

## METABOLIC IMPORTANCE OF Na<sup>+</sup>/K<sup>+</sup>-ATPase ACTIVITY DURING SEA URCHIN DEVELOPMENT

P. K. K. LEONG AND D. T. MANAHAN\*

*Department of Biological Sciences, University of Southern California, Los Angeles, CA 90089-0371, USA*

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### Summary

Early stages of animal development have high mass-specific rates of metabolism. The biochemical processes that establish metabolic rate and how these processes change during development are not understood. In this study, changes in Na<sup>+</sup>/K<sup>+</sup>-ATPase activity (the sodium pump) and rate of oxygen consumption were measured during embryonic and early larval development for two species of sea urchin, *Strongylocentrotus purpuratus* and *Lytechinus pictus*. Total (*in vitro*) Na<sup>+</sup>/K<sup>+</sup>-ATPase activity increased during development and could potentially account for up to 77% of larval oxygen consumption in *Strongylocentrotus purpuratus* (pluteus stage) and 80% in *Lytechinus pictus* (prism stage). The critical issue was addressed of what percentage of total enzyme activity is physiologically active in living embryos and larvae and thus what percentage of metabolism is established by the activity of the sodium pump during development. Early developmental stages of sea urchins are ideal for understanding the *in vivo* metabolic importance of Na<sup>+</sup>/K<sup>+</sup>-ATPase because of their small size and high permeability to radioactive tracers (<sup>86</sup>Rb<sup>+</sup>) added to sea water. A comparison of total and *in vivo* Na<sup>+</sup>/K<sup>+</sup>-ATPase activities revealed that approximately half of the total activity was

utilized *in vivo*. The remainder represented a functionally active reserve that was subject to regulation, as verified by stimulation of *in vivo* Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in the presence of the ionophore monensin. In the presence of monensin, *in vivo* Na<sup>+</sup>/K<sup>+</sup>-ATPase activities in embryos of *S. purpuratus* increased to 94% of the maximum enzyme activity measured *in vitro*. Stimulation of *in vivo* Na<sup>+</sup>/K<sup>+</sup>-ATPase activity was also observed in the presence of dissolved alanine, presumably due to the requirement to remove the additional intracellular Na<sup>+</sup> that was cotransported with alanine from sea water. The metabolic cost of maintaining the ionic balance was found to be high, with this process alone accounting for 40% of the metabolic rate of sea urchin larvae (based on the measured fraction of total Na<sup>+</sup>/K<sup>+</sup>-ATPase that is physiologically active in larvae of *S. purpuratus*). Ontogenetic changes in pump activity and environmentally induced regulation of reserve Na<sup>+</sup>/K<sup>+</sup>-ATPase activity are important factors that determine a major proportion of the metabolic costs of sea urchin development.

Key words: Na<sup>+</sup>/K<sup>+</sup>-ATPase, sodium pump, sea urchin, development, metabolism, *Strongylocentrotus purpuratus*, *Lytechinus pictus*.

### Introduction

Embryos and larvae of marine invertebrates have high mass-specific metabolic rates relative to adult stages (Zeuthen, 1947, 1953; Widdows, 1991). During larval development, metabolic rates per individual can increase by an order of magnitude prior to metamorphosis (e.g. echinoderm and molluscan larvae; Hoegh-Guldberg and Manahan, 1995). It is not known, however, what the biochemical bases are for such high and dynamic metabolic rates and what processes establish the mass-specific metabolic intensity (mass coefficient) of developing stages. For animals in general, the processes that establish the mass coefficient of the metabolic power curve are not fully understood (Heusner, 1991).

Na<sup>+</sup>/K<sup>+</sup>-ATPase is a transmembrane protein found in higher eukaryotes (Skou, 1957) that transports Na<sup>+</sup> and K<sup>+</sup> across the plasma membrane to maintain ionic gradients. These gradients,

in turn, facilitate other secondary active transport systems such as Na<sup>+</sup>/amino acid and Na<sup>+</sup>/glucose cotransport in animals (Crane, 1977; Wright and Manahan, 1989). These important physiological processes are energetically costly to operate – the activity of the sodium pump alone can account for 20% of the energy expenditure of certain mammalian tissues (Milligan and McBride, 1985). In the present study, we measured Na<sup>+</sup>/K<sup>+</sup>-ATPase activity during the early development of the sea urchins *Strongylocentrotus purpuratus* and *Lytechinus pictus* since the small size of sea urchin embryos and larvae renders these stages of development ideal for the study of the importance of Na<sup>+</sup>/K<sup>+</sup>-ATPase in establishing *in vivo* metabolic rates. In addition, because of the high permeability of sea urchin embryos and larvae to specific isotopes added to sea water, *in vivo* measurements of ion flux can be used to

\*Author for correspondence (e-mail: manahan@usc.edu).

determine the physiological level of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity and the ontogenetic changes in this level.

## Materials and methods

### Larval cultures

Adult sea urchins, *Strongylocentrotus purpuratus* (Stimpson) and *Lytechinus pictus* (Verrill), were induced to spawn by intracoelomic injection of 0.5 mol l<sup>-1</sup> KCl. Embryos were suspended in 20 l culture vessels (*S. purpuratus*: 30–40 embryos ml<sup>-1</sup>; *L. pictus*: 10–20 embryos ml<sup>-1</sup>) and reared at 15 °C in filtered sea water (0.2 µm pore size). Prior to experiments, the cultures were concentrated by gently siphoning onto 45 µm or 80 µm mesh screens (the mesh size used depended on the stage of development). Embryos or larvae were washed with filtered sea water, resuspended in a 50 ml polypropylene centrifuge tube (Falcon), and their concentration determined by replicate counts of samples (50–100 µl) placed on 1 ml gridded counting chambers (Sedgewick-Rafter). Known numbers of animals were then removed for the various assays and experiments. Six developmental stages were used for the studies presented here, from the unfertilized egg through to the feeding larval stage (pluteus) reached at approximately 72 h under our culturing conditions. For enzyme analysis, six different cultures of *S. purpuratus* and three of *L. pictus* were studied. Each culture of *S. purpuratus* was started from a different male and female pair. Since smaller numbers of eggs were collected per female, 2–3 females were pooled for each culture of *L. pictus*.

### Measurement of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity: total enzyme activity in tissue homogenates

The measurement of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity at near-maximum rates ( $V_{opt}$ ) in tissue homogenates of embryos and larvae will be referred to as the 'in vitro' assay (cf. 'in vivo' assay, next section). The activity so determined will be referred to as the total ('potential') Na<sup>+</sup>/K<sup>+</sup>-ATPase activity. The total Na<sup>+</sup>/K<sup>+</sup>-ATPase activity was measured as the ouabain-sensitive rate of release of inorganic phosphate (P<sub>i</sub>) in the presence of ATP (Esmann, 1988). Animals at the appropriate stage of development were counted as described above and known numbers were placed in 1.7 ml microcentrifuge tubes. The animals were then pelleted by centrifugation at 12 500 g (Beckman, model-E microfuge) and the sea water was removed by aspiration before samples were stored at -70 °C for subsequent enzyme analysis. All the samples taken at different stages of development from the same culture were stored and later analyzed for enzyme activity. These analyses were performed at the same time to reduce between-stage errors that might have been the result of different assays conditions on different days. For analyses, samples were thawed and immediately resuspended in buffer (10 % sucrose, 5 mmol l<sup>-1</sup> EGTA and 5 mmol l<sup>-1</sup> histidine, pH 7.7) to a final protein concentration of 0.5–1.0 mg ml<sup>-1</sup>. In preliminary experiments, a buffer pH of 7.7 was found to give the maximum enzyme activity (pH range tested 6.5–9.3). The

resulting suspension was homogenized on ice using a Vibra Cell ultrasonicator fitted with a 3 mm probe (model 40, Sonics and Materials) until no visible animal tissue was observed. Similar activities of Na<sup>+</sup>/K<sup>+</sup>-ATPase were measured in larvae when tissue was first treated either by ultrasonication (as above) or by hand-homogenization in a Teflon/glass unit (Wheaton) ( $N=4$  for each method; range of all data for both methods 1.39–1.46 µmol P<sub>i</sub> mg<sup>-1</sup> protein h<sup>-1</sup>; prism-stage larvae used as experimental material). Samples of the sonicated homogenate were taken for enzyme assays (300 µl) and for measurements of protein content (100 µl, modified Bradford assay: Jaeckle and Manahan, 1989). The 300 µl sample was added to a 900 µl reaction mixture (Esmann, 1988) containing a final concentration of 130 mmol l<sup>-1</sup> NaCl, 20 mmol l<sup>-1</sup> KCl, 4 mmol l<sup>-1</sup> MgCl<sub>2</sub>, 3 mmol l<sup>-1</sup> ATP, 30 mmol l<sup>-1</sup> histidine (pH 7.7), with or without ouabain. A concentration of 2 mmol l<sup>-1</sup> ouabain was required for complete inhibition of ouabain-sensitive Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in developing sea urchin tissues (Fig. 1A). The optimum temperature for measurement of enzyme activity was found to be 25 °C (Fig. 1B). This was the lowest temperature tested that gave the best signal-to-noise ratio of Na<sup>+</sup>/K<sup>+</sup>-ATPase to total ATPase activity. Under these conditions of pH, temperature and enzyme concentration, the rate of generation of inorganic phosphate was linear for at least 20 min. After a 20 min incubation at 25 °C, the reaction was stopped by the addition of 100 µl of ice-cold trichloroacetic acid (TCA) (5 % TCA final concentration in reaction mixture). The amount of inorganic phosphate present in the reaction mixture was measured at 700 nm with a DU7 Beckman spectrophotometer (the method of Fiske and Subbarow, 1925; with modifications according to Peterson, 1978).

