Champagne CD, Houser DS, Fowler MA, Costa DP, Crocker DE. Gluconeogenesis is associated with high rates of tricarboxylic acid and pyruvate cycling in fasting northern elephant seals. Am J Physiol Regul Integr Comp Physiol 303: R340–R352, 2012. First published June 6, 2012; doi:10.1152/ajpregu.00042.2012.—Animals that endure prolonged periods of food deprivation preserve vital organ function by sparing protein from catabolism. Much of this protein sparing is achieved by reducing metabolic rate and suppressing gluconeogenesis while fasting. Northern elephant seals (Mirounga angustirostris) endure prolonged fasts of up to 3 mo at multiple life stages. During these fasts, elephant seals maintain high levels of activity and energy expenditure associated with breeding, reproduction, lactation, and development while maintaining rates of glucose production typical of a postabsorptive mammal. Therefore, we investigated how fasting elephant seals meet the requirements of glucose-dependent tissues while suppressing protein catabolism by measuring the contribution of glycogenolysis, glycerol, and phosphoenolpyruvate (PEP) to endogenous glucose production (EGP) during their natural 2-mo postweaning fast. Additionally, pathway flux rates associated with the tricarboxylic acid (TCA) cycle were measured specifically, flux through phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate cycling. The rate of glucose production decreased during the fast (F1,13 = 5.7, P = 0.04) but remained similar to that of postabsorptive mammals. The fractional contributions of glycogen, glycerol, and PEP did not change with fasting; PEP was the primary gluconeogenic precursor and accounted for ~95% of EGP. This large contribution of PEP to glucose production occurred without substantial protein loss. Fluxes through the TCA cycle, PEPCK, and pyruvate cycling were higher than reported in other species and were the most energetically costly component of hepatic carbohydrate metabolism. The active pyruvate recycling fluxes detected in elephant seals may serve to rectify gluconeogenic PEP production during restricted anaplerotic inflow in these fasting-adapted animals.

NEARLY ALL ANIMALS EXPERIENCE PERIODS OF FOOD DEPRIVATION, FREQUENTLY AS A RESULT OF SEASONAL VARIATION IN FOOD AVAILABILITY OR THE REQUIREMENTS OF REPRODUCTION. ANIMALS USUALLY RESPOND TO FASTING BY REDUCING THEIR OVERALL METABOLIC RATE, INCREASING THEIR RELIANCE ON LIPID RESERVES TO MEET ENERGETIC COSTS, AND CONSERVING LEAN TISSUE TO PRESERVE VITAL ORGAN FUNCTION (11). SELECT TISSUES, HOWEVER, CANNOT USE LIPID AS AN ENERGY SOURCE, AND SOME PROTEIN MUST BE DEGRADED TO SUPPLY THE PRECURSORS FOR GLUCONEOGENESIS TO MEET THE ENERGY NEEDS OF THESE TISSUES INCLUDING THE BRAIN, RED BLOOD CELLS, AND RETINAL MEDULLA. THE COMMITMENT OF AMINO ACIDS TO GLUCONEGENIC PATHWAYS DEPLETES PROTEIN RESERVES, COMPROMISING VITAL ORGAN FUNCTION IN FASTING ANIMALS (24). TERMINAL STARVATION OCCURS ONCE LIPID RESERVES ARE DEPLETED OR THE LOSS OF PROTEIN RESULTS IN VITAL ORGAN FAILURE (11, 17, 25). THEREFORE, SURVIVAL AND RECOVERY DEPEND ON METABOLIC ADJUSTMENTS TO MANAGE AVAILABLE ENERGY STORES WHILE FASTING.

