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Energizing an Invertebrate Embryo: Bafilomycin-Dependent Respiration and the Metabolic Cost of Proton Pumping by the V-ATPase

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ABSTRACT

We examine herein the contribution of V-ATPase activity to the energy budget of aerobically developing embryos of *Artemia franciscana* and discuss the results in the context of quiescence under anoxia. ^{31}P-NMR analysis indicates that intracellular pH and NTP levels are unaffected by acute incubation of dechorionated embryos with the V-ATPase inhibitor, bafilomycin A_1. Bafilomycin A_1 also has no significant effect on oxygen consumption by isolated mitochondria. Taken together, these data indicate that bafilomycin does not affect energy-producing pathways in the developing embryo. However, the V-ATPase inhibitor exhibits a concentration-dependent inhibition of oxygen consumption in aerobic embryos. A conservative analysis of respirometric data indicates that proton pumping by the V-ATPase, and processes immediately dependent on this activity, constitutes approximately 31% of the aerobic energy budget of the preemergent embryo. Given the complete absence of detectable Na^+K^+-ATPase activity during the first hours of aerobic development, it is plausible that the V-ATPase is performing a role in both the acidification of intracellular compartments and the energization of plasma membranes. Importantly, the high metabolic cost associated with maintaining these diverse proton gradients requires that V-ATPase activity be downregulated under anoxia in order to attain the almost complete metabolic depression observed in the quiescent embryo.

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Introduction

After only 48 h of anoxia, metabolic heat dissipation by *Artemia franciscana* embryos drops to 0.2% of the aerobic value (Hand 1995), and a nearly ametabolic state is ultimately established (Warner and Clegg 2001; Clegg 1997). One of the primary characteristics of metabolic suppression in this state is a reverse Pasteur effect. Instead of cellular adenylate status being stabilized by an upregulation of substrate-level phosphorylation under anoxia, a net hydrolysis of ATP is experienced in these embryos (Stocco et al. 1972; Busa et al. 1982; Carpenter and Hand 1986). Consequently, within the first 24 h of anoxia, ATP levels drop by 95% while AMP levels increase to nearly 40 times the aerobic value (Anchordoguy and Hand 1994). This absence of a defense of cellular adenylate status during suppression of oxidative energy production poses obvious constraints on energy (ATP)-consuming processes, such as the maintenance of transmembrane ion gradients. In such an energetically depressed context, ATP-dependent proton pumping by the V-ATPase is likely to be downregulated (Covi et al. 2005). We previously demonstrated that V-ATPase inhibition with bafilomycin A_1 halts embryonic development under aerobic conditions (Covi and Hand 2005) without affecting intracellular pH or cellular NTP levels (Covi et al. 2005). Here we employ a combination of respirometry, ^{31}P-NMR, and hatching studies to estimate the energetic savings that would be realized if proton gradients were not defended under anoxia via continued activity of this proton pump. The data presented clearly demonstrate that V-ATPase activity must be downregulated under anoxia and also provide insight into the mechanisms employed to ionically energize an invertebrate embryo under normoxia with the V-ATPase in place of Na^+K^+-ATPase.

As a mechanism for maintaining ion gradients during periods of severe energetic constraint imposed by anoxia, facultative anaerobes appear to coordinate a reduction in ATP-dependent ion pumping with a depression of dissipative pathways for ion gradients. For example, hepatocytes of the western painted turtle demonstrate a 74% decrease in activity of the Na^+K^+-ATPase under anoxia while membrane potential is maintained through an arrest of ion channels (Buck and Hochachka 1993). Under aerobic conditions, the maintenance of Na^+ and K^+ gradients in these cells requires 28% of total ATP turnover (Buck and Hochachka 1993), and the reduction in the magnitude of this energy sink is a large contributor to

the almost 90% drop in total ATP turnover Buck et al. (1993) observed in response to anoxia. Given the extreme energetic constraints present in *Artemia* embryos under anoxia, one might predict that ATP-dependent ion pumping would cease entirely and that complete channel arrest would be required if ion gradients are to be defended (Hand and Hardewig 1996).

Interestingly, Na^+K^+-ATPase activity is undetectable for the first 6 h of development and extremely low for the remainder of preemergence development in encysted *Artemia* embryos (Peterson et al. 1978, 1982). It is unclear how plasma membranes are energized during this period. One possibility is that energization is accomplished using a protonmotive force generated by the V-ATPase. The V-ATPase is a proton pump that is well known for its role in both the constitutive acidification of intracellular compartments (Forgac 2000; Futai et al. 2000) and the energization of specialized plasma membranes in animals (Harvey and Wieczorek 1997; Nelson and Harvey 1999; Forgac 2000; Futai et al. 2000; Nelson et al. 2000; Wieczorek et al. 2000; Kawasaki-Nishi et al. 2003). If this proton pump is acting in such a broad capacity in *A. franciscana* embryos, it should be found in both the plasma membrane and the membranes of intracellular compartments. In accord with such a distribution, we demonstrated presence of the V-ATPase in both heavy membranes (plasma membrane and large organellar fragments) and microsomal vesicles (small intact vesicles for intracellular transport) during the period of development when Na^+K^+-ATPase activity is undetectable (Covi and Hand 2005).

If the V-ATPase acts both in the constitutive acidification of organelles (Golgi, lysosomes, trafficking vesicles, and yolk platelets) and in plasma membrane energization, it is reasonable to predict that it would contribute significantly to cellular ATP turnover, because it would be the main ion motive ATPase within the encysted gastrula. Estimates derived from bafilomycin-sensitive respiration and NADH turnover suggest that the metabolic cost of proton transport in renal proximal tubules can vary greatly (5%–44% of total cellular energy turnover) among species for a single tissue type (Noel et al. 1993; Fleser et al. 1995). The fact that these estimates come from proton-secretory systems, which are expected to have high levels of V-ATPase activity, might suggest that similar estimates for *A. franciscana* embryos would fall toward the lower end of this range. However, it has also been noted that species demonstrating lower ATP turnover resulting from V-ATPase activity also show increased ATP turnover resulting from Na^+K^+-ATPase activity (Noel et al. 1993). Thus, because of the conspicuously low Na^+K^+-ATPase activity of the preemergent embryo, it is reasonable to predict that ATP turnover due to V-ATPase activity might be higher than expected for a nonsecretory and largely undifferentiated cell mass, such as that found in the encysted embryos of *A. franciscana*. Here we use bafilomycin-sensitive respiration to estimate the contribution of V-ATPase activity to the total energy budget of *A. franciscana* embryos. Our results indicate that the relatively high levels of ATP consumption attributable to V-ATPase activity must be downregulated in order to account for the decrease in metabolic heat production observed under anoxia.

Material and Methods

Materials

Instant Ocean was obtained from Aquarium Systems (Eastlake, OH). Oligomycin and gentamycin sulfate were purchased from Sigma (St. Louis, MO). Bafilomycin A_1 (hereafter referred to as bafilomycin) was obtained from either AG Scientific (San Diego, CA) or LC Laboratories (Woburn, MA). Both oligomycin and bafilomycin were dissolved in 100% ethanol, and the stock solutions were stored at −20°C. Low endotoxin sucrose for respirometry experiments was obtained from Pfanstiehl (Waukegan, IL). All other chemicals used were of the highest grade available.