Sodium deoxycholate is considered to be the ideal detergent for unmasking any concealed ('latent') activity of Na<sup>+</sup>/K<sup>+</sup>-ATPase that might be due to the formation of a mixture of inside-out and right-side-out vesicles in the tissue homogenates (Jørgensen and Skou, 1971; Jørgensen, 1988). We tested sodium deoxycholate at six different concentrations (0.02–0.8 mg ml<sup>-1</sup>) on Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in homogenates of embryonic sea urchin tissue. The deoxycholate concentrations tested are known to be sufficient to unmask all vesicle-concealed Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in homogenates of mammalian tissues (Jørgensen and Skou, 1971; Ismail-Beigi *et al.* 1988) and in tissue homogenates of marine vertebrates (Skou and Esmann, 1988; Hwang and Tsai, 1993) and marine invertebrates (Holliday, 1985; Cortas and Edelman, 1988; Corotto and Holliday, 1996). We found that addition of sodium deoxycholate at the concentrations tested did not increase Na<sup>+</sup>/K<sup>+</sup>-ATPase activity (data not shown), suggesting that our protocol measures the total activity of the enzyme in sea urchin tissues. We also tested the effect on Na<sup>+</sup>/K<sup>+</sup>-ATPase activities of alamethicin, a membrane-permeabilizing agent with channel-forming properties (Jones *et al.* 1980; Ritov *et al.* 1993; Xie *et al.* 1989). Addition of alamethicin (at 25–100 µg mg<sup>-1</sup> protein) to sea urchin tissue homogenates did cause an increase in Na<sup>+</sup>/K<sup>+</sup>-ATPase activity; however, this

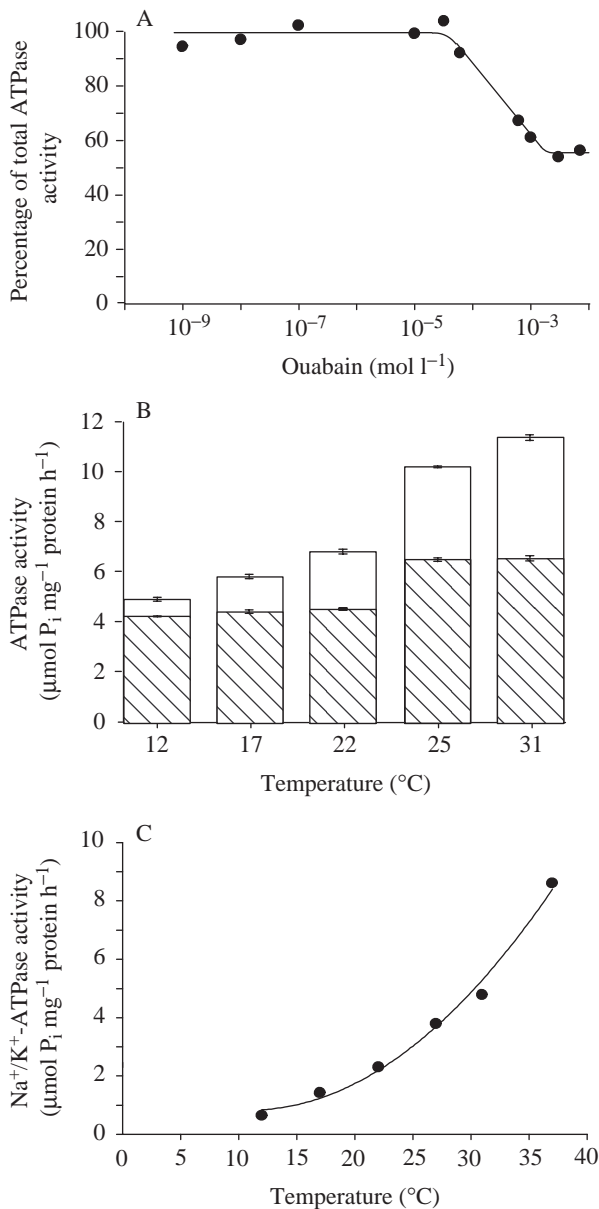


Fig. 1. *Strongylocentrotus purpuratus*, early pluteus-stage larvae. (A) Ouabain inhibition at 2 mmol l<sup>-1</sup> of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity. Each data point represents the mean of three assay replicates. Error bars are not shown because of the subtractive nature of these calculations (for examples, see Table 1). Errors for each different set of assays were as follows: the coefficient of variation for assays without ouabain was 0.3%; with ouabain, the coefficient of variation ranged from a minimum of 0.6% to a maximum of 3.5%. (B) Change in total ATPase activity (given as total height of each histogram bar) and Na<sup>+</sup>/K<sup>+</sup>-ATPase activity as a function of increasing temperature. The open portion of each bar represents Na<sup>+</sup>/K<sup>+</sup>-ATPase (ouabain-sensitive) activity; the hatched portion represents the ouabain-insensitive activity (*N*=6 for each set of assays, with and without ouabain; error bars are S.E.M.). (C) Change in Na<sup>+</sup>/K<sup>+</sup>-ATPase activity as a function of temperature, *N*=6 (*N*=3 for assay at 37°C); the coefficient of variation for assays without ouabain ranged from a minimum of 0.7% to a maximum of 3.8%; the coefficient of variation for assays with ouabain ranged from 1.5% to 4.2%. Calculated *Q*<sub>10</sub> is 2.4 (see text).

increase did not occur in all stages of development tested. The data we present in this paper on total Na<sup>+</sup>/K<sup>+</sup>-ATPase activity during sea urchin development are based on measurements at *V*<sub>opt</sub> of tissue homogenates without deoxycholate, as this detergent had no effect on sea urchin tissues, and without alamethicin as this agent did not consistently increase enzyme activity in all stages studied.

*Measurement of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity: physiologically active enzyme in living embryos and larvae*

The ouabain-sensitive transport rate of <sup>86</sup>Rb<sup>+</sup>, a physiological analog of K<sup>+</sup> (Hilden and Hokin, 1975), from sea water by sea urchin embryos and larvae was used to measure Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in living animals. This approach will be referred to as the 'in vivo' assay of the physiologically active fraction of total Na<sup>+</sup>/K<sup>+</sup>-ATPase enzyme present. A known number of individuals was placed in 10 ml of sea water (20 ml glass vial) to which 1.1 MBq of <sup>86</sup>Rb<sup>+</sup> had been added at the start of the experiment (New England Nuclear, <sup>86</sup>RbCl specific activity 74–740 MBq ml<sup>-1</sup>). At approximately 60 s intervals, a 500 μl sample was removed and filtered through an 8.0 μm (pore-size) filter (25 mm polycarbonate, Nuclepore). The animals on the filter were washed with 15 ml of filtered sea water to remove excess isotope before the filter with animals was transferred to a scintillation vial. A 10 min time course experiment at 15°C (same as the rearing temperature of the animals) was carried out for each assay, during which time the rate of <sup>86</sup>Rb<sup>+</sup> transport was linear. The ouabain-sensitive (Na<sup>+</sup>/K<sup>+</sup>-ATPase-mediated) <sup>86</sup>Rb<sup>+</sup> transport rate was calculated as the difference between the rates in the presence and absence of 2 mmol l<sup>-1</sup> ouabain. Ouabain was added to the sea water 30–45 min before the addition of <sup>86</sup>Rb<sup>+</sup> to facilitate total inhibition of the Na<sup>+</sup>/K<sup>+</sup>-ATPase activity. This preincubation time was chosen as animals were seen to be still active and swimming in the sea water/ouabain solution prior to the start of the experiment. Radioactivity in each vial was measured using a liquid scintillation counter (Beckman, model LS 6000SC) after the addition of 5 ml of scintillation cocktail (Scintisafe, Fisher Scientific). The rate of K<sup>+</sup> transport was calculated from the slope of the linear regression of the increase in [<sup>86</sup>Rb<sup>+</sup>] with time. The slope (disintegrations min<sup>-1</sup> <sup>86</sup>Rb<sup>+</sup> individual<sup>-1</sup> h<sup>-1</sup>) was corrected for the specific activity of <sup>86</sup>Rb<sup>+</sup> in sea water, using the K<sup>+</sup> content of full-salinity sea water (33–34‰). Salinity was measured using a refractometer (AO, Scientific Instruments) and by measuring freezing point depression with an osmometer (Advanced Instruments, model 3D II). The number of moles of K<sup>+</sup> transported was converted to ATP equivalents on the basis of known pump activity: 1 ATP hydrolyzed by the Na<sup>+</sup>/K<sup>+</sup>-ATPase for the efflux of 3 Na<sup>+</sup> from a cell and the influx of 2 K<sup>+</sup>.

