Marine Biology (2005) 146: 923–929
DOI 10.1007/s00227-004-1501-9

RESEARCH ARTICLE

C. D. Lowe · S. J. Kemp · A. D. Bates
D. J. S. Montagnes

Evidence that the rotifer *Brachionus plicatilis* is not an osmoconformer

Received: 21 June 2004 / Accepted: 15 October 2004 / Published online: 27 November 2004

Abstract The rotifer *Brachionus plicatilis* is euryhaline (growing between 2 and 97 ppt) and has previously been considered an osmoconformer. We suggest that *B. plicatilis* is an osmoregulator, exhibiting a pattern of Na^+/K^+ ATPase activity in response to salinity consistent with that of other osmoregulating euryhaline invertebrates. To examine salinity tolerance, growth rates between 5 and 60 ppt were determined. The activity of Na^+/K^+ ATPase was examined, over the same range of salinities, by measuring ATPase activity in rotifer homogenates in the presence and absence of a Na^+/K^+ ATPase inhibitor. Maximum specific growth rate (0.95 day^{-1}) occurred at 16 ppt, highest mean amictic eggs per female (1.41) occurred at 20 ppt, and both parameters decreased rapidly as salinity increased. Egg development time was constant with salinity at 0.92 days. The activity of Na^+/K^+ ATPase per milligram protein increased from 3.9 μmol h^{-1} at 5 ppt to 6.8 μmol h^{-1} at 50 ppt and accounted for 15 and 30% of total ATPase activity, respectively. We suggest that these observations are consistent with increasing stress at high salinities and the occurrence of a hypo-osmoregulatory response. Given the high ATP consumption of Na^+/K^+ ATPase at high salinities, it is possible that a proportion of the corresponding decreases in growth rate and egg production are a direct cost of regulation.

Introduction

Rotifers of the genus *Brachionus* are common fresh- and brackish-water plankton, and consequently they are some of the best-characterised rotifer species. Ecological studies of the *B. plicatilis* species complex have provided insight into the population dynamics and community ecology of aquatic zooplankton (Gomez and Carvalho 2002). In addition, *B. plicatilis* is a critical food in marine aquaculture (Lubzens et al. 2001). Ecological and commercial interest has provided impetus to examine the effects of many environmental factors on *B. plicatilis*. However, in most cases little attention has been paid to the mechanisms underlying these responses. Understanding the basis of mechanisms mediating physiological responses in *B. plicatilis* will undoubtedly allow us to evaluate/improve rotifers used in aquaculture and further understand how environmental gradients affect their biogeography and population dynamics.

For *B. plicatilis*, salinity is an important environmental factor affecting growth and reproduction, having both ecological and aquacultural influences (Lubzens et al. 2001; Derry et al. 2003). *B. plicatilis* is euryhaline, typically occurring at salinities between 2 and 65 ppt, and up to 97 ppt (Walker 1981; Lubzens et al. 1995). It is generally accepted that *B. plicatilis* is an osmoconformer, although present knowledge is limited to a couple of early studies (Epp and Winston 1977, 1978). However, osmoconformation over such a large range of salinities (2–97 ppt, approximately 70–3,300 mosmol) would be exceptional, and almost double the tolerance of the nearest "competitor", the polychaete *Mercierella enigmatica* (Skaer 1974). We suggest that *B. plicatilis* is in fact an osmoregulator.

In this study, we indicate that the activity of the plasma membrane-bound Na^+/K^+ ATPase enzyme changes in response to salinity in *B. plicatilis* and suggest that this is evidence of osmoregulatory ability. Na^+/K^+ ATPase is responsible for the transport of Na^+ and K^+ across the plasma membrane of nearly all animal cells (Lingrel and Kuntzweiler 1994; Axelsen and Palmgren 1998; Palmgren and Axelsen 1998) and is a well-characterised component of both hypo- and hyper-osmoregulation in many halophilic and euryhaline organisms (Holliday 1990; Lima et al. 1997; Lucu and Devescovi

