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SHORT COMMUNICATION

The thermal dependence of Na^+ flux in isolated liver cells from ectotherms and endotherms

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ABSTRACT

The thermal dependence (0–40°C) of Na^+ flux in isolated liver cells of three endotherms (mice, rat and rabbit) was compared with that of ectotherms in the form of a thermally tolerant amphibian (cane toad), a cold-water fish (rainbow trout) and a thermophilic reptile (lizard). Mammals were found to share similar high rates of Na^+ flux (3.0–3.7 nmol Na^+ mg^{-1} protein min^{-1}) at their normal body temperatures (36–39°C). These Na^+ flux rates were significantly greater (P<0.0004–0.0001) than those of the ectotherms, which shared similar low rates of Na^+ flux (0.7–1.3 nmol Na^+ mg^{-1} protein min^{-1}) at their very different normal acclimated body temperatures (15°C for trout, 25°C for toad and 37°C for the lizard species). Trout, which possess highly unsaturated membranes (similar to those of mammals), showed a Na^+ flux with high thermal sensitivity at low temperatures similar to that found in mammals at higher temperatures. The thermal sensitivity of toad Na^+ flux was significantly less (P<0.05–0.01) than that of rat and rabbit. Trout Na^+ flux did not increase with increasing temperature much above 20°C, whereas all other species measured increased their Na^+ flux with increasing temperature up to 40°C. In conclusion, at normal operating body temperatures, the rate of Na^+ flux is much lower in ectotherms.

KEY WORDS: Tissue metabolism, Sodium pump metabolism, Hepatocytes, Lizard, Toad, Rat, Mouse, Trout, Rabbit

INTRODUCTION

The Na^+ and K^+ gradients maintained across the cell membrane of endothermic and ectothermic vertebrates are similar (Else and Hulbert, 1987) and a major cost and contributor to basal metabolic rate (Rolfe and Brown, 1997). These gradients are used to create action potentials and move other ions and substrates across the cell membrane, yet endotherms spend several times more energy on maintaining these gradients than ectotherms (Else and Hulbert, 1987; Hulbert and Else, 1990). The energy spent on maintaining these gradients is due to the activity of the sodium pump, which removes Na^+ from within the cell and reclaims K^+ from outside the cell (Else et al., 1996). In vertebrates the amount of energy spent on sodium pumping alone is estimated to account for ∼20% of basal/standard metabolism (Rolfe and Brown, 1997), and in brain and kidney it can account for up to 50% of tissue metabolism (Else and Hulbert, 1981). The reason endotherms spend several times (up to 5-fold) more energy on maintaining Na^+ and K^+ gradients is the presence of 'leaky' membranes in endotherms (Else and Hulbert, 1987; Hulbert and Else, 1990). 'Leaky' membranes refers to the measurement of much higher rates of passive ionic flux across the cell membranes of endothermic tissues versus those in ectotherms (Else and Hulbert, 1987; Hulbert and Else, 1990).

As endotherms normally maintain high, constant body temperatures (i.e. are homeothermic) whereas ectotherms tend to operate at body temperatures that can be substantially lower and far more variable, it was hypothesized that ectotherms would possess similar low levels of Na^+ flux, irrespective of differences in their normal operating body temperatures. This would ensure that the energy demand of maintaining Na^+ and K^+ gradients would not become excessive in ectotherms with different body temperatures. It was also hypothesized that as membrane lipid unsaturation has been implicated as a determinant of metabolism in vertebrates (Hulbert and Else, 1999), trout with highly unsaturated membranes (similar to those of mammals) would possess a relatively high rate of Na^+ flux at a low body temperature. Therefore, the present study set out to examine the thermal sensitivity of Na^+ flux in a number of ectotherms (toad, trout and lizard) with different normal acclimated body temperatures compared with a group of mammalian endotherms (mouse, rat and rabbit).