Animals

Encysted embryos of the brine shrimp *Artemia franciscana* (Great Salt Lake population) were obtained in the dehydrated state from Sanders Brine Shrimp (Ogden, UT) in 2001 and stored dry at −20°C. These postdiapause embryos were hydrated overnight at 0°C in 0.25 mol L^{-1} NaCl before dechorionation, which was performed as previously described (Kwast and Hand 1993). Both dechorionated and nondechorionated embryos were stored for no more than 3 d in ice-cold 0.125 mol L^{-1} NaCl before experimentation. All solutions were prepared less than 24 h before use, and the length of incubation time in antiformin (0.4 mol L^{-1} NaOH, 60 mmol L^{-1} Na_2CO_3, and household bleach added to a final concentration of 1% sodium hypochlorite) for dechorionation was strictly maintained at 20 min (23°C).

Respirometry

Unless otherwise noted, respiration experiments were conducted in 0.2 mol L^{-1} sucrose. Respiration medium was sterile filtered before use, and oxygen consumption due to any bacterial contamination was limited by the inclusion of 50 μg mL^{-1} gentamycin sulfate. Hatching success for embryos incubated for 67–72 h in 35 ppt artificial sea water containing 50 μg mL^{-1} gentamycin sulfate was not significantly different from control incubations lacking the inhibitor, regardless of whether the cyst chorion was removed ($P > 0.37$; $n = 4$). The final ethanol concentration was 0.5% for all respirometry treatments involving whole embryos.

Immediately before respiration experiments, embryos were blotted dry, weighed in 1.5-mL microcentrifuge tubes (2–3 mg; approximately 230–350 embryos), and resuspended in respiration medium within 4–6 min of blotting. Alternatively, an aliquot of embryos was simply transferred to the respiration

medium without blotting dry and the embryos counted at the end of the experiment. In the latter case, the number of embryos was converted to mass using an empirically determined factor (118 ± 2 embryos mg^{-1} wet weight after blotting dry; mean ± SE, $n = 6$). Oxygen consumption was measured under room lighting in a water-jacketed respiration chamber (model RC350, Strathkelvin Instruments, Glasgow) maintained at 25°C. Oxygen tension was measured in 1.5 mL of medium using a polarographic oxygen electrode (Strathkelvin model 1302), and data were collected with DataCan V acquisition software from Sable Systems (Las Vegas, NV). Raw data were analyzed with DatGraf software from Cyclobios (Innsbruck). Oxygen consumption by the electrodes, back diffusion into the system, and electrode time constants were corrected for as previously described (Kwast et al. 1995).

Oxygen consumption by whole embryos was measured using two methods, for which the critical difference was stirring regime. In the first method, oxygen consumption was recorded continuously for 6.5 h with gentle stirring, and the electrode was raised at regular predetermined intervals to introduce an air pocket for reoxygenation. In this way, oxygen tension was prevented from dropping below 65% air saturation. In the second method, embryos were preincubated in open respiration chambers without stirring for 6.5 h, after which oxygen consumption was measured for a 12–15-min period with gentle stirring. While neither method of respiration measurement resulted in detectable lysis or homogenization of embryos, long-term stirring (method 1) did appear to increase permeability and decrease hatching success of dechorionated embryos (see "Results").

Established protocols were used for mitochondrial isolation (Kwast and Hand 1993) and respiration measurements (Eads and Hand 1999). Measurements of oxygen consumption on isolated mitochondria were made using an Oxygraph-2k high-resolution respirometer (Oroboros Instruments, Innsbruck). Bafilomycin, at a final concentration of 2 μmol L^{-1}, was added to the mitochondrial suspension 5 min before the initiation of state 3 respiration with the addition of ADP. The final concentration of ethanol was 0.1% for bafilomycin treatments. The respiratory control ratio for mitochondria isolated from brine shrimp embryos (5.31 ± 1.2; $n = 10$) demonstrated a high degree of ADP-stimulated respiration.

Hatching Assays

Development was observed for embryos used in select respirometry experiments. Inhibitors and ethanol were removed from the incubation medium after respirometric measurement and before incubation for hatching. To accomplish this, embryos were rinsed in 3 mL of 35 ppt artificial seawater three times at room temperature, and this was followed by a fourth wash in 7 mL. Embryos and seawater from the final wash were transferred to a 12-well culture plate, and counting of developmental stages was conducted 66–69 h after transfer. This period was sufficient to allow for completion of hatching; no further development was observed in individuals that did not reach the naupliar stage by hour 66. Identification of developmental stages used the same criteria described by Conte et al. (1977). Counts of the emergence stages (E1 and E2) were combined into a group termed "emerging." This group also included individuals that exhibited morphologies associated with emergence defects noted by Trotman (1991). Development beyond the encysted stage (%E) was defined as any embryo that began the emergence process (protrusion from the chitinous shell), regardless of whether emergence of the nauplius from the cyst shell and hatching membrane was successful. Conversely, arrest in the encysted stage was defined as any embryo that did not demonstrate any visible signs of initiating emergence.

NMR Spectroscopy

^{31}P-NMR spectroscopy was conducted as described by Covi et al. (2005). In brief, embryos were packed by gravity into a glass NMR tube (10 mm diameter). Mechanical disturbance was prevented by placing a foam plug at the top of the embryo column, and embryos were superfused with 0.2 mol L^{-1} sucrose. Aerobic conditions were maintained with the use of oxygen-saturated medium and a flow rate of 2 mL min^{-1}. As discussed by Kwast et al. (1995), the use of oxygen-saturated medium has no noticeable effect on embryonic development. Perfusion was stopped for <10 s while switching from perfusion with 0.2 mol L^{-1} sucrose to 0.2 mol L^{-1} sucrose containing either 0.2% ethanol (control) or 4 μmol L^{-1} bafilomycin dissolved in ethanol. Peak identification was accomplished by comparison with published spectra (Busa et al. 1982; Kwast et al. 1995).

Statistical Analysis

All data are presented as means ± 1 SEM when error bars are present, and the number of independent embryo preparations used in replicates is indicated for each experiment. Student's *t*-test for paired values was used for comparisons between control and experimental groups. Statistical significance was set at $P < 0.05$ for all analyses.

Results

Exposure of Whole Embryos to Oligomycin

As a test to determine whether the dechorionation procedure served to permeabilize *Artemia franciscana* embryos to the lipid-soluble class of antibiotics known as macrolides, we used an inhibitor with a predictable and measurable physiological effect (oligomycin; respiratory inhibition). An increase in oxygen consumption was observed for dechorionated control em-

bryos during the first 2.25 h after placement in respiration medium. For dechorionated embryos, this increase was also evident for the first 30 min of incubation with the F-ATPase inhibitor, oligomycin. Subsequently, these embryos experienced a progressive and concentration-dependent decrease in oxygen consumption resulting from oligomycin exposure (Fig. 1). After 2.25 h of exposure, the highest concentration of oligomycin tested (4 μmol L^{-1}) reduced the oxygen consumption rate of intact embryos by 77% relative to the control. However, when nondechorionated embryos were incubated in the same concentration of oligomycin, the pattern of oxygen consumption was identical to that of dechorionated control embryos with no oligomycin (Fig. 1). These respiration data are consistent with previous developmental studies, which demonstrated a concentration-dependent decrease in hatching success for dechorionated embryos incubated with oligomycin (Covi and Hand 2005). Indeed, hatching studies carried out on embryos after respirometric measurements also show a concentration-dependent arrest of development in the embryonic stage when expressed as a percentage of the emergence rates for control embryos (1 μM, 89%; 2 μM, 62%; 4 μM, 43%). Unlike the case for longer respiration experiments (see below), this effect does not appear to be related to the use of continuous stirring during the 2.25 h of respiration measurement, because 80% of control embryos hatched successfully after removal from the respiration chamber. Together, these data make clear that removal of the outer proteinaceous layer of the encapsulating cyst shell (dechorionation) confers permeability of the embryonic cuticle to lipid-soluble compounds by demonstrating depression of both whole-embryo respiration and hatching success with oligomycin.