Communicated by J.P. Thorpe, Port Erin

C. D. Lowe (✉) · S. J. Kemp · A. D. Bates · D. J. S. Montagnes
School of Biological Sciences,
University of Liverpool, Biosciences Building,
Crown Street, Liverpool, L69 7ZB, UK
E-mail: clowe@liv.ac.uk
Tel.: +44-151-7954533

1999; Lucu et al. 2000). In osmoregulating marine intertidal crustacea (e.g. *Carcinus maenas*, *Uca pugnax*), Na^+/K^+ ATPase activity increases as external salinities decrease (Holliday 1985; Lucu and Flik 1999). In salt-tolerant freshwater organisms (e.g. *Artemia fransiscana, Macrobrachium olfersii*), Na^+/K^+ ATPase activity increases with increasing salinity (Holliday 1990; Lima et al. 1997). In contrast, in known osmoconformers the activity of the enzyme is constant with changes in salinity (e.g. Lucu et al. 2000). Thus, a change in Na^+/K^+ ATPase activity in response to salinity is a strong indication of osmoregulatory ability. Furthermore, we would expect energetic costs of osmoregulation to reduce energy available for growth. Thus, as a first step towards testing the hypothesis that *B. plicatilis* osmoregulates, we measured Na^+/K^+ ATPase activity, population growth rate, egg production, and egg development time in response to salinity.

Materials and methods

Maintenance of rotifer cultures

A strain of *Brachionus plicatilis*, isolated from the Torre Blanca Marsh ecosystem (provided by S. Manuel, University of Valencia, Spain), was maintained on a diet of the flagellate *Dunaliella salina*. The flagellate was grown on f/2 media (Guillard 1975) enriched artificial seawater (Ultramarine synthetica, Waterlife Industries, UK), in 0.5–4 l, with constant aeration, illumination (120 µmol photons m^{-2} s^{-1}), and temperature (25°C). Flagellates were grown at a range of salinities between 5 and 60 ppt (Fig. 1), by adjusting the mass of sea salt added to the growth media. Flagellates were maintained in exponential growth phase between 1.3×10^6 and 3×10^6 cells ml^{-1} by semi-continuous batch culturing.

Rotifers were maintained under the same conditions as *D. salina*, in 50- to 100-ml cultures, and were fed flagellates at growth-saturating levels of $\geq1\times10^6$ cells ml^{-1} (Lubzens et al. 2001). Rotifer cultures were acclimated to experimental salinities for more than 2 weeks (>10 generations) prior to growth-rate experiments.

