

The spiny dogfish *Squalus acanthias* L. maintains osmolyte balance during long-term starvation

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Over 56 days of starvation, spiny dogfish *Squalus acanthias* lost c. 0.3% body mass per day and condition factor and hepato-somatic index declined, but plasma osmolality levels remained higher than seawater levels for the entire period; major osmolytes in the plasma, such as urea, trimethylamine oxide and inorganic ions did not change appreciably. Urea was always the dominant nitrogen waste and was excreted at a constant rate over the 56 day starvation period, suggesting that a minimum rate of urea loss to the environment is unavoidable. Significant amounts of unknown-nitrogen compounds were also excreted at rates higher than that of ammonia. The dogfish can maintain its osmolytes constant despite losing large amounts of nitrogen-rich urea, and therefore maintains plasma hyperosmotic regulation over long-term starvation.

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INTRODUCTION

It is well known that the plasma of marine elasmobranchs, including sharks, skates and rays, is kept isosmotic or slightly hyperosmotic to their surroundings (Smith, 1931; Holmes & Donaldson, 1969; Haywood, 1973; Hazon *et al.*, 1997). To maintain the high osmolality, elasmobranchs accumulate organic and inorganic components. In the spiny dogfish *Squalus acanthias* L., urea accounts for 29–49% of the total osmolytes in the plasma, with trimethylamine oxide (TMAO) and inorganic ions such as sodium and chloride accounting for most of the remainder (Robertson, 1975; Kajimura *et al.*, 2006). TMAO

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serves as a balancing osmolyte to counteract the toxicity of urea (Yancey, 2001).

While urea is of great importance as an osmolyte to marine elasmobranchs, it also constitutes their main nitrogenous waste, accounting for *c.* 90% of nitrogen (N) excretion in *S. acanthias* (Wood *et al.*, 1995, 2005; Kajimura *et al.*, 2006). The spiny dogfish does not change its urea excretion rate over 48 h after feeding and continues to lose urea to the environment (Kajimura *et al.*, 2006). *Squalus acanthias* also excretes TMAO, apparently *via* the urine (Cohen *et al.*, 1958; Goldstein & Palatt, 1974) and appears to be incapable of *de novo* TMAO synthesis (Baker *et al.*, 1963; Goldstein *et al.*, 1967). Kajimura *et al.* (2006) estimated that wild spiny dogfish would need to feed every 5–6 days just to maintain nitrogen balance.

Field gut content surveys suggest that *S. acanthias* actually feeds at irregular intervals in coastal waters, and many spiny dogfish are caught with empty stomachs (Bonham, 1954; Holden, 1966; Jones & Geen, 1977; Hanchet, 1991; Tanasichuk *et al.*, 1991; Laptikhovskiy *et al.*, 2001; Alonso *et al.*, 2002). Trans-oceanic migrations by *S. acanthias* of up to 7000 km have been reported, but it is not known whether they feed during this movement (McFarlane & King, 2003). In captivity, *S. acanthias* are able to survive for at least 41 days without feeding (Cohen *et al.*, 1958; Leech *et al.*, 1979), while the European lesser spotted dogfish *Scyliorhinus canicula* (L.) withstands a remarkable 150 days (Zammit & Newsholme, 1979). Leech *et al.* (1979) reported that plasma urea levels and osmolality slowly fell during starvation in *S. acanthias*. Haywood (1973) demonstrated similar decreases in the pyjama shark *Pseudocorax africanus* (Gmelin) over a 1 month starvation period such that plasma became hypoosmotic to sea water after 14 days of food deprivation.

In a previous study, the effects of feeding on N metabolism in spiny dogfish were investigated by comparing fed fish to starved fish (Kajimura *et al.*, 2006). The starved fish, however, were deprived of food for only 1 week, a period which may not be long enough to affect urea, TMAO and osmolality. With this observation and the previous field studies in mind, the effect of long-term starvation on N metabolism in *S. acanthias* was investigated. The following were determined: (1) condition factor and hepato-somatic index, (2) nitrogen excretion rates and (3) osmolality and concentrations of organic and inorganic osmolytes in the plasma for fish starved for up to 56 days.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Spiny dogfish [mean \pm s.e. 1900 + 100 g (range 900–3700 g) mass (*M*) and 810 \pm 9 mm (range 620–1000 mm) total length (*L_T*)] were obtained by trawl in Barkley Sound, British Columbia, Canada, between June and August 2005. Spiny dogfish were held at the Bamfield Marine Sciences Centre for 1–3 weeks prior to experimentation in a 155 000 l circular tank served with running sea water at the experimental temperature (11 \pm 1° C), salinity (30 \pm 2), osmolality (924.7 \pm 1.5 mOsmol kg⁻¹) and pH (7.90 \pm 0.15) (values mean \pm s.e.). The animals were fed a ration equivalent to 3% of estimated total biomass of all spiny dogfish in the circular tank every 3 days. The food consisted of dead hake *Merluccius productus* (Ayres) with head and tail removed and cut into

pieces (50–100 mm). For starvation, fish were transferred from the circular tank to a separate 1500 l tank *c.* 1 h after feeding.