It is important to note that just as we found for the plecomacrolide antibiotic, bafilomycin, reversal of the inhibitory effects of oligomycin is not possible. It is exceedingly difficult to remove these lipophilic compounds from *A. franciscana* embryos (J. A. Covi and S. C. Hand, unpublished data). As has been discussed previously (Covi et al. 2005), this could be the result of strong binding between inhibitor and enzyme and/or slow diffusion out of the lipid-rich cell mass of these embryos.

Exposure of Whole Embryos to Bafilomycin

The addition of bafilomycin to the respiration medium after 2.75 h of incubation induced a concentration-dependent decrease in oxygen consumption relative to the control group for dechorionated embryos (Fig. 2*A*). This decrease was very rapid even at concentrations as low as 0.1 μmol L^{-1}. Maximal effect was reached with 0.5 μmol L^{-1} bafilomycin, which produced a steady decline in oxygen consumption matching that of the 10 μmol L^{-1} (saturated) treatment. After 3.75 h of exposure to 0.5 μmol L^{-1} bafilomycin, oxygen consumption by whole embryos had dropped to 24% ± 6% ($n = 3$) of the control value and was still declining.

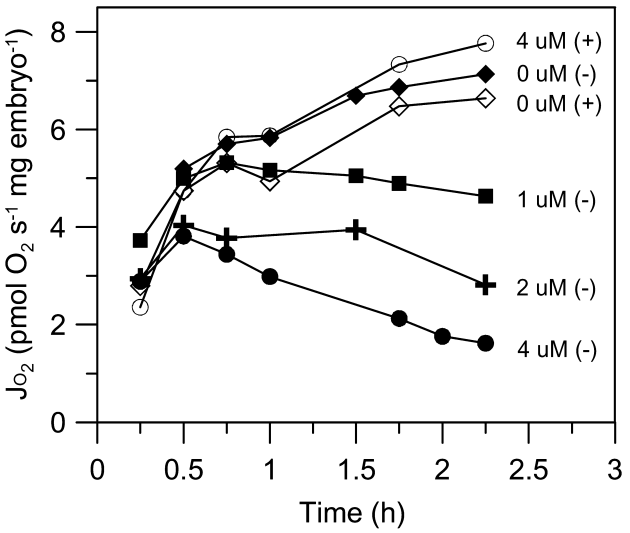

Figure 1. Concentration dependence for and effect of dechorionation on inhibition of oxygen consumption by oligomycin in *Artemia franciscana* embryos. Concentration of oligomycin used is indicated to the right of each plot. Dechorionated embryos are indicated with a minus sign and were incubated in 0 (control), 1, 2, or 4 μmol L^{-1} oligomycin beginning at hour 0. Nondechorionated embryos are indicated with a plus sign and were incubated in 0 or 4 μmol L^{-1} oligomycin.

Exposure of dechorionated embryos to bafilomycin beginning at hour 0 during respiration measurements (Fig. 2*B*) predictably led to a similar pattern of respiratory inhibition. Also, as was observed for embryos incubated with the F-ATPase inhibitor oligomycin, incubation with 10 μmol L^{-1} bafilomycin (saturated) has no effect on oxygen consumption by whole embryos if the chorion is not removed before exposure (Fig. 2*B*), which is further evidence that the chorion is the sole barrier to these lipid-soluble antibiotics.

The large decrease in oxygen consumption induced by submicromolar concentrations of bafilomycin was unexpected, because concentrations ≥2 μmol L^{-1} are required to arrest development in more than 50% of dechorionated embryos (Covi and Hand 2005). An important distinction between these studies, however, is that embryos were incubated under static conditions for hatching, while respiration measurements were conducted in the presence of continuous mechanical stirring. The increased sensitivity to bafilomycin observed in respiration experiments may be the result of mechanical perturbation of the embryonic cuticle by stirring. This possibility is supported by hatching studies performed on embryos after exposure to 6.5 h of gentle stirring in 0.2 mol L^{-1} sucrose. Even though oxygen consumption by control embryos does not decrease during this treatment, 35% ± 2% ($n = 3$) of these embryos failed to initiate emergence after transfer to static artificial seawater (35 ppt), and only 50% ± 10% hatched successfully to produce swimming nauplii. By contrast, average hatching rates are >93%

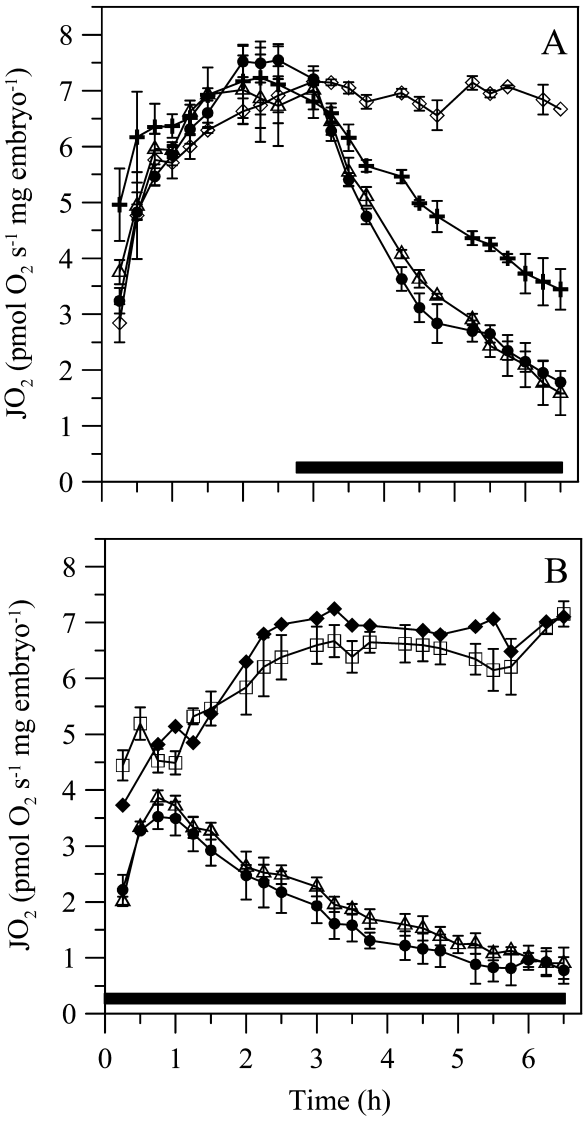

Figure 2. Concentration dependence for inhibition of oxygen consumption by bafilomycin in *Artemia franciscana* embryos exposed to continuous stirring. The presence of bafilomycin is indicated by a solid bar. In *A*, 0 (control; *open diamond*), 0.1 (*plus sign*), 0.5 (*open triangle*), or 10 (*solid circle*) μmol L^{-1} bafilomycin were added 2.75 h after resuspension of dechorionated embryos in respiration medium. In *B*, dechorionated embryos were resuspended directly in medium containing 0.5 (*open triangle*) or 10 (*solid circle*) μmol L^{-1} bafilomycin, and nondechorionated control embryos were resuspended in 0 (*solid diamond*) or 10 (*open square*) μmol L^{-1} bafilomycin. Data represent single measurements when error bars are absent and $n = 3$ when error bars are present.

when dechorionated embryos are incubated under static conditions (Covi and Hand 2005). Because we were unable to find evidence for physical disruption of the embryos with light and scanning electron microscopy (J. A. Covi and S. C. Hand, unpublished data), we can only speculate that a nonspecific increase in permeability of the cuticle occurs during prolonged stirring.

