Alkaline tide and nitrogen conservation after feeding in an elasmobranch (*Squalus acanthias*)

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Summary

We investigated the consequences of feeding for acid–base balance, nitrogen excretion, blood metabolites and osmoregulation in the Pacific spiny dogfish. Sharks that had been starved for 7 days were surgically fitted with indwelling stomach tubes for gastric feeding and blood catheters for repetitive blood sampling and were confined in chambers, allowing measurement of ammonia-N and urea-N fluxes. The experimental meal infused *via* the stomach tube consisted of flatfish muscle (2% of body mass) suspended in saline (4% of body mass total volume). Control animals received only saline (4% of body mass). Feeding resulted in a marked rise in both arterial and venous pH and HCO_3^- concentrations at 3–9 h after the meal, with attenuation by 17 h. Venous $\dot{P}_{O_2}$ also fell. As there were negligible changes in $\dot{P}_{CO_2}$, the response was interpreted as an alkaline tide without respiratory compensation, associated with elevated gastric acid secretion. Urea-N excretion, which comprised >90% of the total, was unaffected, while ammonia-N excretion was very slightly elevated, amounting to <3% of the total-N in the meal over 45 h. Plasma ammonia-N rose slightly. Plasma urea-N, TMAO-N and glucose concentrations remained unchanged, while free amino acid and β-hydroxybutyrate levels exhibited modest declines. Plasma osmolality was persistently elevated after the meal relative to controls, partially explained by a significant rise in plasma Cl^-. This marked post-prandial conservation of nitrogen is interpreted as reflecting the needs for urea synthesis for osmoregulation and protein growth in animals that are severely N-limited due to their sporadic and opportunistic feeding lifestyle in nature.

Key words: gastric acid secretion, metabolic alkalosis, ammonia, urea, osmolality, shark.

Introduction

Feeding is a normal, essential component of the everyday lives of fish. However, most studies on acid–base regulation and nitrogen metabolism in fish have been carried out on starved animals, largely for the sake of experimental convenience, though the approach is often justified based on the need to standardize the metabolic state of the subjects. Feeding is known to have marked effects on acid–base balance in many vertebrate classes (Wang et al., 2001), principally through the phenomenon of the 'alkaline tide' (Niv and Fraser, 2002). This term refers to a significant alkalinization of the blood caused by activation of the acid-secreting cells of the stomach as they secrete HCl to facilitate digestion. Basolateral transport of metabolic base (HCO_3^- ions) into the extracellular fluid occurs on a simultaneous and equimolar basis to the apical transport of metabolic acid (H^+ ions) into the lumen. However, we are aware of no studies on the systemic acid–base consequences of feeding in any fish species. The influence of feeding on nitrogen metabolism in fish has been investigated

in a modest number of studies, mostly from the point of view of aquacultural practice, and this information has recently been reviewed (Wood, 2001). From this review, it is apparent that in teleosts, the simple act of feeding causes alterations in nitrogen metabolism, particularly large increases in ammonia-N excretion, which are more profound than those resulting from any other experimental treatment. It is also apparent that essentially nothing is known about the consequences of feeding in elasmobranchs, not even whether urea-N or ammonia-N excretion increases after feeding in these ureotelic animals. Mommsen and Walsh (1991) speculated that, since urea-N is much more costly to synthesize than ammonia-N, it would make sense for elasmobranchs to excrete excess nitrogen in the form of the latter after feeding, whereas Wood (2001) speculated that, since elasmobranchs feed only sporadically in nature (Jones and Geen, 1977; Cortes and Gruber, 1990; Hanchet, 1991; Tanasichuk et al., 1991), they may be so N-limited as to excrete virtually no 'excess nitrogen' but rather

retain it as urea for osmoregulation and as amino acids for protein growth.

With this background in mind, we pursued the following objectives with the Pacific spiny dogfish, *Squalus acanthias.* The first was to develop a stomach tube feeding model because dogfish will not eat naturally when confined in small boxes, as required for the chronic blood sampling of acid–base studies. The second was to establish whether an alkaline tide occurs after dogfish are fed a realistic ration by this method and, if it does, whether it is modulated by 'respiratory compensation' ($\dot{P}_{CO_2}$ retention to limit pH increases), as in many higher vertebrate carnivores (reviewed by Andrade et al., 2004). The third was to determine the effects of this feeding regimen on nitrogen metabolism, with particular emphasis on ammonia-N and urea-N excretion rates and corresponding blood levels of these two end products. The final goal was to determine changes in associated parameters (blood $\dot{P}_{O_2}$, glucose, trimethylamine oxide, total amino acids, creatinine, osmolality, chloride, glucose, β-hydroxybutyrate) diagnostic of possible osmoregulatory and metabolic phenomena associated with feeding.

Our results demonstrate that feeding causes disturbances in many of the measured parameters, particularly a marked, uncompensated alkaline tide, and reinforce the view that dogfish are strongly N-limited, excreting little 'excess' ammonia-N and no 'excess' urea-N after a meal. The present study forms part of a two-phase investigation on this topic, the other component focussing on some of these same parameters in naturally fed, non-catheterized dogfish and on the accompanying enzymatic changes in various tissues (M.K., P.J.W., T.P.M. and C.M.W., unpublished results).

Materials and methods

Experimental animals

Pacific spiny dogfish (*Squalus acanthias* L., 1.1–3.2 kg) were obtained by trawl netting in Barkley Sound, British Columbia, Canada in July and August 2003. At Bamfield Marine Sciences Centre, they were held in a large 200 000-litre circular tank served with running seawater at the experimental temperature (11–12°C), salinity (30±2‰) and pH (7.9±0.15). Twice weekly, the fish were fed a ration of whole dead trawl fish (mainly flatfish, herring and eelpout) amounting to 2% of the mass of dogfish in the tank, most of which was quickly devoured. One week prior to surgery, fish were transferred in batches of six to a smaller 1500-litre tank where they were fasted.

Each dogfish was anaesthetized with MS-222 (0.2 g l⁻¹), placed on an operating table, weighed, and fitted with indwelling catheters. In Series 1, the catheters consisted of a stomach feeding tube, a caudal artery cannula and a caudal vein cannula, and the anus was tied off to prevent defecation on the supposition that the latter might confound flux measurements (but see below). The anus was ligated with silk suture just anterior to the rectal gland, through a 3 cm incision in the ventral midline In Series 2, only a stomach feeding tube and a caudal artery cannula were inserted, and the anus was not ligated. This change in Series 2 (lack of anal ligation) was adopted because N-excretion rates, particularly those of urea-N, were high and very variable in Series 1.

After Series 1 was completed, a subsequent pilot series with uncannulated animals revealed that anal ligation caused about a 50% increase in urea-N excretion rate, as well as greater variability in both ammonia-N and urea-N excretion rates, probably explained by stress and/or minor blood loss from the incision site. We also noticed that struggling sometimes resulted in fin abrasion against the walls of the fish chambers, another potential source of minor blood loss. Indeed, given the very high levels of urea-N in the bloodstream, blood loss of only ~0.3 ml kg⁻¹ h⁻¹ would be required to cause this elevation. Therefore, only the flux data of Series 2 have been reported, where anal ligation was not employed, and particular care was taken to minimise disturbance.

Based on a number of pilot tests, the final stomach tube design used in both series was individually fitted to each fish *via* the oesophagus, terminating several centimetres anterior to the pylorus. This placement was estimated based on the total length of the fish and was confirmed by autopsy. The key point to successful placement was tailoring the length of the tube to the size of the fish, which we did based on fitting tubes experimentally to a number of dead animals of different lengths. The tube consisted of flexible polyethylene tubing (0.32 cm internal diameter), which was heat polished at the stomach end and heat moulded so as to allow its exit *via* a small puncture wound through the jaw muscle at the side of the mouth and then firmly ligated with silk suture at this point and along the upper jaw, terminating in an upward projection of ~3 cm anterior to the eye. Prior to insertion, the tube was filled with 140 mmol l⁻¹ NaCl, the vehicle for the food slurry (see below), and sealed with a plug at the anterior end. Caudal artery (both Series 1 and 2) and caudal vein catheters (Series 1 only) made of Clay-Adams (Becton-Dickinson, Sparks, MD, USA) polyethylene PE50 tubing were implanted through a small hole in the haemal canal *via* a 5 cm incision through the muscle of the caudal peduncle, as described by DeBoeck et al. (2001). The catheters were filled with heparinised dogfish saline [sodium heparin, 50 i.u. ml⁻¹; saline recipe as in Wood et al. (1994) but with urea level raised to 400 mmol l⁻¹=800 mmol l⁻¹ urea-N]. Wounds were dusted with powdered oxytetracycline to avoid infection, tightly closed with silk ligatures, and sealed with a sheet of rubber dental dam, which was glued to the skin using tissue cement (3M Vetbond, 3M Animal Care Products, St Paul, MN, USA).