MATERIALS AND METHODS

Animals

Animals used to examine Na^+ flux included the rainbow trout [*Oncorhynchus mykiss* (Walbaum 1792)], cane toad [*Rhinella marina* (Linnaeus 1758)], mouse [*Mus musculus* (Linnaeus 1758)], rat [*Rattus norvegicus* (Berkenhout 1769)] and rabbit [*Oryctolagus cuniculus* (Linnaeus 1758)]. Trout were from a local trout farm (ponds at 15°C) and were maintained for up to ∼1 week at 15°C in aerated tanks. Toads were from a Queensland supplier and were maintained for up to 4 weeks in large plastic containers at 25°C. Mouse, rats and rabbits were young adults taken from breeding stock maintained at Deakin University (VIC, Australia). Mammals were maintained in cages at 21°C under a 12 h:12 h light:dark cycle. All animals had access to appropriate food and water. Measurement of tissue metabolism also included use of flathead fish (*Platycephalus bassensis*) caught in Port Phillip Bay, Melbourne, Australia, and maintained in recirculating seawater tanks at 14–17°C for no more than 1 week, and pigeons from a local supplier, used on the day. General body and organ mass data are provided in Table 1. All experiments were approved by animal ethics committees at the University of Melbourne and Deakin University.

Chemicals and materials

$^{22}Na^+$ (as $^{22}NaCl$) was purchased from Amersham Radiochemicals; ouabain and collagenase (type 1) were from Sigma Chemicals. Plastic coverslips, rat collagen and culture plates were from Flow Laboratories. All culture media, sera and antibiotics were purchased from the Australian Commonwealth Serum Laboratories. All general reagents used were of analytical grade and purchase from Sigma, AJAX or BDH Chemicals.

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Table 1. Tissue metabolism of liver, skeletal muscle, kidney and brain from a selection of similar-sized ectotherms and endotherms

Species	M_b (g)	Organ mass (% M_b)	Tissue metabolism (μl O_2 mg^{-1} DM h^{-1})	Na^+-independent metabolism (μl O_2 mg^{-1} DM h^{-1})	Na^+-dependent metabolism (μl O_2 mg^{-1} DM h^{-1})
Liver					
Ectotherms					
‡Trout (*N*=12) (UI 239; PI 164)	275±26	1.00±0.19	2.28±0.19[a]	1.82±0.15[a]	0.48±0.12[a]
‡Flathead (*N*=4)	117±15	0.92±0.02	2.27±0.24[a]	1.87±0.13[a]	0.40±0.21[a]
Cane toad (*N*=6) (UI 137; PI 72)	193±4	3.47±0.41	1.91±0.25[a]	1.36±0.16[a]	0.55±0.20[a]*
Netted dragon (*N*=9)	39±3	3.72±0.26	0.90±0.16[b]	0.68±0.12[b]	0.22±0.13[a]
Bearded dragon (*N*=6) (UI 159; PI 90)	324±43	3.38±0.69	1.04±0.20[b]	0.69±0.11[b]	0.36±0.14[a]
Mean±s.e.m.			1.48±0.44	1.28±0.26	0.40±0.05
Endotherms					
Mouse (*N*=7) (UI 221; PI 153)	30±1	5.48±0.58	4.59±0.36[c]	2.62±0.31[c]	1.97±0.46[b]
Rat (*N*=10) (UI 211; PI 153)	387±41	3.82±0.34	4.51±0.28[c]	2.48±0.19[c]	2.03±0.31[b]
Pigeon (*N*=4)	289±18	6.43±0.44	6.29±0.40[d]	5.07±0.5[d]	1.22±0.12[b]*
Mean±s.e.m.			5.13±0.58	3.39±0.84	1.74±0.26
Ectotherm vs endotherm difference			3.47	2.65	4.35
Muscle§					
Ectotherms					
Cane toad (*N*=6)	193±4	–	0.88±0.09[a]	0.57±0.07[a]	0.31±0.15[a]
Endotherms					
Rat (*N*=5)	387±41	–	2.63±0.22[b]	2.28±0.28[b]	0.35±0.08[a]
Ectotherm vs endotherm difference			2.99	4.00	1.13
Kidney					
Ectotherms					
Cane toad (*N*=6)	193±4	0.51±0.09	9.82±0.68[a]	5.90±0.54[a]	3.92±0.33[a]
Netted dragon (*N*=9)	39±3	0.67±0.08	3.87±1.02[b]	1.96±0.97[b]	1.91±0.95[a]
Bearded dragon (*N*=6)	324±43	0.42±0.07	7.19±0.47[c,*]	3.22±0.41[b,c]	3.96±0.55[a,b]
Mean±s.e.m.			6.96±1.72	3.69±1.16	3.26±0.68
Endotherms					
Mouse (*N*=7)	30±1	1.55±0.10	19.0±1.4[d]	12.2±1.9[d]	6.78±1.1[b,c,*]
Rat (*N*=10)	387±41	0.76±0.03	27.1±0.8[e]	11.5±0.6[d]	15.6±1.0[d]
Mean±s.e.m.			23.1±4.1	11.9±0.35	11.2±4.4
Ectotherm vs endotherm difference			3.31	3.22	3.44
Brain					
Ectotherms					
Cane toad (*N*=6)	193±4	0.06±0.01	13.3±2.2[a]	7.40±0.8[a]	5.90±1.76[a,c]
Netted dragon (*N*=9)	39±3	0.35±0.01	4.97±0.46[b]	4.07±0.36[b]	0.90±0.76[b]
Bearded dragon (*N*=6)	324±43	0.13±0.06	7.38±0.88[c]	5.64±1.57[b]	1.74±0.90[a,b]
Mean±s.e.m.			8.55±2.47	5.70±0.96	2.84±1.55
Endotherms					
Mouse (*N*=7)	30±1	1.45±0.03	11.0±0.6[a,d]	4.53±0.41[b]	6.44±0.53[c]
Rat (*N*=10)	387±41	0.55±0.01	12.0±0.9[a,d]	5.46±0.81[a,b]	6.58±0.53[c]
Mean±s.e.m.			11.5±0.5	5.00±0.47	6.51±0.07
Ectotherm vs endotherm difference			1.34	0.88	2.29 (P<0.01)