To greatly reduce overall stirring time, embryos were preincubated for 6.5 h without stirring, and then brief respiration measurements with stirring were made at the end (Fig. 3*A*). This stirring regime did not markedly perturb embryonic development (12% ± 1% [$n = 3$] of control embryos failed to emerge [Fig. 3*B*] vs. 7% during totally static incubation [Covi and Hand 2005]). At 1 μmol L^{-1} bafilomycin, a concentration higher than the maximally effective level for V-ATPase inhibition in the dose dependency experiment (0.5 μmol L^{-1}; Fig. 2), oxygen consumption was depressed by 31% ± 9% ($n = 3$) relative to the control (Fig. 3*A*) at the end of the 3.75-h exposure period. As expected, the inhibition is significantly less than that

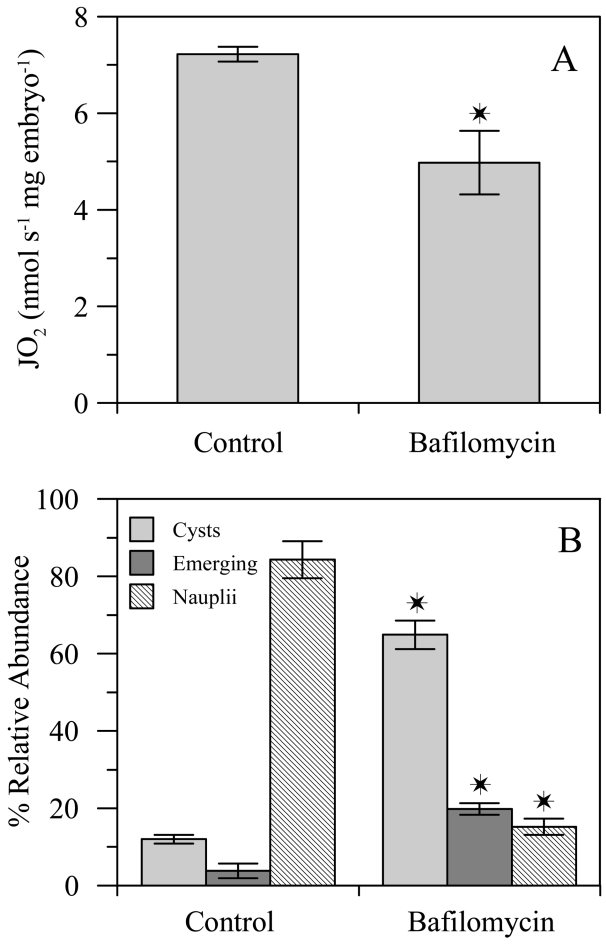

Figure 3. *A*, Effect of static "preincubation" with 1 μmol L^{-1} bafilomycin on oxygen consumption by *Artemia franciscana* embryos. *B*, Developmental success in inhibitor-free medium after respiration measurement. Embryos were incubated in 35 ppt artificial seawater for 6.5 h without stirring before recording oxygen consumption in the same medium. Bafilomycin (1 μmol L^{-1}; treatment group) or ethanol (control) was added at hour 2.75. An asterisk indicates a significant difference from control treatment ($n = 3$).

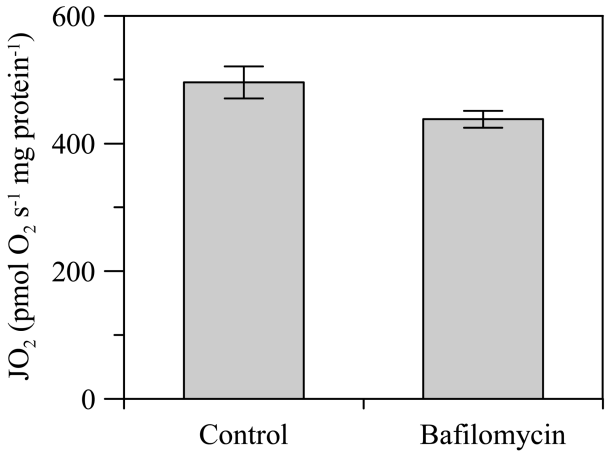

Figure 4. Oxygen consumption by isolated mitochondria from 8-h embryos of the brine shrimp *Artemia franciscana*. No significant difference was found between control and 2 μmol L^{-1} bafilomycin treatments ($P = 0.08$; $n = 3$).

observed with prolonged stirring (Fig. 2; $P = 0.008$), but the estimate is not impacted by excess stirring and thus viewed as more accurate. Using this bafilomycin-sensitive oxygen consumption and assuming a P : O ratio of 2 (Gnaiger et al. 2000), ATP turnover resulting from ATPase proton pumping can be calculated as 9.0 ± 0.4 nmol ATP s^{-1} mg $embryo^{-1}$.

We argue that the above protocol affords a reasonable approximation for the effect of bafilomycin on oxygen consumption in these developing embryos. Permeation of the embryonic cuticle by macrolide antibiotics is not instantaneous. Hours are required to obtain sufficient loading of bafilomycin to arrest embryonic development (Covi and Hand 2005), prevent recovery from anoxia (Covi et al. 2005), and inhibit V-ATPase-dependent oxygen consumption (Fig. 2). In addition, encysted *A. franciscana* embryos develop from a largely undifferentiated cell mass to a swimming nauplius in only 36 h, with emergence and rapid cellular differentiation beginning at 8 h of development. Oxygen consumption increases as development proceeds (Fig. 2*A*). Thus, the use of a 3.75-h incubation with bafilomycin, which ends at a total of 6.5 h of development, provides the best possible compromise between these various factors.

To determine whether the effect of bafilomycin is the result of a direct influence on oxidative phosphorylation (e.g., inhibition of the F_1F_0-ATP synthase), we examined the sensitivity of isolated mitochondria to bafilomycin. Incubation with 2 μmol L^{-1} bafilomycin did not produce a significant decline in state 3 oxygen consumption relative to the control (Fig. 4). This confirms that the bafilomycin-sensitive oxygen consumption observed in whole embryos is not due to inhibition of mitochondrial respiration by this antibiotic.