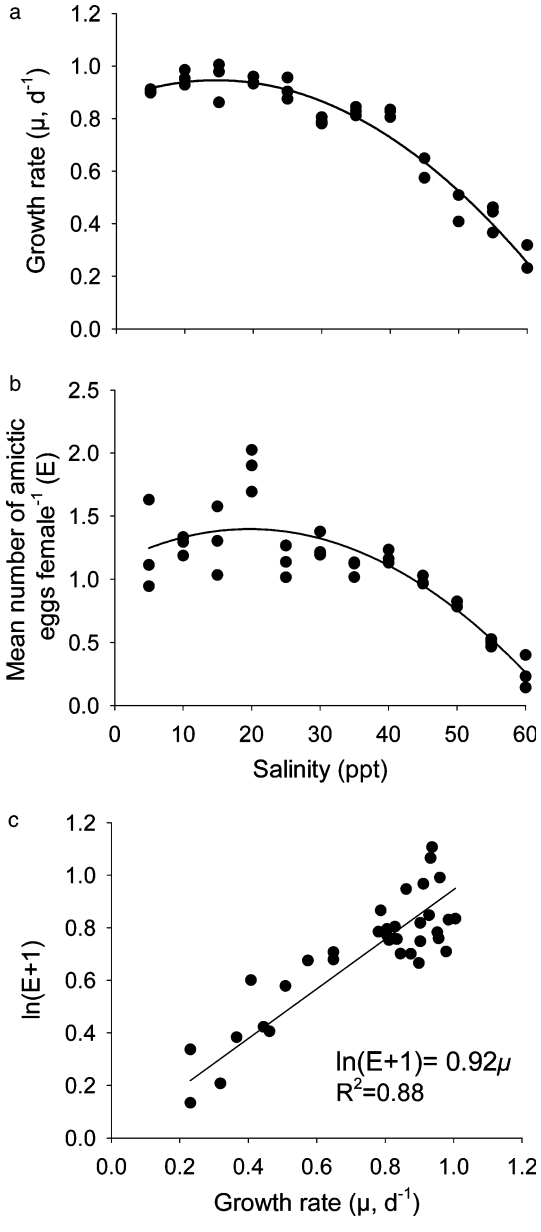

Fig. 1 The response of specific growth rate (**a**) and the mean number of amictic eggs per female (**b**) of *Brachionus plicatilis* to salinity. See Results for the regression equations. **c** The relationship between growth rate and $\ln(E+1)$. E, the egg development time, is 0.92 days and is given as the gradient of the regression line

The effect of salinity on growth rate

The specific growth rate of *B. plicatilis* was examined at salinities between 5 and 60 ppt. Experiments were conducted in 50-ml polypropylene tubes. At each salinity three replicate growth incubations were conducted. For each replicate, five actively swimming amictic females bearing a single egg were transferred from acclimated cultures into tubes containing 10 ml of media and flagellates. Females bearing single eggs were selected to standardise starting conditions at each salinity; this aided comparison between salinity treatments but potentially leads to under- or over-estimation of growth rate; absolute growth-rate values should be interpreted accordingly. Tubes were incubated for 3 days at 25°C under constant illumination and gently agitated twice daily. After 72 h, the contents of tubes were fixed with acidified Lugol's iodine (final concentration 2%), examined using a stereo dissection microscope, and the number of males, mictic and amictic females, and eggs per female were recorded for each replicate. Specific growth rates (µ) were calculated from the change in total number of females as $\mu=\ln(N_t/N_0)/t$, where N_0 is the number of females at the start of the growth incubation, N_t is the number of females after 3 days, and t is time in days.

Na^+/K^+ ATPase activity assay

To examine the response of the Na^+/K^+ ATPase enzyme to changes in salinity, *B. plicatilis* was cultured (as described above) at seven salinities (Fig. 2), in volumes between 0.5 and 1.0 l. For each Na^+/K^+ ATPase assay, a sample of rotifers was fixed in acidified Lugol's iodine (final concentration 2%) and culture density was determined. To remove algal cells and culture media, rotifer cultures were washed onto a 20-µm Nitex mesh filter, first with sterile saline solution (corresponding to the salinity of the growth medium), then with ice-cold homogenising medium (0.25 *M* sucrose, 6.0 m *M* EDTA). Rotifers were concentrated into 2.0 ml of medium and processed on ice in a ground glass homogeniser. Homogenates were kept on ice until assays were performed. All assays were performed within 1 h of harvesting.

ATPase activity was examined as phosphate liberated from ATP by crude homogenates. Assays were conducted at 30°C in a water bath (Grant Instruments, UK). Na^+/K^+ ATPase enzyme activity was determined as the difference between phosphate liberated in the presence of K^+ and in the absence of K^+ but with 1.0 m *M* ouabain (a Na^+/K^+ ATPase inhibitor; Lingrel and Kuntzweiler 1994). Activity was then calculated as phosphate released per milligram protein per hour and phosphate released per rotifer per hour; the patterns of Na^+/K^+ ATPase activity expressed per milligram protein and per rotifer were virtually identical, and only activity expressed as micromoles per milligram protein per hour are presented (Fig. 3a).