We were concerned that our filtering and washing protocol might lyse embryos on filters and release <sup>86</sup>Rb<sup>+</sup>, thereby underestimating the *in vivo* enzyme activity. A comparison of methods showed this was not the case. In a series of parallel time course experiments, one set of 500 μl samples was filtered

as above and a second set of samples was not filtered, but placed on silicone oil and centrifuged to separate the animals from the sea water/isotope mixture (method described in Manahan *et al.* 1989). Two stages of development of *S. purpuratus* were tested in this way, the 12-h-old embryo and the pluteus larval stage. A one-way analysis of variance (ANOVA) showed no significant difference in transport rates between the two methods (embryos,  $P > 0.25$ ,  $N = 4$  experiments; larvae,  $P > 0.05$ ,  $N = 5$ ). All transport assays reported here were performed using filtration, as this method gave a lower background count resulting from the removal of excess isotope by washing with sea water.

#### Regulation of *in vivo* $\text{Na}^+/\text{K}^+$ -ATPase activity

We used the ionophore monensin to stimulate maximum *in vivo*  $\text{Na}^+/\text{K}^+$ -ATPase activity in embryos (7- and 14-h-old) of *S. purpuratus*. Embryos were exposed to  $36 \mu\text{mol l}^{-1}$  monensin in sea water, the concentration found in preliminary experiments to give maximum  $^{86}\text{Rb}^+$  transport rates (concentration range tested  $0$ – $86 \mu\text{mol l}^{-1}$  monensin). Embryos were preincubated in monensin for 5–10 min before experiments started (controls had no monensin), and the transport rate of  $^{86}\text{Rb}^+$  was measured as above.

We tested the effect of transporting the amino acid alanine from sea water on the stimulation of *in vivo*  $\text{Na}^+/\text{K}^+$ -ATPase activity in developing sea urchins (neutral amino acid transport from sea water by marine invertebrates is  $\text{Na}^+$ -dependent; Wright, 1988). Embryos (12 h old) were exposed to an alanine concentration of  $162 \mu\text{mol l}^{-1}$ , a concentration known to produce a maximum transport rate ( $J_{\text{max}}$ ) of alanine into developing stages of *S. purpuratus* (Manahan *et al.* 1989). The transport rate of  $^{86}\text{Rb}^+$  by embryos was measured with and without alanine present in the sea water. In addition, the rate of [ $^{14}\text{C}$ ]alanine and  $^{86}\text{Rb}^+$  transport by embryos was measured by simultaneously exposing the embryos to 1.1 MBq of  $^{86}\text{Rb}^+$  and 74 kBq of [ $^{14}\text{C}$ ]alanine [New England Nuclear, 6.1 MBq  $\mu\text{mol}^{-1}$  alanine with the addition of cold carrier (alanine, Sigma Chemicals) to give the required concentration of  $162 \mu\text{mol l}^{-1}$ ]. The analysis of the transport experiments with dual labels ( $^{14}\text{C}$ ,  $^{86}\text{Rb}^+$ ) was the same as for the other *in vivo* transport assays, except that embryos collected on the polycarbonate filters were first dissolved in 1 ml of tissue solubilizer (Scinti-Gest, Fisher Scientific) for 24 h prior to measuring radioactivity (necessary to measure  $^{14}\text{C}$  accurately by liquid scintillation counting). Dual-label counting with a quench curve (quenching agent, carbon tetrachloride) for each isotope was carried out using a Beckman model LS 6000SC counter.

#### Measurement of oxygen consumption rates

Our previous studies have shown that the accuracy of respiration measurements made on marine invertebrate embryos and larvae in closed-volume microrespiration chambers connected to polarographic oxygen sensors is questionable (Hoegh-Guldberg and Manahan, 1995; Shilling *et al.* 1996). These studies recommended that independent

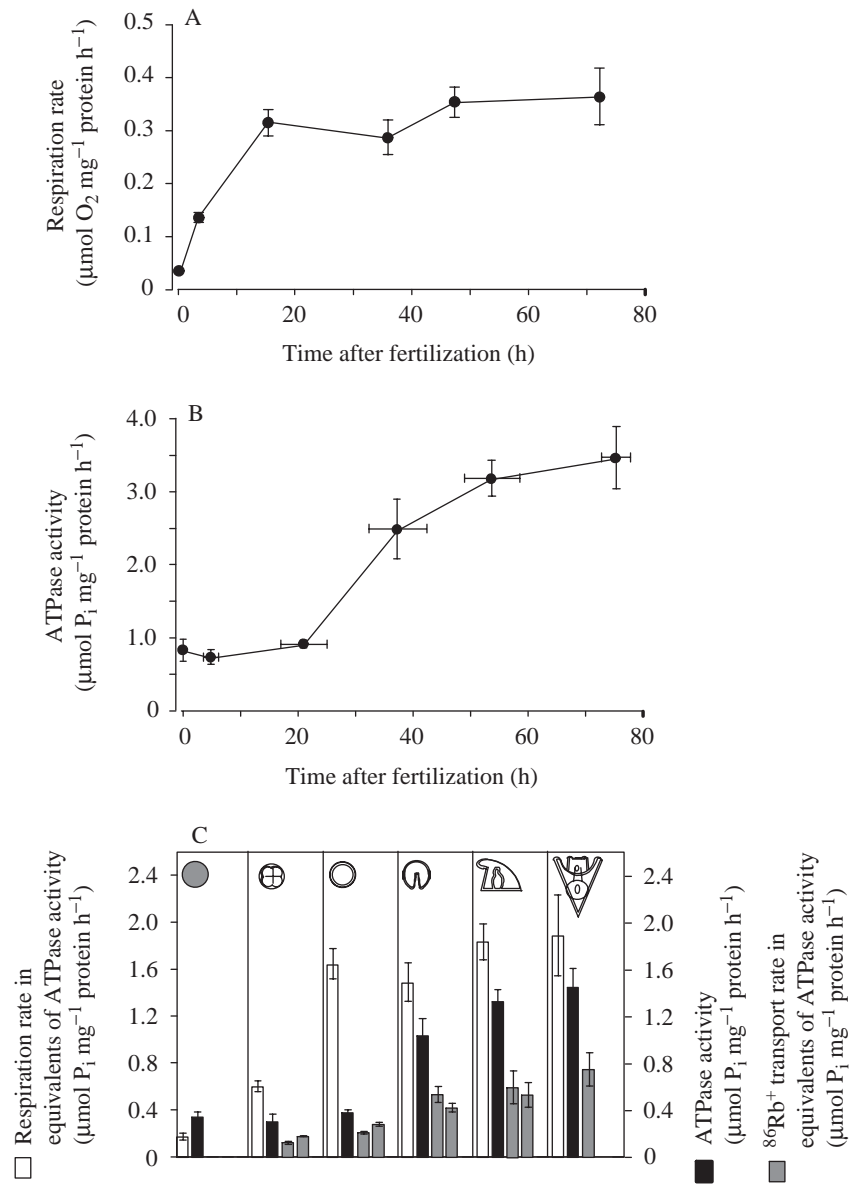
methods should be used to test for accuracy of respiration measurements for developing marine invertebrates. In this study, the rates of oxygen consumption were measured using two different methods: (1) a polarographic oxygen sensor (POS) fitted into a microrespiration chamber (Strathkelvin Instruments, model RC200) to which animals were added, and (2) a modified biological-oxygen-demand (BOD) bottle method. For each method, the range in concentration of animals was 1–3 individuals  $\mu\text{l}^{-1}$ . The first method followed the protocols of Jaeckle and Manahan (1992) with the following modifications: the volume of the microrespiration chambers was set at 175  $\mu\text{l}$ , the temperature was 15 °C, the experimental duration was 45 min, and six replicates were performed per developmental stage. The BOD method (A. G. Marsh and D. T. Manahan, unpublished results) is based on a modification of the standard large-volume BOD method (using Winkler's titration, see Hoegh-Guldberg and Manahan, 1995; Shilling *et al.* 1996). For this second method, animals were incubated for 6–7 h at 15 °C in 500  $\mu\text{l}$  air-tight glass vials (for replication, up to 30 such 'micro-BOD bottles' were used in parallel). At the end of incubation, a 200  $\mu\text{l}$  sample from each glass vial was injected into a low-volume MC100 chamber (70  $\mu\text{l}$ , Strathkelvin Instruments). The oxygen tension of the sea water was then determined using a polarographic oxygen sensor (i.e. for the micro-BOD method, the POS was used only to measure oxygen tension at the end of an incubation in a similar manner to blood-gas analysis systems). We have confidence in our respiration measurements of *S. purpuratus* because similar rates were obtained using both methods. With the micro-BOD method (see Fig. 4B), the respiration rate of 10-h-old embryos was  $4.62 \pm 0.16 \text{ pmol O}_2 \text{ embryo}^{-1} \text{ h}^{-1}$  (mean  $\pm$  95% confidence interval). Expressing the protein-specific respiration rate given in Fig. 2A on a per-embryo basis, the POS-measured respiration rate of 10-h-old embryos was  $4.7 \text{ pmol O}_2 \text{ embryo}^{-1} \text{ h}^{-1}$ .