^{31}P-NMR results show that the NTP and NDP resonances increase throughout early development under the conditions used (see Fig. 5 for select spectra). This increase is consistent with the results found by Carpenter and Hand (1986), who demonstrated that ATP levels rise twofold during the first 4 h of development. Importantly, exposure to 4 μmol L^{-1} bafilomycin or 0.5% ethanol (control) for 3.75 h did not interrupt the predicted ontogenetic increase of NTP (Fig. 5), which is observed during superfusion in the absence of ethanol or bafilomycin (J. A. Covi and S. C. Hand, unpublished data). These data offer in vivo support for the finding that bafilomycin does not have a direct effect on respiration of isolated mitochondria.

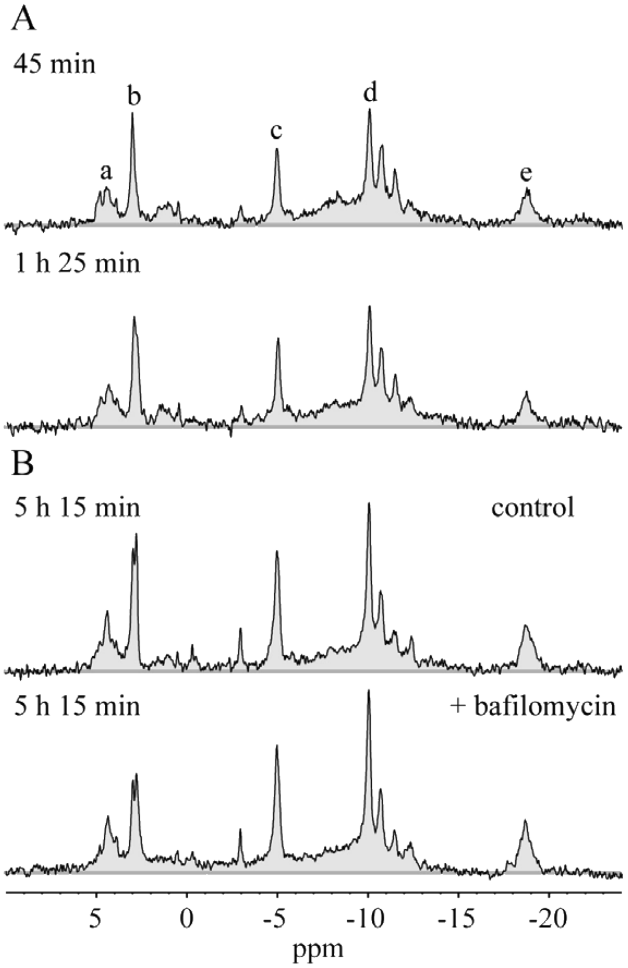

Figure 5. Representative ^{31}P-NMR spectra of intact *Artemia franciscana* embryos during (*A*) aerobic superfusion for 1.5 h with 0.2 mmol L^{-1} sucrose followed by (*B*) an additional 3.75 h after switching superfusion to 0.2 mmol L^{-1} sucrose containing 0.5% ethanol (control) or 4 μmol L^{-1} bafilomycin. Total time of aerobic superfusion is listed in the upper left of each spectrum, and treatment is listed in the upper right. Predominant chemical shifts for phosphate nuclei are identified by letters: *a*, phosphomonoesters; *b*, P_i; *c*, γ-NTP and β-NDP; *d*, α-NTP and NDP; *e*, β-NTP. Baseline for each spectrum is indicated with a gray line.

The continued increase in NTP during superfusion also suggests that the decreased oxygen consumption observed in embryos exposed to bafilomycin can be directly attributed to the effects of V-ATPase inhibition rather than mortality or secondary effects such as altered ATP : ADP ratio.

It is of interest to note that hatching tests conducted on embryos used in ^{31}P-NMR measurements suggest that development is arrested by bafilomycin treatment without short-term effects on NTP balance. A hatching test performed on the embryos superfused with 4 μmol L^{-1} bafilomycin during ^{31}P-NMR demonstrates that 30% of these embryos stop developing in the encysted stage, despite the presence of normal NTP levels after 3.75 h of superfusion (Fig. 5*B*). Similarly, in a previous study, we showed that incubation for 24 h in 4 μmol L^{-1} bafilomycin produced no noticeable decrease in NTP levels during at least 3 h of subsequent aerobic incubation (Covi et al. 2005), despite the fact that up to 84% of these embryos arrested in the encysted stage when incubated in 35 ppt artificial seawater without inhibitors following ^{31}P-NMR measurements (J. A. Covi and S. C. Hand, unpublished data). By comparison, only 6% of control embryos from this study arrested in the encysted stage after ^{31}P-NMR measurement. Thus, it appears that bafilomycin treatment arrests development without affecting adenylate balance during the periods of measurement used here.

Discussion

The primary goal of this study was to evaluate the metabolic impact of V-ATPase inhibition in encysted embryos of the brine shrimp *Artemia franciscana*. We succeeded in demonstrating that (1) cuticular permeability to the F-ATPase inhibitor oligomycin is sufficiently high to demonstrate inhibition of respiration in dechorionated embryos within the first hour of incubation; (2) the arrest of embryonic development and reduction in cellular respiration induced by the V-ATPase inhibitor bafilomycin occurs without affecting energy-producing pathways; and (3) proton pumping by the V-ATPase combined with ATP-consuming processes immediately dependent on this activity constitute at least 31% of the aerobic energy budget of the preemergent embryo. Downregulation of ion pumping by such a costly transporter would be required for concordance with the extreme decrease in energy flow observed under anoxia (Hand and Hardewig 1996).

Approximately a third of ATP turnover in the encysted embryos appears to be either the direct result of or immediately dependent on V-ATPase activity. Under conditions that do not adversely influence viability, incubation of dechorionated embryos with a highly specific inhibitor of the V-ATPase (bafilomycin A_1) induced a 31% drop in oxygen consumption (Fig. 3*A*) without affecting purine di- and triphosphate nucleotide levels (Fig. 5; NTP and NDP) or intracellular pH under constant aerobic incubation (Covi et al. 2005). In light of an apparent lack of bafilomycin-sensitive respiration in isolated mitochondria, the maintenance of high ATP levels suggests that the drop in oxygen consumption is the result of reduced energetic demands resulting from V-ATPase inhibition. Because metabolism in these embryos is fully aerobic under normoxia (Hand and Gnaiger 1988), it is likely that ATP turnover also decreases by 31% with bafilomycin treatment. This drop in cellular energy utilization is likely to be the product of the direct inhibition of ATP consumption by the V-ATPase and, potentially, inhibition of secondary processes immediately dependent on V-ATPase function. Given that these gastrula-stage embryos are composed largely of undifferentiated cells (Hofmann and Hand 1990), these secondary processes would most likely be restricted to constitutive cell functions such as protein trafficking.

