The Journal of Experimental Biology 209, 2929-2938
Published by The Company of Biologists 2006
doi:10.1242/jeb.02329

Metabolic organization and effects of feeding on enzyme activities of the dogfish shark (*Squalus acanthias*) rectal gland

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Accepted 15 May 2006

Summary

In order to investigate the metabolic poise of the elasmobranch rectal gland, we conducted two lines of experimentation. First, we examined the effects of feeding on plasma metabolites and enzyme activities from several metabolic pathways in several tissues of the dogfish shark, *Squalus acanthias,* after starvation and at 6, 20, 30 and 48 h post-feeding. We found a rapid and sustained ten-fold decrease in plasma β-hydroxybutyrate at 6 h and beyond compared with starved dogfish, suggesting an upregulation in the use of this substrate, a decrease in production, or both. Plasma acetoacetate levels remain unchanged, whereas there was a slight and transient decrease in plasma glucose levels at 6 h. Several enzymes showed a large increase in activity post-feeding, including β-hydroxybutyrate dehydrogenase in rectal gland and liver, and in rectal gland, isocitrate dehydrogenase, citrate synthase, lactate dehydrogenase, aspartate amino transferase, alanine amino transferase, glutamine synthetase and Na^+/K^+ ATPase. Also notable in these enzyme measurements was the overall high level of activity in the rectal gland in general. For example, activity of the Krebs' TCA cycle enzyme citrate synthase (over 30 U g^{-1}) was similar to activities in muscle from other species of highly active fish. Surprisingly, lactate dehydrogenase activity in the gland was also high (over 150 U g^{-1}), suggesting either an ability to produce lactate anaerobically or use lactate as an aerobic fuel. Given these interesting observations, in the second aspect of the study we examined the ability of several metabolic substrates (alone and in combination) to support chloride secretion by the rectal gland. Among the substrates tested at physiological concentrations (glucose, β-hydroxybutyrate, lactate, alanine, acetoacetate, and glutamate), only glucose could consistently maintain a viable preparation. Whereas β-hydroxybutyrate could enhance gland activity when presented in combination with glucose, surprisingly it could not sustain chloride secretion when used as a lone substrate. Our results are discussed in the context of the *in vivo* role of the gland and mechanisms of possible upregulation of enzyme activities.

Key words: elasmobranch, shark, ketone body, β-hydroxybutyrate, lipid metabolism.

Introduction

The metabolic biochemistry of elasmobranch fishes is rather different from that of teleost fish. Notably, most elasmobranch tissues show a reliance on ketone bodies[1] (i.e. β-hydroxybutyrate and acetoacetate) as metabolic fuels, especially during food-deprivation when the liver exports ketone bodies for use by other tissues (e.g. muscle, brain, etc.). This pattern is illustrated by a number of prior studies. One of the first comprehensive studies (Zammit and Newsholme, 1979) used enzyme activity measurements to demonstrate that most elasmobranch tissues had a high capacity for oxidation of ketone bodies, but much less so for long chain fatty acids. Furthermore, when examining levels of blood metabolites, they found that ketone body concentrations in plasma of elasmobranchs were much higher than those in teleosts and mammals, and furthermore that they increased markedly after long-term (40–150 days) food deprivation. Subsequent studies reinforced this general view of a ketone body-based metabolism. Notably, Driedzic and Hart (Driedzic and Hart, 1984) demonstrated that little skate [*Raja* (now *Leucoraja*) *erinacea*] hearts performed better when oxidizing ketone bodies as fuels over fatty acids or glucose. Using isolated hepatocytes from the same species, Mommsen and Moon demonstrated that whereas overall hepatic rates of long chain

[1]The term 'body' is an historical artifact; this group of ketone or ketone-derived metabolites are fully soluble in biological fluids (Lehninger et al., 1993).

fatty oxidation were generally low, substrates such as oleate (and leucine for that matter) could support a net export of ketone bodies from the liver (Mommsen and Moon, 1987). Likewise, isolated liver mitochondria of dogfish shark (*Squalus acanthias*) had a great capacity to produce ketone bodies from acetate, palmitoyl-CoA, pyruvate and alanine (Anderson, 1990). In studies using intact and cannulated dogfish, deRoos and colleagues demonstrated that even in tissues such as brain, which in mammals is viewed as a highly glucose-dependent tissue, ketone bodies are an important metabolic fuel in elasmobranchs (deRoos, 1994). Indeed, ketone-body-based metabolism appears to extend also to closely related holocephalans (Speers-Roesch et al., 2006a).

Whereas the very low concentration of serum albumin in elasmobranchs, and thus the low capacity for plasma fatty acid binding and transport, had been reported earlier (Fellows et al., 1980; Fellows and Hird, 1981; Ballantyne et al., 1993), an important connection was later made in an elegant review article (Ballantyne, 1997). Ballantyne reasoned that the strategy of using high concentrations of plasma urea (several hundred millimolar) for osmotic balance (the ureosmotic strategy) would likely disrupt serum albumin structure and function. Thus, this ureosmotic strategy ultimately required a decreased reliance on fatty acids, and an increased reliance on ketone bodies, as circulating metabolic fuels (Ballantyne, 1997).

Clearly, elasmobranchs and holocephalans have an interesting overall metabolic profile compared to teleosts and mammals. In addition, several observations suggest that both circulating glucose and ketone body concentrations are dynamic and can span a large range, depending upon feeding, season, exercise, etc (e.g. Patent, 1973; Zammit and Newsholme, 1979; deRoos et al., 1985; Gutierrez et al., 1988; deRoos and deRoos, 1992; Richards et al., 2003). It is especially the event of feeding, and the large NaCl load that it generates which must be excreted, that is thought to activate the gland through hormonal, acid–base, and other cues (MacKenzie et al., 2002). Indeed, the rectal gland is a highly aerobic organ that has served as a model system for the principles of active NaCl co-transport for almost four decades (for reviews, see Shuttleworth, 1988; Riordan et al., 1994; Silva et al., 1990; Silva et al., 1996; Hazon et al., 2003). However, surprisingly, in the many studies of transport and salt secretion by elasmobranch rectal glands, scant data are available on the substrate(s) that this important gland uses to fuel salt excretion. In most studies of perfused rectal glands glucose is the typical substrate, and in some cases supraphysiological concentrations have been used (e.g. T. J. Shuttleworth, J. Thompson, R. S. Munger and C.M.W., manuscript submitted for publication), but really no systematic published data support this choice of *in vitro* fuel.