EXPERIMENTAL DESIGN

Nitrogen excretion

In order to measure N-excretion, dogfish were transferred to individual 40 l polyurethane-coated wooden boxes served with a seawater flow = 1 l.min⁻¹ and vigorous aeration. Measurements were started after a 1 h settling period and covered periods of *c.* 12 h representing 4–21, 24–36 and 36–48 h (same fish, *n* = 7). Starved fish were also transferred to experimental boxes at 4 (*n* = 11), 14 (*n* = 9), 34 (*n* = 11) and 55 days (*n* = 7) post-feeding (separate groups at each time point) and measured for 24 h. Water samples (15 ml) were drawn from the boxes at *c.* 12 h intervals for ammonia, urea-N and total-N measurements. The boxes were thoroughly flushed after each sampling period by filling and partially emptying (to 8 l) three times over a 15 min period, without exposing the fish to air. Water samples were analysed immediately or frozen at -20° C for later determination of ammonia, urea-N and total-N concentrations. In this study, ammonia refers to the sum of NH₃ and NH₄⁺.

Blood and tissue sampling

Spiny dogfish were taken from the circular tank at 6 (4–8, *n* = 7), 20 (18–23, *n* = 6), 30 (28–32, *n* = 7) and 60 h (50–72 h, *n* = 6) post-feeding, while for later time-points fish were taken directly from the 40 l boxes after completion of the N-excretion measurements, *i.e.* at 5 (*n* = 11), 15 (*n* = 9), 35 (*n* = 11) and 56 days (*n* = 7). At sacrifice, spiny dogfish were terminally anaesthetized in MS-222 (0.2 g l⁻¹), weighed for *M* and measured for *L_T* to allow calculation of condition factor (*K*). Blood samples were taken by caudal puncture with a heparinized 10 ml syringe, and plasma samples, obtained by centrifugation at 9000 g for 2 min, were frozen at -80° C for later analyses. The whole liver excluding the gall bladder was removed and weighed (*M_L*) to estimate the hepatosomatic index (*I_H*).

Analytical techniques

Ammonia in water samples was determined by an indophenol blue method (Ivancic & Degobbis, 1984). Plasma ammonia was measured enzymatically on the first thaw of non-deproteinized plasma using glutamate dehydrogenase (Raichem Kit No. 85446, Raichem, San Diego, CA, U.S.A.). Urea-N and urea concentrations in water and plasma, respectively, were measured with a diacetyl-monoxime method (Rahmatullah & Boyde, 1980). Total nitrogen (total-N) was analysed by a TNM-1 total-N unit interfaced with a Shimadzu TOC analyser using urea as a standard. Unknown nitrogen (unknown-N) was calculated by the following equation: $N_{UN} = N_T - (N_U + A)$, where *N_{UN}* is the unknown-N, *N_T* is the total-N, *N_U* is the urea-N and *A* is the ammonia. Osmolality was determined using vapour pressure osmometry (Wescor 5100C; Wescor, Logan, UT, U.S.A.). Total free amino acid (FAA) levels were measured by ninhydrin assay (Moore, 1968), with subtraction of the previously measured ammonia owing to the partial detection of ammonia by the ninhydrin method (Kajimura *et al.*, 2004); however, plasma contained only a small proportion of ammonia (*c.* 1% relative to FAA). TMAO level was assayed by the ferrous sulphate and EDTA method (Wekell & Barnett, 1991). Chloride concentration was analysed using a CMT 10 chloride titrator (Radiometer, Copenhagen, Denmark). Plasma inorganic ions (Na, K, Mg and Ca) were analysed using a flame atomic absorption spectrophotometer (Varian AA-220; Varian, Walnut Creek, CA, U.S.A.). Plasma glucose levels were measured using the hexokinase method (Infinity Liquid Stable Reagent; ThermoTrace, Noble Park, Victoria, Australia). Plasma β-hydroxybutyrate (β-HB) levels were measured using a β-hydroxybutyrate LiquiColor test (Stanbio Laboratory, Boerne, TX, U.S.A.).

DATA ANALYSIS AND STATISTICAL TREATMENTS

The value of K was calculated from $K = 100M_L^{-3}$. There was a high correlation between L_T and M in the spiny dogfish. The regression of $\log_{10} L_T$ and $\log_{10} M^3$ based on all samples was approximately linear [$r^2 = 0.759$; coefficient (b) = 2.98]. The value of I_H was calculated from $I_H = 100 M_L M^{-1}$. All data are given as mean \pm s.e. (n = number of animals). Multiple comparisons for differences in N excretion and metabolite concentrations at different sampling times were evaluated by one-way ANOVA followed by Fisher's LSD (least significant difference) *post hoc* test. Significance was accepted at $P < 0.05$. All per cent data for K and I_H were arcsine transformed before statistical analyses to avoid potential confounding effects of using mass-specific values (Packard & Boardman, 1999).