Values are means±s.e.m. M_b, body mass; DM, dry mass.

Species are as follows: trout, *Oncorhynchus mykiss*; flathead, *Platycephalus bassensis*; cane toad, *Rhinella marina*; Netted dragon, *Ctenophorus nuchalis*; bearded dragon, *Pogona vitticeps*; mouse, *Mus musculus*; rat, *Rattus norvegicus*; pigeon, *Columba livia*.

*For trout and flathead, experiments were performed at 20°C (the highest tolerable temperature for these species), whereas all other experiments were performed at 37°C. ‡Muscle is intact abdominal muscle for cane toad and intact external oblique of the abdominal muscles for rat (not sliced).

For metabolism values, different superscript letters indicate statistical difference at P<0.02 (to reduce type 1 errors) unless indicated by an asterisk, where P<0.05. Some data were previously published in graphic form (Else and Hulbert, 1987; Hulbert and Else, 1990). Values in parentheses for liver are unsaturation index (UI, derived from membrane composition and expressed as the number of double bonds per hundred fatty acids) and peroxidation index (PI, derived from membrane composition and expressed as the number of bisallylic methylene groups per hundred fatty acids), respectively, from the literature and used in correlations against species where Na^+ flux values were available.

Tissue metabolism

Animals were killed, and organs were removed (renal pelvis removed from kidneys) and cut (if required) to provide a flat base for stable sectioning at 250 μm thickness using a commercial tissue slicer (Sorvall Instruments TC2). Tissue damage was assumed to be similar between species. For skeletal muscle, very thin (estimated at <500 μm) whole abdominal muscle sheet was used (not sliced). Tissue metabolism was measured in duplicate and represents the average oxygen consumption over 2 h (noted every 30 min) in a balanced electrolyte medium using classic Warburg manometry as previously described (Else and Hulbert, 1981). Na^+-independent respiration was determined in the presence of ouabain at 1 mmol l^{-1} (a specific sodium pump inhibitor). Na^+-dependent respiration was calculated as tissue metabolism minus Na^+-independent metabolism. Oxygen consumption is expressed as μl O_2 mg^{-1} dry mass h^{-1}. Dry mass was determined by placing tissue slices in an oven for 12 h at 80°C.