Therefore, in the present study, one goal was to focus particularly on patterns of fuel usage in the rectal gland of the dogfish shark *Squalus acanthias* and how they might be affected by feeding and changes in circulating substrate levels. Furthermore, in order to better characterize the physiological variability of available substrates, and the metabolic scope of the gland, we examined the effects of *natural* feeding on circulating levels of ketone bodies and glucose and the activities of several enzymes of ketone body and general metabolism. This paper is a companion to two others recently published (Wood et al., 2005; Kajimura et al., 2006) that examine the effects of feeding on acid–base balance and nitrogen metabolism and excretion, respectively, in the same species.

Materials and methods

Experimental animals

Spiny dogfish (*Squalus acanthias* L., 1.9–4.0 kg) were obtained by trawl or hook and line in Barkley Sound, British Columbia, Canada, in July and August 2003 and 2005. At Bamfield Marine Sciences Centre, the fish were held for 1–3 weeks prior to experimentation in a 200,000 l circular tank served with running seawater at the experimental temperature (11±1°C), salinity (30±2‰) and pH (7.90±0.15). We found that dogfish would not feed when held in smaller tanks or when isolated, but when held in a large group (approximately 100 fish) in this very large tank, a few dogfish would start feeding after about a week in captivity, and thereafter, the others would quickly learn to do so. Twice weekly, the animals were fed a ration equivalent to 2% of estimated total biomass of all the dogfish in the circular tank, most of which was quickly devoured (fed treatment). Food consisted of whole dead trawl fish (mainly flatfish, herring and eelpout). Samples of food were taken, minced with a food processor and frozen at –20°C for later content analysis, as reported (Wood et al., 2005).

Experimental design

Effects of feeding on plasma metabolites and rectal gland enzyme activities

Some fish (starved treatment) were removed from the circular tank to a separate 1500 l tank 1 week before the start of an experiment, were not fed during this separation period, and then each dogfish was transferred to an individual 40 l polyurethane-coated wooden box (seawater flow=1 l min^{-1}) as described (Wood et al., 1995; Wood et al., 2005). For the fed fish treatment, 1 h after the start of feeding in the large 200,000 l circular tank as described above, fed fish were transferred to these same 40 l boxes. These fish are the same individuals as described (Kajimura et al., 2006); therefore, data on nitrogen excretion, nitrogen metabolizing enzymes, blood nitrogen metabolite levels, etc. are directly comparable to the data of the present study.

This series examined the effect of feeding on blood chemistry and key enzymes of metabolism in the tissues. Dogfish were fed and then transferred to the 40 l boxes as described above. Separate groups were killed at '6 h' (4–8 h, *N*=15), '20 h' (19–22 h, *N*=13), '30 h' (29–31 h, *N*=8) and '48 h' (*N*=10) after feeding. A 1-week starved group (*N*=14) was also examined. At sacrifice fish were anaesthetized with MS-222, weighed, placed on an operating table and blood samples (by caudal puncture with a heparinized 10 ml syringe)

and tissue samples were taken. Ten tissues were dissected; liver, muscle, gills, intestine, rectal gland, esophagus, anterior stomach (stomach 1), posterior stomach (stomach 2), brain and kidney (note that kidney samples were taken only from starved fish and at 48 h post-feeding). Plasma samples, obtained by centrifugation at 9000 ***g*** for 2 min, were stored frozen at –20°C. Tissue samples were frozen immediately with liquid nitrogen, and stored at –80°C for later enzymatic analysis. At sacrifice, the amount of food remaining in the stomach was collected by making a small incision at the distal end of the stomach and gently squeezing the contents into a tared beaker and weighing. Some fish had empty stomachs at sacrifice, and were excluded from the analysis, as we could not be certain that they had fed at 0 h. These fish constituted 4 out of 15 fish at 6 h, 2 out of 13 fish at 20 h, 1 out of 8 fish at 30 h and 1 out of 10 fish at 48 h after feeding. In addition, any solid food that was regurgitated into the boxes during the experiments was collected and weighed.

Perfused rectal gland studies

Dogfish that had starved for 7–10 days were anesthetized with MS-222 (0.2 g l^{-1} seawater) and this dose was maintained by irrigation of the gills with seawater *via* a pump. Mass, length and sex were noted. Rectal glands were cannulated *in situ* until all three ports had been fitted with PE 50 tubing. The artery was first, followed by collecting duct and vein. The artery and vein were flushed with dogfish saline (mmol l^{-1}) (NaCl, 257; Na_2SO_4, 7; $MgSO_4$, 3; KCl, 4; $CaCl_2$, 2; Na_2HPO_4, 0.1; urea 400; trimethylamine oxide (TMAO), 80; $NaHCO_3$ 6; solutions were gassed with 0.275% CO_2 and balance oxygen, yielding a pH of 7.9 to 8.0) with 50 i.u. heparin, while the duct was flushed with 500 mmol l^{-1} NaCl (we also found that heparin was useful in minimizing clots and mucous plugs). All saline solutions were processed through a 0.45 μm filter prior to use. The gland was then removed and connected to a PE-50 line in series with a thermostatted saline reservoir, peristaltic pump, thermostatted windkessel/bubble trap and temperature equilibration coil, similar to the set-up described by Shuttleworth et al. (T. J. Shuttleworth, J. Thompson, R. S. Munger and C.M.W., manuscript submitted for publication). The gland rested on a thermostatted Plexiglass platform (12±1.0°C). Perfusion began at approximately 0.25 ml min^{-1} until the gland was cleared of blood and it was then secured to the platform to prevent movement (by taping the catheters). Input flow was then increased to about 1.0 ml min^{-1} and collection of duct secretion commenced. Typically, a period of 30 min with 5 mmol l^{-1} glucose and no forskolin stimulation preceded substrate tests to allow the gland to equilibrate. Forskolin was then added (from a stock of 25 mg ml^{-1} DMSO to a final concentration of 5 μmol l^{-1}, i.e. 4.1 μl DMSO per 50 ml perfusate) to stimulate gland secretion *via* activation of cAMP-mediated pathways (Morgan and Valentich, 1991). Each substrate/substrate combination test period was 60 min (substrates were added to perfusate as 100-fold concentrated stocks, neutralized when necessary), and duct secretions were collected and venous flow rates were monitored (gravimetrically) every 30 min. Typically venous outflow rates were 60% of inflow rates due to the previously described venous bypass (Kent and Olson, 1982). Experiments typically lasted 5.5 h, allowing five substrate comparisons per gland. Since secretion rates invariably declined slightly over time for a given gland, the order of substrate addition was randomized to prevent bias. Chloride in the duct secretions was determined with a Radiometer CMT 10 Chloride Titrator (Copenhagen, Denmark). Urea was assayed colorimetrically (Rahmatullah and Boyde, 1980). Chloride secretion rates were calculated from the volume of duct secretion, the chloride concentration of the duct secretion, flux time and gland mass to yield mmoles Cl^{-} h^{-1} g^{-1}.