After surgery, the dogfish were revived in anaesthetic-free water and transferred to covered wooden fish boxes, which were coated with polyurethane, the same boxes as used in an earlier study (Wood et al., 1995). The boxes were 105 cm in length, 16.5 cm in width and 25 cm in height, with a flow-through of 1 l min⁻¹. Perimeter aeration over the complete length of the box ensured good mixing during flux measurements. The boxes were bathed in an external running

seawater bath to maintain temperature (11–12°C) when flow-through was suspended for the flux measurements. A recovery period of at least 36 h was allowed before experiments were started.

Preparation and administration of food

Fillets of white muscle were cut from 12 freshly caught flatfish (*Hippoglossoides elassodon* and *Parophrys vetulus*), two of the common species in the trawl-caught fish that were routinely fed to the dogfish in the main holding tank. The fillets were ground to a fine paste in a Waring food blender, then stored frozen at –20°C in small samples until used. A sample was taken for N-analysis. The 'meal' administered to experimental animals consisted of 2% of the dogfish's body mass of the flatfish muscle paste mixed 50:50 with an equal volume of 140 mmol l^{-1} NaCl to create a smooth slurry that could be infused *via* the stomach feeding tube. The rationale for using this vehicle is that it would be representative of the teleost body fluids that are ingested along with muscle when dogfish feed naturally. Thus, the volume infused represented 4% of the body mass. From gut content measurements on naturally feeding dogfish in the large tank, we established that this is well within the range that dogfish can consume on a single feeding event (M.K., P.J.W., T.P.M. and C.M.W., unpublished results). Control animals received 4% of the body mass as 140 mmol l^{-1} NaCl. For feeding, the meal was administered as a bolus down the stomach tube over an approximate 5 min period. A small extra volume of saline was used to flush the tube, which was then re-sealed with a plug.

Flux measurements

For flux measurements, flow-through to the box was stopped and the water level set to a mark representing 32 litres. Water samples (10 ml) for ammonia-N and urea-N measurements were taken at the start and end of each flux period. At times of water renewal ('flushes'), the water level was lowered to the point where the animal's dorsal fin was just exposed, then filled to the top of the box with fresh seawater, a procedure that was repeated three times before the volume was reset to 32 litres.

Blood sampling

Blood samples were withdrawn *via* the catheters into ice-cold gas-tight Hamilton syringes. In Series 1, arterial samples were taken prior to venous samples, and two separate syringes of 0.6 ml and 0.4 ml were taken for each. The first was used for blood gas and pH measurements, and the second was immediately centrifuged (2 min at 9000 *g*), then the plasma was divided into aliquot samples and frozen (–80°C) for metabolite measurements. In Series 1, blood recovered from the electrodes was mixed with the red cell pellet, made up to the original 1.2 ml volume with non-heparinised saline, and re-infused. In Series 2, there were no blood gas measurements but a larger set of metabolite measurements was required, so a volume of 0.9 ml was taken and immediately replaced with an equal volume of non-heparinised saline; the red cells were not re-infused, so as to minimize disturbance. Plasma was obtained

by immediate centrifugation, then divided into four tubes and frozen as above for various metabolite assays.

Experimental series

In Series 1, flow-through to the box was stopped for a 4–6 h period for a pre-feeding measurement of ammonia-N and urea-N flux rates, after which a set of pre-feeding blood samples was taken. A meal of either flatfish muscle slurry (experimental animals, *N*=5) or saline (control animals, *N*=5) was then administered *via* the stomach tube, the box immediately flushed, and a new flux measurement started (0 h). Blood samples were then taken at 1 h, 2 h, 3 h, 4 h, 6 h, 9 h, 15–18 h (nominally 17 h), 24 h and 48 h post-feeding. Water samples were taken at every blood sampling time, with flushes at 9 h, 17 h, 24 h, 36 h and 48 h. Blood samples were analysed for blood gases and pH and plasma ammonia-N, urea-N, glucose and chloride concentrations.

In Series 2, a similar protocol was used but with fewer blood and water samplings; particular care was taken to minimize disturbance. The same feeding methods as in Series 1 were employed for the experimental (*N*=8) and control (*N*=8) animals. Blood samples were taken after the pre-feeding flux period, prior to feeding, and at 2 h, 4 h, 6 h, 9 h, 18 h, 24 h, 34 h and 45 h post-feeding. Water samples were taken simultaneously, with flushes at 9 h, 18 h, 34 h and 45 h. Blood samples were analysed for plasma ammonia-N, urea-N, trimethylamine oxide-N (TMAO-N), creatinine-N, total free amino acids (FAA-N), glucose, β-hydroxybutyrate, chloride and osmolality.

Analytical techniques

Arterial and venous blood oxygen tensions ($\dot{P}a_{O_2}$, $\dot{P}v_{O_2}$) and pHs (pHa, pHv) were measured using Radiometer electrodes kept at the experimental temperature with water jackets. True plasma CO_2 was measured by the method of Cameron (1971) on plasma obtained from blood samples centrifuged in sealed tubes. Outputs of the electrodes (E5036 for $\dot{P}_{O_2}$; GK401C for pH; E5046 for $\dot{P}_{CO_2}$ in the Cameron chamber) were displayed on Radiometer pHM 71 and pHM 72 acid–base analysers. Arterial and venous blood carbon dioxide tensions ($\dot{P}a_{CO_2}$, $\dot{P}v_{CO_2}$) and bicarbonate concentrations ([HCO_3^-]a, [HCO_3^-]v) were calculated using the solubility of carbon dioxide (α_{CO_2}), the apparent pK (pK$_{app}$) for dogfish plasma, and rearrangements of the Henderson–Hasselbalch equation according to Boutilier et al. (1984).

Plasma ammonia-N was measured enzymatically (L-glutamate dehydrogenase; Raichem Ammonia Reagent; Product No. 85446; Mondzac et al., 1965) on the first thaw of frozen plasma. Plasma total free amino acid levels (FAA-N) were measured using the ninhydrin assay (Moore, 1968), with subtraction of previously measured ammonia-N, owing to the partial detection of ammonia by the ninhydrin method (Kajimura et al., 2004). The correction was small, because plasma ammonia-N concentrations were less than 3% of FAA-N concentrations. Plasma urea-N was measured with the diacetyl monoxime method (Rahmatullah and Boyde, 1980).

Plasma TMAO-N levels were assayed by the ferrous sulphate and EDTA method (Wekell and Barnett, 1991). Plasma glucose was determined with hexokinase reagent (Thermotrace kit 1542; ThermoElectron Corp., Waltham, MA, USA). Plasma β-hydroxybutyrate was measured enzymatically (β-hydroxybutyrate dehydrogenase) by the method of McMurray et al. (1984) using Stanbio Laboratory kit 2440 (Boerne, TX, USA). Plasma creatinine-N was measured by the colorimetric assay of Heinegarde and Tiderstrom (1973) (Sigma kit 555; Sigma, St Louis, MO, USA). Plasma chloride was measured by coulometric titration (Radiometer CMT-10; Copenhagen, NV, Denmark) and osmolality by vapour pressure osmometry (Wescor 5100C; Westcor Inc., Logan, UT, USA).

Seawater ammonia-N and urea-N concentrations were determined by the salicylate hypochlorite (Verdouw et al., 1978) and diacetyl monoxime (Rahmatullah and Boyde, 1980) methods, respectively.