Liver cell isolation and culture

Cell isolation and culture methods were performed in a manner similar to that previously described (Else and Hulbert, 1987; Hulbert and Else, 1990). Briefly, each animal was anaesthetized,

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and the liver was cannulated (via the hepatic portal vein) and perfused (at 15°C for trout, 25°C for toad and 37°C for all other species) to clear blood for ∼5 min using a non-recirculating calcium-free cell isolation medium (in mmol l^{-1}: NaCl 137, KCl 5.4, $NaHCO_3$ 25, $MgSO_4{\cdot}7H_2O$ 0.8, Na_2HPO_4 0.85, KH_2PO_4 0.15 and glucose 15, with 0.001% Phenyl Red, pH 7.4). Cell isolation was performed using a recirculating cell isolation medium (as described above except with 1 mmol l^{-1} $CaCl_2$ and 1 mg ml^{-1} of collagenase added) with livers perfused for 20–40 min at 10–20 ml min^{-1}. All livers received a similar volume of medium over the perfusion period. Once perfused, the liver capsule was teased open and the cells were released into the wash medium (cell isolation medium without $NaHCO_3$ or $CaCl_2$). Cells were subsequently filtered (250 µm nylon gauze) and centrifuged at 100 ***g*** (2×4 min) in Dulbecco's modified Eagle's medium (10% calf serum plus antibiotics). Cell viability was assessed using Trypan Blue exclusion and only preparations with >90% exclusion were used. Liver cells were cultured onto collagen (rat-tail 0.07 mg per coverslip)-coated Thermanox® plastic coverslips incubated (5% CO_2) overnight.

Na^+-flux measurement

Coverslips with adherent liver cells were incubated in a physiological salt medium (in mmol l^{-1}: 150 NaCl, 0.8 $MgSO_4$, 1.2 $CaSO_4$, 0.86 K_2HPO_4, 0.14 KH_2PO_4, 10 Hepes, pH 7.4 using KOH, raising K^+ levels to 5 mmol l^{-1}) either with or without ouabain (1 mmol l^{-1}) and 18 kBq ml^{-1} of $^{22}Na^+$. Cell-coated coverslips (in duplicate) were placed at an angle (to prevent rubbing) on either side of a slide holder that formed the bottom of a floating Perspex carousel. The thermal gradient (0–40°C) was created along a 0.9 m long (0.25 m wide) insulated plastic water bath (with plate metal partitioning) using a Ratek RC1 cooler coil (with ice) and a Thermomix 1442D bath heater placed at either end. Steady-state temperatures were normally reached within 2 h. Bath temperatures were monitored throughout the experiment using a series of mercury thermometers together with a calibrated thermistor to assess the temperature of carousel incubation media. Na^+ flux was determined (using $^{22}Na^+$) from the difference in Na^+ entry after 20 min of incubation (a period shown to produce a linear rate of Na^+ entry in liver cells; Else and Hulbert, 1987) either with or without ouabain (as previously performed; Else and Hulbert, 1987; Hulbert and Else, 1990). Extracellular $^{22}Na^+$ was removed using (5×30 s) washes in 20 ml of ice-cold medium. Coverslips, with adherent cells, were cut and placed in 2 ml^{-1} of Lowry base solution (200 mmol l^{-1} Na_2CO_3 and 100 mmol l^{-1} NaOH). $^{22}Na^+$ activity was measured using a high efficiency gamma counter [Wallac Wizard with a 3 in (∼76 mm) sodium iodide crystal] and protein was determined using the Lowry method.

Statistical analyses

Statistical analysis was conducted using GraphPad Prism version 6. The rate of change of Na^+ flux against temperature was determined

