E.M., N=4–6.

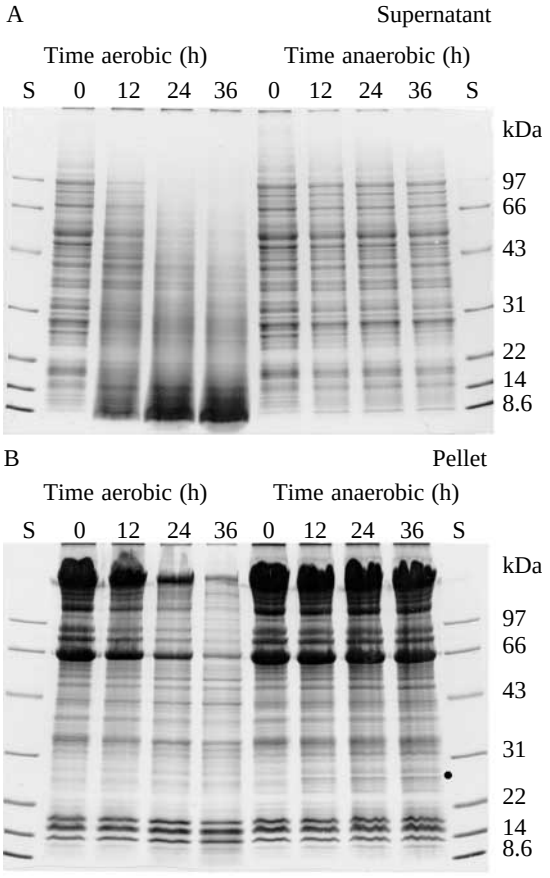

Fig. 4. Analysis of proteins by SDS–polyacrylamide gel electrophoresis of aerobic and anaerobic larvae. Samples of the larval supernatant (A) and pellet (B) fractions shown in Fig. 3 representing 50 larvae per sample were electrophoresed on an SDS–polyacrylamide gel (7% to 18%) and the gel was stained with Coomassie Blue. Standard proteins were included in the outside lanes (S) of each gel, and the molecular mass of these proteins is shown on the right. Note the stability of the protein bands in the yolk platelet fraction (B) of larvae after 24 and 36 h in anaerobic sea water compared with those of larvae in aerobic sea water. The decrease in the amounts of high molecular mass proteins in the supernatant of aerobic larvae with age is due to endogenous serine proteases which have not been inhibited in these extracts. The appearance of a protein band at 26 kDa (●) in the yolk platelet/nuclear fraction of anaerobic embryos has been reported previously (Clegg *et al.* 1994).

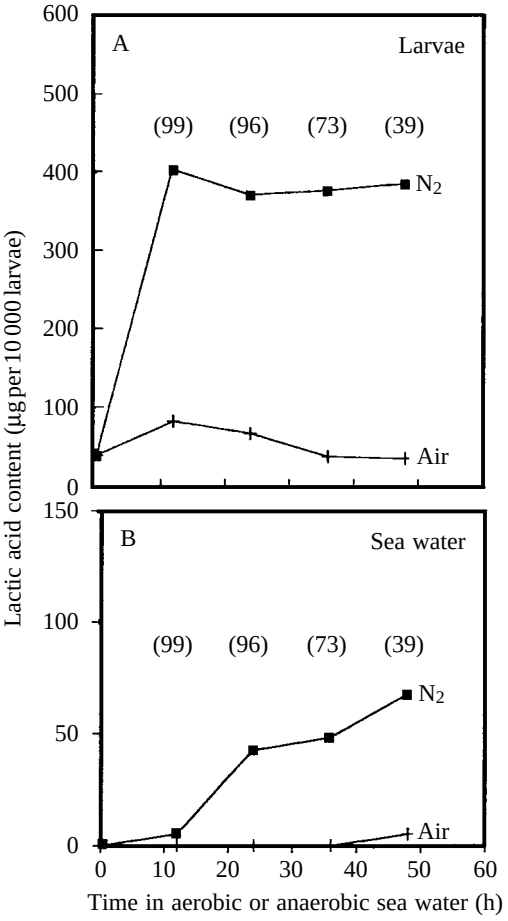

Fig. 5. Lactic acid production in anaerobic and aerobic larvae. Newly hatched nauplius larvae (10 000 per sample) were placed in either aerobic sea water (Air) or anaerobic sea water (N_2) for up to 48 h at 18–20 °C. At the end of the incubation period, the larvae were collected by filtration and the lactic acid content of larvae (A) and sea water (B) was determined as described in Materials and methods. The numbers in parentheses near the top of each panel represent the percentage viability of each sample of larvae collected at the times indicated in anaerobic sea water. *N*=3 for each data point.