The flatfish muscle paste (four replicates) was analysed for N-metabolites in order to estimate the nitrogen load administered in a meal. The frozen paste was pulverized in a mortar and pestle, which was chilled with liquid nitrogen, then homogenized in 0.5 mol l^{-1} $HClO_4$ and centrifuged (4°C, 10 000 g, 5 min). Pellets were washed twice with 0.5 mol l^{-1} $HClO_4$, then the original and wash supernatants were pooled, neutralized with 3 mol l^{-1} KOH and then centrifuged (4°C, 10 000 g, 5 min) to remove precipitated $KClO_4$. The pellet was resuspended in 0.5 mol l^{-1} KOH, incubated for 1 h at 37°C, then analysed for total protein content by the dye-binding assay of Bradford (1976), using Sigma reagent and bovine serum albumin as standard. A standard N-content of 0.16 g-N g^{-1} protein was assumed (Cho, 1990). The final pooled supernatant from the washes was analysed for ammonia-N, urea-N, TMAO-N, FAA-N and creatinine-N using the methods outlined above for plasma.

Statistics

Data are reported as means ± 1 S.E.M. (N). Pre-feeding measurements were first compared using Student's two-tailed t-test (unpaired) to ensure absence of pre-existing differences between experimental and control groups (there were none). The post-feeding data were then subjected to two-way (time, treatment) analysis of variance (ANOVA) to detect overall effects of feeding. Student's t-tests (unpaired) were then applied to detect specific differences between experimental and control groups at the same sampling time, indicated by asterisks. One-way analysis of variance followed by the LSD (least significant difference) test was applied to detect specific differences within a treatment group (control or experimental); means not sharing the same case letters are significantly different. Dunnett's paired multiple comparison test was also employed to detect specific differences within a treatment group (control or experimental), relative to the pre-feeding value, and yielded the same results as those of the more comprehensive LSD test, so only the latter are reported. A significance level of 0.05 was used throughout.

Results

The influence of experimental feeding on blood gases and acid–base status

These parameters were the major focus of Series 1. Pre-feeding arterial pHa (~7.90) was approximately 0.1 unit higher than pHv, while $[HCO_3^-]$a (~3.8 mmol l^{-1}) was ~0.5 mmol l^{-1} lower than $[HCO_3^-]$v. The administration of saline into the stomach in the control treatment had no effect on these parameters (Figs 1A, 2A). However, the administration of a meal of flatfish muscle in the experimental treatment caused
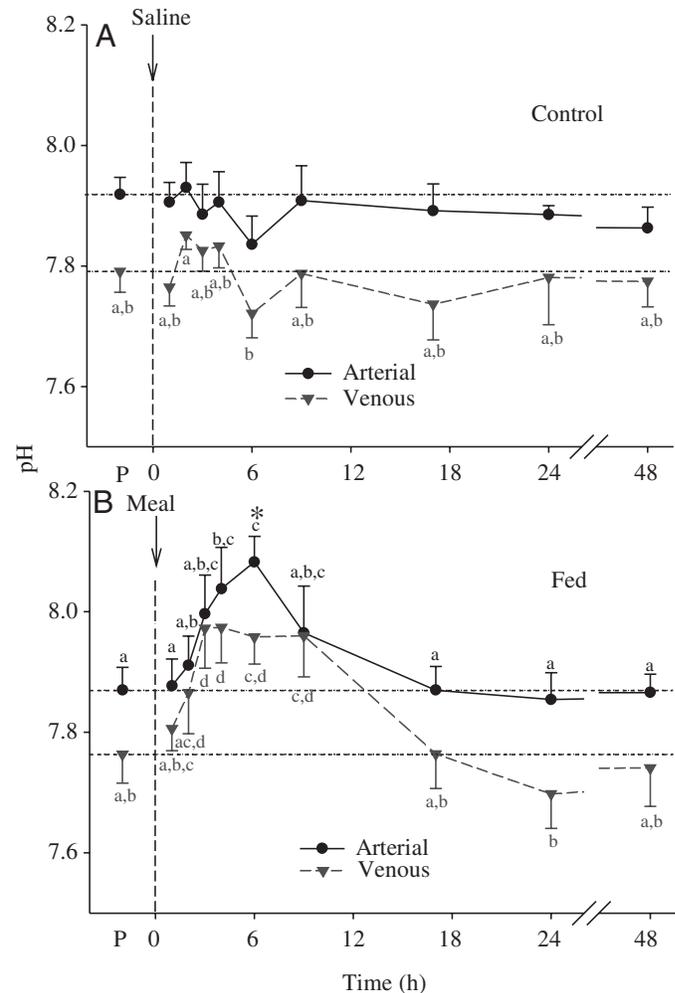

Fig. 1. The influence of (A) the control treatment or (B) experimental feeding on arterial pH (pHa; circles) and venous pH (pHv; triangles) in *Squalus acanthias* of Series 1. The experimental meal was 20 g of flatfish muscle paste in 20 ml of 140 mmol l^{-1} NaCl saline per kg dogfish, administered as a bolus down the stomach tube at time 0 h. The control treatment was comparable gastric loading of 40 ml of 140 mmol l^{-1} NaCl saline per kg dogfish. Values are means ± 1 S.E.M. (N=5). P represents the pre-feeding measurement (horizontal dotted lines). The overall influence of experimental feeding was significant for both pHa and pHv (two-way ANOVA, $P \leq 0.05$). Within each treatment, means not sharing the same letter are significantly different (one way ANOVA followed by the LSD test). Asterisks indicate significant differences between control and experimental treatments for the same parameter at the same time.
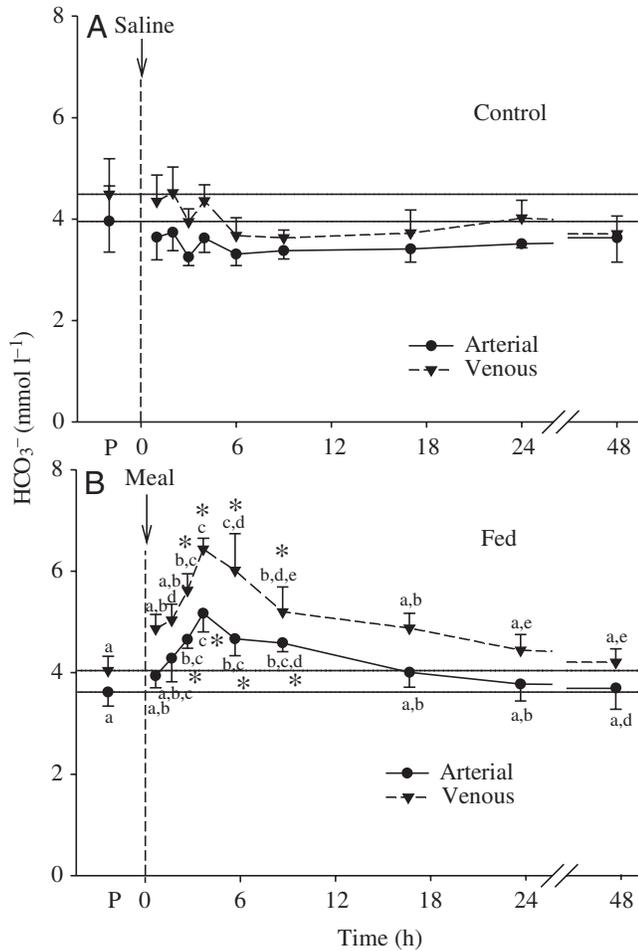
Fig. 2. The influence of (A) the control treatment or (B) experimental feeding on arterial true plasma bicarbonate ([HCO_3^-]a; circles) and venous true plasma bicarbonate ([HCO_3^-]v; triangles) concentrations in *Squalus acanthias* of Series 1. Values are means ± 1 s.e.m. (N=5). Other details as in legend of Fig. 1. The overall influence of experimental feeding was significant for both [HCO_3^-]a and [HCO_3^-]v (two-way ANOVA, $P \le 0.05$).