For rotifers grown at each salinity, Na^+/K^+ ATPase activity was determined from two independent replicates of the assay; each assay was conducted in triplicate, in 5-ml borosilicate glass test tubes. Methods followed Holliday (1985) and Lucu and Devescovi (1999) and were, briefly, as follows. For each homogenate, two reactions, each 333 µl in volume, were prepared: the K^+ reaction contained 20 m *M* Imidazole (pH 7.2), 100 m *M* NaCl, 30 m *M* KCl (final concentrations); the K^-

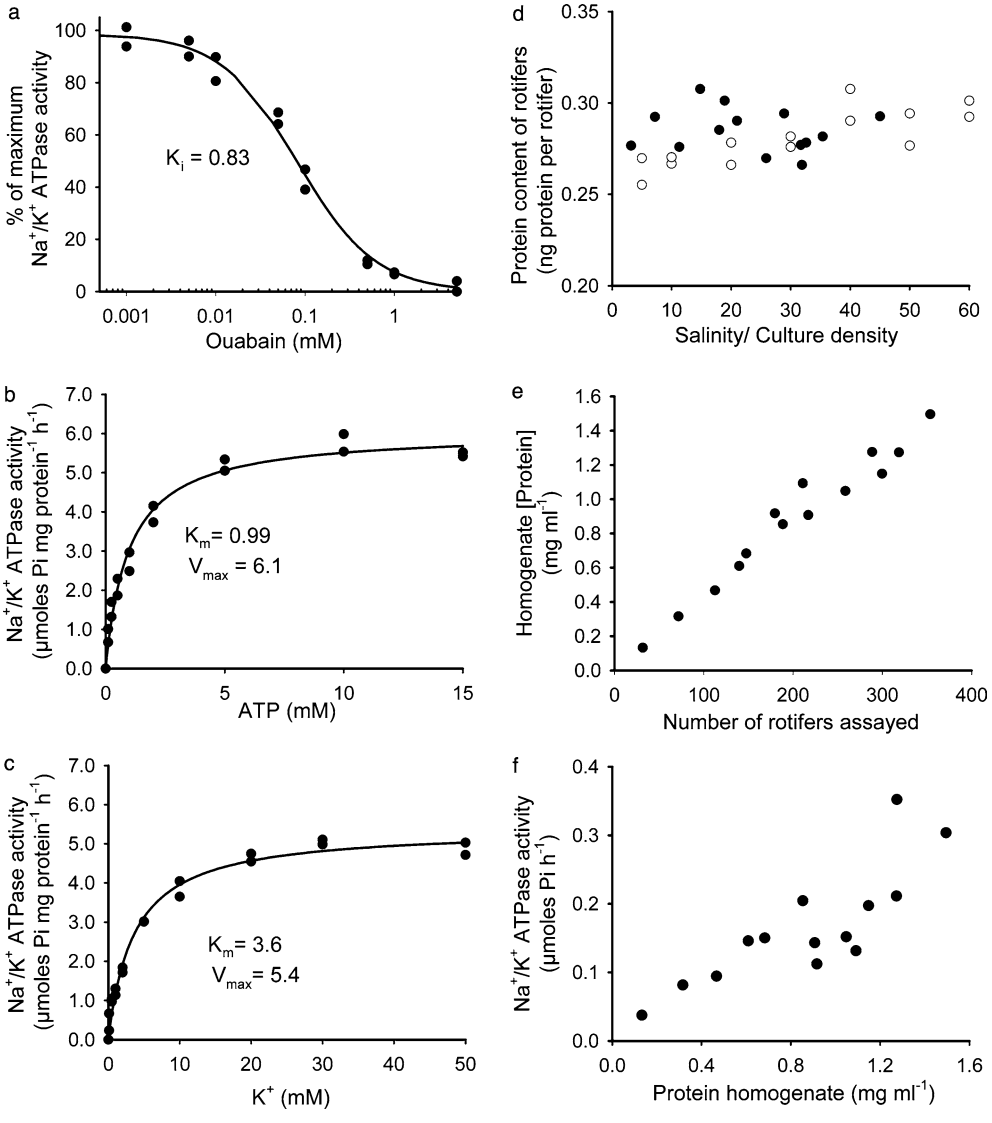

Fig. 2 Characteristics of the enzyme assay system. The kinetic responses of Na^+/K^+ ATPase to ouabain (**a**), ATP (**b**), and K^+ (**c**). Values for K_m, K_i, and V_{max} are given in each panel. **d** The effect of salinity (*open circles*) and culture density (*solid circles*) on the protein content of rotifers. **e** The relationship between protein concentrations of homogenates and the number of rotifers assayed. **f** The relationship between Na^+/K^+ ATPase activity and homogenate protein concentrations