(Fig. 3). In contrast, when N-1 stage larvae were incubated anaerobically, the protein content of the 1630***g*** supernatant decreased at a rate of 4–5 ng h^{-1} $larva^{-1}$ during the first 36 h (at 23 °C). The protein content of the 1630***g*** pellet (representing mainly yolk proteins) remained relatively constant in anaerobic larvae, at a level similar to that observed for anaerobic embryos, suggesting suppression of yolk utilization in both cases. Electrophoresis of larval proteins (Fig. 4) showed that the large decrease in protein in the 1630***g*** pellet of aerobic larvae (but not in anaerobic larvae) was due mainly to yolk protein catabolism. The appearance of a protein of 26 kDa in the 1630***g*** pellet of anaerobic larvae may be due to translocation of this protein from the aqueous cytoplasm into the nucleus in response to anoxia (Clegg *et al.* 1995).

Lactate production in aerobic and anaerobic nauplius larvae

In an early study, Ewing and Clegg (1969) demonstrated that lactic acid is produced in nauplius larvae, but not in encysted embryos of *A. franciscana*, in response to anaerobic conditions. Since pHi may play a key role in the regulation of metabolic activity in both embryos and larvae, we re-examined lactic acid levels in larvae incubated in anaerobic sea water compared with aerobic controls. The results (Fig. 5A) show that lactic acid levels increased rapidly in larvae maintained in anaerobic sea water, reaching a level of 0.04 μg $larva^{-1}$ within 12 h of incubation, while larvae in aerobic sea water showed only a slight increase in lactic acid content (0.004 μg $larva^{-1}$). The high concentration of lactic acid attained in anaerobic larvae is

maintained for several hours, then begins to ‘spill’ into the surrounding sea water (Fig. 5B) as larvae lose viability.

Protease activity in aqueous cytoplasm of embryos and larvae under acidic conditions

Previous studies have shown that embryos and young larvae contain an abundance of cysteine proteases whose activity exhibits an acidic pH optimum and very little activity representing the other major types of proteases (Warner, 1989*b*). Shortly after the larvae hatch, large amounts of serine proteases with an alkaline pH optima are synthesized (Osuna *et al.* 1977). Thus, it seemed likely that lactic acid production in anaerobic larvae might activate endogenous cysteine proteases. To examine this possibility, we incubated aqueous cytoplasm (concentrated) obtained from embryos and larvae at pH 6.0 to determine whether acidification would result in cytoplasmic protein degradation by endogenous proteases. Concentrated aqueous cytoplasm from 12.5 h aerobic embryos, young (N-1) larvae and larvae incubated in anaerobic sea water for 12 h was incubated at pH 6 (and at 30 °C) for up to 16.5 h and the proteins were analyzed by SDS–PAGE. The results in Fig. 6 show that cytoplasmic proteins of 12.5 h embryos resisted degradation *in vitro* for at least 16.5 h at pH 6.0, while in newly hatched nauplius larvae several of the major cytoplasmic proteins were degraded under comparable conditions. The proteins gained during the incubation of concentrated aqueous cytoplasm from 0 h larvae (Fig. 6B) probably represent partially degraded polypeptides generated during the incubation. The addition of naturally occurring and synthetic cysteine protease inhibitors to aqueous cytoplasm from larvae inhibited protein degradation by over 75 % during the 16.5 h of incubation, while the addition of serine protease inhibitors had no effect on the pattern of protein degradation (data not shown). Collectively, our results suggest a role for the major cysteine proteases in the decrease in the amounts of cytoplasmic proteins in anaerobic larvae.

Cysteine protease and cysteine protease inhibitor levels in aerobic and anaerobic embryos and larvae

The cysteine proteases (CP) in *A. franciscana* embryos appear to be under the control of endogenous protease inhibitors (CPI) during early development (Warner, 1989*a*,*b*). Also, the activity of the CPI has been shown to depend on the incubation conditions, being most active at pH 5–6.5. Therefore, we investigated the cytoplasmic CP/CPI ratio in embryos and larvae incubated in aerobic and anaerobic sea water. The results in Fig. 7 show elution profiles of embryo and larval cytoplasmic proteins together with the CP and CPI proteins. Of interest was the observation that embryo CP eluted from the column as a 95 kDa complex, while larval CP eluted at a position of 60 kDa, close to the observed molecular mass of purified embryo and larval CP (Warner, 1989*b*). The high molecular mass of the embryo CP from the gel filtration column suggested that it might be complexed with other proteins while larval CP is not. A similar 95 kDa complex was detected in anaerobic embryos but not in anaerobic larvae. The