## Results

#### Assay conditions for measurement of total $\text{Na}^+/\text{K}^+$ -ATPase activity

The monophasic ouabain inhibition curve for  $\text{Na}^+/\text{K}^+$ -ATPase activity suggests the presence of only a single class of ouabain-binding site in larvae of *S. purpuratus* (Fig. 1A). The occurrence of ouabain inhibition of  $\text{Na}^+/\text{K}^+$ -ATPase activity was also supported by independent experiments on living embryos. In the presence of  $2 \text{ mmol l}^{-1}$  ouabain, 10-h-old embryos had a respiration rate of  $1.55 \pm 0.21 \text{ pmol O}_2 \text{ embryo}^{-1} \text{ h}^{-1}$  (mean  $\pm$  S.E.M.), whereas controls with no ouabain present had a respiration rate of  $4.62 \pm 0.08 \text{ pmol O}_2 \text{ embryo}^{-1} \text{ h}^{-1}$  (mean  $\pm$  S.E.M.) (after a 6 h exposure to ouabain, respiration rates were significantly different: *t*-test,  $P < 0.001$ , d.f. = 18). The assay temperature had a differential effect on ouabain-sensitive ( $\text{Na}^+/\text{K}^+$ -ATPase) and ouabain-insensitive ATPase activities, with the ouabain-sensitive  $\text{Na}^+/\text{K}^+$ -ATPase component being more temperature-sensitive (Fig. 1B). The  $Q_{10}$  of  $\text{Na}^+/\text{K}^+$ -ATPase was found to be 2.4 for *S. purpuratus* (Fig. 1C) over the

Fig. 2. *Strongylocentrotus purpuratus*. (A) Protein-specific change in the rate of oxygen consumption during early development. The rate of oxygen consumption was measured at 15 °C (the rearing temperature) for six developmental stages (see diagrams in C): unfertilized egg, 4-h-old embryo, hatched blastula (hatching at 18h), early gastrula, prism and early pluteus larva. For each data point, *N*=6; error bars are S.E.M. (B) Change in activity of Na<sup>+</sup>/K<sup>+</sup>-ATPase during development, measured at 25 °C by an *in vitro* enzyme assay (at *V*<sub>opt</sub>, see text for details of the protocol). Enzyme activity was determined for the six developmental stages for which rates of oxygen consumption were measured. Each data point is the mean ± S.E.M. for measurements made on six different cultures of embryos and larvae (each culture was started with different gametes collected from one female and one male). Vertical error bars are S.E.M.; horizontal bars represent the maximum and minimum sampling time range for each of the six cultures. (C) Comparison of protein-specific rate of oxygen consumption and specific activity of Na<sup>+</sup>/K<sup>+</sup>-ATPase during development. All data are expressed as equivalents of ATPase activity at 15 °C (see text for methods of data conversion using measured values of *Q*<sub>10</sub>). Open bars represent rates of oxygen consumption in equivalents of ATPase activity, filled bars represent the corresponding total *in vitro* Na<sup>+</sup>/K<sup>+</sup>-ATPase activities (error bars are S.E.M., *N*=6). *In vivo* Na<sup>+</sup>/K<sup>+</sup>-ATPase activities are presented as stippled bars. For *in vivo* measurements, each error bar represents the rate of K<sup>+</sup> transport calculated from a time course transport experiment with 6–7 individual data points (K<sup>+</sup> transport was measured as <sup>86</sup>Rb<sup>+</sup> flux with correction for specific activity of K<sup>+</sup> in sea water, see text). The error bars for the *in vivo* rates are S.E.M. of the slope of the linear regression line. Where two stippled bars are shown for a single stage of development, each represents a separate time course experiment on the same group of animals. Rates of K<sup>+</sup> transport were also measured for unfertilized eggs (far left histograms), but rates were below the limit of detection (see text).



temperature range tested (12–37 °C) and 2.1 for *Lytechinus pictus* (range 15–37 °C, results not shown). All enzymatic assays were conducted at pH 7.7 and 25 °C. During the measurement of *Q*<sub>10</sub> at different temperatures, the assay pH varied (histidine-based buffer) from 7.34 to 7.77. The effect of this slight temperature-dependent change in pH was investigated further in another set of assays (pH values of 7.34 and 7.77, each buffer at 25 °C) and was found to have no significant effect on the measurement of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity (*t*-test, *P*>0.5, *N*=5).

*Change in rate of oxygen consumption and Na<sup>+</sup>/K<sup>+</sup>-ATPase activity through early development of S. purpuratus*

The rate of oxygen consumption, given as a rate per milligram protein for comparison with enzyme activity, showed a significant increase (*t*-test, *P*<0.001) from

0.03 µmol O<sub>2</sub> mg<sup>-1</sup> protein h<sup>-1</sup> before fertilization to 0.12 µmol O<sub>2</sub> mg<sup>-1</sup> protein h<sup>-1</sup> for 4-h-old embryos (Fig. 2A). The increase continued during development until after hatching (18 h), when the rate of oxygen consumption reached its peak at 0.32 µmol O<sub>2</sub> mg<sup>-1</sup> protein h<sup>-1</sup>, and remained unchanged (ANOVA, *P*>0.5) through to the early pluteus stage at 72 h old. These changes in protein-specific respiration rates for different stages were not caused by the calculation of the respiration rate on a per milligram protein basis (i.e. there was no change in respiration rate per individual, but a rapid stage-specific decrease in protein content resulting in an apparent increase in respiration rate for that stage of development when expressed on a per unit protein basis). During the 72 h period studied, protein content per individual decreased at a gradual and linear rate of -1.2 ng individual<sup>-1</sup> day<sup>-1</sup> from a starting

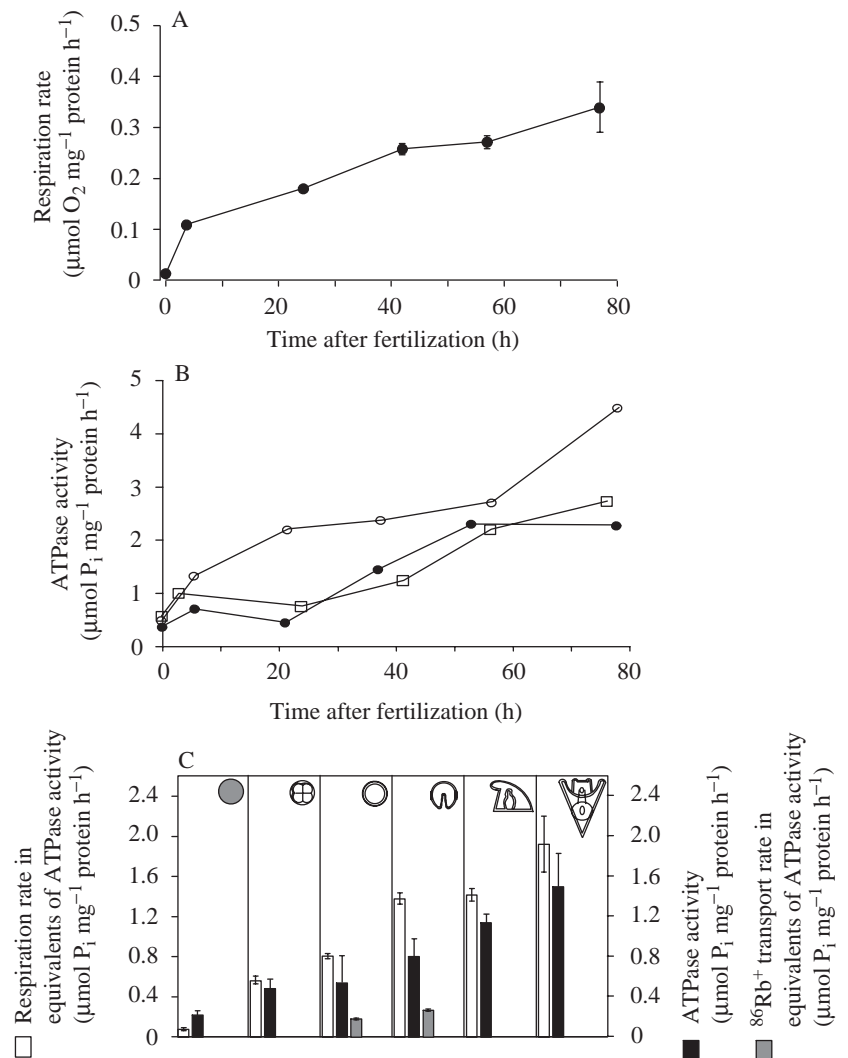
protein content in the egg of 21 ng (by ANOVA, the decrease in protein content with time was significant,  $P < 0.002$ ,  $N = 34$ ).