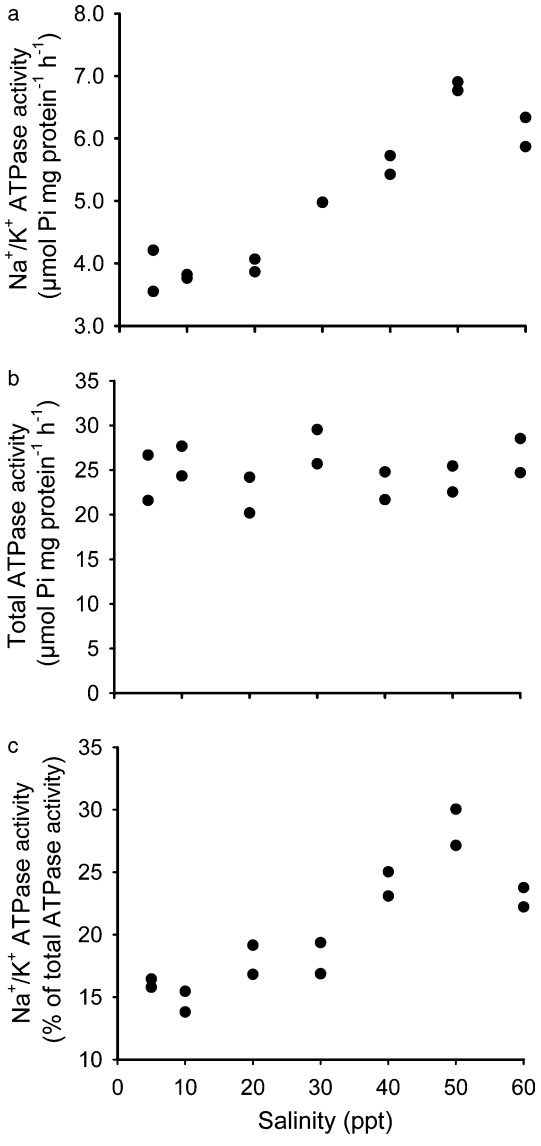

Fig. 3 Na^+/K^+ ATPase activity in *B. plicatilis* grown at salinities between 5 and 60 ppt. **a** Na^+/K^+ ATPase activity. **b** Total ATPase activity, measured as the activity of all ATPases present in the uninhibited reaction, expressed as phosphate released from K^+ reactions (50 m *M* K^+, no ouabain); see Materials and methods. **c** Na^+/K^+ ATPase as a percentage of total ATPase activity in homogenates

reaction contained 20 m *M* Imidazole (pH 7.2), 130 m *M* NaCl, 1.0 m *M* ouabain (final concentrations); and each reaction contained 66.7 µl of homogenate. After addition of homogenate, tubes were equilibrated to 30°C for 5 min. Blank (homogenising medium) and phosphate standards (0.60 m *M* Na_2HPO_4) were prepared in duplicate and also incubated. Reactions were started by adding 66.7 µl of a solution containing 15 m *M* Na_2ATP and 30 m *M* $MgCl_2$ (final concentrations) and incubated for 15 min. Reactions were stopped by adding 1.5 ml Bonting's colour reagent (H_2SO_4, ammonium molybdate, $FeSO_4$) and allowed to develop for 20 min. Absorbance was measured at 700 nm (Ultrospec 2000, Pharmacia Biotech).

Protein concentration

Protein concentrations of crude homogenates were determined by the Bradford method (Bradford 1976; Sambrook and Russell 2001). Duplicate assays were conducted for each homogenate and standard, using 2.5 ml Bradford reagent (Sigma, Michigan, USA) and 50 µl homogenate or bovine serum albumin (BSA) standard. Reactions were incubated at 25°C for 20 min and absorbance measured at 595 nm. For each homogenate assay, a BSA standard reaction (1.0 mg ml^{-1} BSA) was included to check consistency. The absorbance (595 nm) of crude homogenates was standardised against a BSA standard curve (0.1–1.5 mg ml^{-1}).