Fig. 3. The influence of (A) the control treatment or (B) experimental feeding on arterial CO_2 tension ($\dot{P}a_{CO_2}$; circles) and venous CO_2 tension ($\dot{P}v_{CO_2}$; triangles) in *Squalus acanthias* of Series 1. Values are means ± 1 s.e.m. (N=5). (1 torr=0.1333 kPa.) Other details as in legend of Fig. 1. There was no overall significant influence of experimental feeding for either parameter (two-way ANOVA, $P > 0.05$).

marked increases (0.2–0.25 units) in both pHa and pHv that were significant overall (two-way ANOVA) and at individual time points from 3 h to 9 h post-feeding but that had attenuated by 17 h (Fig. 1B). [HCO_3^-]a and [HCO_3^-]v increased significantly and then declined over the same time course (Fig. 2B), indicative of a classic alkaline tide. The increase in [HCO_3^-]v (~2.5 mmol l^{-1}) was larger than that (~1.5 mmol l^{-1}) in [HCO_3^-]a, so the arterial–venous difference approximately doubled (Fig. 2B).

Pre-feeding arterial $\dot{P}a_{CO_2}$ (~1 torr) was ~0.5 torr below $\dot{P}v_{CO_2}$, while $\dot{P}a_{O_2}$ (~100 torr) was much higher than $\dot{P}v_{O_2}$ (~20 torr; note that 1 torr=0.1333 kPa). These parameters were again unaffected by the loading of saline into the stomach (Figs 3A, 4A). Experimental feeding had negligible influence on $\dot{P}a_{CO_2}$ and $\dot{P}v_{CO_2}$; the former was very slightly elevated at 2 h only, and there were no overall effects of treatment (two-way ANOVA), indicating a lack of respiratory compensation
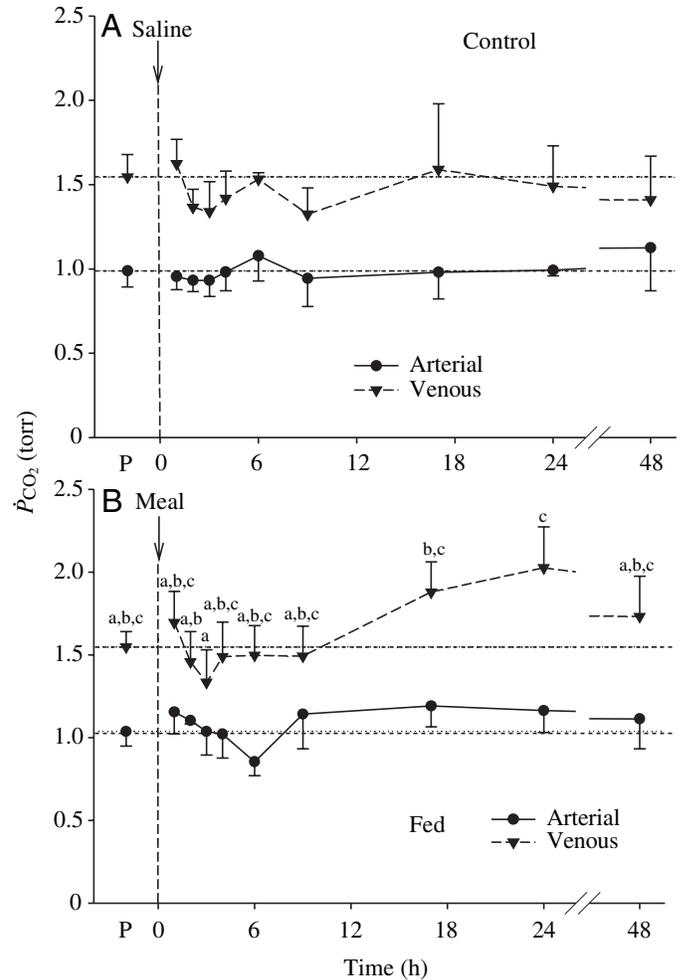
for the alkaline tide (Fig. 3B). $\dot{P}a_{O_2}$ also remained constant at pre-feeding levels, but $\dot{P}v_{O_2}$ declined by ~50% from 1 h to 6 h post-feeding (significant relative to the control group at 3 h and 6 h; Fig. 4B), reflecting greater O_2 utilization from the blood.

The influence of experimental feeding on ammonia-N and urea-N excretion

No differences were detected in N-excretion rates between the control and experimental treatments in Series 1, but the rates, particularly those of urea-N, were high and variable, probably due to minor blood loss associated with anal ligation, struggling and/or fin abrasion. The protocol was modified in Series 2 to avoid these problems (see Materials and methods). Figs 5 and 6 present data from those fish of Series 2 (control N=5, experimental N=4) where there was no struggling or potential for blood loss from fin abrasion. When data from all the fish of Series 2 (control N=8, experimental N=8) were
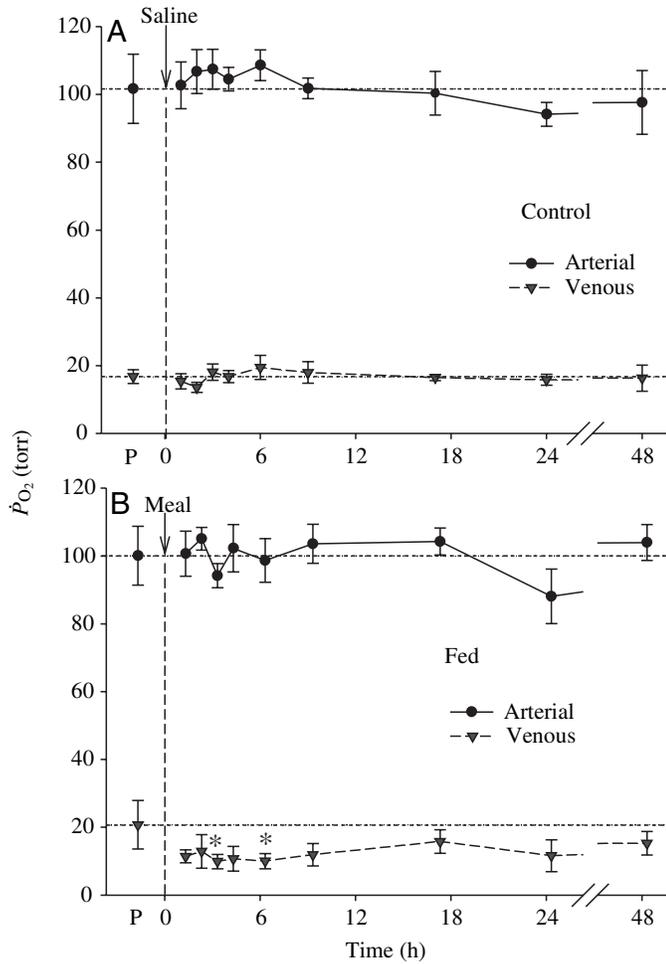
Fig. 4. The influence of (A) the control treatment or (B) experimental feeding on arterial O_2 tension ($\dot{P}a_{O_2}$; circles) and venous O_2 tension ($\dot{P}v_{O_2}$; triangles) in *Squalus acanthias* of Series 1. Values are means ± 1 S.E.M. (*N*=5). (1 torr=0.1333 kPa.) Other details as in legend of Fig. 1. There was a significant overall influence of experimental feeding only for $\dot{P}v_{O_2}$ (two-way ANOVA, $P \leqslant 0.05$).
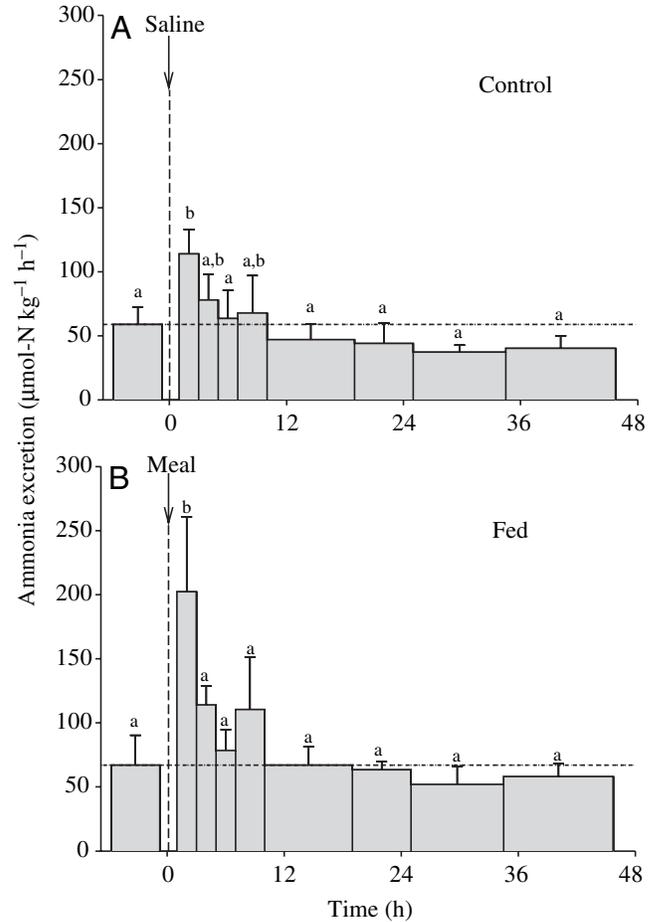