RESULTS

SURVIVAL, K AND I_H

No mortality occurred in any of the groups, and even the seven fish starved for 56 days stayed healthy, although one fish from the 35 day starvation group stayed at the bottom of the tank without any active swimming. By the end of the experiment, however, the fish in the 35 and 56 day groups had become noticeably more slender. The K fell quickly over the first 60 h after feeding and then decreased gradually during prolonged starvation [Fig. 1(a)]. Although the I_H tended to decrease slightly over the first month of the starvation period, the values dropped substantially between the 35 and 56 day measurements

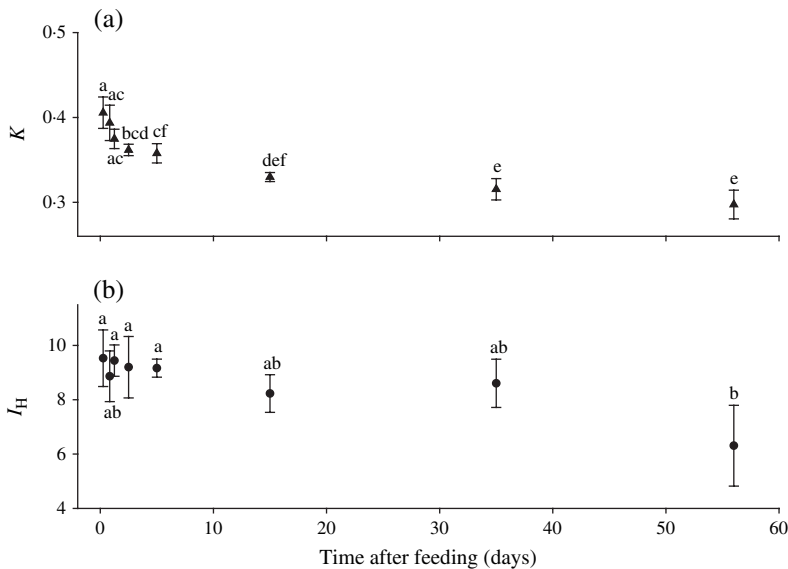


FIG. 1. Changes in (a) condition factor (K) and (b) hepato-somatic index (I_H) in response to starvation for 56 days in *Squalus acanthias*. Data are presented as means \pm s.e. ($n = 5$ to 9 at each time). Means sharing the same lower case letter within the same variable are not significantly different from one another (one-way ANOVA followed by LSD *post hoc* test, $P > 0.05$). The data were arcsine transformed before statistical analyses.

[Fig. 1(b)]. The I_H values for the 56 day starvation group was significantly lower than for the fish groups from 6 h to 5 days after feeding with the exception of the 20 h group. Since the I_H values of the spiny dogfish were quite variable in the present study, this lack of significance is probably due to the small sample size at 20 h after feeding ($n = 5$). The maximum and minimum I_H in individual fish were 13.3 and 2.1%, found at 6 h and 56 days after feeding, respectively.

PATTERN OF NITROGEN EXCRETION AFTER FEEDING

Total-N excretion was $1641 \mu\text{mol-N kg}^{-1} \text{h}^{-1}$ at 4–21 h after feeding, but decreased quickly by 24–36 h to *c.* $1014 \mu\text{mol-N kg}^{-1} \text{h}^{-1}$ and then did not change thereafter [Fig. 2(a)]. Urea-N excretion was constant over the entire experiment [Fig. 2(b)]. Ammonia-N excretion fell rapidly by 5 days after feeding, and then remained constant beyond 5 days [Fig. 2(c)]. Unknown-N excretion was high just after feeding, then decreased quickly and remained relatively constant [Fig. 2(d)]. At most of the time-points, spiny dogfish excreted more than half of total-N as urea-N (53–82%), although fish at the first time point (4–21 h) after the feeding excreted 50% of the total-N as unknown-N. In general, all of the excretions were stable by 15 days post-feeding. From 15 to 56 days, urea-N represented 72%, ammonia-N 2% and unknown-N 27% of the total-N excretion. The spiny dogfish excreted total-N at a rate of $791 \mu\text{mol-N kg}^{-1} \text{h}^{-1}$ at the end of starvation period.

