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## Regulation of branchial $\text{Na}^+/\text{K}^+$ -ATPase in common carp *Cyprinus carpio* L. acclimated to different temperatures

Juriaan R. Metz, Erwin H. van den Burg, Sjoerd E. Wendelaar Bonga and Gert Flik\*

Department of Animal Physiology, Faculty of Science, University of Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands

\*Author for correspondence (e-mail: gertflik@sci.kun.nl)

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### Summary

Isogenic carp *Cyprinus carpio* L. were acclimated to water temperatures of 15, 22 and 29°C for at least 8 weeks. The acclimations consistently resulted in slightly, but significantly, different plasma osmolality, sodium, potassium and chloride concentrations between the groups studied. Plasma total and ionic calcium levels were unaffected, indicating successful adaptation. The apparent changes in set point for plasma ion levels are explained by altered sodium pump activity and hormonal control of branchial permeability to water and ions. It appears that in 15°C-acclimated fish, a lower apparent  $\text{Na}^+/\text{K}^+$ -ATPase activity is compensated by strongly enhanced  $\text{Na}^+/\text{K}^+$ -ATPase expression (determined biochemically and immunohistochemically). In 29°C-acclimated fish, the higher ambient temperature activates the enzyme. Arrhenius plots for branchial  $\text{Na}^+/\text{K}^+$ -ATPase preparations of the three groups of fish suggest the occurrence of different enzyme isoforms or protein (in)stability as explanations for differences in apparent enzyme activities, rather than temperature-dependent changes in membrane fluidity. As for hormonal control over permeability, prolactin mRNA expression (and

anticipated production and release) is lower in fish kept at 29°C, suggesting that control over branchial permeability to water and ions needs to be downregulated at higher temperatures. In so doing, enhanced sodium pump activity is balanced by a controlled passive ion loss to fine-tune plasma sodium levels. Basal plasma cortisol levels did not correlate positively with  $\text{Na}^+/\text{K}^+$ -ATPase expression, but doubling plasma cortisol levels in control fish by administering exogenous cortisol (for 7 days, using implanted minipumps and thus stress-free) enhanced  $\text{Na}^+/\text{K}^+$ -ATPase expression. This effect must be the result of a glucocorticoid action of the steroid: in fish, mineralocorticoid receptors have higher affinity for cortisol than glucocorticoid receptors. At a lower ambient temperature, branchial  $\text{Na}^+/\text{K}^+$ -ATPase expression is upregulated to counteract the temperature-inhibited activity of the sodium pump, perhaps via a mineralocorticoid receptor.

Key words: temperature acclimation, osmoregulation,  $\text{Na}^+/\text{K}^+$ -ATPase, cortisol, prolactin, chloride cell, immunohistochemistry, real-time RT-PCR, common carp, *Cyprinus carpio*.

### Introduction

The common carp *Cyprinus carpio* L. is a stenohaline freshwater species. Carp originated in the Danube basin and their huge ability to adapt allowed them to extend their habitats throughout the temperate zone of the northern hemisphere, in water temperatures ranging from 0 to 30°C (Billard, 2001). The optimal temperature for carp growth is around 25°C. Temperature strongly influences all biochemical processes and, obviously, when a set of crucial physiological processes is subjected to changes in water temperature, internal homeostasis may become at risk. Being a eurythermal fish, carp must have adaptive mechanisms to control internal homeostasis over a broad range of ambient temperatures. Yet, little is known about the physiology of temperature adaptation in carp.

Some physiological phenomena in fish that depend critically on temperature are oxygen consumption (Becker et al., 1992),

immune competence (Le Morvan-Rocher et al., 1995), growth and metabolism (Burel et al., 1996; Fine et al., 1996). Also it may be anticipated that osmoregulation, involving energised ion pumps in the gills (and in other osmoregulatory organs such as intestine and kidney), is a critically temperature-sensitive process. Ambient temperature determines the hydromineral status of the body fluids of freshwater fish by influencing both passive and active ion-transport mechanisms. For example, a rise in temperature will increase passive processes such as loss of ions and gain of water, thus requiring enhanced osmoregulatory activity. Higher temperatures will activate enzyme-driven ion transport (defined for example by a  $Q_{10}$  value or activation energy). Not all components of a physiological process are equally sensitive to temperature, yet may still require secondary adjustment to altered key enzyme activities, and thus eurythermal fish must have developed



adequate adaptation strategies to cope successfully with varying environmental temperatures.

Gills play a crucial role in hydromineral homeostasis of a fish. In freshwater fish, the function of chloride cells in the branchial epithelium is uptake of ions ( $\text{Na}^+$ ,  $\text{Cl}^-$ ,  $\text{Ca}^{2+}$ ) from the surrounding water (Flik et al., 1994; Perry, 1997). The most important and extensively studied enzyme in the chloride cell is sodium/potassium-activated adenosine triphosphatase ( $\text{Na}^+/\text{K}^+$ -ATPase), the enzymatic expression of the sodium pump, which is under multiple hormonal control (McCormick, 1995; Young et al., 1995; Evans, 2002). Cortisol and prolactin are considered the most important endocrine factors.

Cortisol is the end product of the hypothalamic-pituitary-interrenal axis (HPI-axis; Wendelaar Bonga, 1997) and stimulates  $\text{Na}^+/\text{K}^+$ -ATPase activity in many freshwater teleost fish species, including cichlids (Dange, 1986; Dang et al., 2000a), salmonids (Richman and Zaugg, 1987) and cyprinids (Abo Hegab and Hanke, 1984). Cortisol enhances chloride cell numbers (Richman and Zaugg, 1987; McCormick, 1990) and size (Madsen, 1990; McCormick, 1990; Dang et al., 2000a). Prolactin, on the other hand, exerts a mainly inhibitory control over branchial  $\text{Na}^+/\text{K}^+$ -ATPase activity (Pickford et al., 1970; Madsen and Bern, 1992), and reduces chloride cell numbers and activity (Foskett et al., 1982). Moreover, prolactin stimulates  $\text{Ca}^{2+}$  uptake (Flik et al., 1994) and limits branchial permeability to water and ions (Hirano, 1986; Wendelaar Bonga et al., 1990; Evans, 2002).