Fig. 5. The influence of (A) the control treatment or (B) experimental feeding on the rate of ammonia-N excretion in *Squalus acanthias* of Series 2. Other details as in legend of Fig. 1. There was a significant overall influence of experimental feeding (two-way ANOVA, $P \leqslant 0.05$). There were no significant differences between control and experimental treatments at the same time. Values are means ± 1 S.E.M. Data were taken from those dogfish of Series 2 (control *N*=5, experimental *N*=4) where there was no struggling or potential for blood loss from fin abrasion.

analysed, trends and statistical significance were identical, although variability was somewhat greater.

Prior to feeding, urea-N excretion (~750 μmol-N kg^{-1} h^{-1}) was approximately 13-fold greater than ammonia-N excretion (~60 μmol-N kg^{-1} h^{-1}). The administration of saline into the stomach of control dogfish caused a small elevation in ammonia-N excretion at 0–2 h (Fig. 5A). Experimental feeding caused a slightly larger increase in ammonia-N excretion that was significant at 0–2 h and also significantly greater overall relative to the control treatment (two-way ANOVA), although there were no significant individual differences between the two treatments at particular time points (Fig. 5B). Over 45 h, the elevation in ammonia-N excretion amounted to ~700 μmol-N kg^{-1}.

Urea-N excretion was unaffected by saline loading in the control group apart from an unexplained increase in the latter at 6–9 h post-infusion (Fig. 6A). Urea-N excretion was not increased by experimental feeding. Overall urea-N excretion

over 45 h was actually lower in the experimental fish by ~3200 μmol-N kg^{-1}, although two-way ANOVA revealed no significant effect of treatment (Fig. 6B).

The measured N-content in the meal of flatfish muscle (Table 1) helps put these small differences in ammonia-N and urea-N excretion into perspective. The total N-content amounted to 1625 μmol-N g^{-1} muscle, or ~32 500 μmol-N kg^{-1} dogfish, in the meal, more than 90% of which was in the form of protein (Table 1). At the pre-feeding N excretion rates (ammonia-N=60 μmol-N kg^{-1} h^{-1}, urea-N=750 μmol-N kg^{-1} h^{-1}), the N-content of the meal would be lost in ~40 h.

The influence of experimental feeding on plasma metabolites and ionoregulatory status

Absolute levels and responses of plasma metabolites were similar in Series 1 and 2, so the more extensive data set of the
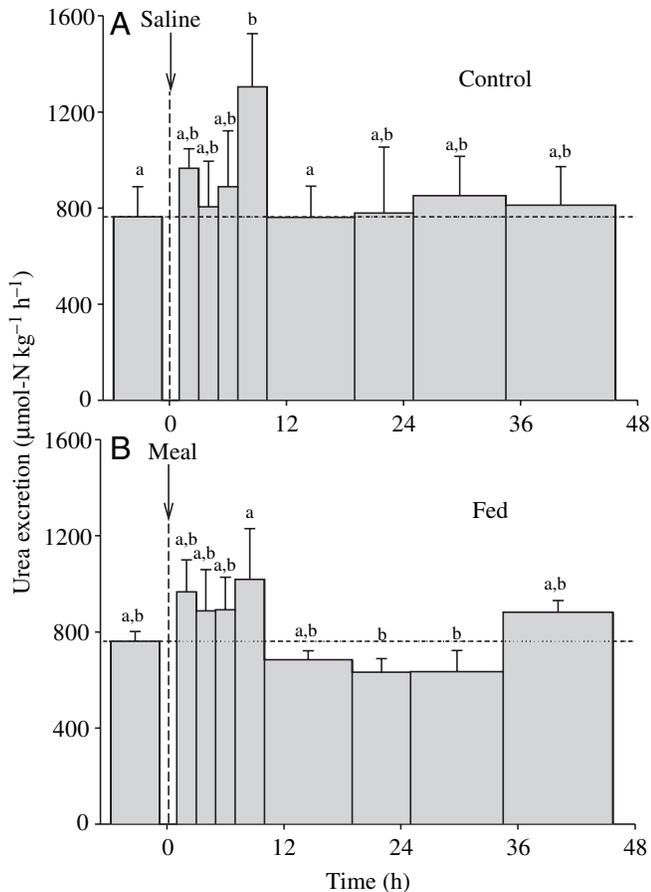
Fig. 6. The influence of (A) the control treatment or (B) experimental feeding on the rate of urea-N excretion in *Squalus acanthias* of Series 2. Values are means ± 1 S.E.M. (control *N*=5, experimental *N*=4). Other details as in legends of Figs 1 and 5. There was no significant overall influence of experimental feeding (two-way ANOVA, *P*>0.05). There were no significant differences between control and experimental treatments at the same time.

latter (arterial values) is presented. Of the plasma parameters measured in Series 1 (ammonia-N, urea-N, glucose, Cl$^-$), the only detectable arterial–venous difference was in ammonia-N, which was approximately 50 μmol l^{-1} higher on the venous side.

Pre-feeding plasma ammonia-N levels were extremely low (~150 μmol-N l^{-1}) relative to plasma urea-N (~800 mmol-N l^{-1}) and plasma TMAO-N levels (~70 mmol-N l^{-1}; Fig. 7). The administration of saline caused no change in plasma ammonia-N in the control treatment but there was a persistent

elevation in plasma ammonia-N associated with experimental feeding, which was significant overall (two-way ANOVA), with individual significant differences at 4 h, 18 h and 45 h post-feeding (Fig. 7B). Plasma urea-N levels tended to rise slightly in both treatments (significant only in the control group at 18 h), but there was no overall effect of feeding (two-way ANOVA) and no significant differences at any time between the two treatments (Fig. 7A). Plasma TMAO-N concentration was marginally affected by feeding, with a significantly higher value at 4 h in the fed group but no overall effect (two-way ANOVA) or other specific differences (Fig. 7C). Other plasma N-compounds (FAA-N ~12 mmol-N l^{-1}, creatinine-N ~0.15 mmol-N l^{-1}), as well as glucose (~6 mmol l^{-1}) and β-hydroxybutyrate (~4.5 mmol l^{-1}) were similarly little affected by the treatments (Table 2). However both FAA and β-hydroxybutyrate were lower after feeding overall (by two-way ANOVA), although there were no significant differences at specific time points (Table 2).

Plasma osmolality (~940 mOsmol kg^{-1}) exhibited a clear response to experimental feeding, with a post-feeding elevation that was significant overall relative to the control treatment (two-way ANOVA), with individual significant differences at 2 h, 4 h, 6 h, 9 h, 18 h and 34 h (Fig. 8A). There was a slight tendency for osmolality to decline in the control group. Post-feeding differences averaged ~25 mOsmol kg^{-1} between the two treatments. Changes in plasma Cl$^-$ concentration (~255 mmol l^{-1} prior to feeding) explained part of this difference (Fig. 8B). Cl$^-$ tended to rise in the experimentally fed dogfish and fall initially in the saline-loaded control animals. The difference was ~10 mmol l^{-1}, accounting for ~40% of the osmolality difference, and was highly significant overall (two-way ANOVA), although the only individual significant difference was at 18 h.