Assay optimisation

Before assays of Na^+/K^+ ATPase activity were conducted, the kinetic responses of the Na^+/K^+ ATPase enzyme to K^+, ATP, and ouabain were examined to optimise assay conditions. Homogenates were prepared (as described above) from rotifers grown at 20 ppt. The kinetic properties of Na^+/K^+ ATPase in *B. plicatilis* were examined in response to K^+ (0–50 m *M* KCl), ATP (0–15 m *M* Na_2ATP), and ouabain (0.001–5 m *M*). For assays of ouabain inhibition, concentrations of K^+ and ATP were 30 and 15 m *M*. For assays of inhibition and kinetic responses, substrate concentrations of the non-assayed substrate were as for the standard assay (i.e. 30 m *M* K^+, 15 m *M* ATP, and 1.0 m *M* ouabain).

Statistical analyses

All curves were fit using the curve-fitting function in SigmaPlot V8.0 (SPSS, UK). The relationships for growth rate versus salinity and egg production versus salinity (Fig. 1a, b) were modelled using second-order quadratic functions; regression models were used to estimate maximum growth rate and mean number of eggs per female. Egg development time was calculated, using model II linear regression (Sokal and Rohlf 1995), from growth rate and mean number of eggs per female data as $\mu = \ln(E+1)/\mathrm{EDT}$, where EDT is egg development time (Fig. 1c). To determine whether egg development time was constant the slope of logμ versus log[ln($E+1$)] was tested for significant deviation from 1.

For enzyme kinetic responses (K^+, ATP, and ouabain) V_{max}, K_m, and K_i were calculated from regression fits of standard equations for enzyme responses to titrations of substrate and inhibitor (Stryer 1995). To test for the potential confounding effects of culture density and salinity on the protein content of rotifers, the slopes of the plot of protein concentration versus salinity and culture density were tested for deviations from zero (*t*-test, $\alpha = 0.05$).

Results

The effect of salinity on rotifer growth

Specific growth rate (μ) was positive at all salinities (S) examined. Growth rate versus salinity was modelled as $\mu = -3.0\times10^{-4}\times S^2 + 9.8\times10^{-3}\times S + 0.87$ ($R^2 = 0.94$). Lowest growth rate occurred at 60 ppt and from the regression model maximum growth was predicted to be 0.95 at 16 ppt (Fig. 1a). Total number of eggs (not shown) and mean number of eggs per female (Fig. 1b) exhibited similar trends. Mean number of eggs per female (E) versus salinity (S) was modelled as $E = -7.0\times10^{-4}\times S^2 + 2.8\times10^{-2}\times S + 1.13$ ($R^2 = 0.73$). The predicted maximum mean eggs per female occurred at approximately 20 ppt (Fig. 1b) and decreased sharply with increasing salinity. Males and resting eggs were not observed in cultures during growth experiments (data not shown). Egg development time was invariant with salinity; the slope of logμ versus log [ln($E+1$)] was not significantly different from 1, ($t_{34} = -1.16$, $P < 0.05$). From the regression model (Fig. 1c) egg development time was 0.92 days.

Validation and optimisation of Na^+/K^+ ATPase assay

To detect the Na^+/K^+ ATPase enzyme in *B. plicatilis* and to optimise concentrations of K^+, ATP, and ouabain, assays of substrate concentration gradients were performed. Na^+/K^+ ATPase activity was detected in rotifer homogenates, that is, ouabain inhibitable ATPase activity occurred (Fig. 2a). The Na^+/K^+ ATPase enzyme exhibited typical responses to substrates and inhibitor (Fig. 2a–c). Values of K_m and V_{max} for ATP and K^+, and values of K_i for ouabain (Fig. 2a–c) are consistent with values reported in the literature for the Na^+/K^+ ATPase enzyme in other osmoregulating invertebrates (e.g. Holliday 1985, 1990; Lucu et al. 2000).