Total  $\text{Na}^+/\text{K}^+$ -ATPase activity at  $V_{\text{opt}}$  (Fig. 2B) did not change significantly from fertilization to hatching (ANOVA,  $P > 0.5$ ) and had a mean value of  $0.8 \mu\text{mol P}_i \text{ mg}^{-1} \text{ protein h}^{-1}$ . A rapid increase in activity from 0.8 to  $2.5 \mu\text{mol P}_i \text{ mg}^{-1} \text{ protein h}^{-1}$  was observed later in development from the hatching blastula to the early gastrula. This higher enzyme activity was maintained through to the early pluteus stage with no further statistically significant increase in activity (ANOVA,  $P > 0.2$ ).

Fig. 2C shows the total and *in vivo*  $\text{Na}^+/\text{K}^+$ -ATPase activities compared with respiration rate for each of the six stages of development studied. All data shown in Fig. 2C are expressed as equivalents of ATPase activity at  $15^\circ\text{C}$  to facilitate direct comparisons. The diagrams of each of the developmental stages in Fig. 2C correspond to the six stages of development presented in the upper figures (six different time points in Fig. 2A,B). To

compare metabolic rate with  $\text{Na}^+/\text{K}^+$ -ATPase activity, the rates of oxygen consumption were converted to ATP equivalents using a mean value for lipid and protein (McGilvery, 1979;  $5.2 \text{ pmol ATP pmol}^{-1} \text{ O}_2$ ). Carbohydrate was not included in this calculation as it is a minor component of the biochemical composition of embryos and larvae of *S. purpuratus* (Shilling and Manahan, 1990). In the unfertilized egg (Fig. 2C, far left histograms), the potential activity of total  $\text{Na}^+/\text{K}^+$ -ATPase was higher than could be accounted for by the respiration rate, suggesting that a large fraction of the  $\text{Na}^+/\text{K}^+$ -ATPase is physiologically inactive in the unfertilized egg (verified using *in vivo* measurements of enzyme activity in eggs, see below). The rapid increase in respiration rate after fertilization (Fig. 2A) and the slower increase in total  $\text{Na}^+/\text{K}^+$ -ATPase activity (Fig. 2B) resulted in the enzyme activity being potentially 50% of respiration rate for 4-h-old embryos, decreasing to 23% for hatching blastulae. Later in development, total  $\text{Na}^+/\text{K}^+$ -ATPase activity could potentially account for over 70% of the metabolic

Fig. 3. *Lytechinus pictus*. (A) Protein-specific change in the rate of oxygen consumption during early development. The rate of oxygen consumption was measured at  $15^\circ\text{C}$  (the rearing temperature) for six developmental stages (see diagrams in C): unfertilized egg, 4-h-old embryo, hatched blastula (hatching at 20 h), early gastrula, prism and early pluteus larva ( $N = 6$ , error bars are S.E.M.). (B) Change in activity of  $\text{Na}^+/\text{K}^+$ -ATPase during development, measured at  $25^\circ\text{C}$  using *in vitro* enzyme assay (at  $V_{\text{opt}}$ , see text for details of protocol). Activity was determined for the six developmental stages for which the rate of oxygen consumption was measured. Data for three separate cultures (represented by different symbols) are shown to illustrate variations between cultures for this species. The enzyme activity for animals from each culture is shown as the mean of at least three assay replicates. Error bars are not shown because of the subtractive nature of these calculations (for examples, see Table 1). Errors for each set of assays were as follows: the coefficient of variation for assays without ouabain ranged from a minimum of 0.9% to a maximum of 3.4%; the coefficient of variation for assays with ouabain ranged from 1.5% to 4.2%. (C) Comparison of protein-specific rate of oxygen consumption and specific activity of  $\text{Na}^+/\text{K}^+$ -ATPase during development. All data are expressed as equivalents of ATPase activity at  $15^\circ\text{C}$  (see text for methods of data conversion using measured values of  $Q_{10}$ ). Open bars represent rates of oxygen consumption in equivalents of ATPase activity, filled bars represent corresponding total  $\text{Na}^+/\text{K}^+$ -ATPase activities (error bars for both data sets are S.E.M.). The *in vivo*  $\text{Na}^+/\text{K}^+$ -ATPase activities of hatching blastula and early gastrula are presented as stippled histogram bars. Each bar represents the rate of  $\text{K}^+$  transport (measured as  $^{86}\text{Rb}^+$  transport, see text) calculated from a time course transport experiment with 6–7 individual data points. The error bars shown for these experiments are S.E.M. of the slope of the linear regression line. Rates of  $\text{K}^+$  transport were also measured for unfertilized eggs (far left histograms), but rates were below the limit of detection (see text).



rates of post-hatched embryos and larvae (early gastrula, 70%; prism, 72%; pluteus, 77%).

*Comparison of total and in vivo Na<sup>+</sup>/K<sup>+</sup>-ATPase activities*

The *in vivo* Na<sup>+</sup>/K<sup>+</sup>-ATPase activity of *S. purpuratus*, measured as the rate of <sup>86</sup>Rb<sup>+</sup> transport by embryos and larvae, was substantially lower throughout development than the corresponding total Na<sup>+</sup>/K<sup>+</sup>-ATPase activity (Fig. 2C). No *in vivo* Na<sup>+</sup>/K<sup>+</sup>-ATPase activity was measurable as ouabain-sensitive <sup>86</sup>Rb<sup>+</sup> transport in unfertilized eggs from three different females. Approximately half (range 42–64%) of the total Na<sup>+</sup>/K<sup>+</sup>-ATPase activity was physiologically active in the post-fertilization stages. The protein-specific physiologically active Na<sup>+</sup>/K<sup>+</sup>-ATPase increased during development from blastula to pluteus larval stage (ANOVA, comparison of slopes for rates of <sup>86</sup>Rb<sup>+</sup> transport by blastulae and pluteus larvae; variance ratio 8.46, *F*<sub>0.05(1,9)</sub>=7.21, using the conservative, higher, of the two blastula rates given in Fig. 2C).

*Change in rate of oxygen consumption and Na<sup>+</sup>/K<sup>+</sup>-ATPase activity during early development of L. pictus*

As was observed for *S. purpuratus*, a rapid increase in metabolic rate occurred by 3–5 h after fertilization in embryos of *L. pictus* (Fig. 3A). Unfertilized eggs had a respiration rate of 0.015 μmol O<sub>2</sub> mg<sup>-1</sup> protein h<sup>-1</sup> that increased to 0.11 μmol O<sub>2</sub> mg<sup>-1</sup> protein h<sup>-1</sup> at 3–5 h after fertilization. A further increase in respiration rate occurred until the late gastrula stage was reached (at 40 h), when metabolic rate remained unchanged through to the early pluteus stage (ANOVA, *P*>0.2). During the period of development studied (to approximately 80 h post fertilization), the protein content of *L. pictus* did not decrease significantly from the initial protein content in the egg of 37 ng (ANOVA of slope; variance ratio=3.07, *F*<sub>0.05(1,15)</sub>=6.20).

The ontogenetic change in total Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in *L. pictus* was found to be more variable between cultures than was found for *S. purpuratus*. The results from three different cultures, each started from different males and females, are presented in Fig. 3B, where the general pattern shows an ontogenetic increase, with a lower Na<sup>+</sup>/K<sup>+</sup>-ATPase activity before hatching (20 h) followed by an increase in the later stages.