Discussion

The alkaline tide

Experimental feeding clearly induced an alkaline tide in both the arterial and venous blood of *Squalus acanthias*, evidenced by marked increases in pH (Fig. 1) and plasma [HCO$_3^-$] (Fig. 2) from 3 to 9 h after the meal. To our knowledge, this is the first report of an alkaline tide in elasmobranchs, or indeed in any fish species, although it is a commonly reported phenomenon in amphibians, reptiles and mammals (Wang et al., 2001). The response (reviewed by Hersey and Sachs, 1995; Sachs et al., 1995; Niv and Fraser, 2002) reflects the addition of metabolic base to the blood by parietal cells of the gastric

Table 1. *The concentrations (μmol-N g^{-1}) of various nitrogen compounds in the paste of flatfish muscle, prior to dilution with saline vehicle[1]*

Protein-N	FAA-N[2]	Ammonia-N	TMAO-N[3]	Urea-N	Creatinine-N
1534.9	46.0	6.7	23.1	13.2	1.1

[1]An experimental meal consisted of 20 g of muscle paste per kg dogfish, diluted in 20 ml of 140 mmol l^{-1} NaCl vehicle per kg dogfish; [2]free amino acids; [3]trimethylamine oxide.
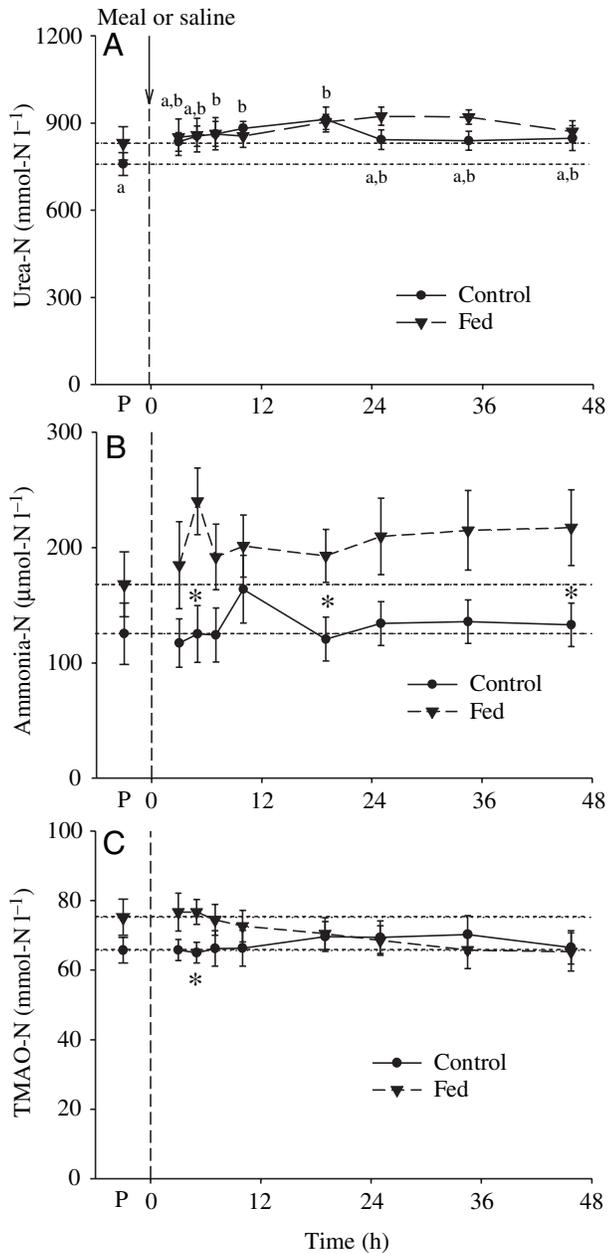
Fig. 7. The influence of experimental feeding (triangles) or the control treatment (circles) on (A) plasma urea-N (B) plasma ammonia-N and (C) plasma trimethylamine oxide-N (TMAO-N) concentrations in *Squalus acanthias* of Series 2. Note the different units (μmol l^{-1} for ammonia-N *versus* mmol l^{-1} for urea-N and TMAO-N). Values are means ± 1 S.E.M. (*N*=8). Other details as in legend of Fig. 1. There was a significant overall influence of experimental feeding only on plasma ammonia-N (two-way ANOVA, $P \leq 0.05$) but not on plasma urea-N or TMAO-N.

mucosa. When these cells are activated to secrete HCl into the stomach to facilitate digestion, basolateral efflux of HCO_3^- (metabolic base) into the extracellular fluid compartment matches the rate of apical H^+ secretion into the lumen. H^+ and HCO_3^- are created by CO_2 hydration, catalysed by intracellular carbonic anhydrase. A K^+-stimulated, H^+-translocating

ATPase is responsible for the apical H^+ secretion, and a Cl^-/HCO_3^- exchanger for the basolateral HCO_3^- export and Cl^- entry. Cl^- is believed to exit apically *via* a Cl^- channel, such that there is a net secretion of HCl into the stomach. A K^+-stimulated, H^+-ATPase very similar to that of mammals has been localized in gastric gland cells of the elasmobranch *Dasyatis sabina* (Smolka et al., 1994), and carbonic anhydrase is abundant in the gastric tissue of *S. acanthias* (Maren, 1967).

Unlike members of other vertebrate classes (see Andrade et al., 2004), the dogfish exhibited virtually no evidence of respiratory compensation (i.e. elevation of $\dot{P}_{CO_2}$ to offset the rise in pH; Fig. 3). The likely reason for this difference is that the dogfish breathes exclusively with its gills. Because of the much lower solubility of O_2 relative to CO_2 in water, the gills of most water-breathing animals are greatly hyperventilated with respect to CO_2 excretion, such that ventilatory adjustments have only a small effect on blood $\dot{P}_{CO_2}$ levels (Perry and Wood, 1989). Furthermore, although ventilation was not measured in the present study, it was our impression that the dogfish were ventilating more deeply after a meal, during the period when $\dot{P}_{V_{O_2}}$ was decreased (Fig. 4B). This would tend to enhance rather than inhibit CO_2 excretion. The dogfish gill is particularly efficient at CO_2 excretion because the HCO_3^- dehydration reaction is catalysed in part by extracellular carbonic anhydrase, which is both present on the endothelial surfaces of gill blood vessels and circulates freely in the blood plasma (Wood et al., 1994; Gilmour et al., 1997, 2001; Henry et al., 1997; Wilson et al., 2000). It may be virtually impossible for the dogfish to 'retain CO_2'. In future studies, use of a K^+-stimulated, H^+-ATPase inhibitor such as omeprazole (see Andrade et al., 2004) may prove informative to separate changes in acid–base status resulting from augmented gastric acid secretion from those associated with altered ventilation and metabolic rate.

While ventilatory control of acid–base status appears to be minimal, the ability of elasmobranchs to correct acid–base disturbances *via* ion *versus* acidic/basic equivalent exchange mechanisms at the gills is well established (e.g. Heisler, 1988). Their ability to resist the alkalinizing influence of basic equivalent loading is particularly impressive (Wood et al., 1995; Tresguerres et al., 2005). Thus while the alkaline tide had been corrected by 17 h post-feeding (Figs 1, 2), this does not necessarily mean that the gastric H^+ secretion responsible for the phenomenon had subsided, but rather that branchial base excretion mechanisms had fully caught up with the rate of metabolic base addition to the blood by the acid-secreting cells of the gastric mucosa. Indeed, autopsies revealed that digestion continued right up to 48 h (and probably longer) and that some food paste still remained in the stomach by this time. Interestingly, food remnants had not reached the anus by this time, so initial concerns (Series 1) about defecation contaminating N-flux measurements proved unfounded. A slow time course (relative to that of teleosts) for digestion, assimilation and gut passage appears to be characteristic of elasmobranchs (Jones and Geen, 1977; Wetherbee et al., 1987; Schurdak and Gruber, 1989; Wetherbee and Gruber, 1990; Cortes and Gruber, 1990; Sims et al., 1996).