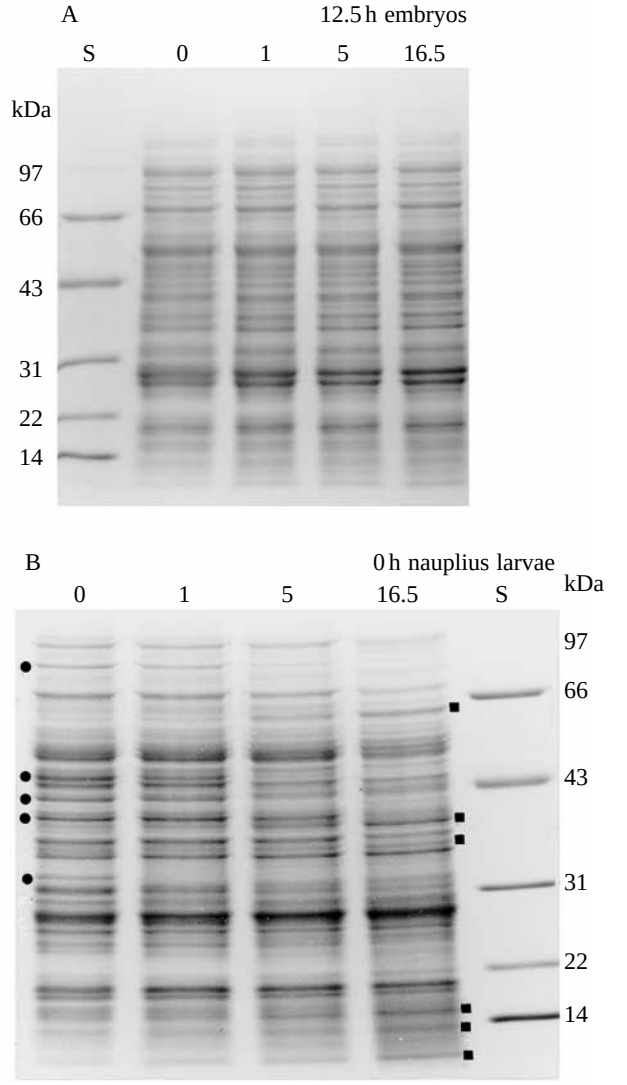

Fig. 6. Endogenous protease activity in the aqueous cytoplasm of embryos and larvae as shown by SDS–polyacrylamide gel electrophoresis. Concentrated aqueous cytoplasm from 12.5 h embryos (A) and 0 h larvae (B) was incubated in a buffer containing 50 mmol l^{-1} sodium acetate, 1 mmol l^{-1} dithiothreitol and 1 mmol^{-1} EDTA, pH 6.0 at 30 °C, and samples were taken at the times (in h) indicated above each lane for protein analysis on SDS–polyacrylamide gels (7 % to 18 %). Standard proteins were also included in one lane (S) of each gel. The filled circles in B indicate the proteins lost during incubation, while the the filled squares represent the protein bands gained. Each lane in A contains approximately 30 μg of protein, and each lane in B contains approximately 35 μg of protein.

separation of cysteine protease (CP) and cysteine protease inhibitor (CPI) activity by gel filtration allowed us to quantify the CP and CPI activities, and to determine the CP/CPI ratio in aerobic and anaerobic embryos and larvae. The data in Table 1 show that embryos have a CP/CPI ratio of 0.46–0.71, while in young larvae this ratio is greater than 1. The increase in CP/CPI in newly hatched nauplius larvae appears to be due mainly to a decrease in their CPI content. During anaerobiosis,

Table 1. *Cysteine protease and cysteine protease inhibitor activity in* Artemia franciscana *embryos and larvae under aerobic and anaerobic conditions*

Stage	CP (mEU 10^{-4} animals)	CPI (mIU 10^{-4} animals)	CP(M+L) (mEU 10^{-4} animals)	CP/CPI,* cytoplasmic
0 h embryos	73.7±9.7	104±15	4.5±2.4	0.71
0 h embryos + 4 weeks of anoxia	67.2	115	14.8	0.58
10–13 h embryos	45.1±5.7	97.9±9.0	7.0±4.4	0.46
10–12 h embryos + 4–6 weeks of anoxia	67.0±4.3	83.3±20.4	10.0±3.3	0.80
0 h larvae	33.7±2.7	31.5±9.2	16.8±1.2	1.07
0 h larvae + 12 h of anoxia	79.0	50.8	3.6	1.55

All assays were carried out at pH 5.0 and 30 °C using pooled cysteine protease (CP) and cysteine protease inhibitor (CPI) fractions from the G-150 Sephadex column (see Fig. 7).