Our previous experiments show that carp acclimated to increasing water temperature show increased basal plasma cortisol levels (Arends et al., 1998). In the present study, we evaluate the hydromineral and endocrine consequences of acclimation to water at 15, 22 and 29°C. We anticipate that during temperature acclimation, cortisol and prolactin activities may change to warrant hydromineral homeostasis *via* up- or downregulation of  $\text{Na}^+/\text{K}^+$ -ATPase activity. Plasma osmolality and ion composition, gill  $\text{Na}^+/\text{K}^+$ -ATPase activity and expression were assessed to establish the end points of the adaptation strategy. The possible roles of plasma cortisol and pituitary prolactin (the latter quantified by real-time polymerase chain reaction) in the adaptation process will be discussed. It appears that the common carp is a species with subtle mechanisms and unexpected strategies for temperature adaptation.

## Materials and methods

### Animals

Adult male isogenic carp *Cyprinus carpio* L., strain E4xR3R8 (Bongers et al., 1997), were obtained from the Wageningen University fish culture facility (De Haar Vissen; The Netherlands). Fish weighed approximately 150 g and were kept in 150 l tanks, with recirculating filtered tapwater, under a 16 h:8 h light:dark regime at 22°C. Fish were fed commercial fish food (Trouvit, Trouw, Putten, The Netherlands) at a ration of 1.5% of the estimated body mass per day. At least 8 weeks before sampling, three groups of 20 fish each were transferred

from a common 400 l stock tank to acclimation tanks in which the ambient temperature was gradually changed (by 1°C per day) to final water temperatures of 15, 22 or 29°C. Experiments were carried out in the spring of 2001 and 2002.

### Plasma parameters

Fish were anaesthetised in 0.1% (v/v) 2-phenoxyethanol (Sigma, St Louis, USA). Immediately thereafter (always within 2 min), 1 ml blood was taken from the caudal vessels, using a 1 ml syringe that contained 20 µl of 2% (w/v)  $\text{Na}_2\text{EDTA}$  (Sigma) as anti-coagulant. Blood was transferred to ice-cold Eppendorf tubes containing 1 trypsin-inhibiting unit (t.i.u.) of aprotinin (Sigma). After 5 min centrifugation (1000 g, 4°C), plasma was separated from blood cells and stored at -20°C until analysis.

Plasma cortisol was measured by radioimmunoassay (RIA) as described by Arends et al. (1998). Plasma osmolality was measured on a freezing-point depression osmometer (Gonotec, Berlin, Germany). Plasma  $\text{Na}^+$  and  $\text{K}^+$  concentrations were determined by flame photometry (Radiometer Copenhagen FLM3 flame photometer);  $\text{Cl}^-$  was measured spectrophotometrically by the formation of ferrothiocyanate (O'Brien, 1962).  $\text{Ca}^{2+}$  was measured spectrophotometrically with a commercial kit (Sigma). The free  $\text{Ca}^{2+}$  fraction was determined after filtering heparinised blood plasma over a 10 kDa membrane.

### $\text{Na}^+/\text{K}^+$ -ATPase

The specific,  $\text{Na}^+$ - and  $\text{K}^+$ -dependent, ouabain-sensitive ATPase activity was measured in crude gill homogenates containing saponin, to obtain optimal substrate availability, as described by Flik et al. (1983). Homogenates (final protein content 1 mg ml<sup>-1</sup>) were divided into 12 fractions and triplicate 10 µl samples were incubated for either 15 min at 37°C, 20 min at 29°C, 30 min at 22°C or 45 min at 15°C. The specific activity was calculated by subtracting the  $\text{K}^+$ -dependent, ouabain-sensitive ATPase activity from the total ATPase activity. ATP hydrolysis was assessed by the amount of inorganic phosphate formed min<sup>-1</sup> mg<sup>-1</sup> protein under each incubation condition. Sample protein content was assayed using a commercial protein kit (BioRad, Hercules, CA, USA).

Histological preparation of the gills was carried out according to standard protocols (e.g. Dang et al., 2000b). Briefly, sections were collected from a comparable area in the trailing edge of the filament where the chloride cells reside. Care was taken to orient the filaments in a standardised way to obtain cross-sections through the secondary lamellae perpendicular to the axis of the filament. After dewaxing, blocking of endogenous peroxidase with 2% (v/v)  $\text{H}_2\text{O}_2$  and blocking of non-specific sites with 10% (v/v) normal goat serum, the slides were incubated overnight with a monoclonal antibody against chicken  $\text{Na}^+/\text{K}^+$ -ATPase (IgG $\alpha$ 5, Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, USA) at a final dilution of 1:500 (v/v). Goat anti-mouse (Nordic



Immunology, Tilburg, The Netherlands) was used as secondary antibody at 1:150 (v/v) dilution. The slides were subsequently incubated with 1:150 (v/v) diluted mouse peroxidase anti-peroxidase (M-PAP; Nordic). Staining was performed in 0.025% (w/v) 3,3'-diaminobenzidine (DAB) and 0.0005% (v/v)  $\text{H}_2\text{O}_2$ .

#### Cortisol administration

To determine the effect of exogenous cortisol on  $\text{Na}^+/\text{K}^+$ -ATPase activity, a miniosmotic pump (Model 1007D, Alzet, California, USA) was implanted into the peritoneal cavity of the anaesthetised fish. This allowed for a stable elevation of plasma cortisol levels for 1 week without repetitive handling. This approach was used instead of cortisol injection, which evokes stress responses due to repetitive handling, or feeding, which yields high individual variation. The minipumps were filled with cortisol (hydrocortisone; Sigma) at  $6 \text{ mg kg}^{-1}$  body mass using 30% (w/v) 2-hydroxypropyl- $\beta$ -cyclodextrin (Sigma) as vehicle. Control animals received vehicle only. After 1 week, the fish were killed, blood sampled, and gills were taken and analysed as described above.