PLASMA NITROGEN AND OSMOTIC CONSTITUENTS

Plasma osmolality was rather stable (means ranging from 932 to 972 mOsmol kg^{-1}) during long-term starvation [Fig. 3(a)], and slightly higher than that of seawater (925 mOsmol kg^{-1}). Plasma urea was elevated 20 h after feeding, decreased by 60 h, and remained constant at $>406 \text{ mM}$ [Fig. 3(b)] for the remainder of the experiment. Plasma TMAO similarly remained more or less stable (72–87 mM) during the long-term starvation [Fig. 3(c)]. The molar ratio of urea to TMAO in plasma was relatively stable during the entire period (five- to six-fold). Plasma glucose was lowest 6 h after feeding (mean 2.9 mM), then increased with starvation time, and increased more than two-fold (mean 6.3 mM) at 15 days [Fig. 4(a)]. Although plasma β -hydroxybutyrate followed similar qualitative trends to plasma glucose, it increased 56-fold from 20 h (mean 0.3 mM) to 35 days [mean 16.9 mM; Fig. 4(b)]. The maximum β -hydroxybutyrate value observed was 23 mM in a 35 day starved fish. Although plasma FAA tended to decrease after feeding, the levels hardly changed during the long-term starvation period (Table I). Relative to all other measured compounds, plasma ammonia levels were very low, with the highest concentrations at 20 h, and falling to <0.05 this level from 15 days onwards (Table I). In general, plasma inorganic osmolytes remained constant (Table I). The inorganic ion concentrations of seawater measured by Wood *et al.* (2007) in a parallel study at the same time were 452 mM Na^+ , 515 mM Cl^- , 9.8 mM K^+ , 9.5 mM Ca^{2+} and 52 mM Mg^{2+} . The spiny dogfish therefore maintained plasma inorganic ions at levels of 56–59% for Na^+ , 47–49% for Cl^- , 36–41% for

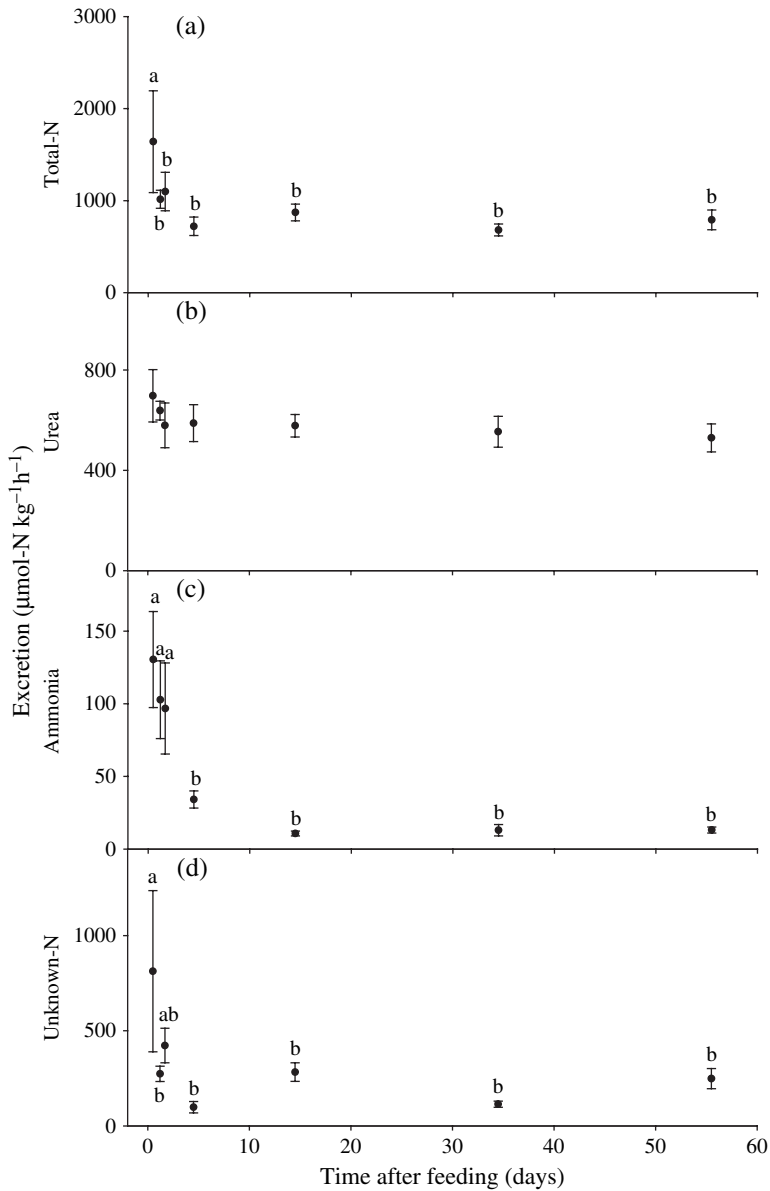


FIG. 2. Nitrogen (N) excretion rates of *Squalus acanthias*: (a) total-N, (b) urea-N, (c) ammonia and (d) unknown-N. Data are presented as means \pm s.e. ($n = 7$ to 11 at each time). Means sharing the same lower case letter within the same variable are not significantly different from one another (one-way ANOVA followed by LSD *post hoc* test, $P > 0.05$).

K^+ , 45–51% for Ca^{2+} and 2–3% for Mg^{2+} relative to sea water levels. The sum of the plasma inorganic osmolytes (Na^+ , Cl^- , K^+ , Ca^{2+} and Mg^{2+}) was 512–530 mM during the long-term starvation and did not show any significant differences at any time points (ANOVA). Na^+ and Cl^- were clearly the dominant inorganic osmolytes in the spiny dogfish.