M+L represent the crude mitochondria+lysosome fraction collected by centrifugation as described in Materials and methods.

Standard deviations were calculated for all samples with $N>3$. All other values were the mean of two separate experiments.

*There was a highly significant difference ($P<0.05$) between the CP/CPI ratio of larvae and embryos using the Wilcoxon two-sample test (Sokal and Rohlf, 1995).

mEU and mIU are defined in Materials and methods.

a high CP/CPI is maintained, due in part to an increase in CP activity. To test the hypothesis that CP exists as a multiprotein complex in embryos but not in larvae, we analyzed samples of CP directly from the Sephadex G-150 column (see Fig. 7) from both embryos and larvae by SDS–PAGE and western blotting. A duplicate sample (run on a second gel and blotted) was examined for the presence of a 26 kDa protein, which functions as a stress protein in these embryos (Clegg *et al.* 1994, 1995; Jackson and Clegg, 1996). The results (Fig. 8A) showed that both embryos and larvae contain CP of identical composition, consisting of subunits of 29 and 32 kDa as determined by immunoblotting with anti-CP antibodies. In addition, embryo CP fractions contained a 19 kDa fragment of the 26 kDa stress protein in column fractions with the highest CP activity, while neither the 19 kDa nor the 26 kDa protein was detected in CP fractions from larvae (Fig. 8B). Since the 19 kDa band (Fig. 8A) is not present in unfractionated embryo cytoplasm, or in column fractions eluted with buffer containing the cysteine protease inhibitor Z-Phe-Ala-CH_2F, we conclude that the 19 kDa band arises from CP activity on the 26 kDa protein after CPI removal by gel filtration. Cysteine protease inhibitor (CPI) protein (11–14 kDa) may also be a component of the 95 kDa embryo CP complex since heat treatment of the 95 kDa CP complex yielded a soluble fraction with CP inhibitor activity. These data suggested that the 95 kDa CP (complex) consists of proteins in addition to the CP and that the complex may contain both the CPI and 26 kDa stress protein.

Discussion

Resistance to anaerobic conditions is a characteristic of many aquatic invertebrates, but little is known about the details of the molecular mechanisms involved. From early work (Ewing and Clegg, 1969) and the study reported here, we know that the ability of *A. franciscana* embryos to withstand anaerobic conditions is developmentally regulated: hydrated encysted embryos display vastly greater tolerance to anoxia than pre-nauplius and nauplius larvae. The mechanisms that permit survival of *A. franciscana* embryos in anaerobic environments have not been described in detail, but they appear to be inactivated or switched off during the embryonic–larval transition period of development. Thus, the stability of these embryos in anaerobic sea water is lost abruptly, over a period of a few hours, between emergence of the E-1 stage (pre-nauplius) and subsequent hatching of the N-1 larvae. Remarkably, the LT_{50} falls from over 4 years for encysted embryos (Clegg, 1997) to 1–4 days in larvae, depending on the temperature of the anaerobic sea water used for incubations (see Figs 1, 2). This embryonic–larval transition period is also characterized by increased metabolic activity, including enhanced rates of carbohydrate metabolism and the synthesis of proteins and nucleic acids (reviewed by Clegg and Conte, 1980; Slegers, 1991).

An important mechanism that regulates *A. franciscana* development involves changes in intracellular pH (pHi) (Busa, 1985). When larvae are transferred to anaerobic sea water immediately after the embryonic–larval transition point, there is rapid production and accumulation of lactic acid (see Fig. 5), an event not observed in anaerobic encysted embryos over periods of years. On the basis of measurements showing that young larvae (12 h post-hatch) contain 76 % water by mass, and assuming uniform distribution in the larvae, it appears that