Analytical techniques and statistics

Plasma glucose levels were measured using an infinity glucose hexokinase liquid stable reagent (ThermoTrace, Noble Park, Victoria, Australia). Plasma β-hydroxybutyrate levels were measured using a β-hydroxybutyrate LiquiColor test (Stanbio Laboratory, Boerne, TX, USA). Plasma acetoacetate levels were measured according to published methods (Williamson et al., 1962).

For analysis of enzyme activity, tissues were homogenized on ice in 5–10 volumes of homogenization buffer (20 mmol l^{-1} K_2HPO_4, 10 mmol l^{-1} Hepes, 0.5 mmol l^{-1} EDTA, 1 mmol l^{-1} dithiothreitol, 50% glycerol, adjusted with NaOH to pH 7.5 at 24°C) using a Brinkman Polytron. Homogenates were centrifuged at 8000 ***g*** for 20 min at 4°C. The supernatant, or a 1:9 or 1:99 dilution in homogenization buffer was used directly for assaying the activity of β-hydroxybutyrate dehydrogenase (EC 3.1.1.30; BHBDH, ketone body metabolism), malic enzyme (EC 1.1.140; ME, NADPH generation), 6-phosphogluconate dehydrogenase (EC 1.1.1.44; 6-PGDH, pentose shunt pathway), lactate dehydrogenase (EC 1.1.1.27; LDH, anaerobic glycolysis), NADP-isocitrate dehydrogenase (EC 1.1.1.41; ICDH, oxidative metabolism) and citrate synthase (EC 2.3.3.1; CS, mitochondrial abundance) by previously described methods (Mommsen et al., 1980). To assess nitrogen metabolism, glutamate dehydrogenase (EC 1.4.1.3; GDH), alanine amino transferase (EC 2.6.1.2; AAT) and aspartate amino transferase (EC 2.6.1.1; DAT, formerly ASP AT) were assayed by the methods of Walsh and Henry (Walsh and Henry, 1990) and glutamine synthetase (transferase units, EC; GS) by the methods of Webb and Brown (Webb and Brown, 1980). Not all tissues were assayed for all enzymes. The protocol used for the determination of Na^{+}/K^{+} ATPase activity followed that of McCormick (McCormick, 1993). In all cases assay temperature was 22°C.

All data are reported as mean ± s.e.m. All data were normally distributed, and multiple comparisons for differences in metabolite concentrations and enzyme activities at different sampling times were evaluated by one-way ANOVA followed by Dunnett's paired multiple comparison test (Figs 2, 3, 4 and 6) and a LSD *post-hoc* test for all the other data. Significance was accepted at $P<0.05$. For rectal gland substrate preference experiments, because of inter-preparation variability, it was

often convenient to express data as a percentage of the control period, and if so, arcsine-transformed values were used in statistical tests.

Results

Effects of feeding on plasma metabolites and enzyme activities

Plasma β-hydroxybutyrate concentrations in starved and fed dogfish were in the same range as prior studies, and feeding resulted in a >fivefold decrease in plasma β-hydroxybutyrate in the first and all subsequent sampling periods (Fig. 1A). Plasma acetoacetate (Fig. 1B) and glucose (Fig. 1C) concentrations were relatively stable over all treatments with the exception of a noticeable decrease in glucose during the first post-prandial period, i.e. in the 4–8 h group (6 h group; Fig. 1C).

For metabolic enzymes, absolute activity levels and effects of feeding thereon, were tissue dependent. For β-hydroxybutyrate dehydrogenase, the order of tissue-specific activity in starved dogfish was rectal gland>>anterior stomach>intestine>kidney>liver>posterior stomach>brain> muscle, gill and esophagus (Fig. 2). BHBDH activity in rectal gland increased significantly 4–8 h after feeding; this increase was sustained throughout much of the following time period (Fig. 2). Liver BHBDH activity peaked at 19–22 h, and returned to control levels by 29–31 h (Fig. 2). Anterior stomach activity of BHBDH increased slightly by 19–22 h, and declined thereafter. Other tissues exhibited no significant changes (muscle, gill, intestine, kidney) or only minor changes (Fig. 2). For malic enzyme, activities were highest in intestine and rectal gland, and most tissues showed no significant changes with feeding (liver, muscle, intestine, rectal gland, anterior stomach) (Fig. 3). The most pronounced variation of ME was in brain, where activities peaked at 19–22 h post-feeding (Fig. 3).

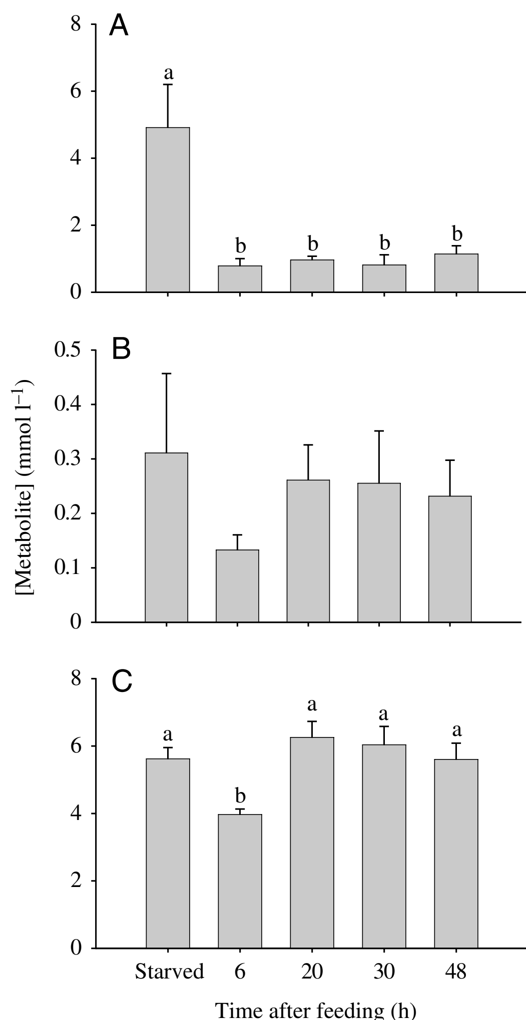

Fig. 1. Plasma concentrations of metabolites at various times post-feeding in dogfish shark (*Squalus acanthias*). (A) β-hydroxybutyrate; values are means ± s.e.m. (N=7–14 per time point). (B) Acetoacetate; values are means ± s.e.m. (N=6 per time point). (C) Glucose; values are means ± s.e.m. (N=7–14 per time point). Different letters above the bars indicate significant differences (P<0.05).