#### Prolactin expression

Relative expression of prolactin was assessed using real-time quantitative polymerase chain reaction (PCR). Pituitary glands, rapidly removed after anaesthesia, were brought into 250  $\mu\text{l}$  Trizol reagent (Gibco BRL, Gaithersburg, USA), immediately followed by total RNA extraction according to the manufacturer's instructions. To ensure complete removal of traces of genomic DNA, a sample equivalent to 1  $\mu\text{g}$  of total RNA was incubated with 1 unit DNase I (amplification grade; Gibco BRL) for 15 min at room temperature. To inactivate DNase, 1  $\mu\text{l}$  of 25  $\text{mmol l}^{-1}$  EDTA was added and the sample was incubated for 10 min at  $65^\circ\text{C}$  to simultaneously linearise RNA. Thereafter, the RNA was reverse transcribed (RT) with 300 ng random primers (Gibco BRL), 0.5  $\text{mmol l}^{-1}$  dNTPs, 10 units RNase Inhibitor (Gibco BRL), 10  $\text{mmol l}^{-1}$  dithiothreitol and 200 units Superscript<sup>TM</sup> RT (Gibco BRL) for 50 min at  $37^\circ\text{C}$ . For quantitative PCR analysis, 5  $\mu\text{l}$  of 50 $\times$  diluted RT-mix was used as template in 25  $\mu\text{l}$  amplification mixture, containing 12.5  $\mu\text{l}$  SYBR Green Master Mix (Applied Biosystems Benelux, Nieuwerkerk aan den IJssel, The Netherlands) and 3.75  $\mu\text{l}$  of each primer (final concentration 600  $\text{nmol l}^{-1}$ ). The primer sets used in the PCR were, for prolactin (165 bp; 303 bp in the case of genomic DNA): forward 5'-CAT CAA TGG TGT CGG TCT GA-3', reverse 5'-TGA AGA GAG GAA GTG TGG CA-3', and for  $\beta$ -actin (154 bp; 255 bp in case of genomic DNA): forward 5'-GCC CCC AGC ACA ATG AAA A-3', reverse 5'-GGT GGA CGA TGG ATG GTC-3'. After an initial step at  $95^\circ\text{C}$  for 10 min, a real-time quantitative PCR of 40 cycles was performed (GeneAmp 5700, Applied Biosystems), each cycle consisting of 15 s denaturation at  $95^\circ\text{C}$  and 1 min annealing and extension at  $60^\circ\text{C}$ . Cycle threshold ( $C_T$ ) values were determined and expression of prolactin was calculated as a percentage of  $\beta$ -actin expression.

#### Statistical analyses

In all experiments, differences among groups were assessed by the non-parametric Mann-Whitney  $U$ -test. Linear regression for correlation data was based on the least squares method. Significance was accepted at  $P < 0.05$ . All values are expressed as means  $\pm$  standard error of the mean (S.E.M.).

#### Results

As shown in Table 1, plasma osmolality differed significantly among groups and was highest in the  $15^\circ\text{C}$ -acclimated group. The differences in plasma osmolality were paralleled by comparable differences in  $\text{Na}^+$  and  $\text{Cl}^-$  levels in the plasma. Plasma  $\text{K}^+$  was highest in the  $29^\circ\text{C}$ -acclimated group. Total and free  $\text{Ca}^{2+}$  levels did not differ among groups.

$\text{Na}^+/\text{K}^+$ -ATPase activity assayed under conditions of  $V_{\text{max}}$  (i.e. at  $37^\circ\text{C}$ ) was twofold higher in gill homogenates from the cold-acclimated group compared to those of the  $22^\circ\text{C}$ - and  $29^\circ\text{C}$ -adapted group (Fig. 1;  $N=8$ ,  $P < 0.01$ ). However,  $\text{Na}^+/\text{K}^+$ -ATPase activity measured at the acclimation temperature of the fish ( $V_{\text{apparent}}$ ), revealed that the homogenates from the  $29^\circ\text{C}$ -acclimated group had the highest apparent  $\text{Na}^+/\text{K}^+$ -ATPase activity ( $N=8$ ;  $P < 0.05$ ).  $\text{Na}^+/\text{K}^+$ -ATPase activity did not differ significantly between the sham-treated group and the untreated group at  $22^\circ\text{C}$ , but implantation for 7 days of with a miniosmotic pump filled with cortisol resulted in a significant increase in  $\text{Na}^+/\text{K}^+$ -ATPase activity ( $N=8$ ,  $P < 0.05$ ).