DEPRESSING METABOLIC RATE IS A COMMON CONSEQUENCE OF FASTING (26, 51, 75, 82). THIS OPTION, HOWEVER, IS NOT AVAILABLE TO ANIMALS THAT UNDERGO FASTS IN COMBINATION WITH ENERGETICALLY DEMANDING PROCESSES, INCLUDING MIGRATION (52, 86), REPRODUCTION (21, 22, 63), AND DEVELOPMENT (4, 83). THUS FASTING IN NEONATES IS RARE DUE TO THE HIGH ENERGY AND MATERIAL REQUIREMENTS OF GROWTH AND DEVELOPMENT. THESE CONTINUED REQUIREMENTS USUALLY DEMAND SIGNIFICANT NUTRIENT CONSUMPTION BY THE NEONATE. NEVERTHELESS, SOME SPECIES INTEGRATE FASTING WITH POSTNATAL DEVELOPMENT; KING PENGUIN CHICKS (APTONETIDES PATAGONICA) UNDERGO A FAST OF 5 MO (18) AND MANY PHOcid SEALS (E.G., GREY SEAL, HALichoerus grypus; HARP SEAL, Phoca groenlandica; MONK SEALS Monachus schauinslandi; AND NORTHERN ELEPHANT SEALS, Mirounga angustirostris) UNDERTAKE PROLONGED FASTS OF 2–12 WK AFTER WEANING (19).

NORTHERN ELEPHANT SEALS PROVIDE AN IDEAL STUDY SYSTEM TO INVESTIGATE LONG-DURATION FASTING AND ONE OF THE FEW OPPORTUNITIES TO STUDY FASTING DURING DEVELOPMENT. AFTER BEING WEANED, PUPS FAST FOR 2–3 MO WHILE METABOLIC REQUIREMENTS ARE MET PRIMARILY THROUGH FATTY ACID OXIDATION (64). POSTNATAL DEVELOPMENT CONTINUES AND THE SWIMMING AND DIVING ABILITIES NECESSARY FOR FORAGING AT SEA ARE ACQUIRED DURING THE FAST (76). THIS DEVELOPMENTAL PROGRESSION IS EVIDENT IN THE INCREASES IN HEMATOCRIT, HEMOGLOBIN AND MYOGLOBIN CONCENTRATIONS, AND MASS-SPECIFIC BLOOD VOLUME THAT OCCUR ACROSS THE FAST (81). PROTEIN CATABOLISM CONtributes less than 4% to the average metabolic rate while fasting and declines with the progression of the fast (1, 32, 70), SUGGESTING THAT THE CONTRIBUTION OF PROTEIN TO GLUCONEOGENESIS MAY DECLINE ACROSS THE FAST. PREVIOUS WORK FOUND THAT ELEPHANT SEALS ONLY SLIGHTLY DECREASE ENDOGENOUS GLUCOSE PRODUCTION (EGP) AND MAINTAINED CIRCULATING GLUCOSE LEVELS ABOVE 140 MG/DL DURING THE POSTWEANING FAST (15) COINCIDENT WITH PREVIOUSLY MEASURED DECREASES IN METABOLIC RATE AND PROTEIN CATABOLISM (1, 32, 74). THIS CONTRASTS WITH THE MARKED SUPPRESSION OF GLUCOSE PRODUCTION IN RESPONSE TO FASTING SEEN IN MOST SPECIES (11, 16, 20, 39, 80).

UNCHARACTERISTIC OF FASTING MAMMALS, ELEPHANT SEAL WEANLINGS DEMONSTRATE MODERATE FASTING HYPERGLYCEMIA (GLUCOSE CONCENTRATIONS ABOVE 140 MG/DL) AND RATES OF EGP THAT ARE GREATER THAN THOSE OBSERVED IN NONFASTING-ADAPTED SPECIES UNDERGOING SIMILAR DURATION FASTS (14, 15). THE RATES OF EGP OBSERVED IN FASTING WEANLINGS ARE, IN FACT, COMPARABLE TO THOSE OF SIMILARLY SIZED POSTABSORPTIVE MAMMALS. FURTHERMORE, THE RATE OF EGP EXCEEDS THE ESTIMATED ENERGETIC REQUIREMENTS OF GLUCOSE-DEPENDENT TISSUES BY SEVERAL TIMES (15). GIVEN ELEPHANT SEALS’ RELIANCE ON LIPID CATABOLISM AND HIGH RATE OF LIPOLYSIS, GLUCONEOGENESIS FROM GLYCEROL (GNGglycerol)
was the expected dominant pathway in glucose production. Measures of GNGglycerol in this species, however, suggest that glycerol is a relatively minor contributor to glucose production during lactation (31).