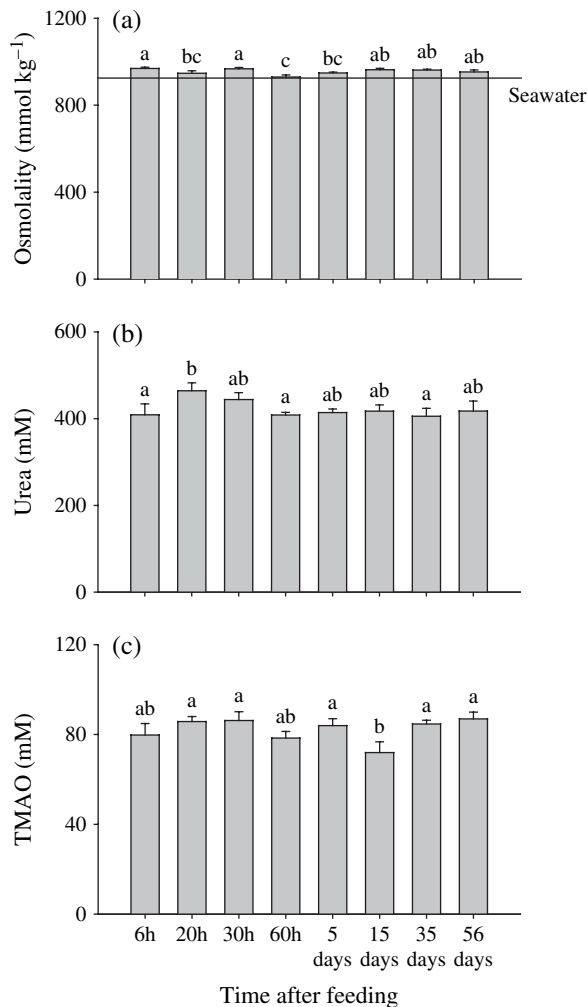


FIG. 3. Changes in plasma (a) osmolality, (b) urea and (c) trimethylamine oxide (TMAO) in starved and fed *Squalus acanthias*. Values are means \pm s.e. ($n = 6$ to 9 at each time). Means sharing the same lower case letter within the same compound are not significantly different from one another (one-way ANOVA followed by LSD *post hoc* test, $P > 0.05$).

DISCUSSION

Cohen *et al.* (1958) and Leech *et al.* (1979) reported that *S. acanthias* are able to survive prolonged starvation for at least 41 days, although they also observed high levels of ill health or mortality over the prolonged starvation. In the present study, 10 of the 11 spiny dogfish starved for 35 days and all seven fish starved for 56 days remained healthy. Possible reasons for the lower health and higher mortality in these earlier studies are probably that the fish were stressed from having blood samples taken every 7 days during starvation (Cohen *et al.*, 1958), which was not done here, and that the fish had higher metabolic demands due to high water temperatures, up to 17° C in Cohen *et al.*

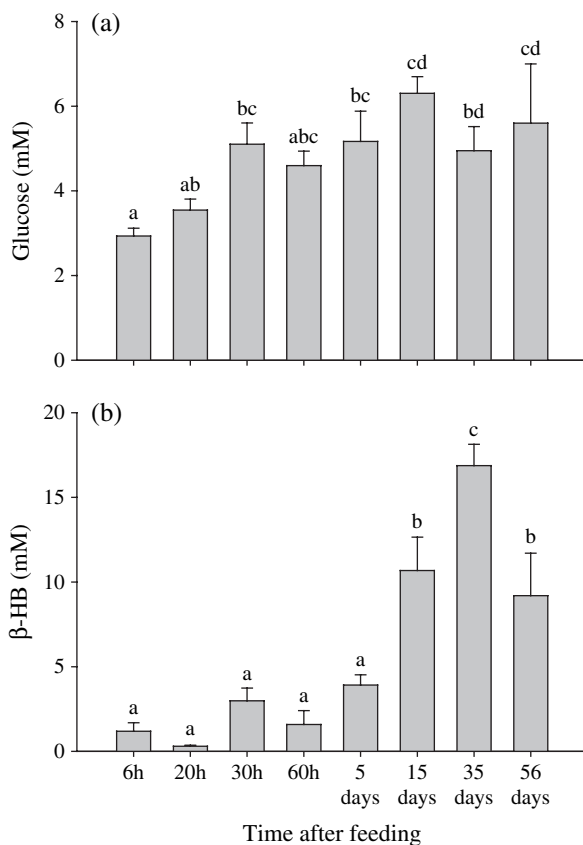


FIG. 4. Changes in plasma (a) glucose and (b) β -hydroxybutyrate (β -HB) in starved and fed *Squalus acanthias*. Values are means \pm S.E. ($n = 6$ to 9 at each time). Means sharing the same lower case letter within the same compound are not significantly different from one another (one-way ANOVA followed by LSD *post hoc* test, $P > 0.05$).

(1958) and from 12.5°C to 14°C in Leech *et al.* (1979) v. $11 \pm 1^{\circ}\text{C}$ in the present study.