The  $\text{Na}^+/\text{K}^+$ -ATPase activity of gill homogenates from carp

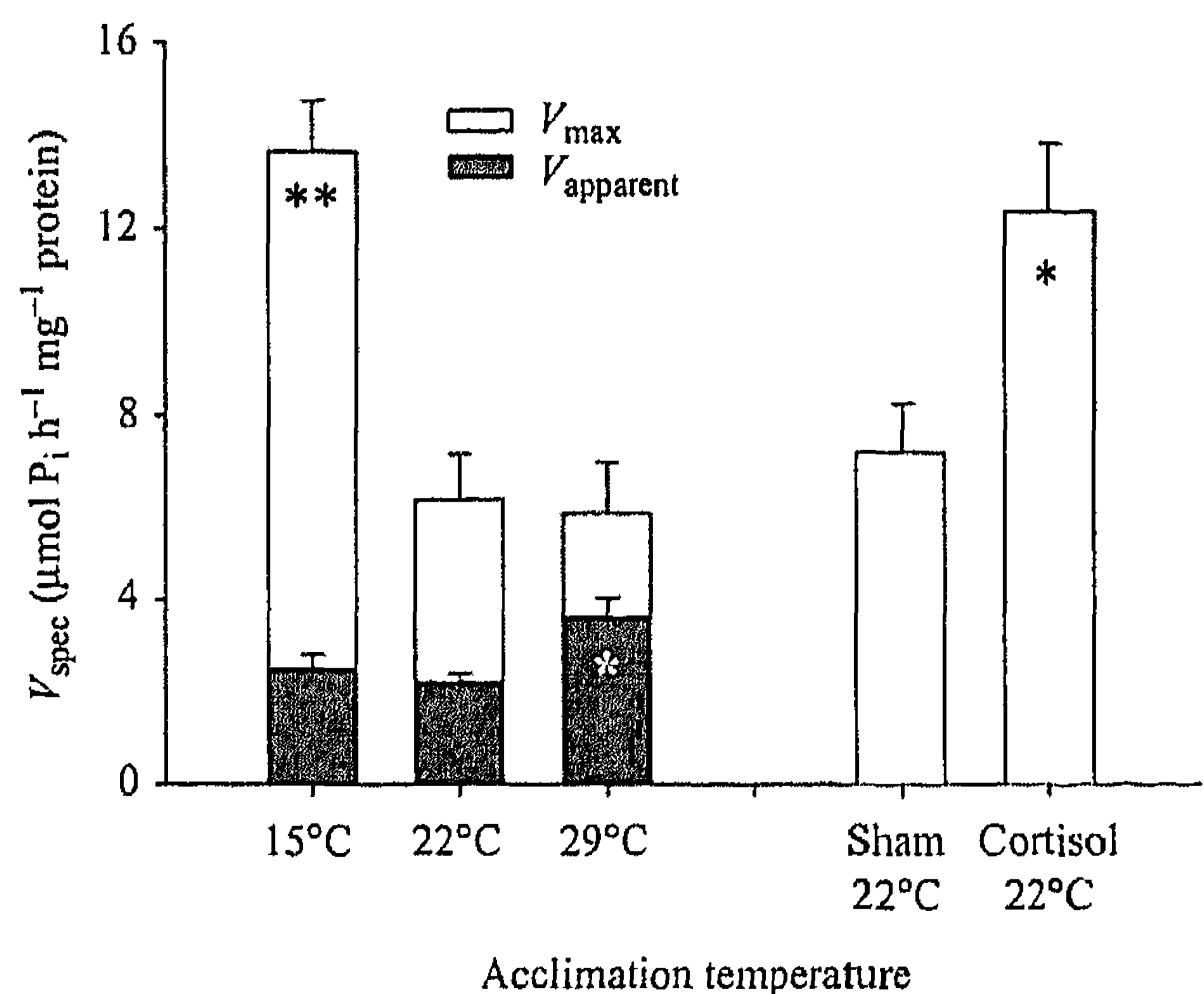


Fig. 1. Specific branchial  $\text{Na}^+/\text{K}^+$ -ATPase activity ( $V_{\text{spec}}$ ) in carp acclimated for 8 weeks to water temperatures of 15, 22 or  $29^\circ\text{C}$ , and in  $22^\circ\text{C}$ -acclimated carp that had a minipump filled with vehicle or cortisol implanted for 7 days. Activity was assayed at optimal temperature ( $V_{\text{max}}$ ; white bars) and at the acclimation temperature of the fish ( $V_{\text{apparent}}$ ; grey bars). Values are means  $\pm$  S.E.M. Asterisks indicate statistically significant differences among groups (\* $P < 0.05$ ; \*\* $P < 0.01$ ).

Table 1. Plasma osmolality and ion composition of fish acclimated for 8 weeks to water temperatures of 15, 22 and 29°C

|  | Water temperature |           |           | Significance test<br>(water temperatures) | P                        |
|--|-------------------|-----------|-----------|---|--------------------------|
|  | 15°C              | 22°C      | 29°C      |   |                          |
| Plasma osmolality (mosmol kg <sup>-1</sup> ) | 289±2.3           | 264±1.3   | 278±2.6   | 22 vs 29<br>22 vs 15<br>15 vs 29          | <0.01<br><0.001<br><0.05 |
| Plasma ions (mmol l <sup>-1</sup> )          |                   |           |           |   |                          |
| Na <sup>+</sup>                              | 149.2±1.9         | 132.4±3.2 | 143.7±2.8 | 22 vs 29<br>22 vs 15<br>15 vs 29          | <0.01<br><0.001<br><0.05 |
| Cl <sup>-</sup>                              | 147.7±1.7         | 111.5±3.3 | 131.2±3.7 | 22 vs 29<br>22 vs 15<br>15 vs 29          | <0.01<br><0.001<br><0.01 |
| K <sup>+</sup>                               | 3.87±0.14         | 3.45±0.15 | 4.54±0.19 | 22 vs 29<br>22 vs 15<br>15 vs 29          | <0.001<br><0.01<br><0.01 |
| Total Ca <sup>2+</sup>                       | 1.99±0.07         | 1.99±0.06 | 1.85±0.09 | n.s.                                      |                          |
| Free Ca <sup>2+</sup>                        | 0.87±0.04         | 0.87±0.05 | 0.87±0.06 | n.s.                                      |                          |

Values are means ± S.E.M., N=8.

n.s., not significant.

acclimated to 15, 22 or 29°C at different *in vitro* assay temperatures is shown in Fig. 2. Discontinuity in the Arrhenius plots of enzyme activity were evident in homogenates from the 15°C-acclimated fish at 29°C and the 22°C-acclimated fish at

22°C. No break in the plot of the 29°C fish was observed over the range of the temperatures tested. Calculated Q<sub>10</sub>-values for Na<sup>+</sup>/K<sup>+</sup>-ATPase activity were approximately 1.7 for the 15 and 22°C-acclimated fish at the higher temperatures and for the 29°C fish at all temperatures tested. At temperatures below the breakpoint, Q<sub>10</sub>-values were significantly higher and increased up to 2.3.