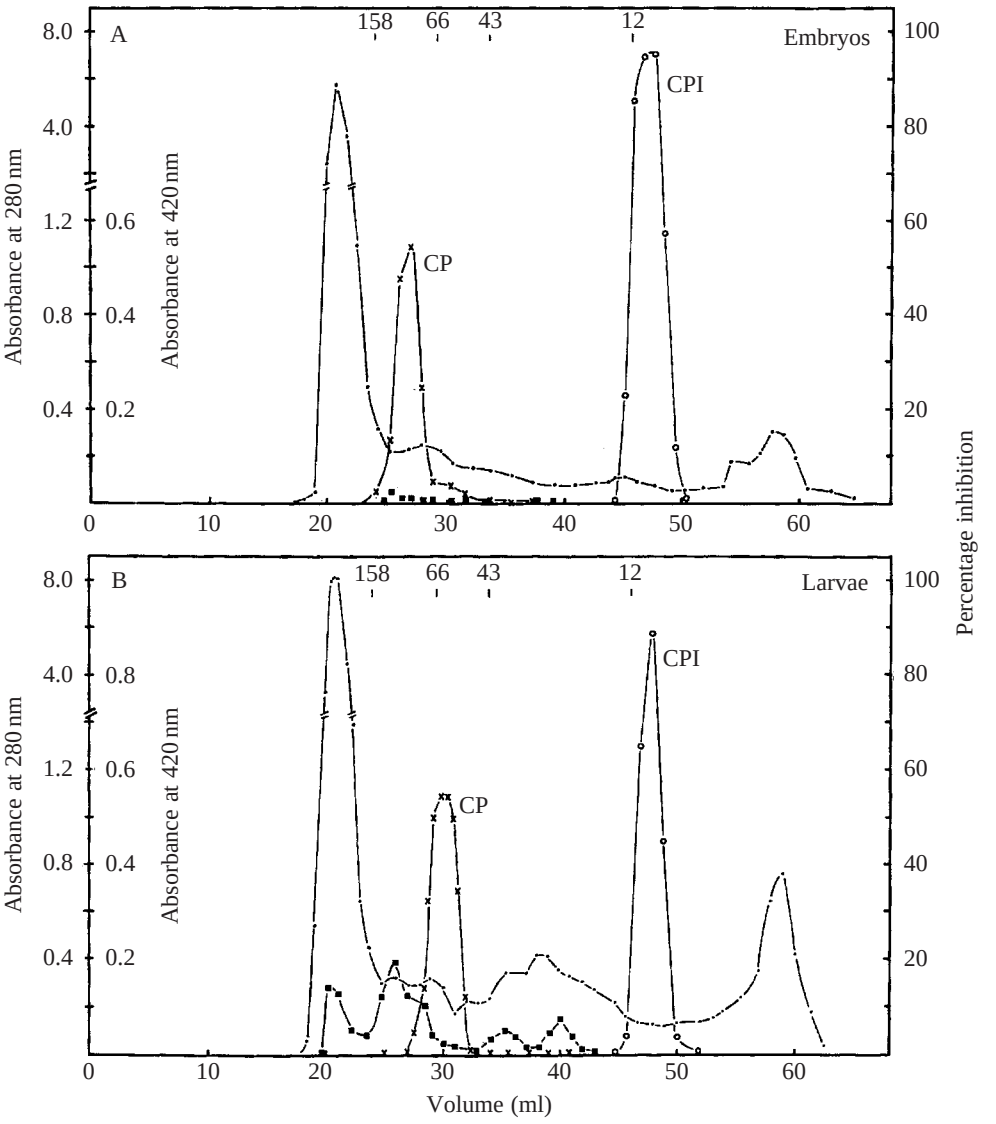

Fig. 7. Separation of cysteine protease and cysteine protease inhibitors from the aqueous cytoplasm of embryos and larvae by gel filtration. The post-mitochondrial supernatants of 12.5 h embryos (A) and 0 h larvae (B) were chromatographed on a Sephadex G-150 (SF) column (1 cm×77 cm) equilibrated with a buffer containing 15 mmol l^{-1} potassium phosphate, 25 mmol l^{-1} KCl and 10 % glycerol, pH 6.8. Column fractions were analyzed for protein at 280 nm (●), protease activity (CP) at pH 4.0 (×) and pH 7.4 (■) (absorbance at 420 nm) and cysteine protease inhibitor (CPI) activity (○) expressed as percentage inhibition of a standard cysteine protease reaction assay. The numbers at the top of each panel represent the molecular mass (kDa) of standard proteins eluting at these positions. In A, the cysteine protease (CP) from 12.5 h embryos eluted at a position of 95 kDa, whereas in B the cysteine protease (CP) from 0 h larvae eluted at a position of 60 kDa. The cysteine protease inhibitor (CPI) from both 12.5 h embryos and 0 h larvae eluted at a position of 11 kDa.