Table 2. *Changes in arterial plasma concentrations of free amino acid-N, creatinine-N, glucose and β-hydroxybutyrate in dogfish subjected to either experimental feeding or the control treatment*

	Pre-feeding	2 h	4 h	6 h	9 h	18 h	24 h	34 h	45 h
FAA-N (mmol-N l^{-1})									
Fed	11.8±0.6	11.8±0.6†	12.0±0.6†	11.7±0.5†	11.7±0.5†	11.5±0.5†	11.5±0.5†	11.4±0.5†	11.3±0.4†
Control	12.7±0.6	13.1±0.6	13.0±0.8	13.2±0.6	12.7±0.6	12.4±0.6	12.4±0.6	12.2±0.6	12.0±0.6
Creatinine-N (mmol-N l^{-1})									
Fed	0.14±0.01	0.15±0.01	0.15±0.02	0.14±0.01	0.14±0.01	0.14±0.02	0.13±0.01	0.13±0.01	0.13±0.02
Control	0.15±0.1	0.14±0.01	0.14±0.01	0.15±0.01	0.14±0.01	0.14±0.01	0.14±0.01	0.14±0.01	0.13±0.01
Glucose (mmol l^{-1})									
Fed	6.7±0.5	6.9±0.6	6.4±0.5	6.6±0.7	6.2±0.7	6.5±0.8	7.1±0.7	7.4±1.0	6.8±0.9
Control	5.7±0.9	6.3±0.8	6.0±0.7	5.8±0.7	5.9±0.7	6.0±0.7	6.1±0.7	6.1±0.6	6.4±0.7
β-HB (mmol l^{-1})									
Fed	3.8±1.0	3.8±1.0†	3.7±1.1†	3.4±1.0†	3.4±1.1†	3.4±1.1†	3.2±1.2†	2.6±1.2†	2.2±0.9†
Control	5.2±0.9	4.5±1.0	4.6±0.9	4.7±1.0	4.8±1.0	4.7±1.1	4.6±1.3	4.3±1.2	4.6±1.2

An experimental meal comprised a gastric loading of 20 g flatfish muscle paste in 20 ml of 140 mmol l^{-1} NaCl saline per kg dogfish, while the control treatment comprised a gastric loading of 40 ml of 140 mmol l^{-1} NaCl saline per kg dogfish.

Values are means ± 1 S.E.M. (N=8); † designates a significant overall effect of feeding (two-way ANOVA, $P \leq 0.05$). There were no significant differences ($P>0.05$) within treatment groups or between control and experimental treatments at specific times. FAA-N, free amino acid-N; β-HB, β-hydroxybutyrate.

Using several approaches, it is possible to make a rough estimate of the rate at which metabolic base was added to the blood by the acid-secreting cells of the stomach. One approach is simply by comparison to the recent work on *S. acanthias* of Tresguerres et al. (2005), who found that a HCO$_3^-$ infusion rate of 1000 µmol kg^{-1} h^{-1} induced a slightly larger metabolic alkalosis than seen in the present study (Figs 1, 2). Another approach is to employ the traditional technique pioneered by Rune (1965, 1966) and now widely used in humans (Niv and Fraser, 2002), whereby the rate at which 'base excess' is generated in the blood is measured, factored by a distribution space (0.3 of body mass). Applying this method, and a blood non-HCO$_3^-$ buffer capacity of 9 slykes (9 µmol pH unit^{-1} g^{-1}) for *S. acanthias* (Lenfant and Johansen, 1966), to the data of Figs 1 and 2 from hours 1–4 post-feeding yields a rate of ~475 µmol kg^{-1} h^{-1}. This is undoubtedly an underestimate as some metabolic base excretion across the gills would have already started during this period (Wood et al., 1995). Thus, the rate of endogenous metabolic base generation associated with digesting the experimental meal in the dogfish was in the range of 475–1000 µmol kg^{-1} h^{-1}. By way of comparison, the reported rate in human subjects after a normal meal of mixed composition is ~425 µmol kg^{-1} h^{-1} (Rune, 1966). It would be interesting to test whether an even larger experimental meal would cause a larger alkaline tide in the dogfish, or whether a maximal rate has been achieved in the present study.

The literature is unclear with respect to the pattern of gastric acid secretion in carnivorous elasmobranchs. Some reports (Sullivan, 1905; Caira and Jolitz, 1989) indicate that stomach pH is close to neutrality in fasting animals, with much lower values in fed animals, including *S. acanthias* (Sullivan, 1905), in accord with the present results indicating a sharp increase in secretion after feeding. Others have reported a markedly acidic

gastric pH regardless of the presence or absence of food in the stomach (Babkin et al., 1935; Menon and Kewalramani, 1959; Williams, 1970). A recent detailed study (Papastamiatou and Lowe, 2004) using indwelling, continuously recording pH sensors in the stomachs of naturally feeding leopard sharks (*Triakis semifasciata*) reported an extremely acid stomach (pH ~1.5) in starved animals, while feeding was associated with a sharp rise in pH (to ~3.5) followed by a progressive slow acidification thereafter. The data were interpreted as reflecting a continuous low level of acid secretion during fasting, which was accelerated after feeding. The buffering action of the food was responsible for the initial rapid rise in pH and for retarding the later fall in pH. It seems probable that all elasmobranchs increase gastric acid secretion after feeding but that there may be inter-species differences in whether it is completely turned off between meals. In future studies, it will be of interest to track the pH and buffer capacity of the stomach contents over time after feeding in *S. acanthias* to evaluate the magnitude and temporal relationship between the rate of gastric acid secretion and the alkaline tide in the blood.

Nitrogen conservation after feeding

Experimental feeding of the ureotelic dogfish resulted in no increase in urea-N excretion (indeed, a non-significant decrease; Fig. 6) and only a very small rise in ammonia-N excretion (Fig. 5), amounting to less than 3% of the total-N in the meal. The increase in ammonia-N excretion was accompanied by a modest increase in plasma ammonia-N concentration (Fig. 7B). This is very different from the situation in ammoniotelic teleosts, where ammonia-N excretion and plasma ammonia-N may rise many-fold after a meal, and even urea-N excretion may increase modestly (reviewed by Wood, 2001). These results therefore support the
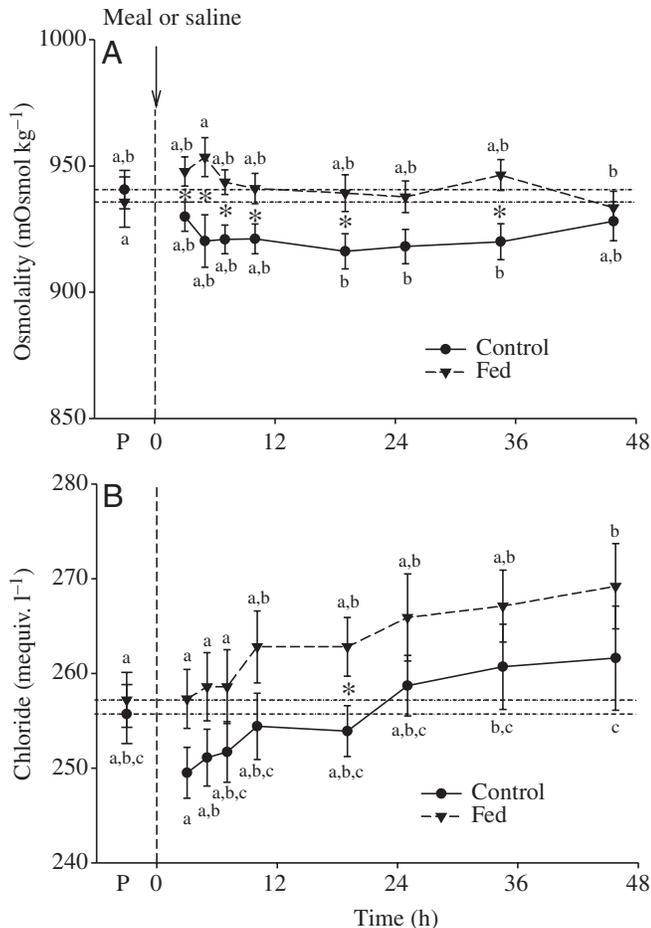
Fig. 8. The influence of experimental feeding (triangles) or the control treatment (circles) on (A) plasma osmolality and (B) plasma chloride concentration in *Squalus acanthias* of Series 2. Values are means ± 1 S.E.M. (*N*=8). Other details as in legend of Fig. 1. There were significant overall influences of experimental feeding on both osmolality and chloride (two-way ANOVA, $P \leqslant 0.05$).

prediction of Wood (2001) that elasmobranchs may be so N-limited as to excrete virtually no 'excess nitrogen' after feeding. Inasmuch as ammonia-N excretion increased slightly, they also support the prediction of Mommsen and Walsh (1991) that any elevation in N-loss that does occur should be in the form of ammonia-N, because of the high cost of urea-N synthesis.