Light microscopical analysis showed that the gill filaments of 15°C-acclimated fish were thicker and the lamellae slightly shorter and thicker than those of 29°C-acclimated fish (Fig. 3). Na<sup>+</sup>/K<sup>+</sup>-ATPase immunopositive cells (chloride cells, CCs) were located only in the interlamellar spaces in all fish analysed. CCs in 15°C-acclimated fish were more abundant (105±12 CCs mm<sup>-1</sup> versus 62±8 CCs mm<sup>-1</sup>; N=8, P<0.05), appeared larger and contained visibly more immunoreactive Na<sup>+</sup>/K<sup>+</sup>-ATPase than those of 29°C-acclimated fish. The gill structure of 22°C-acclimated fish was very similar to that of the 29°C-acclimated fish, with respect to number and structure of immunoreactive cells (not shown).

Fig. 4A shows plasma cortisol values of fish acclimated for 8 weeks to 15, 22 and 29°C, and 22°C-acclimated carp containing an implanted miniosmotic pump filled with cortisol or vehicle (sham-treated). Basal cortisol levels correlated positively with the acclimation temperature within the range of temperatures tested and were, within the range, best-fitted to the equation: plasma cortisol (nmol l<sup>-1</sup>)=3.86T-51 (r<sup>2</sup>=0.99, P<0.01), where T=temperature. Cortisol-treated fish had 2.4-fold

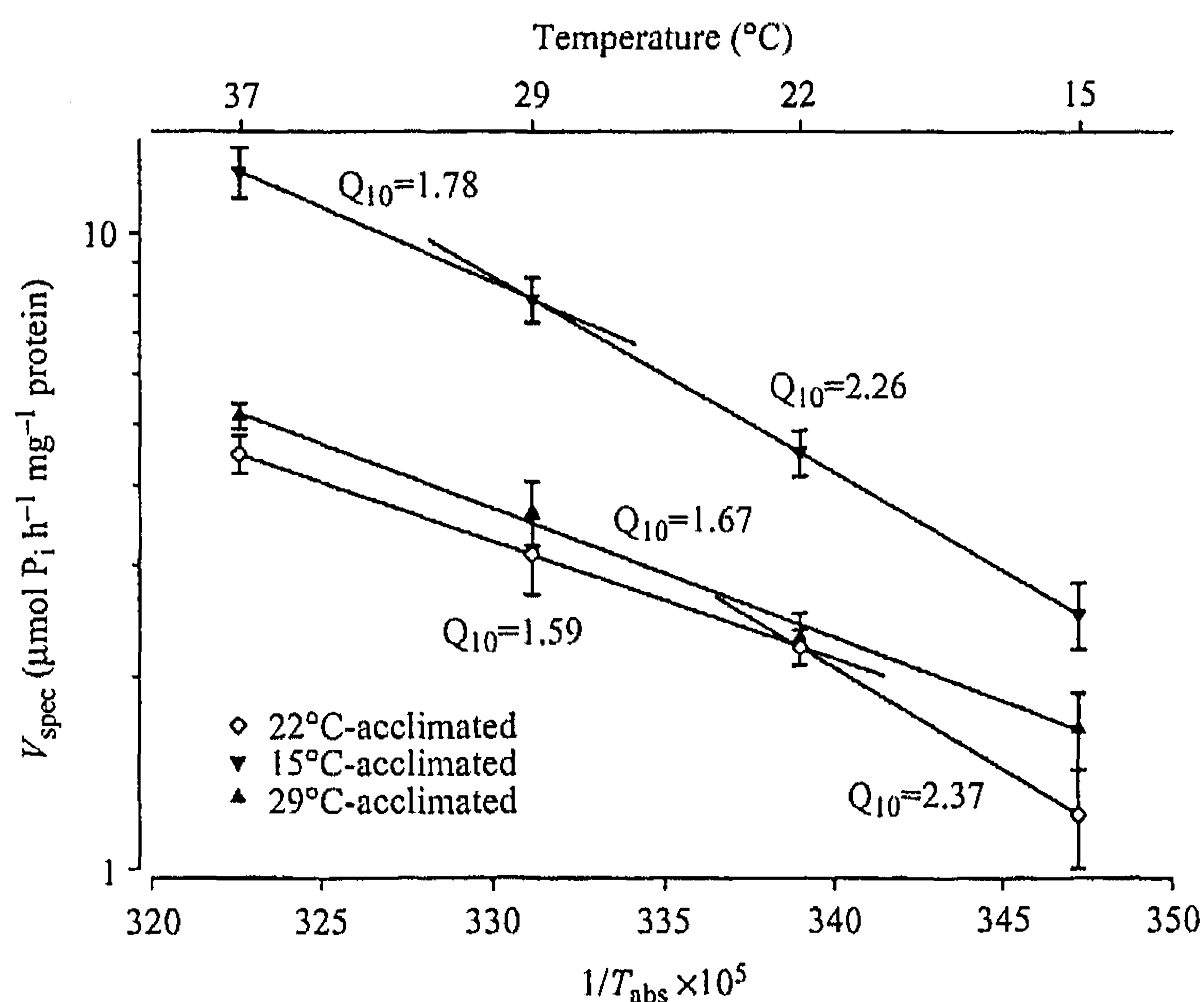


Fig. 2. Arrhenius plot of the specific branchial Na<sup>+</sup>/K<sup>+</sup>-ATPase activity ( $V_{\text{spec}}$ ) of carp acclimated to water temperatures of 15, 22 or 29°C. Q<sub>10</sub>-values are indicated in the graph. Breaks in the plot occur at 29°C in the 15°C-acclimated fish and at 22°C in the plot of the 22°C-acclimated fish. No apparent break in the plot of the 29°C fish is observed over the range of the temperatures tested. Values are means ± S.E.M.



higher circulating cortisol levels than vehicle-treated fish ( $N=8$ ,  $P<0.01$ ).

Pituitary prolactin expression relative to the housekeeping gene  $\beta$ -actin, quantified by real-time RT-PCR, was one third as high in the 29°C-acclimated group as in the other two groups (Fig. 4B;  $N=8$ ,  $P<0.01$ ).