lactic acid reaches a concentration of 40–45 mmol l^{-1} in larvae after 12 h in anaerobic sea water, or the equivalent of 143 μmol g^{-1} dry mass. Remarkably, nearly 50 % of anaerobic larvae survive for about 2 days (18–20 °C) under these conditions (see Fig. 2). While no pHi measurements have been reported for anaerobic larvae of *A. franciscana*, the protons generated in anaerobic larvae probably result in a pHi considerably below that observed in anaerobic embryos using ^{31}P-NMR analysis. Using this technique, the pHi was observed to fall from ⩾7.9–6.5 after approximately 24 h in anaerobic sea water (Busa *et al.* 1982; Busa and Crowe, 1983) and then to remain relatively constant, falling only by approximately 0.1 pH unit, if at all, during the next 5 months in anaerobic sea water (Clegg *et al.* 1995). The protons which lower the pHi in these embryos are thought to arise mainly from dephosphorylation of ATP and partial hydrolysis of the large store of guanosine nucleotides (Stocco *et al.* 1972; Busa *et al.* 1982; Busa, 1985) and not from the accumulation of the end products of anaerobic carbohydrate metabolism (Ewing and Clegg, 1969; Clegg and Jackson, 1989*b*). In anaerobic larvae, 45 mmol l^{-1} lactic acid would probably, by itself, be sufficient to lower the pHi by approximately 1.4 units, taking the intracellular buffering capacity to be 32 mmol H^+ pH^{-1} unit (Busa, 1985). Thus, in anaerobic larvae, we speculate that pHi would fall below that observed in anaerobic embryos (i.e. pH 6.3) due to protons generated during lactic acid production and nucleotide dephosphorylation. Treatment of N-1 stage larvae with sea water containing 40 mmol l^{-1} NH_4Cl, which had been shown to raise the pHi by at least 0.9 unit in *A. franciscana* embryos (Busa and Crowe, 1983), did not promote recovery of larvae from the toxic effects of anaerobic sea water. However, the lack of effect of NH_4Cl could be because this compound is toxic to the *A. franciscana* larvae (but not to the embryos) in aerobic sea water (Busa and Crowe, 1983).

The onset and extent of lactic acid production are dramatic in *A. franciscana* larvae, but are not unique. The anoxia-tolerant mud shrimp *Calocaris macandreae* also produces lactate, which reaches concentrations of 40–50 mmol l^{-1} in

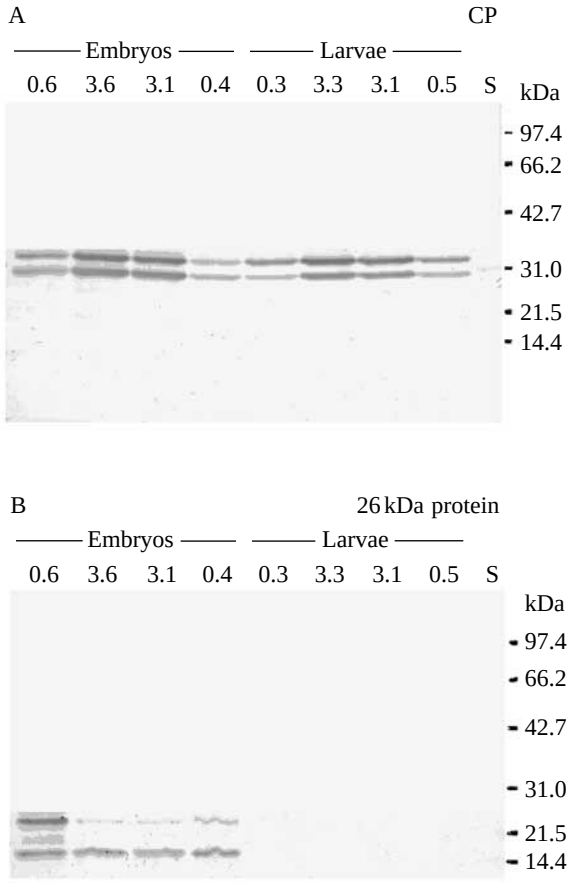

Fig. 8. Western blot analysis of the 26 kDa protein in the cysteine protease (CP) fraction of encysted embryos and larvae of *Artemia franciscana*. Samples (in duplicate) from the CP fractions shown in Fig. 7 were analyzed for both the CP and 26 kDa protein using SDS–PAGE followed by immunochemical analysis using anti-CP and anti-26 kDa protein antibodies on separate western blots. (A) Analysis of four column fractions from 12.5 h embryos and newly hatched (0 h) nauplius larvae using anti-CP antibodies. (B) Analysis of the same fractions as those shown in A with anti-26 kDa protein antibodies. The numbers above each lane represent the amount of CP (in mEU, see Materials and methods) from each column fraction that were applied to each lane. The numbers on the right represent the molecular mass of standard proteins (S) in lane 9 (far right) migrating to these positions on the gel. The CP bands (A) from both embryos and larvae have molecular masses of 32 and 29 kDa, while the anti-26 kDa protein antibody detects a band at 26 kDa and also one at 19 kDa, which is the dominant band in fractions with the highest CP activity.