Oxidative metabolism and/or activity of hydrogen shuttles, as indicated by ICDH activity was similar in most tissues of starved dogfish, with the highest activity in anterior stomach, liver and posterior stomach (Fig. 4). ICDH activity incurred a massive (>10-fold) increase in rectal gland such that by 19–22 h, this enzyme clearly had the highest activity of tissues examined. This increase was followed by a rapid reduction to starved control levels by 29–31 h (Fig. 4). Notably, liver, anterior stomach and intestine also exhibited similar, yet less pronounced patterns of peak ICDH activity at 19–22 h (Fig. 4). Because of these high levels of ICDH induced by feeding in the rectal gland, we also measured a more direct indicator of oxidative metabolism in the rectal gland (only), mitochondrial citrate synthase (Fig. 5), for comparison. The values of CS measured were rather high in comparison to tissues for other species (see Discussion) and showed a significant increase beginning at 20 h post-feeding (Fig. 5).

The activity of lactate dehydrogenase, generally considered to be an indicator of anaerobic glycolysis (or alternatively for the utilization of lactate) in the cytosolic compartment was, as expected, highest in muscle tissue, followed by rectal gland and intestine, and then several other tissues with liver being the lowest (Fig. 6). LDH activity in liver, intestine and esophagus did not change post-feeding, and a significant peak in rectal gland and brain was noticed in LDH activity by 19–22 h (Fig. 6). Muscle exhibited a peak by 29–31 h, whereas other significant variation was less remarkable (Fig. 6).

The pentose shunt enzyme 6-PGDH showed significant increases in liver, gill, intestine, esophagus and anterior stomach with feeding, peaking at 19–22 h at ~1 U g^{-1} in gill (results not shown). Interestingly, rectal gland, a tissue in which other enzymes showed plasticity had rather constant levels of 6-GPDH at ~0.6 U g^{-1} in all treatments (results not shown).

The activation of secretory function of the rectal gland was reflected in the initial activation of Na^+/K^+ ATPase shortly after feeding (Fig. 7). This was followed by a return to fasting enzyme activity levels at 20 h and a subsequent slow increase of activity at 48 h (Fig. 7).

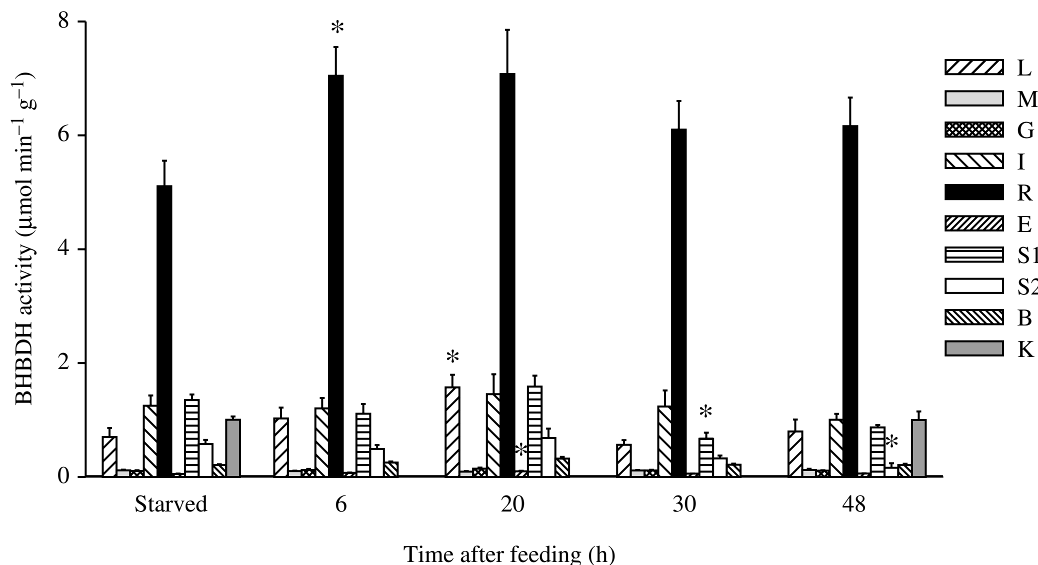

Fig. 2. Activities of β-hydroxybutyrate dehydrogenase (BHDBH; μmol min^{-1} g^{-1}) in various tissues at various times post-feeding in dogfish shark (*Squalus acanthias*). Values are means ± s.e.m. (*N*=3–12 per time point). L, liver; M, muscle; G, gill; I, intestine; R, rectal gland; E, esophagus; S1, anterior stomach; S2, posterior stomach; B, brain and K, kidney. NB kidney samples were taken only from starved fish and at 48 h post-feeding. Increases in activity in rectal gland at all times post-feeding were significantly different from that of starved fish ($P<0.05$). Significant changes in enzyme activities for other tissues are indicated by an asterisk ($P<0.05$).

Activities of four enzymes of nitrogen metabolism (AAT, DAT, GDH and GS) are shown in Table 1, and there were significant increases in activity associated with feeding in three of them (AAT, DAT and GS). However, these activities are relatively unremarkable in their overall levels in comparison with a variety of other dogfish tissues presented in Kajimura et al. (Kajimura et al., 2006).

Rectal gland metabolic substrate preferences

In general, the perfused rectal gland preparation in our hands showed good viability, and secretion rates/activation by forskolin compared well with previous studies. For all preparations, chloride concentration of rectal gland secretions ranged from 454 to 582 mmol l^{-1}, and urea concentration from 7 to 28.8 mmol l^{-1}.