### Discussion

In this study, three major observations were made. (1) When acclimated to water temperatures of 15, 22 or 29°C, carp appear to reset their plasma mineral composition at each temperature. (2) Carp enhance  $\text{Na}^+/\text{K}^+$ -ATPase expression at low temperatures to counteract temperature-dependent inactivation of  $\text{Na}^+/\text{K}^+$ -ATPase. At higher temperatures, the fish may rely on ambient temperature-driven activation of the  $\text{Na}^+/\text{K}^+$ -ATPase pool. (3) Basal plasma cortisol levels correlate positively with apparent  $\text{Na}^+/\text{K}^+$ -ATPase activity but not with total enzyme expression. Also the prolactin status of the fish fits with classical inhibitory control over the sodium pump activity, but not over pump abundance. Increased  $\text{Na}^+/\text{K}^+$ -ATPase activity at higher temperatures is consistent with the decreased prolactin expression and consequent enhanced branchial permeability to  $\text{Na}^+$ . These three key findings will be discussed separately.

#### Hydromineral status

Acclimation temperature affects the hydromineral status of common carp, reflected by different plasma  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Cl}^-$  profiles and total plasma osmolality. We interpret the

observation that plasma  $\text{Ca}^{2+}$  levels are perfectly regulated to indicate successful adaptation of the fish. Changes in plasma  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{K}^+$  as a result of temperature acclimation have been established already in many fish species (Burton, 1986), but at variance with our data, a constant plasma ion composition was reported for carp acclimated to temperatures between 2 and 30°C (Houston et al., 1970; Houston and Smeda, 1979). We observed consistently that plasma osmolality as well as  $\text{Na}^+$  and  $\text{Cl}^-$  levels (the major determining ions for osmolality) are elevated at both 15°C and 29°C when compared to the values seen in fish at 22°C. We have no clearcut explanation for our observations, but suggest that the altered endocrine status of the fish (see below) could at least form the basis for such differences. The adaptive response and resetting could represent an energetically more profitable condition for the fish as a result of multiple altered processes in the gills, as well as in other osmoregulatory organs (e.g. intestine and kidney).

#### $\text{Na}^+/\text{K}^+$ -ATPase activity

When maximum velocity (i.e. under optimal conditions for substrate concentrations and accessibility as well as temperature, viz. 37°C, yielding an estimate of the total amount of enzyme) was assessed, a very large rise in  $\text{Na}^+/\text{K}^+$ -ATPase activity was observed in the gills from the 15°C-acclimated fish. When we performed immunohistochemistry on the gills, more and larger chloride cells, containing more  $\text{Na}^+/\text{K}^+$ -ATPase immunoreactivity, were observed in the 15°C-acclimated fish. The immunohistochemical picture is thus in agreement with the maximum velocity determination of  $\text{Na}^+/\text{K}^+$ -ATPase activity. However, note that when the enzyme activity was assayed at the acclimation temperature of the fish, the reverse effect was seen: the lowest activity was at 15 and 22°C, and the apparent highest activity at 29°C. Thus it is important to assay the enzyme at the acclimation temperature of the fish in order to provide a physiological interpretation. Furthermore, an intact cell may contain a latent pool of membrane vesicles carrying the enzyme that would be released and assayed when a homogenate is made, so biochemical analysis would tend to overestimate enzyme activity.

It has been demonstrated for many membrane-bound enzymes that a break in an Arrhenius plot is often caused by a change of membrane fluidity. Membrane fluidity is largely determined by the lipid composition. Fish acclimated to lower ambient temperatures respond by lowering the membrane melting point (Wodtke,