Total Na<sup>+</sup>/K<sup>+</sup>-ATPase activity is potentially a major metabolic component during early development of *L. pictus* (Fig. 3C). As was found for eggs of *S. purpuratus*, the total Na<sup>+</sup>/K<sup>+</sup>-ATPase, if active, would exceed the rate of oxygen consumption in unfertilized eggs of *L. pictus*. None of the enzyme was active, however, as no *in vivo* activity of the enzyme was measurable as ouabain-sensitive <sup>86</sup>Rb<sup>+</sup> transport in unfertilized eggs (eggs from three different females were tested). Total enzyme activity could potentially account for over 50% of the metabolic rates for all stages examined (4-h-old embryo, 86%; hatching blastula, 67%; early gastrula, 58%; prism, 80%; pluteus, 78%). The *in vivo* Na<sup>+</sup>/K<sup>+</sup>-ATPase activity was also measured for blastulae and gastrulae: 32%

and 33%, respectively, of the total Na<sup>+</sup>/K<sup>+</sup>-ATPase was found to be physiologically active in these embryonic stages (Fig. 3C).

*Increase of in vivo Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in the presence of monensin*

Maximum *in vivo* Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in embryos of *S. purpuratus* was measured in the presence of 36 μmol l<sup>-1</sup> monensin and was compared with the total *in vitro* Na<sup>+</sup>/K<sup>+</sup>-ATPase activity. The monensin-stimulated *in vivo* Na<sup>+</sup>/K<sup>+</sup>-ATPase activity increased in all experiments to a mean of 94% of the total Na<sup>+</sup>/K<sup>+</sup>-ATPase activity (Table 1). These experiments show that the measured differences between *in vivo* and total Na<sup>+</sup>/K<sup>+</sup>-ATPase activities (Fig. 2C) represent reserve activity of the enzyme that can be utilized *in vivo*.

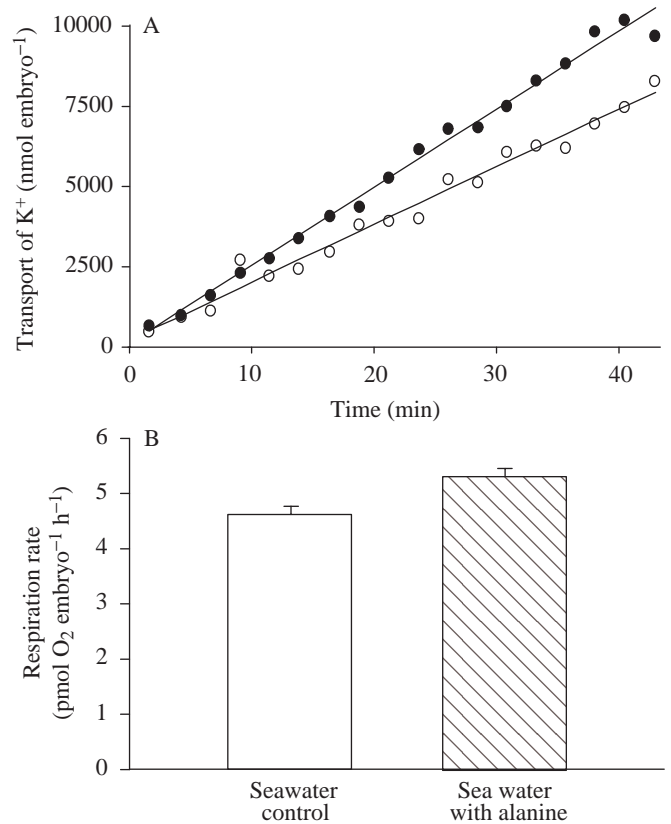


Fig. 4. *Strongylocentrotus purpuratus*. (A) Transport of K<sup>+</sup> by 12-h-old embryos in the absence (open circles) and presence (shaded circles) of alanine in sea water. K<sup>+</sup> transport was measured as <sup>86</sup>Rb<sup>+</sup> flux, with correction for specific activity from K<sup>+</sup> in sea water (see text). When present, a final concentration of 162 μmol l<sup>-1</sup> alanine was added to sea water 5 min before the assay began. The *r*<sup>2</sup> values for the two regression lines were 0.98 in the absence and 0.99 in the presence of alanine. Transport of K<sup>+</sup> in the presence of alanine was significantly greater than that in the absence of alanine (ANOVA of comparison of slopes, variance ratio 58.4, *F*<sub>0.001(1,30)</sub>=13.3). (B) Rate of oxygen consumption for 10-h-old embryos in the absence (open bar, *N*=15) and presence (hatched bar, *N*=15) of 162 μmol l<sup>-1</sup> alanine. Error bars are 95% confidence limits.

Table 1. Comparison of maximum  $\text{Na}^+/\text{K}^+$ -ATPase activities measured *in vitro* and *in vivo* for embryos of *Strongylocentrotus purpuratus*

	Total <i>in vitro</i> activity at $V_{\text{opt}}$			Maximum <i>in vivo</i> activity			% <i>in vivo</i> activity of <i>in vitro</i> activity <sup>c</sup>
	ATPase activity, no ouabain	ATPase activity, 2 mmol l <sup>-1</sup> ouabain	$\text{Na}^+/\text{K}^+$ -ATPase specific activity <sup>a</sup>	ATPase activity, 36 $\mu\text{mol l}^{-1}$ monensin	ATPase activity, 2 mmol l <sup>-1</sup> ouabain	$\text{Na}^+/\text{K}^+$ -ATPase specific activity <sup>b</sup>	
Culture 1							
7-h-old embryo	2.57±0.03	2.04±0.01	0.53	0.47±0.03 0.55±0.03	0.10±0.01	0.37 0.45	70 85
14-h-old embryo	2.70±0.01	2.10±0.02	0.60	0.74±0.03 0.84±0.01	0.11±0.01	0.63 0.73	105 122
Culture 2							
7-h-old embryo	2.72±0.03	2.11±0.01	0.61	0.73±0.07 0.78±0.03	0.18±0.01	0.55 0.60	90 98
14-h-old embryo	3.87±0.03	3.14±0.03	0.73	0.81±0.05 0.92±0.07	0.20±0.03	0.61 0.72	84 99
							Mean 94

All activities are presented as  $\mu\text{mol P}_i \text{mg}^{-1} \text{protein h}^{-1}$ ; total *in vitro* activities are expressed as mean  $\pm$  S.E.M. ( $N=7$ ); *in vivo* activities are presented as mean  $\pm$  S.E. of the slope of the linear regression line ( $N=6$  or 7 data points) used to determine the transport rates of  $^{86}\text{Rb}^+$  at 15 °C.

<sup>a</sup>Total *in vitro*  $\text{Na}^+/\text{K}^+$ -ATPase activity: ATPase activity (no ouabain) minus ATPase activity with 2 mmol l<sup>-1</sup> ouabain. Activities were measured at 25 °C and converted to the corresponding values at 15 °C ( $Q_{10}=2.4$ , from Fig. 1C) for direct comparison with *in vivo* measurements made at 15 °C.

<sup>b</sup>*In vivo*  $\text{Na}^+/\text{K}^+$ -ATPase activity: ATPase activity with 36  $\mu\text{mol l}^{-1}$  monensin (no ouabain) minus ATPase activity with 2 mmol l<sup>-1</sup> ouabain.

<sup>c</sup>*In vivo*  $\text{Na}^+/\text{K}^+$ -ATPase activity expressed as a percentage of total *in vitro*  $\text{Na}^+/\text{K}^+$ -ATPase activity at the same developmental stage.



Table 2. Comparison of total *in vitro* Na<sup>+</sup>/K<sup>+</sup>-ATPase activity (*V<sub>opt</sub>*) and *in vivo* Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in the presence of alanine for 9-h-old embryos of *Strongylocentrotus purpuratus*

Total <i>in vitro</i> activity at <i>V<sub>opt</sub></i>			<i>In vivo</i> activity		
ATPase activity, no ouabain	ATPase activity, 2 mmol l <sup>-1</sup> ouabain	Na <sup>+</sup> /K <sup>+</sup> -ATPase specific activity <sup>a</sup>	ATPase activity, 162 μmol l <sup>-1</sup> alanine	ATPase activity, 2 mmol l <sup>-1</sup> ouabain	Na <sup>+</sup> /K <sup>+</sup> -ATPase specific activity <sup>c</sup>
2.73±0.01	2.33±0.01	0.40	0.54±0.01	0.17±0.03 <sup>b</sup>	0.37
			0.48±0.06		0.31
			0.50±0.03		0.33
			0.52±0.06		0.35
					Mean 0.34

Ratio of *in vivo* to *in vitro* activity 0.34/0.40=0.85

All activities presented as μmol P<sub>i</sub> mg<sup>-1</sup> protein h<sup>-1</sup>; total *in vitro* activities are expressed as mean ± S.E.M. (*N*=7); *in vivo* activities are expressed as mean ± S.E. of the slope of the linear regression line (*N*=6 or 7 data points) used to determine the transport rates of <sup>86</sup>Rb<sup>+</sup> at 15 °C.