It is appropriate to note that while the acidification of intracellular organelles is likely to play a critical role for the V-ATPase in *A. franciscana* embryos, this enzyme could also serve to energize the plasma membranes during early preemergence development. While the Na^+K^+-ATPase is almost ubiquitously employed for the energization of plasma membranes in animal cells, it is conspicuously absent from encysted *A. franciscana* embryos. Biochemical analyses show no measurable Na^+K^+-ATPase activity whatsoever until hour 6 of aerobic development (Fisher et al. 1986), and activity is exceedingly low between hour 6 and hatching (Peterson et al. 1978). It is plausible that the V-ATPase is the ion transporter that functions to energize embryonic membranes before expression of the Na^+K^+-ATPase. Certainly, during preemergence development, the V-ATPase could serve in both the energization of plasma membranes and the acidification of intracellular compartments. Such a dual function could explain why the ATP-dependent activity of this proton pump appears to represent such a substantial fraction of total cellular ATP turnover in the encysted embryo. In support of this argument, approximately 10%–16% of total cellular ATP turnover results from a combination of V-ATPase (acidification of organelles) and Na^+K^+-ATPase (energization of the plasma membrane) activity in the nonexcitable cells of mammalian vascular endothelium (Gruwel et al. 1995; Culic et al. 1997). In the isolated body wall musculature of *Sipunculus nudus* (marine invertebrate), these two ion pumps are responsible for almost 30%–40% of total cellular ATP turnover. The combined activity of these two pumps can even consume more than 80% of the cellular energy budget in mammalian proximal tubules, where it has been noted that the inhibition of Na^+K^+-ATPase with ouabain increases the amount of ATP turnover inhibited by bafilomycin (Noel et al. 1993; Fleser et al. 1995). An examination of the energetic requirements of V- and Na^+K^+-ATPase activity in a large variety of tissue types from a broad array of species illustrates the range of V-ATPase activity that might be expected if this enzyme were serving to both acidify intracellular compartments and energize plasma membranes (Table 1). The mean bafilomycin-sensitive oxygen consumption observed in this analysis is 14% ± 3% of

Table 1: Comparative analysis of Na^+K^+-ATPase and V-ATPase contributions to total energy turnover

		Percent of Total Energy Turnover[a]			Inhibitor Concentration		
Organism	Tissue	NaK-ATPase	V-ATPase	Combined	Ouabain (mM)	Bafilomycin (μM)	References
Dog	Cortical tubules	34–45	15–32	49–77	1	.5–1	Noel et al. 1993; Duplain et al. 1995; Fleser et al. 1995
Pig	Cortical tubules	35	19	54	1	1	Duplain et al. 1995
Rat	Cortical tubules	47	9	56	1	1	Duplain et al. 1995
Rabbit	Cortical tubules	50–58	4	54–62	1	1	Noel et al. 1993; Duplain et al. 1995
Hamster	Cortical tubules	49–55	2	51–57	1	1	Noel et al. 1993; Duplain et al. 1995
Marine worm	Muscle	17–25	12–14	29–39	1	.1	Portner et al. 2000
Fish	Gill	18–37	19	37–56	.01–.5	1	Stagg and Shuttleworth 1982; Morgan and Iwama 1999
Fish	Hepatocytes	15–45	…	…	1–6.67	…	Krumschnabel et al. 1994*a*, 1994*b*; Mark et al. 2005
Rat	Kidney	57	…	…	1	…	Else and Hulbert 1987
Rat	Liver	45	…	…	1	…	Else and Hulbert 1987
Rat	Brain	55	…	…	1	…	Else and Hulbert 1987
Rat	Distal colon	32	…	…	1	…	Saravi et al. 2003
Lizard	Kidney	69	…	…	1	…	Else and Hulbert 1987
Lizard	Liver	35	…	…	1	…	Else and Hulbert 1987
Lizard	Brain	24	…	…	1	…	Else and Hulbert 1987
Frog	Skin	15–24	…	…	2	…	Flanigan and Guppy 1997
Frog	Brain	30–34	…	…	2	…	Flanigan and Guppy 1997
Brine shrimp	Embryos	0[b]	31	31	…	1	Peterson et al. 1978; this study
Mean ± SE		36 ± 3	14 ± 3	60 ± 4			All species/tissues[c]
Range		15–58	2–32	29–77			All species/tissues[c]

Note. Ellipses indicate no available data.

[a] All values were derived from ouabain- or bafilomycin-sensitive respiration and corrected to represent percentage of total oxygen consumption.

[b] NaK-ATPase activity is undetectable for the first 6 h of aerobic development (Peterson et al. 1978). Thus, we assume oxygen consumption is negligible.

[c] Values for brine shrimp embryos were not included in calculation of mean, SE, or range.

total respiration, which is half of that observed for encysted *Artemia* embryos. In addition, the oxygen consumption supporting V-ATPase activity in *Artemia* embryos is within the range of combined V- and Na^+K^+-ATPase activity observed in other systems (Table 1; 29%–77%). Given that these encysted embryos are surrounded by a permeability barrier and do not need to osmoregulate with respect to the environment, it is not unexpected that the value would fall toward the lower end of this range. Notably, this range includes the activity required for both membrane energization by the V- or Na^+K^+-ATPase and compartment acidification by the V-ATPase. Thus, our estimate that V-ATPase activity accounts for 31% of total ATP turnover in *A. franciscana* embryos fits with a model whereby this enzyme functions in both membrane energization and the acidification of intracellular organelles during preemergence development.

While the concentrations of bafilomycin required to produce a maximal physiological response in *A. franciscana* embryos were very similar in hatching (Covi and Hand 2005) and ^{31}P-NMR studies (Covi et al. 2005), that is, 4 μmol L^{-1}, we observed in this study that a lower concentration was required to produce a maximal response in respirometry experiments (0.5 μmol L^{-1} [Fig. 2]). The reason for this difference is not fully clear, although as discussed earlier, the requirement for mechanical stirring in the respiration studies is a likely factor.

The data presented here demonstrate that after >5 h of incubation with 4 μmol L^{-1} bafilomycin, NTP levels still remain high (Fig. 5*B*); pH_i status is also unaffected by this treatment (Covi et al. 2005). Together, these data demonstrate that the embryos remain viable throughout the period of bafilomycin exposure used here. However, because emergence does not occur following such exposures (Covi and Hand 2005), it is also clear that developmental arrest is promoted. We propose that inhibition of yolk degradation by bafilomycin precludes a progression of developmental events required for emergence, thereby delaying embryonic development. Lysosomal-mediated degradation of yolk platelets peaks for the first time at emergence (Perona and Vallejo 1989), and the 25% of yolk stores

degraded during preemergence development (Utterback and Hand 1987) provide more than a simple supply of energy and molecular building blocks in encysted *Artemia* embryos. Yolk degradation is responsible for the programmed release of hydrolytic enzymes and substances implicated in the onset of protein synthesis (Slegers 1991). It has also been suggested to play a role in determining the sequence of events involved in preemergence development (Drinkwater and Clegg 1991). Thus, it is not unexpected that incubation of *Artemia* embryos with lysosomotrophic agents reversibly inhibits both yolk degradation and embryonic development (Perona et al. 1987). Incubation of cultured mammalian cells with bafilomycin induces a similar developmental arrest, the kinetics of which closely match a concurrent lysosomal alkalinization (Ohkuma et al. 1993). Blockage of yolk utilization by bafilomycin treatment also inhibits cellular differentiation in isolated cells from *Xenopus laevis* embryos (Fagotto and Maxfield 1994). In short, lysosomal degradation of yolk is critical to embryonic development for a diverse array of species.