both the hemolymph and tissues at the LT_{50} (43 h, 10 °C) of this organism (Anderson *et al.* 1994). What are the consequences, in terms of pHi effects, of the rapid increase in lactic acid level and other potential proton-generating mechanisms in anaerobic larvae? Protein synthesis *in vitro* is reduced markedly as the pH of the reaction mixture falls below 7.0 (Hoffman and Hand, 1994; Hand and Hardewig, 1996), and the low pHi in anaerobic embryos and larvae could contribute to the down-regulation of cytoplasmic and mitochondrial protein synthesis during anaerobiosis (Hofmann and Hand, 1990; Clegg and Jackson, 1989*b*; Kwast and Hand, 1993, 1996*a*,*b*; Kwast *et al.* 1995). It would be interesting to determine the effect of low pHi on hexokinase activity in *A. franciscana* larvae, since it is believed that inhibition of embryo hexokinase by low pHi and other endogenous factors contributes to the inhibition of carbohydrate metabolism during anaerobiosis (Rees *et al.* 1989). Organic acids do not accumulate in anaerobic embryos of *A. franciscana* (while they do in anaerobic larvae), despite the the large stores of carbohydrates and measurable amounts of lactic acid dehydrogenase in embryos (Ewing and Clegg, 1969; Clegg and Jackson, 1989*b*). Finally, it could also be argued that as lactic acid accumulates in anaerobic larvae, reducing the pHi to below that found in anaerobic embryos, the intracellular milieu could easily activate latent/inhibited cysteine proteases in the larval cytoplasm leading to the 'low' LT_{50} of anaerobic larvae (Warner, 1989*a*,*b*).

A remarkable aspect of the ability of the embryos of *A. franciscana* to survive extremely long periods of anaerobiosis concerns the mechanisms by which enzymes and other proteins are protected from unfolding, aggregation and proteolytic degradation following removal of molecular oxygen. During aerobic development of *A. franciscana*, cytoplasmic proteins are believed to be degraded (as part of the normal turnover process) by at least two pathways, an ATP-dependent, ubiquitin-mediated multicatalytic pathway (Anchordoguy and Hand, 1994, 1995) and one involving the major cysteine proteases found predominantly in the aqueous cytoplasm (Warner, 1989*b*; Warner *et al.* 1995). The observation by Anchordoguy and Hand (1994) that ubiquitin conjugates disassemble in anaerobic embryos of *A. franciscana* suggests that this mechanism is not functional in anaerobic embryos and, therefore, is unable to participate in cytoplasmic protein turnover. A similar observation (A. H. Warner, S. A. Jackson and J. S. Clegg, unpublished results) has been made using anaerobic larvae. It appears that the stabilization of cytoplasmic proteins probably involves additional strategies designed to protect the embryo from the usually deleterious effects of oxygen lack. The 26 kDa stress protein described recently (Clegg *et al.* 1994, 1995; Jackson and Clegg, 1996) and cysteine protease inhibitors (Warner, 1989*a*,*b*; Warner and Sonnenfeld-Karcz, 1992) probably also serve to protect cytoplasmic enzymes and proteins against unfolding and proteolysis during prolonged anaerobiosis of encysted embryos. One major feature distinguishing anaerobic larvae from anaerobic embryos of *A. franciscana* is that protein degradation mechanisms are inhibited in anaerobic embryos (Anchordoguy and Hand, 1994, 1995), but not in anaerobic larvae (Fig. 3). In larvae, cytoplasmic protein degradation is apparent within a few hours of placing them in anaerobic sea water. Nevertheless, larvae withstand an approximately 20 % reduction (overall) in their aqueous cytoplasmic proteins during the first 12 h of anaerobiosis without loss of viability. Further reductions of an additional 10–15 % of cytoplasmic proteins were found to be associated with a rapid loss in viability. It seems that death occurs only after sufficient

damage has accumulated during prolonged anaerobiosis. In contrast, yolk platelet utilization is inhibited in both anaerobic larvae and anaerobic embryos (Figs 3, 4). Since yolk platelet utilization during development appears to require an alkaline pHi to mobilize yolk proteins from the platelet to the site of degradation (Utterback and Hand, 1987), it is not surprising that yolk utilization is inhibited in both anaerobic embryos and larvae because of their low pHi.