<sup>a</sup>Total *in vitro* Na<sup>+</sup>/K<sup>+</sup>-ATPase: ATPase activity (no ouabain) minus ATPase activity with 2 mmol l<sup>-1</sup> ouabain. Activities were measured at 25 °C and converted to the corresponding values at 15 °C (*Q*<sub>10</sub>=2.4, from Fig. 1C) for direct comparison with *in vivo* measurement made at 15 °C.

<sup>b</sup>This value used to convert (by subtraction) the four rates given in column labeled ATPase activity, 162 μmol l<sup>-1</sup> alanine to Na<sup>+</sup>/K<sup>+</sup>-ATPase specific activity.

<sup>c</sup>*In vivo* Na<sup>+</sup>/K<sup>+</sup>-ATPase: ATPase activity with 162 μmol l<sup>-1</sup> alanine (no ouabain) minus ATPase activity with 2 mmol l<sup>-1</sup> ouabain.

Table 3. Comparison of *in vivo* Na<sup>+</sup>/K<sup>+</sup>-ATPase in the presence of alanine (column IV) and the simultaneous rate of alanine transport (column I) expressed as equivalent of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity for 9-h-old embryos of *Strongylocentrotus purpuratus* (column II)

I	II	III	IV	V (column IV minus column III)
Alanine transport rate (pmol Ala embryo <sup>-1</sup> h <sup>-1</sup> )	Alanine transport rate <sup>a</sup> (expressed as predicted ATPase equivalent) (pmol P <sub>i</sub> embryo <sup>-1</sup> h <sup>-1</sup> )	<i>In vivo</i> Na <sup>+</sup> /K <sup>+</sup> -ATPase activity (control) (pmol P <sub>i</sub> embryo <sup>-1</sup> h <sup>-1</sup> )	<i>In vivo</i> Na <sup>+</sup> /K <sup>+</sup> -ATPase activity in the presence of 162 μmol l <sup>-1</sup> alanine (pmol P <sub>i</sub> embryo <sup>-1</sup> h <sup>-1</sup> )	Increase of <i>in vivo</i> Na <sup>+</sup> /K <sup>+</sup> -ATPase activity in the presence of alanine (pmol P <sub>i</sub> embryo <sup>-1</sup> h <sup>-1</sup> )
2.81±0.19	2.81	3.03±0.09	5.76±0.15	2.73
2.57±0.22	2.57		4.73±0.09	1.70
2.89±0.26	2.89		5.09±0.30	2.06
2.15±0.18	2.15		5.37±0.61	2.34
Mean 2.61	Mean 2.61		Mean 5.24	Mean 2.21

ANOVA: column II and column V: variance ratio=2.10 (not significant), *F*<sub>0.05(1,7)</sub> = 8.07.

All transport rates (columns I, III and IV) are expressed as mean ± S.E. of the slope of the linear regression line (*N*=7 or 8 data points) used to determine the corresponding rates.

<sup>a</sup>Alanine transport rates from column I were converted to predicted ATPase equivalents (column II) by assuming that an influx of 3 Na<sup>+</sup> is required for cotransport of 1 alanine molecule and that active efflux of 3 Na<sup>+</sup> by the sodium pump will result in hydrolysis of 1 ATP; i.e. 1 ATP is required for the transport of 1 alanine molecule (see text for details).

*Increase of in vivo Na<sup>+</sup>/K<sup>+</sup>-ATPase activity induced by alanine*

In the presence of dissolved alanine (162 μmol l<sup>-1</sup>) in sea water, the *in vivo* Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in embryos of *S. purpuratus* increased relative to controls with no alanine addition (Fig. 4A). The *in vivo* Na<sup>+</sup>/K<sup>+</sup>-ATPase activity increased in the presence of alanine to 85 % (Table 2, mean of four experiments 0.34 μmol P<sub>i</sub> mg<sup>-1</sup> protein h<sup>-1</sup>) of the total Na<sup>+</sup>/K<sup>+</sup>-ATPase activity measured by the *in vitro* enzymatic assay at *V<sub>opt</sub>* (0.40 μmol P<sub>i</sub> mg<sup>-1</sup> protein h<sup>-1</sup>). This result suggests that the increased cotransport of Na<sup>+</sup>, caused by the

alanine transporter operating at *J<sub>max</sub>*, increased the *in vivo* Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in embryos to near-maximum rates, at 85 % of the total activity measured at *V<sub>opt</sub>*. This up-regulation was further investigated by measuring simultaneously the transport of <sup>86</sup>Rb<sup>+</sup> and [<sup>14</sup>C]alanine by embryos (i.e. by measuring the *in vivo* Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in the presence of alanine and the corresponding alanine transport rate). Rates of [<sup>14</sup>C]alanine transport were converted to equivalents of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity based on a 3:1 relationship between amino acid and Na<sup>+</sup> transport in marine invertebrates (i.e. 3 Na<sup>+</sup> required to cotransport an

amino acid molecule against the large uphill gradient from sea water to intracellular concentrations; see Stevens and Preston, 1980; Wright, 1988). At  $J_{\max}$ , the mean transport rate was 2.61 pmol alanine embryo<sup>-1</sup> h<sup>-1</sup> (Table 3, column I). At 3 Na<sup>+</sup> per amino acid transported, 7.83 pmol Na<sup>+</sup> embryo<sup>-1</sup> h<sup>-1</sup> would be transported. One ATP molecule would be hydrolyzed by the Na<sup>+</sup>/K<sup>+</sup>-ATPase to remove those 3 Na<sup>+</sup>, giving an alanine transport rate expressed as Na<sup>+</sup>/K<sup>+</sup>-ATPase equivalent of 2.61 pmol P<sub>i</sub> embryo<sup>-1</sup> h<sup>-1</sup> (Table 3, column II). The Na<sup>+</sup>/K<sup>+</sup>-ATPase activity of embryos in sea water with no alanine present (control) was 3.03 pmol P<sub>i</sub> embryo<sup>-1</sup> h<sup>-1</sup> (column III) and increased to 5.24 pmol P<sub>i</sub> embryo<sup>-1</sup> h<sup>-1</sup> in the presence of alanine (column IV). The increase in Na<sup>+</sup>/K<sup>+</sup>-ATPase activity *in vivo* due to the presence of alanine in sea water is the difference between these two rates (column IV minus column III) and was 2.21 pmol P<sub>i</sub> embryo<sup>-1</sup> h<sup>-1</sup> (column V). Thus, the predicted *in vivo* increase in Na<sup>+</sup>/K<sup>+</sup>-ATPase activity from [<sup>14</sup>C]alanine and Na<sup>+</sup> cotransport was 2.61 pmol P<sub>i</sub> embryo<sup>-1</sup> h<sup>-1</sup> (column II), and the actual increase in activity, measured by the increase in <sup>86</sup>Rb<sup>+</sup> transport, was 2.21 pmol P<sub>i</sub> embryo<sup>-1</sup> h<sup>-1</sup> (column V). These rates do not differ (ANOVA) (see Table 3), demonstrating that embryonic Na<sup>+</sup>/K<sup>+</sup>-ATPase is up-regulated instantaneously (Fig. 4A) in direct proportion to changes in the physiological activity of amino acid transporters.

A direct metabolic test of the alanine/Na<sup>+</sup> and Na<sup>+</sup>/K<sup>+</sup>-ATPase stoichiometry presented in Table 3 was made by stimulating oxygen consumption of embryos in the presence of alanine in sea water. The rate of oxygen consumption of 10-h-old embryos of *S. purpuratus* increased significantly (*t*-test,  $P < 0.001$ , d.f.=28) by 0.70 pmol O<sub>2</sub> embryo<sup>-1</sup> h<sup>-1</sup>, from 4.62±0.16 pmol O<sub>2</sub> embryo<sup>-1</sup> h<sup>-1</sup> (mean ± 95 % confidence interval) in sea water alone (control) to 5.32±0.15 pmol O<sub>2</sub> embryo<sup>-1</sup> h<sup>-1</sup> in the presence of 162 μmol l<sup>-1</sup> alanine (Fig. 4B). The smallest difference between the 95 % confidence limits was 0.39 pmol O<sub>2</sub> embryo<sup>-1</sup> h<sup>-1</sup>, calculated as the difference between the maximum control value of 4.78 pmol O<sub>2</sub> embryo<sup>-1</sup> h<sup>-1</sup> and the minimum value in the presence of alanine of 5.17 pmol O<sub>2</sub> embryo<sup>-1</sup> h<sup>-1</sup>. When converted to ATP equivalents, these respiratory rate increments of 0.7 and 0.39 pmol O<sub>2</sub> embryo<sup>-1</sup> h<sup>-1</sup> are 2.03 and 3.64 pmol P<sub>i</sub> embryo<sup>-1</sup> h<sup>-1</sup>, respectively (5.2 pmol ATP pmol<sup>-1</sup> O<sub>2</sub>). The increase in Na<sup>+</sup>/K<sup>+</sup>-ATPase activity (Table 3, column V) had a mean value of 2.21 pmol P<sub>i</sub> embryo<sup>-1</sup> h<sup>-1</sup> in the presence of alanine. This value falls within the statistical confidence limits of the respiration measurements, showing that the stimulation of oxygen consumption in the presence of alanine can be explained by the increased ATP demand of the sodium pump.