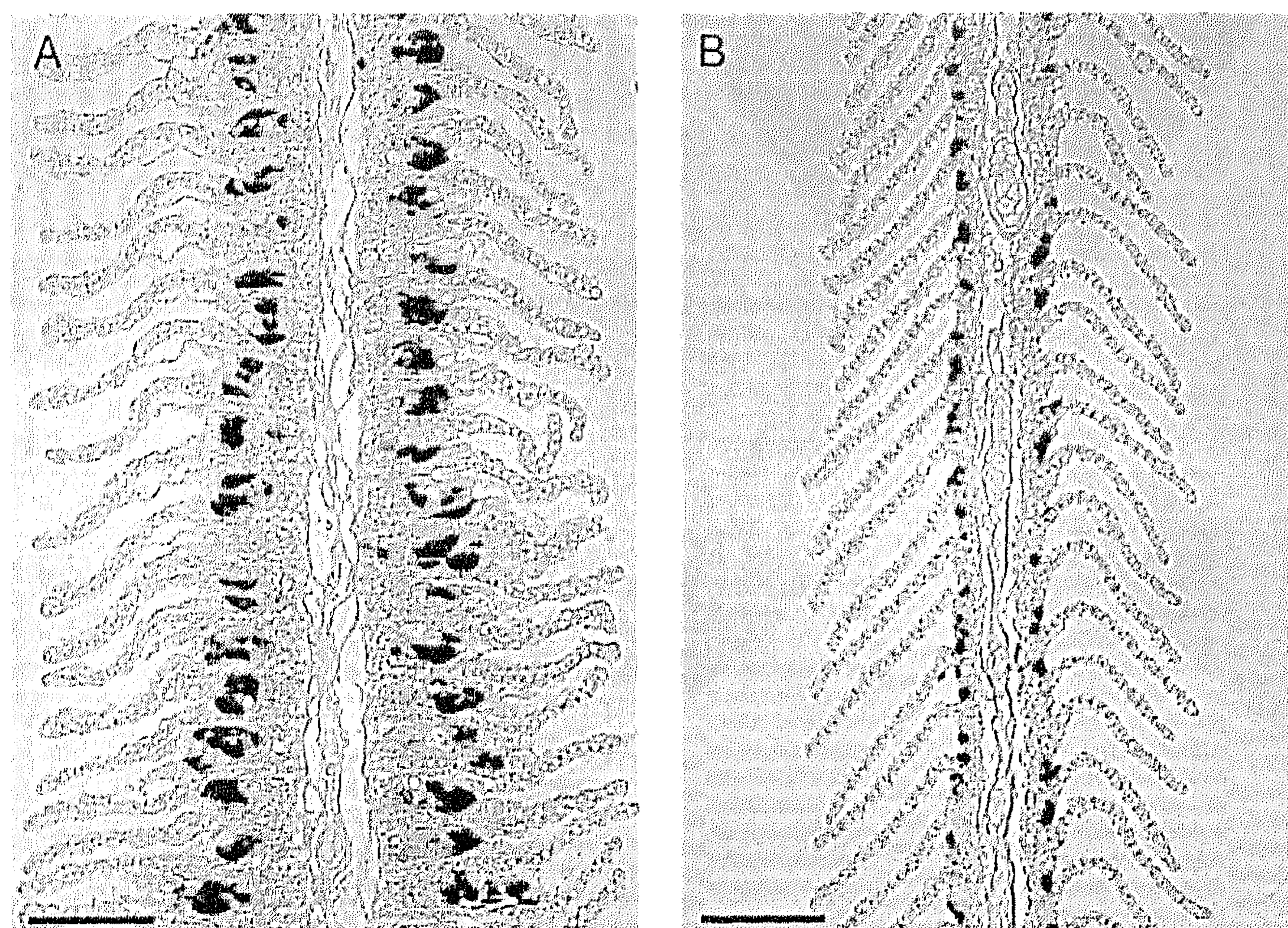


Fig. 3. Immunohistochemical demonstration of  $\text{Na}^+/\text{K}^+$ -ATPase immunoreactive cells (chloride cells) in gills of (A) 15°C-acclimated and (B) 29°C-acclimated carp. Chloride cells are more abundant and larger in the gills of 15°C-acclimated carp. Representative examples are shown at the same magnification. Scale bars, 100  $\mu\text{m}$ .



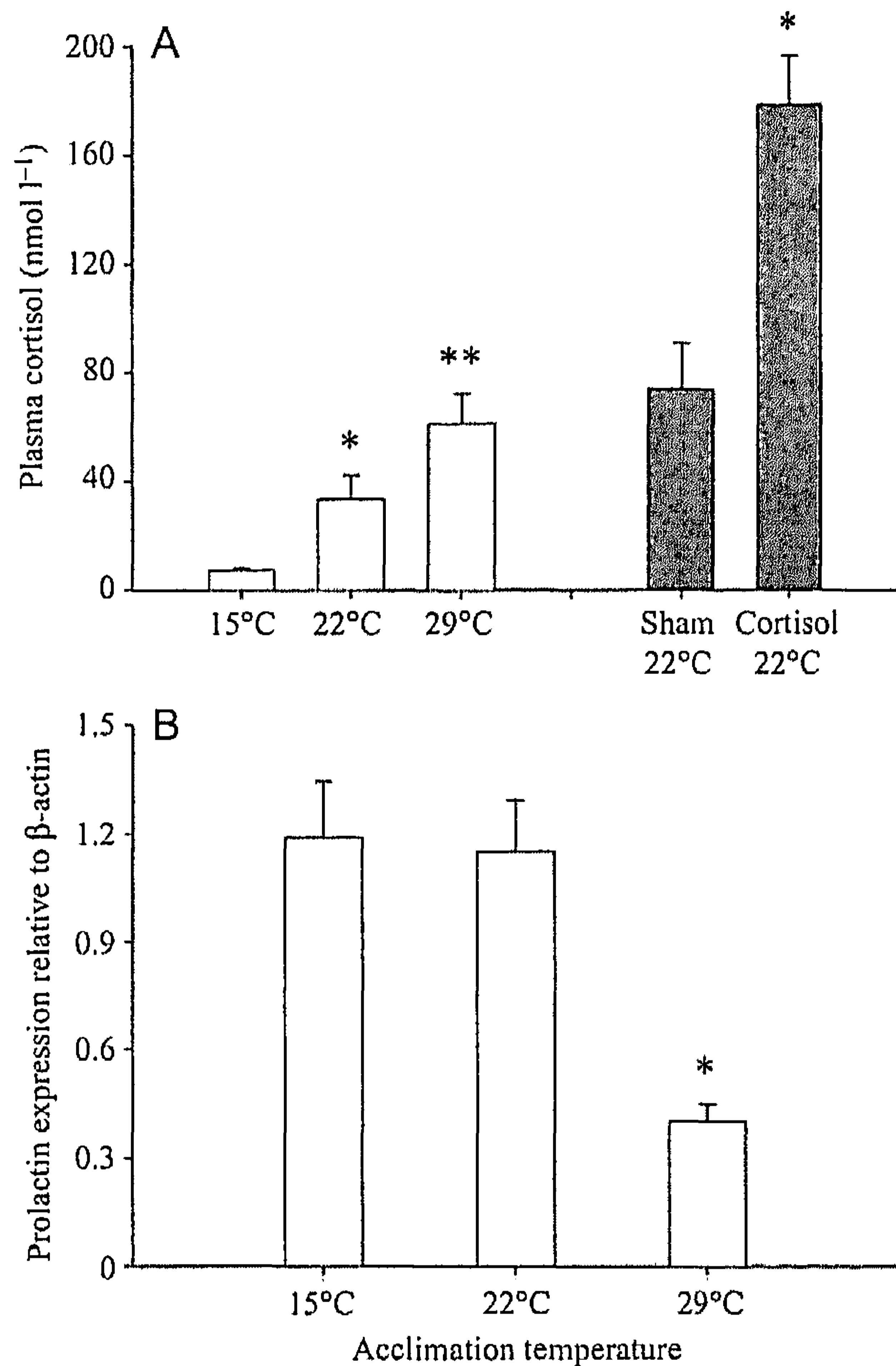


Fig. 4. (A) Plasma cortisol levels in carp acclimated for 8 weeks to water temperatures of 15, 22 or 29°C and in 22°C-acclimated carp that had a minipump filled with vehicle or cortisol implanted for 7 days. (B) Pituitary prolactin expression, relative to the housekeeping gene  $\beta$ -actin, in 15, 22 or 29°C-acclimated carp quantified by real-time PCR. Values are means  $\pm$  S.E.M. Asterisks indicate statistically significant differences among groups (\* $P$ <0.05; \*\* $P$ <0.01).