The mean L_T of the spiny dogfish in the present study was 810 mm. Based on the mean L_T and K [Fig. 1(a)], the M at each time point can be backcalculated, and then the amount of body mass the fish lost per day can be estimated. The K quickly fell until 60 h and then decreased only slowly thereafter. Wood *et al.* (2007) found that the food in the gastrointestinal tract was greatly reduced 60 h after feeding and was nearly completely cleared by 5 days. In the present study, K was therefore probably elevated in the first 5 days due to food in the gastrointestinal tract. Indeed, the fish lost *c.* 4.6% of their body mass per day from 6 to 60 h, whereas from 5 to 56 days post-feeding, which is after the gastrointestinal tract was completely devoid of food, the fish lost only 0.3% of body mass per day. Leech *et al.* (1979) reported that the per cent of body mass lost per day in the spiny dogfish was *c.* 0.5% over a 43 day starvation period.

It is well known that elasmobranchs exhibit high I_H values compared to those of teleosts. In *Centroscymnus coelolepis* Barbosa du Bocage & de Brito

TABLE I. Mean \pm S.E. organic and inorganic components of the plasma of *Squalus acanthias*. The numbers of fish were between six and nine per time interval. Means sharing the same lower case letter in the same tissue are not significantly different from one another (one-way ANOVA followed by LSD *post hoc* test, $P > 0.05$)

	6 h	20 h	30 h	60 h	5 days	15 days	35 days	56 days
FAA (mmol l ⁻¹)	9.0 \pm 1.0 ^a	7.4 \pm 0.6 ^{abc}	8.6 \pm 0.4 ^{ab}	7.0 \pm 0.5 ^{bc}	5.8 \pm 0.3 ^c	7.4 \pm 0.5 ^{bd}	6.3 \pm 0.6 ^{cd}	5.7 \pm 0.5 ^c
Ammonia (μ mol-N l ⁻¹)	113.1 \pm 28.3 ^a	193.1 \pm 65.5 ^b	19.2 \pm 6.1 ^{cd}	92.7 \pm 42.4 ^{ac}	29.2 \pm 18.0 ^{cd}	7.2 \pm 3.2 ^d	3.9 \pm 1.6 ^d	7.3 \pm 2.8 ^d
Na ⁺ (mmol l ⁻¹)	258.6 \pm 4.0 ^{ab}	257.5 \pm 4.1 ^{ab}	258.7 \pm 3.3 ^{ab}	259.4 \pm 6.4 ^{ab}	254.1 \pm 3.2 ^a	268.6 \pm 3.1 ^b	266.0 \pm 2.3 ^b	260.1 \pm 6.5 ^{ab}
Cl ⁻ (mmol l ⁻¹)	246.4 \pm 3.7 ^{ab}	248.3 \pm 1.4 ^{ab}	243.7 \pm 1.7 ^a	251.0 \pm 2.8 ^{ab}	249.1 \pm 2.1 ^{ab}	251.1 \pm 3.0 ^{ab}	248.7 \pm 3.9 ^{ab}	254.7 \pm 4.2 ^b
K ⁺ (mmol l ⁻¹)	4.0 \pm 0.2 ^a	3.9 \pm 0.2 ^{ab}	3.9 \pm 0.1 ^{ab}	3.6 \pm 0.1 ^{ab}	3.5 \pm 0.1 ^b	3.8 \pm 0.1 ^{ab}	3.5 \pm 0.1 ^b	3.7 \pm 0.1 ^{ab}
Ca ²⁺ (mmol l ⁻¹)	4.6 \pm 0.2 ^{ab}	4.0 \pm 0.1 ^a	4.8 \pm 0.2 ^b	4.5 \pm 0.3 ^{ab}	4.3 \pm 0.2 ^{ab}	4.8 \pm 0.2 ^b	4.7 \pm 0.2 ^b	4.6 \pm 0.3 ^{ab}
Mg ²⁺ (mmol l ⁻¹)	1.0 \pm 0.1 ^a	1.0 \pm 0.0 ^a	1.4 \pm 0.1 ^b	1.4 \pm 0.1 ^b	1.4 \pm 0.1 ^b	1.5 \pm 0.1 ^b	1.5 \pm 0.1 ^b	1.6 \pm 0.1 ^b

Capello the lipid content in the liver is 40–50%, whereas it ranges from 1 to 5% in other tissues (Remme *et al.*, 2006). The liver seems to be the principal site of lipid storage in elasmobranchs. It was found that the spiny dogfish had a mean I_H of *c.* 9% until 5 days after feeding [Fig. 1(b)], similar to values found in previous research on spiny dogfish (7–11%) (Leech *et al.*, 1979; Bellamy & Hunter, 1997). The I_H tended to decrease over the starvation period [Fig. 1(b)]; however, as with the K , food in the gastrointestinal tract adds some additional mass to the actual body mass and therefore affects the calculation of I_H , resulting in an underestimation (maximum 0.7% at 20 h after feeding) of I_H at the early times. For the values after 5 days post-feeding, the I_H seems to be stable until day 35, but dropped at 56 days. This suggests that the liver lipids in the spiny dogfish may be used in the later stages of starvation.