While it is tempting to suggest that the inhibitory effect of bafilomycin A1 on the embryonic development of animals is mediated by altering lysosomal pH, the actual mechanisms are likely to be more complicated. The effect of macrolide antibiotics like bafilomycin could involve a number of nonexclusive mechanisms including alkalinization of membrane-bound compartments, altered transmembrane pH gradients, changes in transmembrane protein conformation, impaired membrane fusion, and disruption of protein trafficking/sorting, all of which would ultimately affect lysosomal function (cf. Mukherjee et al. 1997; Nishi and Forgac 2002; Bonifacino and Traub 2003; Weisz 2003; Ni et al. 2006). By way of example, recent work by Komazaki and Hiruma (1999) demonstrates that bafilomycin inhibits yolk degradation in early amphibian embryos by preventing the fusion of yolk platelets with late endosomes/lysosomes. Work by van Weert et al. (1995) demonstrates that bafilomycin strongly inhibits transport from late endosomes to lysosomes in HepG2 cells as well. The effect of bafilomycin could also be more direct. Inhibition of V-ATPase activity would produce an alkalinization of late endosomes and lysosomes, ultimately preventing the release of lysosomal acid hydrolases from mannose-6-phosphate receptors in late endosomes (cf. Mukherjee et al. 1997; Bonifacino and Traub 2003; Weisz 2003; Ni et al. 2006) and inhibiting the activity of these enzymes in lysosomes.

In conclusion, the respiration data presented for aerobically developing embryos of the brine shrimp *A. franciscana* demonstrate that inhibition of proton pumping by the V-ATPase significantly reduces oxygen consumption. ^{31}P-NMR of whole embryos and respiration assays on isolated mitochondria demonstrate that energy-producing pathways continue to function in the absence of V-ATPase activity. Together, these data suggest that the lowered oxygen consumption in whole embryos treated with bafilomycin is caused by decreased energy utilization by the V-ATPase and, potentially, processes immediately dependent on V-ATPase proton pumping. Bafilomycin-sensitive respiration in *A. franciscana* embryos is approximately double that of the average value for other species/tissues and is within the range expected if this enzyme were to be solely responsible for all membrane energization. Because of the absence of detectable Na^+K^+-ATPase activity during early development, it seems reasonable to suggest that proton pumping by the V-ATPase is responsible for both the constitutive acidification of intracellular compartments and the energization of plasma membranes during the early stages of preemergence development. Importantly, the magnitude of this metabolic cost requires that V-ATPase activity be downregulated under anoxia in order to attain the almost complete metabolic depression observed in the intact embryo.

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Literature Cited

Anchordoguy T.J. and S.C. Hand. 1994. Acute blockage of the ubiquitin-mediated proteolytic pathway during invertebrate quiescence. Am J Physiol 267:R895–R900.

Bonifacino J.S. and L.M. Traub. 2003. Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu Rev Biochem 72:395–447.

Buck L.T. and P.W. Hochachka. 1993. Anoxic suppression of Na^+-K^+-ATPase and constant membrane potential in hepatocytes: support for channel arrest. Am J Physiol 265: R1020–R1025.

Buck L.T., S.C. Land, and P.W. Hochachka. 1993. Anoxia-tolerant hepatocytes: model system for study of reversible metabolic suppression. Am J Physiol 265:R49–R56.

Busa W.B., J.H. Crowe, and G.B. Matson. 1982. Intracellular pH and the metabolic status of dormant and developing *Artemia* embryos. Arch Biochem Biophys 216:711–718.

Carpenter J.F. and S.C. Hand. 1986. Arrestment of carbohydrate metabolism during anaerobic dormancy and anaerobic acidosis in *Artemia* embryos: determination of pH-sensitive control points. J Comp Physiol B 156:451–459.

Clegg J. 1997. Embryos of *Artemia franciscana* survive four years

of continuous anoxia: the case for complete metabolic rate depression. J Exp Biol 200:467–475.

Conte F.P., P.C. Droukas, and R.D. Ewing. 1977. Development of sodium regulation and de novo synthesis of Na+K-activated ATPase in larval brine shrimp, *Artemia salina*. J Exp Zool 202:339–362.

Covi J.A. and S.C. Hand. 2005. V-ATPase expression during development of *Artemia franciscana* embryos: potential role for proton gradients in anoxia signaling. J Exp Biol 208:2783–2798.

Covi J.A., S.C. Hand, and W.D. Treleaven. 2005. V-ATPase inhibition prevents recovery from anoxia in *Artemia franciscana* embryos: quiescence signaling through dissipation of proton gradients. J Exp Biol 208:2799–2808.

Culic O., M.L. Gruwel, and J. Schrader. 1997. Energy turnover of vascular endothelial cells. Am J Physiol 273:C205–C213.

Drinkwater L.E. and J.S. Clegg. 1991. Experimental biology of cyst diapause. Pp. 93–117 in R.A. Browne, P. Sorgeloose, and C.N.A. Trotman, eds. *Artemia* Biology. CRC, Boca Raton, FL.

Duplain M., J. Noel, A. Fleser, V. Marshansky, A. Gougoux, and P. Vinay. 1995. Mechanisms of proximal proton secretion in BBM of herbivorous, omnivorous, and carnivorous species. Am J Physiol 269:R104–R112.

Eads B.D. and S.C. Hand. 1999. Regulatory features of transcription in isolated mitochondria from *Artemia franciscana* embryos. Am J Physiol 277:R1588–R1597.

Else P.L. and A.J. Hulbert. 1987. Evolution of mammalian endothermic metabolism: leaky membranes as a source of heat. Am J Physiol 253:R1–R7.

Fagotto F. and F.R. Maxfield. 1994. Changes in yolk platelet pH during *Xenopus laevis* development correlate with yolk utilization: a quantitative confocal microscopy study. J Cell Sci 107:3325–3337.

Fisher J.A., L.A. Baxter-Lowe, and L.E. Hokin. 1986. Regulation of Na, K-ATPase biosynthesis in developing *Artemia salina*. J Biol Chem 261:515–519.

Flanigan J.E. and M. Guppy. 1997. Metabolic depression and sodium-potassium ATPase in the aestivating frog, *Neobatrachus kunapalari*. J Comp Physiol B 167:135–145.

Fleser A., V. Marshansky, M. Duplain, J. Noel, A. Hoang, A. Tejedor, and P. Vinay. 1995. Cross-talk between the Na^+-K^+-ATPase and the H^+-ATPase in proximal tubules in suspension. Renal Physiol Biochem 18:140–152.

Forgac M. 2000. Structure, mechanism and regulation of the clathrin-coated vesicle and yeast vacuolar H^+-ATPases. J Exp Biol 203:71–80.

Futai M., T. Oka, G. Sun-Wada, Y. Moriyama, H. Kanazawa, and Y. Wada. 2000. Luminal acidification of diverse organelles by V-ATPase in animal cells. J Exp Biol 203:107–116.

Gnaiger E., G. Mendez, and S.C. Hand. 2000. High phosphorylation efficiency and depression of uncoupled respiration in mitochondria under hypoxia. Proc Natl Acad Sci USA 97: 11080–11085.

Gruwel M.L., C. Alves, and J. Schrader. 1995. Na^+-K^+-ATPase in endothelial cell energetics: ^{23}Na nuclear magnetic resonance and calorimetry study. Am J Physiol 268:H351–H358.

Hand S.C. 1995. Heat flow is measurable from *Artemia franciscana* embryos under anoxia. J Exp Zool 273:445–449.