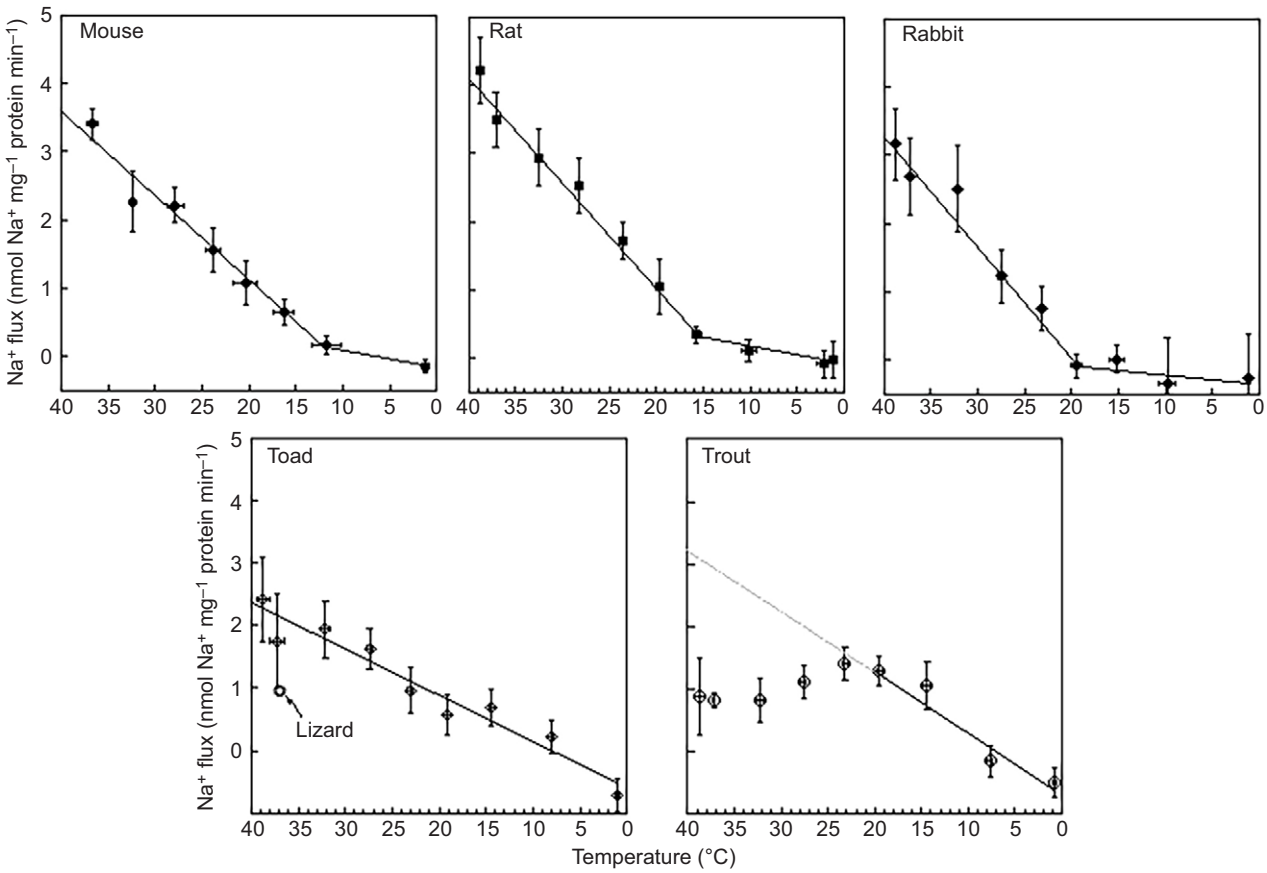

Fig. 1. The thermal dependence of Na^+ flux between 0 and 40°C for liver cells isolated from endotherms and ectotherms. Endotherms: mouse (*Mus musculus*), *N*=8; rat (*Rattus norvegicus*), *N*=7; rabbit (*Oryctolagus cuniculus*), *N*=6; ectotherms: cane toads (*Rhinella marina*), *N*=6; rainbow trout (*Oncorhynchus mykiss*), *N*=6. A single value at 37°C for a lizard species (*Pogona vitticeps*) has been added to the cane toad panel (Else and Hulbert, 1987). The grey line for trout is an extrapolation of the change in Na^+ flux rate measured between 0 and 20°C. Values are means±s.e.m.

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for each animal and combined as species groups for one-way ANOVA and Tukey's multiple comparisons test. Unpaired *t*-tests were used for other general comparisons. Values are means±s.e.m.