In embryos of *A. franciscana*, the only proteolytic activity measurable *in vitro* in aqueous cytoplasm incubated at acidic pH is that attributable to cysteine proteases (CP) (Warner and Shridhar, 1985). Moreover, CP activity is believed to be stringently controlled during early development by multiple endogenous cysteine protease inhibitor (CPI) proteins which are present in amounts greater than the CP (Warner, 1989*b*; Warner and Sonnenfeld-Karcz, 1992; see also Table 1). As development proceeds, the CPI content declines rapidly in the cytoplasm during the embryonic–larval transition period, such that in newly hatched larvae the CP activity is more abundant than the CPI activity. We believe that the changes in CP/CPI ratio in developing embryos, especially during the embryonic–larval transition period, combined with a decrease in pHi as lactic acid accumulates in anaerobic larvae are the major factors contributing to the degradation of cytoplasmic proteins in anaerobic larvae (Fig. 3). *In vitro* experiments suggest that certain cytoplasmic proteins of *A. franciscana* larvae (but not embryos) are more susceptible to degradation at pH 6.0 then others (see Fig. 6). In an attempt to compare cytoplasmic protein degradation in anaerobic larvae (*in vivo*) with cytoplasmic protein degradation *in vitro* (at pH 6.0, 30 °C), we performed densitometric scans of the SDS–PAGE gels in Fig. 4 and Fig. 6. While the cytoplasmic proteins in anaerobic larvae (Fig. 4A) did not show the same pattern of degradation as observed *in vitro* (Fig. 6B), scans of the cytoplasmic proteins showed a disproportionate loss of proteins of 26 kDa and 32 kDa and an increase in amounts of protein of approximately 15 kDa in anaerobic larvae over time. Moreover, quantification of the scanned cytoplasmic proteins in Fig. 4 showed a 50 % reduction in protein during the first 36 h in anaerobic sea water, consistent with data in Fig. 3A on total cytoplasmic protein levels in anaerobic larvae. Thus, it would appear that the cysteine protease is not acting alone *in vivo* to degrade cytoplasmic proteins in intact anaerobic larvae.

We have also observed that two abundant cytoplasmic proteins, elongation factor 2 and a 26 kDa protein with demontrated stress-related functions, are very sensitive to inactivation by CP at pH 6.0 (Warner, 1987; A. H. Warner, unpublished observations). Given that degradation of the most protease-sensitive proteins in the cytoplasmic extracts of larvae can be inhibited *in vitro* by the addition of *A. franciscana* CPI protein, effectively restoring the CP/CPI ratio to the level found in embryos, the CP in the aqueous cytoplasm of larvae must be a major contributor to the loss of cytoplasmic proteins in larvae during anaerobiosis. The effectiveness of CPI in the regulation of CP activity is also pH-dependent, showing greatest inhibitory activity at approximately pH 6–7 (Warner 1989*a*). As the pH falls, CPI becomes less inhibitory and eventually, at approximately pH 4.0, it becomes a substrate for CP (Warner, 1989*a*). Collectively, the data in the present paper suggest that the loss of proteins from the aqueous cytoplasm in anaerobic larvae is due, to a large extent, to activation of cytoplasmic cysteine proteases by the reduced pHi and decrease in CPI content in larvae compared with the situation in embryos, where pHi and CPI activity are higher.

Finally, it may be instructive to evaluate the quaternary structure of the cysteine protease (CP) which is demonstrably different in *A. franciscana* embryos (95 kDa) and larvae (60 kDa), despite the fact that both embryonic and larval CP have the same subunit composition (i.e. subunits of 29 and 32 kDa). The transition of CP from a 95 kDa complex to a 60 kDa protein occurs abruptly during the embryonic–larval transition period of development, at the same time that the embryo loses its greatest tolerance to anoxia. At the cellular level, no major differences in the distribution of CP have been detected between embryos and young larvae (Warner *et al.* 1995). However, the fact remains that CP from embryos is associated with other macromolecules, probably including the 26 kDa stress protein described recently (Clegg *et al.* 1994, 1995; Jackson and Clegg, 1996). Whether the 26 kDa protein is associated (non-covalently) with CP in *A. franciscana* embryos is not yet certain, but in the absence of the CPI protein the 26 kDa protein is a substrate for CP in slightly acidic (pH 6.8) conditions. The 26 kDa protein has no known CP inhibitor activity, but its possible association with CP in embryos suggests that it may be involved in some way with CP regulation during anaerobiosis. Although we have focused on the role that the cysteine proteases may play in the response of embryos and larvae to anaerobiosis, it is recognized that other processes are also probably involved, notably in the larvae.

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