In the pyjama shark, Haywood (1973) showed that plasma osmolality and urea levels decreased gradually and simultaneously during 1 month of starvation, and that osmolality fell *c.* 20–50 mOsm kg⁻¹ below the level of seawater. Haywood (1973) suggested that plasma osmolality is directly related to plasma urea levels. In contrast to the pyjama shark, plasma osmolality and urea levels in *S. acanthias* were well maintained for nearly 2 months of starvation [Fig. 3 (a), (b)], and osmolality was kept slightly higher than that of seawater at all times. It was found that not only plasma urea, but also the other major osmolytes, such as plasma TMAO, inorganic ions and FAA, barely changed over the starvation period [Fig. 3(c) and Table I]. The spiny dogfish seemed to keep its osmotic balance constant during the 56 day starvation period. Recently, Treberg & Driedzic (2006) showed that winter skate *Leucoraja ocellata* (Mitchill) also maintain their plasma osmolality as well as plasma urea and TMAO for a 45 day starvation period. In the present study, spiny dogfish plasma urea levels increased 20 h after feeding and then returned to the baseline levels. This is in agreement with previously reported data (Kajimura *et al.*, 2006) that showed a peak of plasma urea at 20 h after feeding, probably associated with increased urea synthesis at this time due to an increase in ornithine urea cycle enzyme activities for nitrogen conservation after feeding.

Surprisingly, the spiny dogfish exhibited a virtually constant rate of urea excretion during 56 days of starvation [Fig. 2(b)], and yet maintained constant plasma urea levels [Fig. 3(b)]. The average urea excretion rate was 591 $\mu\text{mol-N kg}^{-1} \text{h}^{-1}$ over the period studied. This observation therefore indicates that the spiny dogfish lost a total of 397 mmol of urea per 1 kg body mass during 56 days of starvation. Assuming that protein-N content is regarded as 0.16 g of nitrogen per g protein (Kajimura *et al.*, 2004), 69.5 g of protein would need to be broken down to provide 397 mmol of urea per 1 kg body mass. It has been reported that urea content in the muscle is 332 mmol per kg in the spiny dogfish (Robertson, 1975), and that white muscle accounts for 50% of the body mass of the salmon shark *Lamna ditropis* Hubbs & Follett (Bernal *et al.*, 2003). Using these variables, it can be estimated that the urea content in the muscle is 166 mmol per 1 kg spiny dogfish. The spiny dogfish therefore lost 2.4 times more urea to the environment than they have in their muscles during the 56 day starvation period. Presumably, this must be replaced by the breakdown of 69.5 g of protein. Although the excretory organs of elasmobranchs (kidney and gills) are specialized to reduce urea permeability and to

actively retain urea (Schmidt-Nielsen *et al.*, 1972; Pärt *et al.*, 1998; Fines *et al.*, 2001; Janech *et al.*, 2003, 2006; Morgan *et al.*, 2003; Hill *et al.*, 2004), the urea leakage to the environment apparently cannot be further reduced even during long-term starvation. Since 5 mol of ATP are needed to synthesize 1 mol of urea *via* the ornithine urea cycle (Anderson, 2001), the spiny dogfish presumably requires a considerable amount of energy to keep its urea level, and therefore osmolality, constant during long-term starvation.

Urea and ammonia are the primary forms of nitrogen excreted in fishes. Most studies examining nitrogenous wastes have focused on these end products. It is also known, however, that a considerable amount of excreted nitrogen is found in waste products other than ammonia and urea, such as trimethylamine (TMA) and TMAO, creatine and creatinine, amino acids, protein and uric acid (Smith, 1929; Wood, 1958; Olson & Fromm, 1971; McCarthy & Whitley, 1972; Goldstein & Palatt, 1974; Cockcroft & Du Preez, 1989; Kajimura *et al.*, 2004). In the present study, the rate of unknown-N excretion at the first time point between 4 and 21 h after feeding was exceptionally high ($813 \mu\text{mol-N kg}^{-1} \text{h}^{-1}$) compared to the other time points (between 98 and $423 \mu\text{mol-N kg}^{-1} \text{h}^{-1}$), and varied in individual fish [Fig. 2(d)]. Spiny dogfish have large amounts of ingested food with some liquid chyme in the cardiac stomach at 6 and 20 h after feeding, and vomiting has been observed both in the fish boxes and the feeding tank (Kajimura *et al.*, 2006; Wood *et al.*, 2007). It is possible that some fish vomited chyme just after feeding and that the partially digested protein or amino acids in the vomit was detected by the nitrogen analyser. Studies using inert markers in the food would be instructive in quantifying vomited chyme.