### Discussion

Total Na<sup>+</sup>/K<sup>+</sup>-ATPase activity is potentially a very significant metabolic component of sea urchin development. Early ontogenetic changes in the total Na<sup>+</sup>/K<sup>+</sup>-ATPase activity

for *Strongylocentrotus purpuratus* can be described as a step function (Fig. 2B), with near-constant activities from the unfertilized egg to the hatching blastula followed by a rapid increase to the early gastrula. Activity remained unchanged to the larval stage (pluteus). This general pattern of ontogenetic increase in activity during development of *Strongylocentrotus purpuratus* and *Lytechinus pictus* is similar to that reported during development of the sea urchin *Hemicentrotus pulcherrimus* (Mitsunaga-Nakatsubo *et al.* 1992), suggesting that increases in total Na<sup>+</sup>/K<sup>+</sup>-ATPase occur from blastulae to gastrulae. The large component of total ATPase activity that is ouabain-insensitive in larvae of *S. purpuratus* (Fig. 1B) has been found for other developmental forms; for example, over 90 % of total ATPase is ouabain-insensitive in embryos of the amphibian *Xenopus laevis* (Han *et al.* 1991) and approximately 50 % in eggs (Ciapa *et al.* 1984) and embryos (Mitsunaga-Nakatsubo *et al.* 1986) of other species of sea urchin. Although not characterized further in our study, this component is commonly referred to as non-specific Mg<sup>2+</sup>-ATPase activity (Stekhoven and Bonting, 1981; Han *et al.* 1991).

If physiologically active, the total Na<sup>+</sup>/K<sup>+</sup>-ATPase activity could potentially account for 23–77 % (stage-dependent) of the rates of oxygen consumption of *S. purpuratus* (Fig. 2C) and 58–86 % of the rates of *L. pictus* (Fig. 3C). Values that exceed total respiration rate based on Na<sup>+</sup>/K<sup>+</sup>-ATPase operating at its maximum potential are known (e.g. kidney; and our values for unfertilized eggs, Fig. 2C). In the mammalian kidney, for instance, Soltoff and Mandel (1984, their Table 3) reported that total Na<sup>+</sup>/K<sup>+</sup>-ATPase activity of the proximal tubules was over 130 % of the respiratory capacity of the tissue. If all such ATP-requiring processes, including the large non-Na<sup>+</sup>/K<sup>+</sup>-ATPase activity (Fig. 1B), were operating at maximum rates, the potential metabolic demand would far exceed the metabolic capacity of the organism. Usually, however, metabolic calculations of this kind based on the physiologically active fraction of the enzyme show that less than 100 % of respiration can be accounted for (e.g. *in vivo* activity of Na<sup>+</sup>/K<sup>+</sup>-ATPase is 70 % of respiration rate in kidney cells, Balaban *et al.* 1980, and 45 % in liver cells, McBride and Milligan, 1985).

During sea urchin development, the *in vivo* rates of Na<sup>+</sup>/K<sup>+</sup>-ATPase could account for up to 40 % of the metabolic rate (value for pluteus-stage larvae, Fig. 2C). Because we obtained similar respiration rates for developing stages of *S. purpuratus* using two different methods, we have confidence in the values. However, this finding does not negate the need to confirm the accuracy of polarographic oxygen sensors for other species, as suggested previously (Hoegh-Guldberg and Manahan, 1995; Shilling *et al.* 1996). Clearly, the cost of ion regulation consumes a very large percentage of the larva's metabolism, but even this is a conservative estimate. The *in vivo* Na<sup>+</sup>/K<sup>+</sup>-ATPase activity, determined using <sup>86</sup>Rb<sup>+</sup> as a tracer, is a good measure of the net flux of K<sup>+</sup> from sea water into the animals. However, we cannot be sure that this method also measures the Na<sup>+</sup>/K<sup>+</sup>-ATPase activity involved in between-cell K<sup>+</sup> flux, a process that also requires ATP.

A comparison between total Na<sup>+</sup>/K<sup>+</sup>-ATPase activities and

*in vivo* rates during development of *S. purpuratus* revealed that, on average, 51 % of the total Na<sup>+</sup>/K<sup>+</sup>-ATPase activity was physiologically active (Fig. 2C). We verified that the difference between total and *in vivo* Na<sup>+</sup>/K<sup>+</sup>-ATPase activities in embryos does represent reserve activity that can be up-regulated *in vivo* (Table 1). Monensin increases the influx of Na<sup>+</sup> (Pressman, 1976) which, in turn, causes the stimulation of *in vivo* Na<sup>+</sup>/K<sup>+</sup>-ATPase activity to restore the ionic gradients (Ismail-Beigi *et al.* 1988). In embryos of *S. purpuratus*, the addition of monensin stimulated the *in vivo* Na<sup>+</sup>/K<sup>+</sup>-ATPase activity to values close (94 %, Table 1) to the maximum rate of enzyme activity measured at  $V_{opt}$ . This finding shows that the difference between total and *in vivo* Na<sup>+</sup>/K<sup>+</sup>-ATPase activities represents a reserve that is functionally active in sea urchin embryos.

What process(es) might stimulate reserve activity of the sodium pump in developing sea urchins? Na<sup>+</sup>-dependent nutrient transport is one such process. Sea urchin embryos and larvae can transport dissolved free amino acids from sea water (Epel, 1972; Manahan *et al.* 1983). Neutral amino acid transport is Na<sup>+</sup>-dependent in developing echinoderms (Epel, 1972; Davis *et al.* 1985), as free amino acids are transported against a concentration that is approximately 10<sup>6</sup> times greater intracellularly than is found in sea water (Stephens, 1988). We tested to what extent an experimentally induced increase in amino acid transport rate would increase the physiological activity of Na<sup>+</sup>/K<sup>+</sup>-ATPase in sea urchin embryos. At a concentration known to cause a maximum transport rate of alanine ( $J_{max}$ ) in sea urchin embryos (Manahan *et al.* 1989), the addition of alanine to sea water significantly stimulated *in vivo* Na<sup>+</sup>/K<sup>+</sup>-ATPase activity to 85 % of the maximum available enzyme activity (Table 2). The observed increase in activity of Na<sup>+</sup>/K<sup>+</sup>-ATPase in the presence of alanine was not significantly different from the predicted increase in pump activity that would be required to remove the Na<sup>+</sup> that was cotransported with the alanine from sea water (assuming 3 Na<sup>+</sup> cotransported per molecule of alanine, Table 3). The change in sodium pump activity in the presence of alanine occurred rapidly (Fig. 4A) and shows that reserve pump activity in embryos can be activated in response to a change in amino acid concentration in sea water. The increased *in vivo* activity of Na<sup>+</sup>/K<sup>+</sup>-ATPase would also increase ATP demand, and the predicted stoichiometry of this increase was measurable as an increase in respiration rate when alanine was present in sea water (Fig. 4B). Stimulation of respiration by dissolved organic substrates in sea water has been reported previously (Jaeckle and Manahan, 1992, and references therein), but in marine invertebrates the phenomenon has not been considered in the context of transport physiology. In a fluctuating environment, where embryos and larvae may encounter rapid changes in amino acid concentrations over small scales (Manahan, 1990), the down-regulation of *in vivo* activity of the sodium pump in an environment low in nutrients would conserve energy. Conversely, the rapid up-regulation of the pump in response to a high nutrient concentration would permit

increased nutrient transport rates while maintaining the required intracellular ionic concentrations.

In conclusion, Na<sup>+</sup>/K<sup>+</sup>-ATPase activity is a major metabolic component during early sea urchin development. Approximately half of the total Na<sup>+</sup>/K<sup>+</sup>-ATPase activity is in reserve at physiological rates, but this can be activated rapidly in response to process(es) that involve changes in the Na<sup>+</sup> gradient. With its large metabolic importance and its tight coupling with other transmembrane processes, such as amino acid transport, Na<sup>+</sup>/K<sup>+</sup>-ATPase activity could be a useful index to measure the physiological state of larval forms of marine invertebrates.

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