RESULTS AND DISCUSSION

At the normal acclimated body temperature for each species (15°C for trout, 25°C for cane toad, 37°C for the thermophilic lizard and mammals), the flux of Na^+ into liver cells of ectothermic species averaged ∼1 nmol Na^+ mg^{-1} protein min^{-1} whereas for the mammalian species it was much higher at ∼3.3 nmol Na^+ mg^{-1} protein min^{-1} (P<0.0004–0.0001). This difference is not solely due to the overall lower normal body temperature of ectotherms, as the thermophilic lizard value was measured at 37°C (Else and Hulbert, 1987) and shows a level of Na^+ flux similar to that of other ectotherms, and several times lower than that measured for mammals. The rate of change in Na^+ flux with increasing temperature (i.e. thermal sensitivity) was statistically higher in the rat and rabbit compared with the toad (P<0.05–0.01), whereas no difference was found between the trout and the mammalian species measured.

Apart from possessing a Na^+ flux with a relatively high thermal sensitivity (similar to that of mammals), trout liver cells also possessed a relatively high rate of Na^+ flux at low temperature. Trout membranes are known to be highly polyunsaturated (Hazel, 1979) and subsequently highly unsaturated. High levels of membrane unsaturation have been associated, via the membrane pacemaker theory of metabolism (Hulbert and Else, 1999), with high levels of metabolism found in endotherms. Some fish species seem to contradict this theory by possessing high levels of membrane unsaturation and yet are ectothermic. However, in the case of trout, they show a relatively high level of Na^+ flux at a low body temperature. Trout also possess a higher than expected level of proton leak across their mitochondria at low temperature (Brookes et al., 1998), with this 'leak' another major source of energy consumption that would fit with their high membrane unsaturation. This suggests that high levels of membrane unsaturation allow trout to maintain a relatively high level of Na^+ flux at low body temperature, similar to other ectotherms operating at higher body temperatures (0.70±0.18 nmol Na^+ mg^{-1} protein min^{-1} at 15°C for trout, 1.26±0.21 nmol Na^+ mg^{-1} protein min^{-1} at 25°C for cane toad and 0.91±0.15 nmol Na^+ mg^{-1} protein min^{-1} at 37°C for a thermophilic lizard). However, the Na^+ flux of all ectotherms measured remained much lower at their normal operating body temperatures compared with those of mammals [between 3.0 and 3.7 (±0.2–0.4) nmol Na^+ mg^{-1} protein min^{-1}].

The source of the difference in Na^+ flux between ectotherms and endotherms is not resolved. A previous study using rat and toad found that the Na^+/H^+ exchanger was the only major source of Na^+ flux that could be inhibited (Else and Mansfield, 2008). This exchanger accounted for 50% of a high Na^+ flux in rat and ∼90% of a lower Na^+ flux in toad liver cells. One novel finding of the present study was that toad and trout liver cells, at very low temperature (∼1°C) in ouabain, had a level of Na^+ flux that was lower than that of normal cells (values slightly below zero). One possible explanation for this is that at very low temperature the ATPase pump may become inactive and act as a channel, with ouabain binding to the pump inhibiting some Na^+ flux.

The thermal sensitivities of Na^+ flux for mammals show a transition with increasing temperature going from a very low flux at low temperature to a high flux at higher temperatures (Fig. 1). For mammals, these transitions take place at approximately 14, 16 and 19°C in mouse, rat and rabbit liver cells, respectively. These may be related to phase transitions linked to the unsaturation of the mammalian membranes (Raison et al., 1971). Trout and toad liver cells appear not to show a phase transition point like mammals, even though trout membranes are highly polyunsaturated. In trout, Na^+ flux does not continue to increase in a linear fashion much beyond 20°C, which is only slightly above the 15°C at which the animals were acclimated. This is presumably due to some instability in the membrane and/or sources of ionic leak. There is limited information on what stabilizes a membrane at higher temperature. Changes in heat shock proteins have be explored as a potential mechanism in the thermal tolerance of membranes in endotherms (Shabtay and Arad, 2005). That study used the transition to endothermy in an avian developmental model to examine thermoprotection of membranes associated with endothermy, but little indication of any heat shock protein response was found. Alternative mechanisms, such as an increase in fatty acid synthase (FAS) at higher body temperatures, have been found (Shabtay and Arad, 2005).