In the first series of experiments, we wished to determine potential effects of glucose concentration and we compared the typically used (and indeed physiological) 5 mmol l^{-1} to the supraphysiological concentration of 30 mmol l^{-1} used in some studies. We found no effect of

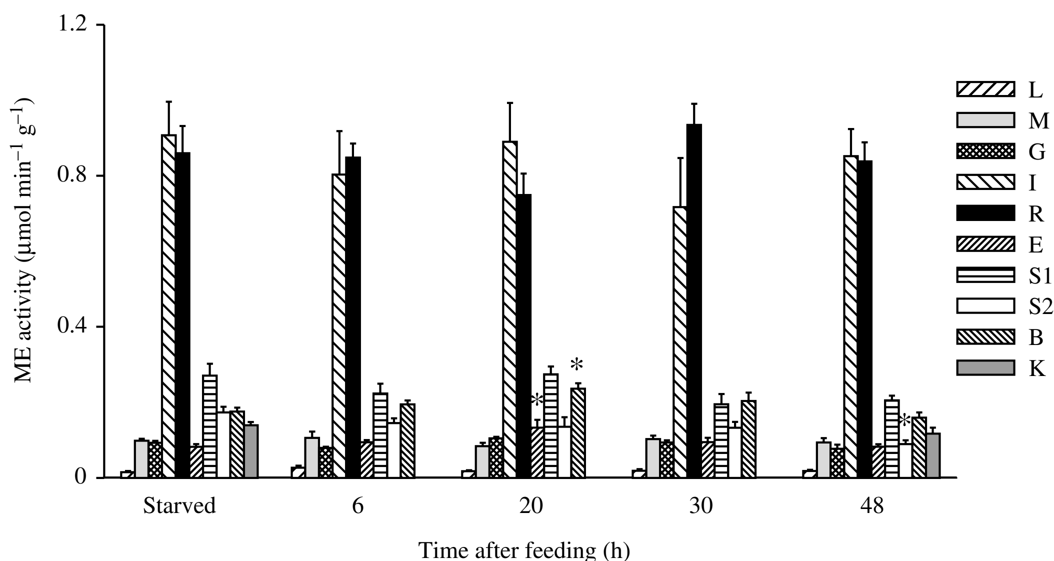

Fig. 3. Activities of malic enzyme (ME; μmol min^{-1} g^{-1}) in various tissues at various times post-feeding in dogfish shark (*Squalus acanthias*). Values and symbols as in Fig. 2. Significant changes in enzyme activities are indicated by an asterisk ($P<0.05$).

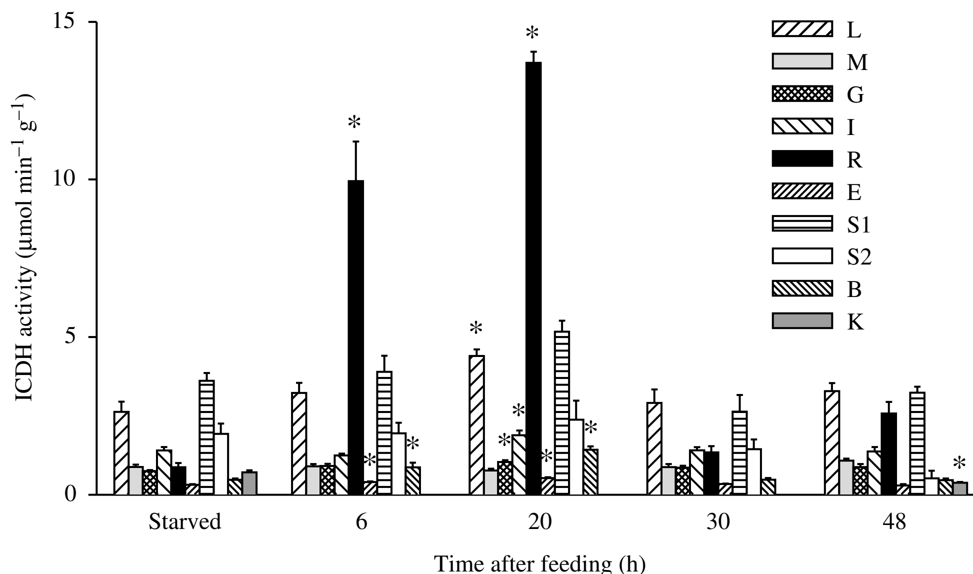

Fig. 4. Activities of NADP-isocitrate dehydrogenase (ICDH; μmol min^{-1} g^{-1}) in various tissues at various times post-feeding in dogfish shark (*Squalus acanthias*). Values and symbols as in Fig. 2. Significant changes in enzyme activities are indicated by an asterisk (P<0.05).

glucose concentration in this range (5 mmol l^{-1} glucose = 0.594±0.232 mmol Cl^{-} h^{-1} g^{-1}; 30 mmol l^{-1} glucose = 0.592± 0.094 mmol Cl^{-} h^{-1} g^{-1}).

In the next series of experiments, we wished to examine if the addition of other substrates to the baseline of 5 mmol l^{-1} glucose could alter chloride secretion. Among a variety of substrates (lactate, alanine, glutamate, acetoacetate), only β-hydroxybutyrate (BHB) had the ability to increase secretion rate by 60-87%, without any discernable difference between the effects of 0.5 to 5 mmol l^{-1} BHB (Table 2). Notably, the augmentation of secretion rate by BHB had some residual effect in that when some glands were switched back to glucose only, elevated secretion rate was maintained for at least another hour (Table 2).

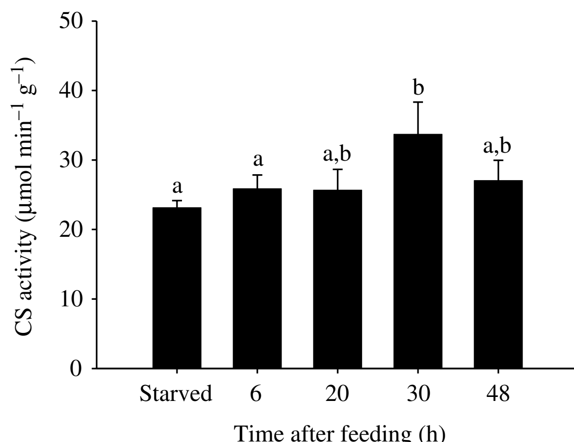

Fig. 5. Activities of citrate synthase (CS; μmol min^{-1} g^{-1}) in rectal gland at various times post-feeding in dogfish shark (*Squalus acanthias*). Values and symbols as in Fig. 2 (N=5–12 per time point). Different letters above the bars indicate significant differences (P<0.05).