1981; Hazel, 1984). As the breaks in the Arrhenius plot in this study appear to move rightwards with increasing acclimation temperature, we speculate that the breaks are related to protein thermo(in)stability or differences in isozyme activities rather than membrane fluidity. Indeed, different  $\text{Na}^+/\text{K}^+$ -ATPase isozymes have been identified in fish gills (Cutler et al., 1995, 2000).

It would seem that carp acclimated to 15°C increase chloride cell numbers and  $\text{Na}^+/\text{K}^+$ -ATPase expression to compensate for the lower reaction rate that occurs at this temperature. Slight overcompensation might explain the enhanced plasma  $\text{Na}^+$  and  $\text{Cl}^-$  levels seen in these fish.

#### Cortisol and prolactin

It was predicted that the altered hydromineral status of carp upon acclimation to different temperatures results from

changes in their endocrine status, since the ion pumps in the gills are under strict hormonal control. Cortisol and prolactin are the two classical  $\text{Na}^+/\text{K}^+$ -ATPase regulators in fish, having opposite effects. Cortisol is a stimulator of  $\text{Na}^+/\text{K}^+$ -ATPase activity, and is often therefore referred to as a 'seawater-adapting hormone', as branchial  $\text{Na}^+$  secretion in seawater requires enhanced  $\text{Na}^+/\text{K}^+$ -ATPase activity (Epstein et al., 1980; McCormick, 1995). Prolactin, on the other hand, is referred to as the 'freshwater-adapting hormone', inhibiting  $\text{Na}^+/\text{K}^+$ -ATPase activity and ensuring low water- and ion-permeability of the gills (Hirano, 1986; Wendelaar Bonga et al., 1990; Bern and Madsen, 1992). A critical role for cortisol, not just in seawater fish but also in freshwater fish, cannot be denied as both cortisol and prolactin appear to be necessary to maintain ionic homeostasis in hypophysectomised freshwater catfish (Parwez and Goswami, 1985).

Our data confirm the results of Arends et al. (1998) and Van den Burg et al. (2003), who showed that rising temperatures induce increased basal plasma cortisol levels. Unexpectedly, we found no relationship between basal plasma cortisol levels and branchial  $\text{Na}^+/\text{K}^+$ -ATPase abundance. Yet, after 1 week of exogenously administered cortisol,  $\text{Na}^+/\text{K}^+$ -ATPase activity was clearly enhanced, which is in agreement with earlier studies (Abo Hegab and Hanke, 1984; De Boeck et al., 2001). It would seem that circulating cortisol levels must exceed a certain threshold level to act as a stimulator of branchial  $\text{Na}^+/\text{K}^+$ -ATPase activity. Indeed, it has been shown in freshwater rainbow trout that chloride cells contain at least two cortisol receptors, a mineralocorticoid receptor (MR) having a high affinity and a glucocorticoid receptor (GR) with a lower affinity (Ducouret et al., 1995; Colombe et al., 2000; Sloman et al., 2001). Assuming a similar situation in carp, it follows that the enhanced  $\text{Na}^+/\text{K}^+$ -ATPase activity after exogenously administered cortisol results from a GR-mediated effect of cortisol. An attractive hypothesis proposed by Sloman et al. (2001), based on GR and MR-pharmacological studies, is that the more sensitive MR is upregulated in situations when chloride cell proliferation is required, for example in ion-deficient water. A similar activation of a silent MR would explain our observations in the 15°C-acclimated carp, while the circulating basal cortisol levels are too low for a GR-mediated effect. The differences in basal cortisol levels observed here may thus reflect more so the metabolic status of the fish at different temperatures.

Pituitary prolactin expression was downregulated in the 29°C-acclimated group. Assuming that 8 weeks of acclimation results in a steady resetting of the protein expression machinery in a cell, we speculate that differences in mRNA levels reflect equivalent differences in protein production and secretion, implying that 29°C-acclimated carp have lower plasma prolactin levels (which we cannot determine directly by radioimmunoassay) than the 15 and 22°C-acclimated fish. This is in agreement with plasma measurements in rainbow trout *Oncorhynchus mykiss* (Rand-Weaver et al., 1995) and newt *Cynops pyrrhogaster* (Takahashi et al., 2001) kept at different ambient temperatures. Since prolactin limits membrane



permeability to water and ions (Hirano, 1986; Wendelaar Bonga et al., 1990; Evans, 2002), our observation that 29°C-acclimated fish have a higher apparent Na<sup>+</sup>/K<sup>+</sup>-ATPase activity is in accordance with an enhanced branchial permeability as a result of downregulated prolactin expression, leaving plasma mineral composition within a physiological range.

#### Concluding remarks

Taken together, the hydromineral status of carp appears to be reset when the fish is acclimated to different temperatures. To cope with different temperatures, the eurythermal carp exploits various strategies resulting in subtle yet significant readjustments of hydromineral balance. Clearly, branchial Na<sup>+</sup>/K<sup>+</sup>-ATPase activity is differentially regulated. We interpret our data to indicate that carp, at higher ambient temperatures, rely on a temperature-enhanced branchial enzymatic pump activity. A higher apparent Na<sup>+</sup>/K<sup>+</sup>-ATPase activity, combined with enhanced branchial permeability, ensures ionic homeostasis. At a lower ambient temperature, by contrast, branchial Na<sup>+</sup>/K<sup>+</sup>-ATPase expression is upregulated to counteract the temperature-inhibited activity of the sodium pump, perhaps *via* a mineralocorticoid receptor. Understanding the role of cortisol in temperature adaptation requires further study of the regulation of cortisol receptors in the gills.

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