The impact of Na^+ flux rate on metabolism was investigated by measuring normal tissue oxygen consumption plus the sodium-independent and sodium-dependent components of metabolism in a variety of organs from ectotherms and endotherms (Table 1). Although based on a minimal number of species, in liver, kidney and brain respectively, the sodium-dependent metabolism was 4.4-, 3.4- and 2.3-fold higher in endotherms than in ectotherms whereas in skeletal muscle the difference was negligible at 1.1-fold. This demonstrates what has previously been observed, that metabolism associated with Na^+ flux in the major organs is much larger in endotherms but represents a similar proportion of overall metabolism in the tissues of both vertebrate groups (Hulbert and Else, 1999).

For liver, where both flux and the metabolic cost of sodium pumping (i. e. sodium-dependent metabolism) are available, it is possible to correlate one against the other for the species examined at their normal acclimated body temperatures (rabbit not included as no tissue metabolism). This produced a correlation coefficient of 0.987, suggesting a very close association. Correlating the rate of

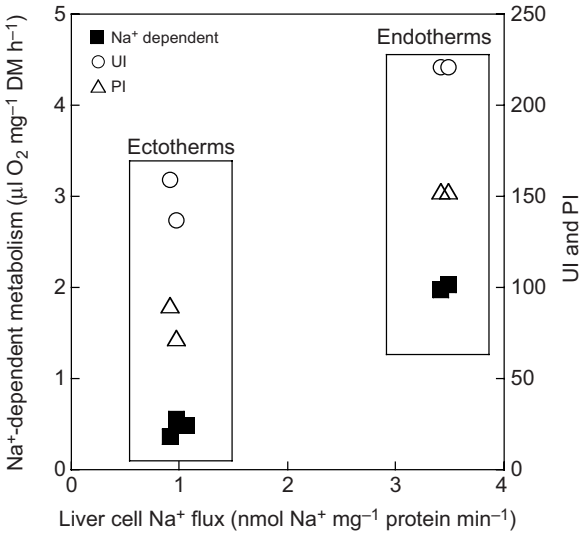

Fig. 2. Rate of Na^+ flux in liver cells plotted against Na^+-dependent metabolism of liver tissue slices and membrane fatty acid unsaturation and peroxidation indices. Values are means. Rainbow trout (N=6), Na^+ flux measured at 15°C and tissue metabolism at 20°C; cane toads (N=6), Na^+ flux measured at 25°C and tissue metabolism at 37°C; mice (N=7–8), rats (N=5–7) and rabbits (N=3), flux and metabolism measured at 37°C. Trout values are not included in unsaturation index (UI) and peroxidation index (PI) plots. DM, dry mass.

Na^+ flux at normal acclimated body temperatures for the cane toad at 25°C and for the thermophilic lizard and endotherms at 37°C against membrane unsaturation (i.e. unsaturation index UI=total number of double bonds per 100 fatty acids) or peroxidation index (PI=total number of bisallylic bonds per 100 fatty acids) gives correlation coefficients of 0.91 and 0.95, again suggesting a potential association. However, the addition of the rate of Na^+ flux for trout (at 15°C) reduces these correlations to 0.35 and 0.46, respectively, whereas if Na^+ flux is recalculated at 37°C for all species (including the extrapolated flux for trout), the correlation coefficients against UI and PI increase to 0.73 and 0.79. Fig. 2 shows how Na^+ flux, pump metabolism and membrane UI and PI for the species examined separated into groups.

In conclusion, ectotherms possess rates of Na^+ flux that are severalfold lower at their normal body temperatures than those of endotherms. The one ectotherm that has a high membrane unsaturation (like the endotherms examined), i.e. trout, managed to maintain a rate of Na^+ flux that was relatively high at low temperature and was thermally sensitive, as found for mammals at higher temperatures. However, the rate of Na^+ flux measured in trout at its normal acclimated body temperature was still similar to that of other ectotherms and did not continue to increase when measured at higher temperatures.

Competing interests
The author declares no competing or financial interests.

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