Based on the above result of BHB augmentation of the effect of glucose, and on the high BHBDH values obtained for rectal gland (Fig. 2), in the next experimental series we wished to determine if BHB could support chloride secretion alone, or alternatively, if glucose was strictly required by the gland. Clearly, preparations were not viable when perfusion was begun with BHB only, as their chloride secretion rates were low in comparison to most preparations (Table 3). Even when augmented with 5 mmol l^{-1} glucose in the second flux period, secretion rates declined (Table 3). We also attempted starting the gland with 5 mmol l^{-1} glucose and then switching to BHB alone, or subsequently adding back glucose, but preparations so treated also exhibited decline (Table 3). In all cases in these preparations, we could detect an 'on the spot' substantial decline in the volume secreted in the second 30-min portion of each hour-long flux when glands were presented with only BHB as substrate (results not shown).

Table 1. *Activities of selected enzymes of nitrogen metabolism in rectal gland of dogfish shark, starved and post-feeding*

Time post feeding (h)	N	Enzyme activity (μmol min^{-1} g^{-1})			
		AAT	DAT	GDH	GS
0 (Starved)	8	1.75±0.21[a]	72.10±2.82[a,b]	7.32±0.38	1.45±0.21[a]
6	12	2.59±0.23[b,c]	78.68±3.22[a]	7.86±0.39	3.32±0.39[b]
20	5	3.12±0.48[b]	67.01±1.97[b]	7.92±0.57	3.88±0.34[b]
30	7	2.07±0.25[a,c]	67.37±2.75[b]	7.55±0.41	1.69±0.33[a]
48	6	2.15±0.24[a,c]	72.07±2.64[a,b]	7.43±0.22	1.85±0.42[a]

AAT, alanine amino transferase; DAT, aspartate amino transferase; GDH, glutamate dehydrogenase; GS, glutamine synthetase.

Different lowercase letters indicate significant differences (P<0.05).

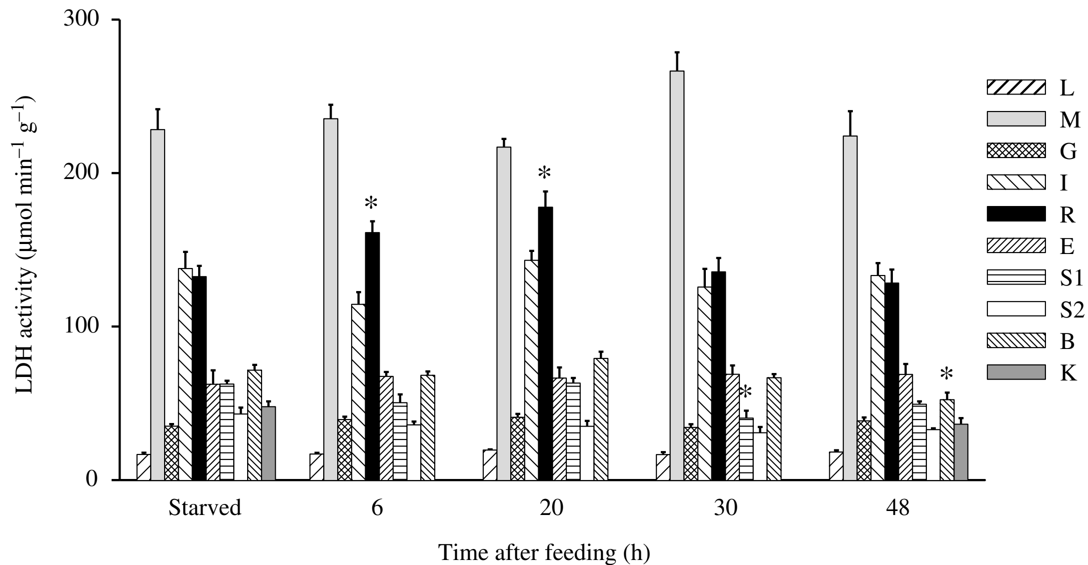

Fig. 6. Activities of lactate dehydrogenase (LDH; μmol min^{-1} g^{-1}) in various tissues at various times post-feeding in dogfish shark (*Squalus acanthias*). Values and symbols as in Fig. 2. Significant changes in enzyme activities are indicated by an asterisk ($P<0.05$).

Discussion

Several prior studies had shown that starvation in elasmobranchs increases the levels of circulating ketone bodies, which is especially notable after long-term starvation. To our knowledge, the present study is the first to examine the reverse and to demonstrate just how rapidly ketone bodies can be cleared from the circulation following feeding in elasmobranchs. Indeed during the first 4–8 h postprandial period, plasma BHB values declined 10-fold (Fig. 1A) and remained at characteristic 'fed' values of approximately 0.5 mmol l^{-1}. Interestingly, these dramatic changes in plasma BHB were not evident in acetoacetate (Fig. 1B), the oxidized counterpart of BHB, probably reflecting altered redox status of tissues involved in ketone body metabolism. The decline in BHB 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. In support of increased utilization, we observed significant elevations in BHBDH activities in rectal gland and brain (Fig. 2). The observation of a transient increase in liver BHBDH following feeding (Fig. 2) is interesting in light of the prior study (Zammit and Newsholme, 1979) where *starvation* was shown to increase liver BHBDH in related species, and the expectation that feeding would turn

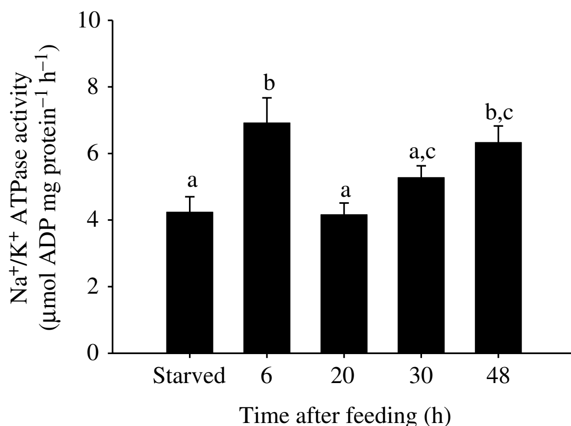

Fig. 7. Activities of Na^+/K^+ ATPase (μmol ADP mg^{-1} protein h^{-1}) in rectal gland at various times post-feeding in dogfish shark (*Squalus acanthias*). Values and symbols as in Fig. 4 (N=5–11 per time point). Different letters above the bars indicate significant differences ($P<0.05$).