Hand S.C. and E. Gnaiger. 1988. Anaerobic dormancy quantified in *Artemia* embryos: a calorimetric test of the control mechanism. Science 239:1425–1427.

Hand S.C. and I. Hardewig. 1996. Downregulation of cellular metabolism during environmental stress: mechanisms and implications. Annu Rev Physiol 58:539–563.

Harvey W.R. and H. Wieczorek. 1997. Animal plasma membrane energization by chemiosmotic H^+ V-ATPases. J Exp Biol 200: 203–216.

Hofmann G.E. and S.C. Hand. 1990. Subcellular differentiation arrested in *Artemia* embryos under anoxia: evidence supporting a regulatory role for intracellular pH. J Exp Zool 253:287–302.

Kawasaki-Nishi S., T. Nishi, and M. Forgac. 2003. Proton translocation driven by ATP hydrolysis in V-ATPases. FEBS Lett 545:76–85.

Komazaki S. and T. Hiruma. 1999. Degradation of yolk platelets in the early amphibian embryo is regulated by fusion with late endosomes. Dev Growth Differ 41:173–181.

Krumschnabel G., S. Malle, P.J. Schwarzbaum, and W. Wieser. 1994*a*. Glycolytic function in goldfish hepatocytes at different temperatures: relevance for Na^+ pump activity and protein synthesis. J Exp Biol 192:285–290.

Krumschnabel G., P.J. Schwarzbaum, and W. Wieser. 1994*b*. Coupling of energy supply and energy demand in isolated goldfish hepatocytes. Physiol Zool 67:438–448.

Kwast K.E. and S.C. Hand. 1993. Regulatory features of protein synthesis in isolated mitochondria from *Artemia* embryos. Am J Physiol 265:R1238–R1246.

Kwast K.E., J.I. Shapiro, B.B. Rees, and S.C. Hand. 1995. Oxidative phosphorylation and the realkalinization of intracellular pH during recovery from anoxia in *Artemia franciscana* embryos. Biochim Biophys Acta 1232:5–12.

Mark F.C., T. Hirse, and H.O. Portner. 2005. Thermal sensitivity of cellular energy budgets in some Antarctic fish hepatocytes. Polar Biol 28:805–814.

Morgan J.D. and G.K. Iwama. 1999. Energy cost of NaCl transport in isolated gills of cutthroat trout. Am J Physiol 277: R631–R639.

Mukherjee S., R.N. Ghosh, and F.R. Maxfield. 1997. Endocytosis. Physiol Rev 77:759–803.

Nelson N. and W.R. Harvey. 1999. Vacuolar and plasma membrane proton-adenosinetriphosphatases. Physiol Rev 79:361–385.

Nelson N., N. Perzov, A. Cohen, K. Hagai, V. Padler, and H.

Nelson. 2000. The cellular biology of proton-motive force generation by V-ATPases. J Exp Biol 203:89–95.

Ni X., M. Canuel, and C.R. Morales. 2006. The sorting and trafficking of lysosomal proteins. Histol Histopathol 21:899–913.

Nishi T. and M. Forgac. 2002. The vacuolar (H^+)-ATPases: nature's most versatile proton pumps. Nat Rev Mol Cell Biol 3:94–103.

Noel J., P. Vinay, A. Tejedor, A. Fleser, and R. Laprade. 1993. Metabolic cost of bafilomycin-sensitive H^+ pump in intact dog, rabbit, and hamster proximal tubules. Am J Physiol 264:F655–F661.

Ohkuma S., S. Shimizu, M. Noto, Y. Sai, K. Kinoshita, and H. Tamura. 1993. Inhibition of cell growth by bafilomycin A1, a selective inhibitor of vacuolar H^+-ATPase. In Vitro Cell Dev Biol Anim 29A:862–866.

Perona R., B. Ezquieta, and C.G. Vallejo. 1987. The degradation of yolk in *Artemia*. Pp. 105–123 in W. Decleir, L. Moens, H. Slegers, E. Jaspers, and P. Sorgeloos, eds. *Artemia* Research and Its Applications. Vol. 2. Universa, Wetteren.

Perona R. and C.G. Vallejo. 1989. Mechanisms of yolk degradation in *Artemia*: a morphological study. Comp Biochem Physiol 94:231–242.

Peterson G.L., L. Churchill, J.A. Fisher, and L.E. Hokin. 1982. Structural and biosynthetic studies on the two molecular forms of the ($Na^+ + K^+$)-activated adenosine triphosphatase large subunit in *Artemia salina* nauplii. J Exp Zool 221:295–308.

Peterson G.L., R.D. Ewing, and F.P. Conte. 1978. Membrane differentiation and de nova synthesis of the ($Na^+ + K^+$)-activated adenosine triphosphatase during development of *Artemia salina* nauplii. Dev Biol 67:90–98.

Portner H.O., C. Bock, and A. Reipschlager. 2000. Modulation of the cost of pHi regulation during metabolic depression: a ^{31}P-NMR study in invertebrate (*Sipunculus nudus*) isolated muscle. J Exp Biol 203:2417–2428.

Saravi F.D., T.A. Saldena, C.A. Carrera, J.E. Ibanez, L.M. Cincunegui, and G.E. Carra. 2003. Oxygen consumption and chloride secretion in rat distal colon isolated mucosa. Dig Dis Sci 48:1767–1773.

Slegers H. 1991. Enzyme activities through development: a synthesis of the activity and control of the various enzymes as the embryo matures. Pp. 37–73 in R.A. Browne, P. Sorgeloos, and C.N.A. Trotman, eds. *Artemia* Biology. CRC, Boca Raton, FL.

Stagg R.M. and T.J. Shuttleworth. 1982. Na^+,K^+ ATPase, ouabain binding and ouabain-sensitive oxygen consumption in gills from *Platichthys flesus* adapted to seawater and freshwater. J Comp Physiol 147:93–99.

Stocco D.M., P.C. Beers, and A.H. Warner. 1972. Effect of anoxia on nucleotide metabolism in encysted embryos of the brine shrimp. Dev Biol 27:479–493.

Trotman C.N.A. 1991. Normality and abnormality in early development. Pp. 75–92 in R.A. Brown, P. Sorgeloos, and C.N.A. Trotman, eds. *Artemia* Biology. CRC, Boca Raton, FL.

Utterback P.J. and S.C. Hand. 1987. Yolk platelet degradation in preemergence *Artemia* embryos: response to protons in vivo and in vitro. Am J Physiol 252:R774–R781.

van Weert A.W.M., K.W. Dunn, H.J. Gueze, F.R. Maxfield, and W. Stoorvogel. 1995. Transport from late endosomes to lysosomes, but not sorting of integral membrane proteins in endosomes, depends on the vacuolar proton pump. J Cell Biol 130:821–834.

Warner A.H. and J.S. Clegg. 2001. Diguanosine nucleotide metabolism and the survival of *Artemia* embryos during years of continuous anoxia. Eur J Biochem 268:1568–1576.

Weisz O.A. 2003. Acidification and protein traffic. Int Rev Cytol 226:259–319.

Wieczorek H., G. Grber, W.R. Harvey, M. Huss, H. Merzendorfer, and W. Zeiske. 2000. Structure and regulation of insect plasma membrane H^+V-ATPase. J Exp Biol 203:127–135.