The spiny dogfish excreted significant amounts of unknown-N not only just after the feeding event, but also during the prolonged starvation period (56 days) at rates lower than urea excretion but higher than ammonia excretion at all time points. In little skate *Raja erinacea* (Mitchill), Goldstein & Palatt (1974) measured TMAO excretion using an injecting radio isotopic tracer procedure and reported that $0.82 \text{ mmol kg}^{-1} \text{ day}^{-1}$ (*i.e.* $34.2 \mu\text{mol-N kg}^{-1} \text{h}^{-1}$) was excreted to the environment. They also estimated that the spiny dogfish lost 10.4% of plasma TMAO per day based on the radio isotopic tracer method (Goldstein & Palatt, 1974). Cohen *et al.* (1958) also reported that some TMAO in the spiny dogfish is lost to the environment *via* the renal system. Presumably, TMAO constitutes some of the unknown-N excretion in the spiny dogfish. Treberg & Driedzic (2006), however, have pointed out that the methodology used by Goldstein & Palatt (1974) resulted in overestimated values, and reported that winter skates lose <1% of whole body TMAO per day.

Although some TMAO in the body may be lost to the environment, plasma TMAO was maintained at a relatively stable level over the 56 day starvation period. It is generally believed that *S. acanthias* is not capable of *de novo* TMAO synthesis (Baker *et al.*, 1963; Goldstein *et al.*, 1967). Robertson (1975) reported that *S. acanthias* accumulates large amounts of TMAO and urea in the muscle. TMAO may therefore be supplied from the body during long-term starvation.

Plasma ammonia was highest at 20 h, and then quickly decreased (Table I). Similar changes in plasma ammonia were observed in a previous study, which showed that the plasma ammonia was lowest at 30 h during a 48 h starvation

period, although there were no significant differences (Kajimura *et al.*, 2006). In contrast to urea and TMAO, however, plasma ammonia was negligible; the concentration of ammonia in plasma was <0.02% of urea-N and <0.2% of TMAO [Table I and Fig. 3(b), (c)].

Similar to plasma ammonia, ammonia excretion was greatly reduced by 5 days after feeding and minimized by 15 days [Fig. 2(c)]. In the spiny dogfish, ammonia is mainly excreted across the gills (*c.* 96% of total ammonia excretion), apparently by diffusion (Wilkie, 2002), and not *via* the urine (Wood *et al.*, 1995). Ammonia excretion and plasma ammonia seem to change simultaneously. Ammonia excretion, however, accounted for only 2% of total nitrogen excretion after 15 days. The above observations would suggest that low ammonia loss rates are accomplished by whole-body ammonia scavenging by a variety of metabolic pathways and organs (*e.g.* hepatic glutamate dehydrogenase) to maintain low plasma to environmental partial pressure gradients, rather than by ammonia trapping mechanisms specific to the gill or other integumental surfaces.

Urea, TMAO and inorganic ions are major osmolytes in marine elasmobranchs, and therefore have considerable influence on osmolality. The osmotic coefficient of urea is 0.96, that of TMAO is 1.19 and that of inorganic ions in plasma is 0.92 (Robertson, 1975, 1989). Using these coefficient values, the respective osmolalities contributed by these osmolytes can be calculated. In this study, plasma urea levels, TMAO concentrations and total inorganic ions accounted for 389–446 mOsmol kg⁻¹ [Fig. 3(b)], 86–103 mOsmol kg⁻¹ [Fig. 3(c)] and 469–485 mOsmol kg⁻¹ (Table I), respectively. Therefore, urea comprised *c.* 40–47%, TMAO *c.* 9–11% and total inorganic ions *c.* 48–51% of total plasma osmolality [Fig. 3(a)]. These ratios were quite stable for a 2 month starvation period. These results suggest that *S. acanthias* is able to keep major plasma osmolyte levels constant, and therefore plasma osmolality is also maintained constant above the level of seawater for 56 days of starvation.

It was previously shown that plasma β -HB levels were high in starved fish and declined 10-fold after feeding, and that plasma glucose levels showed a slight and transient decrease after feeding (Walsh *et al.*, 2006). In the present study, plasma β -HB [Fig. 4(b)] and glucose [Fig. 4(a)] showed similar trends, with low levels after feeding and progressive increases over the starvation period. Plasma glucose gradually increased, whereas plasma β -HB remained low until day 5 of starvation and then increased rapidly at day 15. Walsh *et al.* (2006) suggested that the decline of plasma β -HB levels after feeding could reflect either a decrease in the export rate by the liver, an increase in utilization by other tissues, or a combination of both factors. The present results for β -HB concentrations when taken together with prior results (Zammit & Newsholme, 1979; Ballantyne, 1997; Walsh *et al.*, 2006) reinforce the overall concept of a ketone body based metabolism as a general feature of elasmobranchs, and notably that this reliance on ketone bodies is particularly pronounced during periods of starvation. In contrast to the results for glucose, Zammit & Newsholme (1979) reported that plasma glucose levels in *S. canicula* do not change during long-term starvation.

In conclusion, the spiny dogfish can probably maintain its osmolytes constant despite losing large amounts of urea as N-waste, and can therefore retain its hyperosmotic plasma over long-term starvation. This ability may be

adaptive in light of its irregular feeding habits, and may facilitate long trans-ocean journeys away from the food-rich coastal shelf. Although urea in the body is excreted to the environment, the spiny dogfish must synthesize urea, probably through sacrifice of body protein stores, in order to avoid a decrease in osmolality over the starvation period.

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