Table 2. *Ability of various substrates at physiological concentrations to augment the ability of glucose to support dogfish shark rectal gland chloride secretion*

(A) Effects of substrates	Activity (%)
Glucose (5 mmol l^{-1})	100
Lactate (1 mmol l^{-1})	98.7±11.7
Alanine (1 mmol l^{-1})	88.5±11.0
Glutamate (0.1 mmol l^{-1})	87.3±4.5
Acetoacetate (0.25 mmol l^{-1})	91.2±0.8
BHB (0.5 mmol l^{-1})	160±12.5*
BHB (5 mmol l^{-1})	187±15.7*
(B) Residual effect of BHB	
Glucose only, no prior BHB	103±13.9
Glucose + 5 mmol l^{-1} BHB	200.5±1.6*
Glucose only, 1 h after glucose + 5 mmol l^{-1} BHB	191±54*

BHB, β-hydroxybutyrate.

Substrates are in addition to 5 mmol l^{-1} glucose.

Values are expressed as percentages (means ± s.e.m., N=3) of the 5 mmol l^{-1} glucose value (0.621±0.203 mmol Cl^- h^{-1} g^{-1}).

*Significantly different from control, $P<0.05$.

Table 3. *Ability of β-hydroxybutyrate (0.5 mmol l^{-1}) to support dogfish shark rectal gland chloride secretion*

	Time after addition of BHB (h)			
	0–1	1–2	2–3	3–4
(A) Effect of initiating forskolin stimulation in the presence of 0.5 mmol l^{-1} BHB alone and with glucose (5 mmol l^{-1})				
BHB alone	0.128±0.059			
BHB + glucose		0.192±0.027	0.098±0.054*	0.018±0.018*
(B) Effect of initiating forskolin stimulation in the presence of 5 mmol l^{-1} glucose alone before switching to 0.5 mmol l^{-1} BHB				
Glucose	0.360±0.110			
BHB alone		0.292±0.029		
BHB + glucose			0.250±0.028*	

BHB, β-hydroxybutyrate.
Values are Cl^- secretion (mmol Cl^- h^{-1} g^{-1} tissue wet mass) (means ± s.e.m.) (*N*=3).
*Significantly different from rate in 0–1 h period (*P*<0.05).

down hepatic BHB production. We can only speculate that this unexpected observation relates either to species differences or the very different time courses of the two studies [several days in the present *vs* 40 to 150 days (Zammit and Newsholme, 1979)], or the complexity of the response of hepatic BHBDH to feeding regimes.

Plasma glucose concentration remained relatively stable following feeding (Fig. 1C), although it underwent a temporary decrease of about 30% during the 4-8 h post-feeding period. A similar decrease in blood glucose was shown when dogfish were exposed to exogenous insulin (deRoos et al., 1985). It would be interesting to determine if feeding release of insulin is responsible for this decrease in plasma glucose. Although peptide sequences have been determined for shark insulins and sharks respond to injection of homologous insulins with decreases in plasma glucose [but no changes in plasma BHB or acetoactate (Anderson et al., 2002)], post-prandial responses in plasma insulin and subsequent metabolic changes are yet to be described.

In terms of enzyme activity measurements, we noted increases in Na^+/K^+ ATPase activity at 6 h and 48 h (Fig. 7) that are consistent with activation of the gland by feeding. Notably, the increase in activity is biphasic, a point that we return to below. One of the most striking results to emerge from this study are the overall enzyme activity values for the rectal gland. It has been known for some time that the tissue is extremely metabolically active when in secretion mode; for example, even unstimulated glands have oxygen consumption rates of 0.1–0.3 μmol g^{-1} min^{-1}, whereas stimulated glands can achieve rates of 1.4 μmol g^{-1} min^{-1} (Silva et al., 1980; T. J. Shuttleworth, J. Thompson, R. S. Munger and C.M.W., manuscript submitted for publication). Our results demonstrate that these rates are also reflected in enzyme activities. Notably, per mass, enzyme activities for rectal gland are higher than all other tissues measured (for BHBDH and ICDH, Figs 2, 4) or are equivalent to those of the highest tissues measured (for ME and LDH, Figs 3, 6). The CS data (Fig. 5) are also notable in that the 20 to 30 U g^{-1} we report are similar to values for red muscle in tuna (Guppy et al., 1979)!

The LDH data are particularly interesting in light of prior measurements of substantial lactate in the gland (7 mmol l^{-1}) (Silva et al., 1980), values that are close to those in white muscle in this species (Richards et al., 2003). The inability of lactate to augment glucose-based secretion rates (Table 2) indicates that the high LDH activity seen in the rectal gland is probably not for *use* of lactate from the circulation, but for production of lactate. Given the rather high oxygen consumption rate of the gland, we speculate that there may be brief periods when demand for chloride secretion outpaces the ability of the circulation to deliver oxygen to fuel aerobic metabolism, and that a certain fraction of the gland's secretion output is met periodically by anaerobic glycolysis.

In addition to having generally high metabolic rates reflected in enzyme activity, the metabolic poise of the rectal gland seems to be rather plastic at the molecular level. Short-term (i.e. 4–8 h) increases in enzyme activity were noted for BHBDH, ICDH, LDH and Na^+/K^+ ATPase. A similar observation of short-term activation of Na^+/K^+ ATPase activity was made for European dogfish *Scyliorhinus canicula* at 3–12 h postfeeding (MacKenzie et al., 2002). These changes in both studies take place presumably during a time course when mechanisms other than transcriptional or translational control would predominate [and indeed MacKenzie et al. did not find an increase in Na^+/K^+ ATPase message in the short term (MacKenzie et al., 2002)]. It would be interesting to examine the mechanisms of short-term enzyme activation in this gland, especially considering that there is not precedent for typical post-translational modifications (e.g. phosphorylation/dephosphorylation) for many of the enzymes we examined.

We also observed changes in enzyme activities after a longer time course post-feeding (for ICDH, CS, and Na^+/K^+ ATPase) where it is more likely that translational and transcriptional control could be involved. Control by transcription for Na^+/K^+

ATPase was examined in European dogfish *Scyliorhinus canicula* (MacKenzie et al., 2002), and changes in mRNA were only observed 1 day after feeding at a time when enzyme activities were not elevated. It will be interesting to determine if a similar lack of transcript elevation is seen for the three genes showing late enzyme activation in the present study, namely CS, ICDH and Na^+/K^+ ATPase.