Under the culturing strategy used to grow rotifers for the assay, protein content per rotifer was independent of both salinity (slope not different from zero, Fig. 2d) and rotifer culture density (2 to 50 rotifers ml^{-1}; slope not different from zero, Fig. 2d). For the enzyme assay, estimates of protein concentration were linear with the number of rotifers homogenised (Fig. 2e), and Na^+/K^+ ATPase activity in homogenates was linear with homogenate protein concentration (Fig. 2f). Thus, there was no bias associated with non-linear relationships between protein content of rotifers and culture density or salinity, or between protein concentration and enzyme activity.

Na^+/K^+ ATPase activity response to salinity

Having detected Na^+/K^+ ATPase activity, the effect of salinity on enzyme activity in rotifers was examined. Na^+/K^+ ATPase activity varied in response to culture salinity. Lowest activity occurred at 5 and 10 ppt (3.9 μmol mg $protein^{-1}$ h^{-1}); activity increased linearly between 20 ppt and a maximum activity of 6.8 μmol mg $protein^{-1}$ h^{-1} at 50 ppt (Fig. 3a). Total ATPase activity remained constant with salinity (Fig. 3b). As a result, the percentage of total ATPase activity, accounted for by Na^+/K^+ ATPase, increased from approximately 15% to 30%, between 5 and 50 ppt (Fig. 3c). At 60 ppt Na^+/K^+ ATPase activity decreased to 6.0 μmol mg $protein^{-1}$ h^{-1} and approximately 23% of total ATPase activity (Fig. 3a–c). The activity of Na^+/K^+ ATPase was inversely correlated to both growth rate (Spearman's $r = -0.90$, $P < 0.05$) and mean number of eggs per female (Spearman's $r = -0.86$, $P < 0.05$; Fig. 4a, b).

Discussion

Several environmental gradients influence the distribution of and competition between rotifers in the *Brachionus plicatilis* species complex (Gomez et al. 1997; Ortells et al. 2003). Salinity has been identified as an important factor; both seasonal and spatial fluctuations in salinity influence rotifer abundance and species competition in natural habitats (Ciros-Pérez et al. 2001). In addition, salinity is an important factor in rotifer aquaculture systems. Consequently, understanding salinity tolerance mechanisms will increase our understanding of the ecological and economic impacts of salinity on *B. plicatilis*. It is commonly accepted that

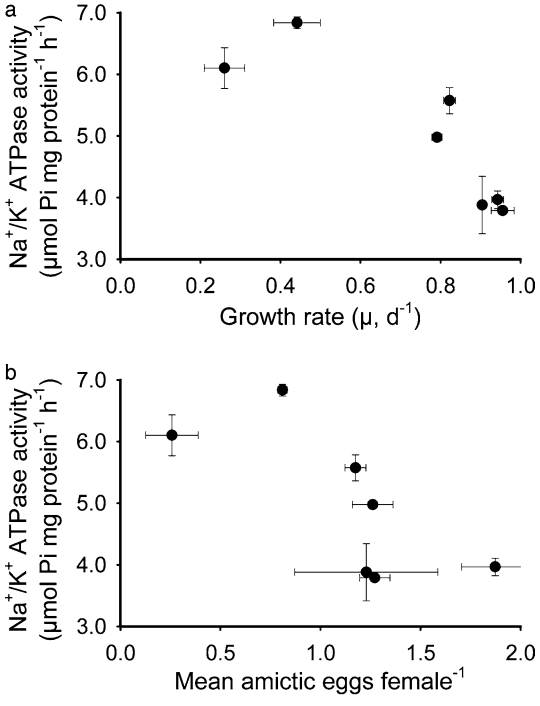

Fig. 4 The relationship between Na^+/K^+ ATPase activity and **a** growth rate and **b** egg production

B. plicatilis is a euryhaline osmoconformer (Epp and Winston 1978; Walker 1981; Frolov et al. 1991). While there are ample data that document salinity tolerance in *B. plicatilis*, only one early study has examined the occurrence of osmoconformity (Epp and Winston 1977). In this article we provide a strong indication that *B. plicatilis* is an osmoregulator, potentially expending large amounts of energy on this process.