This virtual absence of a post-prandial rise in N-excretion was not an artifact of the experimental feeding approach or disturbance, because a very similar result (no change in urea-N excretion, small rise in ammonia-N excretion) was seen in our companion study on naturally feeding, uncannulated dogfish (M.K., P.J.W., T.P.M. and C.M.W., unpublished results), where plasma urea-N, ammonia-N and TMAO-N concentrations were similar to those of the present study. Notably, in that study, total N-excretion rates were only ~60% of those reported here, both before and after feeding, principally due to a 40% lower rate of urea-N excretion. Despite the precautions taken to minimise disturbance in Series

2, it appears that cannulation and experimental handling inevitably increase the urea-N 'leakiness' of the animals. Similarly elevated baseline rates were reported in an earlier cannulation study on *S. acanthias* (Wood et al., 1995) and possibly may result from an inhibitory action of catecholamines on the active urea retention mechanism in the gills (Pärt et al., 1998), discussed below.

The urea-dependent osmoregulatory strategy of elasmobranchs, as first documented by Smith (1930), entails the maintenance of a massive urea-N gradient from body fluids to seawater, and a substantial, continuous loss of urea-N across the gills is unavoidable even under normal conditions (Boylan, 1967; Perlman and Goldstein, 1988). At the pre-feeding N-excretion rates (ammonia-N=60 μmol-N kg^{-1} h^{-1}, urea-N=750 μmol-N kg^{-1} h^{-1}) measured in the present study (Figs 5, 6), the N-content of the meal (32 500 μmol-N kg^{-1} dogfish) would be lost in ~40 h, or in ~67 h at the lower excretion rates measured (M.K., P.J.W., T.P.M. and C.M.W., unpublished results) for uncannulated animals. These simple calculations, coupled with an extensive literature indicating that feeding is sporadic, irregular and opportunistic in sharks (Jones and Geen, 1977; Cortes and Gruber, 1990; Hanchet, 1991; Tanasichuk et al., 1991), suggest that these elasmobranchs may be severely N-limited in nature, such that N-conservation after feeding would be a high priority. Indeed, dietary protein restriction eliminated the ability of the dogfish *Scyliorhinus stellaris* to raise plasma urea-N in response to osmotic challenge (Armour et al., 1993). Plasma urea-N levels and osmolality progressively fell during prolonged starvation in *S. acanthias* (Leech et al., 1979) and in the pyjama shark, *Poroderma africanum* (Haywood, 1973), and rose rapidly upon re-feeding in the latter. In the present study, plasma osmolality increased significantly after feeding (Fig. 8A), but without any detectable post-prandial rises in plasma urea-N (Fig. 7A). However, in naturally feeding *S. acanthias*, osmolality and urea-N concentrations in the blood plasma were significantly elevated at 20 h after a meal (M.K., P.J.W., T.P.M. and C.M.W., unpublished results); increases in plasma urea-N, TMAO-N and Cl$^-$ concentrations (discussed below) all contributed to the phenomenon.

Several mechanisms probably contribute to N-conservation, particularly after feeding. Firstly, the elasmobranch gill is uniquely designed to minimize both urea-N and ammonia-N permeability, while maintaining permeability to respiratory gases (Boylan, 1967). Wood et al. (1995) calculated that branchial urea-N permeability in *S. acanthias* was only ~7% of that in a typical teleost, while branchial ammonia-N permeability was only ~4%. A high cholesterol:phospholipid ratio in the basolateral membranes of the gill epithelium appears to play an important role in this selective permeability (Fines et al., 2001). Urea-N retention is also achieved by a low apical membrane permeability and a 'back-transport' mechanism for urea in the gill epithelium (Pärt et al., 1998) that is basolaterally located, ATP-dependent and Na$^+$-coupled (Fines et al., 2001). It would be interesting to test whether the activity of this transporter increases after feeding. Ammonia-

N retention has been attributed to ammonia scavenging by high-affinity glutamine synthetase in gill cells (Wood et al., 1995). However, more importantly, glutamine synthetase activities increase substantially to trap ammonia-N in a number of other tissues, particularly the liver, and there is a clear activation of the ornithine urea cycle enzymes for urea-N synthesis in both the liver and extra-hepatic tissues after natural feeding (M.K., P.J.W., T.P.M. and C.M.W., unpublished results). Finally, by analogy to teleosts, it is likely that protein synthesis for growth is strongly stimulated after feeding (reviewed by Carter and Houlihan, 2001; Wood, 2001), although to our knowledge, this has never been evaluated in elasmobranchs. One indication that this may be occurring is the decrease in $\dot{P}_{V_{O_2}}$ of venous blood returning from the trunk musculature (Fig. 4), suggestive of increased metabolic expenditures in muscle at this time.

Changes in plasma composition associated with feeding

Creatinine-N and TMAO-N were measured because of recent interest in the metabolism of these N-products (see Walsh and Mommsen, 2001) and the importance of the latter as a stabilizing osmolyte (Yancey, 2001). Creatinine is a breakdown product of muscle creatine, while TMAO is probably of exogenous origin. Creatinine-N levels did not change after experimental feeding (Table 2). However, plasma TMAO-N levels rose very slightly (Fig. 7C) both in this study and in naturally feeding dogfish (M.K., P.J.W., T.P.M. and C.M.W., unpublished results). Some elasmobranchs can synthesize TMAO, but it appears that *S. acanthias* cannot (Goldstein et al, 1967; Goldstein and Palatt, 1974), so TMAO-N is thought to come from the diet, where it may be more abundant than urea-N (e.g. Table 1).

In general, aerobic metabolism in elasmobranchs appears to rely heavily on the oxidation of ketone bodies and, to a lesser extent, amino acids, with reduced reliance on carbohydrate and almost no usage of fatty acids (Ballantyne, 1997). Zammit and Newsholme (1979) reported that prolonged starvation (up to 150 days) caused no change in plasma glucose but a marked rise in plasma β-hydroxybutyrate levels in *Sc. stellaris*, while deRoos et al. (1985) described similar responses in *S. acanthias* during a much shorter period of fasting (up to 9 days). Richards et al. (2003) reported an increased uptake of β-hydroxybutyrate but no change in glucose uptake associated with elevated post-exercise metabolism in a perfused trunk muscle preparation of *S. acanthias*. The constancy of blood glucose and the modest declines in β-hydroxybutyrate and FAA-N levels in plasma after feeding in the present study (Table 2) are in accord with this scenario. Plasma FAA-N levels did not show an initial surge after experimental feeding (Table 2), in marked contrast to teleosts, where large increases occur (reviewed by Wood, 2001). Only a small delayed rise in plasma FAA-N levels at 30 h after natural feeding was detected (M.K., P.J.W., T.P.M. and C.M.W., unpublished results). However, plasma levels are not necessarily representative of flux. It is possible that relatively slow digestion and absorption in the dogfish, coupled with increased usage of amino acids as

an oxidative substrate and for protein synthesis, resulted in little apparent change in FAA-N concentrations.

Perhaps the most surprising change in plasma composition was the progressive rise in Cl⁻ (Fig. 8B), which explained ~40% of the rise in plasma osmolality (Fig. 8A). The same phenomenon was seen in naturally feeding dogfish (M.K., P.J.W., T.P.M. and C.M.W., unpublished results). We hypothesise that the explanation lies in the powerful acid–base exchange mechanisms in the gills of marine elasmobranchs (Payan and Maetz, 1973; Bentley et al., 1976; Heisler, 1988; Claiborne and Evans, 1992). Apical Na^+/H^+ exchange powered by basolateral Na^+/K^+-ATPase appears to occur in one type of mitochondria-rich cell, and apical Cl^-/HCO_3^- exchange powered by basolateral $V-H^+$-ATPase in another (Wilson et al., 1997, 2002; Piermarini and Evans, 2001; Piermarini et al., 2002; Claiborne et al., 2002; Edwards et al., 2002; Tresguerres et al., 2005). An ongoing activation of net Cl^- uptake in exchange for the export of metabolic base by the latter mechanism – i.e. compensation of the alkaline tide – would explain the increase in plasma Cl^-. In future studies, it will be of interest to evaluate whether the rectal gland (Shuttleworth, 1988) is activated to handle this Cl^- load indirectly associated with feeding.

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