Given the above results on rapid clearance of BHB, and the high enzyme activities of the gland (particularly for BHBDH), we wished to determine substrate preferences of the gland in an *in vitro* perfused system. Indeed, several parameters of gland function (responsiveness of chloride transport to forskolin stimulation, secretory flow rate, chloride secretion rate, ability to exclude urea from the secretion, etc.) indicated that our preparation was viable for several hours after isolation, in accordance with previous studies (e.g. T. J. Shuttleworth, J. Thompson, R. S. Munger and C.M.W., manuscript submitted for publication). Prior to testing of several substrates, we examined the use of a more realistic glucose concentration as a baseline substrate, and indeed found that a typical plasma glucose value of 5 mmol l^{-1} could support gland function as effectively as 30 mmol l^{-1} (see Results); therefore subsequent experiments were conducted at this concentration.

Several substrates representing carbohydrate (lactate), amino acid (alanine, glutamate) and ketone body (acetoacetate, β-hydroxybutyrate) metabolism were examined for their ability to augment the chloride secretion rate supported by 5 mmol l^{-1} glucose. Indeed, only BHB was able to further stimulate the gland (Table 2), and it did so equally at concentrations typical of fed (0.5 mmol l^{-1}) or starved (5 mmol l^{-1}) dogfish plasma.

However, BHB alone was unable to sustain the chloride transport function of the gland. Even in different combinations/arrangements (glucose first, followed by BHB, etc), the viability of the gland was compromised after only a short period when the sole fuel source provided was BHB. Thus we conclude that glucose is one of the likely primary substrates for the gland *in vivo*, but that BHB can supplement the glucose-based metabolism. This arrangement makes sense from more than one perspective. First, at the biochemical level, the reactions of glycolysis using glucose as a substrate prior to entry of acetyl CoA into the mitochondria will provide two additional ATP molecules per mole of substrate, as well as two NADH equivalents per mole at the glyceraldehyde-3-phosphate dehydrogenase step, and two NADH equivalents at the pyruvate dehydrogenase step (all four of which can be transferred to the electron transport system). One mole of BHB will provide only one NADH equivalent (*via* BHBDH) *en route* to acetyl CoA. Thus, on a per mole basis, glucose is a more efficient substrate than BHB. Another advantage of using primarily glucose as a fuel is that, if $NADH/NAD^+$ ratios skew unfavorably, or if oxygen demand simply outpaces supply, some of the pyruvate could be shunted towards lactate production by LDH to replenish cytoplasmic NAD^+ and maintain redox balance. Interestingly, the ability of BHB to augment glucose-fueled chloride secretion would imply that, at least under our experimental conditions, the utilization of acetyl-CoA by rectal gland mitochondria did not reach its maximum. It is likely that exogenous delivery of BHB can increase the rate of chloride transport, linked to rapid delivery of intramitochondrial electrons through the BHBDH reaction and perhaps minor adjustments in intramitochondrial redox. An analogous situation would be the rapid, partial, utilization of α-glycerophosphate by insect flight muscle mitochondria. However, it appears that the rectal gland mitochondria will only respond if already primed with glucose. At this point, and using analogous mammalian models, we can assume that the subsequent steps in BHB metabolism are relatively slow delivering acetyl-CoA to citrate synthase *via* a CoA transferase, using mitochondrial succinyl-CoA as CoA donor, and thiolase. By the same token, BHB alone should have provided at least a short oxidative surge, but the time course of our experiments was not designed to detect such short-term effects.

Often the activity of citrate synthase (our choice here) or cytochrome c oxidase are used as a proxy for mitochondrial abundance. Considering that CS in insect flight muscle is between 40 and 120 U g^{-1} of thorax (scaling effects!) at 25°C (Heinrich and Mommsen, 1985), the 25–40 U in rectal gland also compare favorably with other highly aerobic tissues, such as bluefin tuna heart [77–95 U at 25°C (Blank et al., 2004)]. Although these highly aerobic tissues are usually characterized by relatively modest anaerobic capacities, as indicated by the activity of LDH, it appears that the dogfish rectal gland has the best of both worlds: a high aerobic capacity to drive transport processes, backed up by substantial anaerobic capacity. Considering the relative inefficiency of anaerobic glycolysis, the rectal gland seems to follow the motto: transport at any cost! Of course, taking into consideration the small relative size of the rectal gland (at 0.05% of body mass), it is unlikely that consumption rates of glucose or BHB, or production rates of lactate, will make major contributions to the metabolic turnover of the whole dogfish. It would be interesting to repeat these experiments under conditions where rectal gland secretion was maximal to determine if the mitochondria could be saturated, and to examine if significant amounts of lactate were produced. As noted above, significant levels of lactate have been reported previously in rectal gland (Silva et al., 1980).

The choice of glucose as one apparent primary fuel for the rectal gland, with the ability to supplement with BHB, also makes sense in the context of the whole animal. When the gland is stimulated by neuroendocrine signals and the alkaline tide (Wood et al., 2005; C.M.W., R. S. Munger, J. Thompson, T. J. Shuttleworth, manuscript submitted for publication) following feeding, glucose concentrations are relatively stable, whereas BHB concentrations are rapidly changing. Thus, the rectal gland would have a constant substrate supply and not necessarily be competing with other tissues for BHB to perform its vital physiological functions. A final caveat to our analysis is that lipid metabolism was not examined. Indeed, recent enzymatic evidence suggests that a few selected

peripheral tissues in elasmobranches, including rectal gland, may in fact be able to oxidize lipids as indicated by activities of enzymes such as carnitine palmitoyl transferase (Speers-Roesch et al., 2006b). The perfused rectal gland preparation would be ideal to examine the ability of substrates such as palmitate to support chloride secretion.

For decades, the elasmobranch rectal gland has proved to be an exciting physiological system for the study of transport processes. Further studies of its metabolic physiology and biochemistry in this context will surely yield similarly exciting insights into the mechanisms of rapid upregulation of metabolism.

We thank the staff at the Bamfield Marine Sciences Centre, especially Dr Bruce Cameron, for their exceptional level of support, without whose help these studies could not have been accomplished. The research was supported by an NSF grant (IOB-0455904) and NIH grant (ES05705) to P.J.W. and an NSERC Discovery Grant to C.M.W., who are also supported by the Canada Research Chair Program.

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