B. plicatilis occurs in aquatic habitats from freshwater lakes and ponds to hypersaline lagoons (Walker 1981), tolerating salinities up to 97 ppt (Lubzens et al. 1995). The Torre Blanca Marsh strain of *B. plicatilis* examined in this study exhibited maximum growth at 16 ppt and grew at all salinities between 5 and 60 ppt; these values agree with previous data on this isolate from its natural habitat (Gomez et al. 1997). In this article we do not examine the direct ecological consequences of salinity tolerance in *B. plicatilis* (but see Derry et al. 2003; Ortells et al. 2003). Instead, our purpose is to provide an indication of an osmoregulatory mechanism in this organism.

The activity of the Na^+/K^+ ATPase enzyme in *B. plicatilis* increased almost twofold over salinities between 5 and 60 ppt (Fig. 3); there was a significant correlation between this increase and decreases in growth rate and egg production (Fig. 4). Thus, enzyme activity was lowest (Figs. 3, 4) at salinities where growth rate and egg production were highest (15–20 ppt; Fig. 1a, b). This comparison of responses suggests that at high salinities, stress (measured as a reduction in growth) increases. However, from our data, the mechanisms of stress are unclear. An ion-regulatory response certainly occurs, but we have not measured how effective this is. If regulation is not perfect, increasing internal ion concentrations could lead to biochemical disruption and protein denaturation associated with high salt concentrations (Oren 1999) and a subsequent reduction in growth rate. Potentially this explains why at high salinities (60 ppt) Na^+/K^+ ATPase activity decreases (Fig. 3). Alternatively, decreases in growth rate and reproductive output might occur as a direct cost of increased regulation at high salinities. Egg development time was constant (0.92 days) and thus was not influenced by variation in salinity. Given this, decreases in growth rate associated with increases in salinity are likely to result from a decreased investment in egg production, potentially occurring as an energetic trade-off against the increasing costs of osmoregulation.

The Na^+/K^+ ATPase enzyme in *B. plicatilis* accounted for up to 30% of total ATP consumption at high salinities (50 ppt, Fig. 3a, b). However, total ATPase activity was constant (Fig. 3c); thus at high salinities Na^+/K^+ ATPase activity represents a large energetic cost. Unfortunately, comparisons of this observation with other organisms are difficult. For the majority of larger animals, specific tissues are assayed, and enzyme-specific activity is expressed as a percentage of total activity for that tissue; obviously for rotifers this approach was not possible. However, given the large proportion of energy used by the Na^+/K^+ ATPase enzyme in *B. plicatilis*, it is likely that at least a proportion of the decreases in population growth at high salinity is a cost associated with ion regulation. Recognition of these high energetic costs raises questions concerning the ecological advantages/disadvantages of osmoregulation in a small r-strategic organism. Rapid adaptation to new habitats, underpinned by wide environmental tolerances, is potentially an important component of dispersal and colonisation in rotifers (DeMeester et al. 2002). Similarly, competition between closely related *Brachionus* species and strains, and the maintenance of complex spatial and temporal distributions (Gomez et al. 2002), are liable to be influenced by subtle differences in physiological tolerances and mechanisms such as ion- and osmoregulation. We make no further speculation here. Instead, now that changes in Na^+/K^+ ATPase activity have been established, we are currently comparing activity between several strains of *B. plicatilis* and *B. rotundformis*.

Acknowledgements This study forms part of a PhD thesis by C.D. Lowe at the University of Liverpool, funded by the Natural Environment Research Council (grant no. NER/S/A/2001/0630). Thanks are offered to Dr. D. Parry who provided advice, equipment, and reagents for enzyme assays. Pierre Ferrer, Stephanie Swift, and Jamie Watson provided valuable assistance with laboratory work. Drs. H. Noyes, I. Saccheri, and P. Watts provided valuable discussion. We also thank two anonymous reviewers for constructive comments.

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