The objective of this study was to examine the pathways involved in glucose production during fasting in northern elephant seals. Most tracer studies in wildlife use single or, perhaps, dual-label isotope techniques; these techniques allow the assessment of only one or two pathway fluxes per tracer. Analysis by nuclear magnetic resonance (NMR) allows for the simultaneous determination of the enrichment of isotopes at multiple atomic positions. This positional tracer enrichment can then be used to determine multiple pathway fluxes simultaneously from a single tracer study (36). We applied stable isotope tracers and positional isotope analysis to study carbohydrate metabolism during natural, prolonged fasts in northern elephant seals (see Fig. 1 for a diagram of pathways investigated). We aimed to determine the sources responsible for continued glucose production during fasting and characterize pathway flux rates related to the tricarboxylic acid (TCA) cycle. Rates of EGP were measured using [1,6-13C2]glucose; the contributions of glycogen, glycerol, and PEP were determined using deuterated water (2H2O). Pathway flux rates relating to the TCA cycle were simultaneously assessed using [U-13C3]propionate.

METHODS

Study animals and sedation. This study was conducted at Año Nuevo state park, San Mateo county, California. Measurements were made in eight pups (5 female, 3 male) early (18, sd 2.4, days) and late (53, sd 5.0, days) in their postweaning fast between February and April, 2009. We determined fasting duration by monitoring mother-pup pairs during lactation and marking pups on their day of weaning using hair dye (Lady Clairol, Stamford, CT) and by inserting identification tags in rear flippers (jumbo roto-tags, Dalton, Oxon, UK). During the initial measurement, a VHF radio transmitter (Advanced Telemetry Systems, Isanti, MN) was attached with 5-min epoxy to the dorsal pelage of study animals to facilitate recapture at the end of the fast; the transmitter was removed during the second measurement.

Mass measurements were made by suspending the study animal, while restrained in a custom-made cage, from a tripod and scale (MSI tension dynamometer, ±2 kg; Seattle, WA).

Study animals were chemically immobilized in the field using 1 mg/kg tiletamine-zolazepam (Telazol) as previously described (15, 32), and maintenance doses of ketamine (50–100 mg) and diazepam (2.5–5 mg) were administered intravenously throughout the procedure as needed. All drugs were from Fort Dodge Animal Health (Fort Dodge, IA). This anesthetic protocol does not significantly influence carbohydrate metabolism in elephant seals (13). This work was conducted under National Marine Fisheries Permit no. 14636, approved by the UC Santa Cruz Institutional Animal Care and Use Committee, and was in accordance with the Guide for the Care and Use of Laboratory Animals.

Infusion protocol. Three isotope tracers, 2H2O, [U-13C3]-propionate, and [1,6-13C2]glucose (all from Cambridge Isotope Laboratories, Andover, MA), were used to examine carbohydrate metabolism in seals following the methods of Jones et al. (38). Each measurement was performed over 2 days. In the evening of the first day, weaned pups were chemically immobilized in the field as described above. 2H2O (480 ml) was injected into the intraperitoneal space to achieve ~1% isotope enrichment of body water. After 2H2O administration, study animals were held in an enclosure overnight while the isotope equilibrated.

The infusion procedure was performed the following day. Weaned pups were immobilized as before. Two catheters were inserted into the extradural vessel to facilitate blood sampling and to administer isotopic tracers and anesthetic agents. First, a 14-gauge × 13.3-cm catheter (BD Angiocath no. 382259, Franklin Lakes, NJ) was inserted dorsal to the pelvis, approximately one-quarter body length anterior from the tail. Initial blood samples were collected for hormone and metabolite analysis. [U-13C3]propionate (2.5 g) dissolved in 30 ml sterile water was slowly administered intravenously over ~15 min. The second catheter, 14-gauge × 20 cm (no. 1610-2P; MILA International, Erlanger, KY), was inserted about 25 cm anterior to the initial catheter to administer [1,6-13C2]glucose. A primed continuous infusion of [1,6-13C2]glucose dissolved in sterile water was given using a Pegasus VARIO micropump infusion pump (Instech Solomon, Plymouth Meeting, PA). The priming dose was 500 mg, and the infusion rate was 500 mg/h over 3 h; we began all infusion procedures between 10:30 AM and 12:00 PM to minimize the potential for diel

Fig. 1. Pathways of carbohydrate metabolism investigated in this study. The tracer methods used in the present study investigate pathways shown in bold, these include: endogenous glucose production (EGP, v1), glycolysis (v2), gluconeogenesis from glycogen (GNGglycogen, v3), gluconeogenesis from phosphoenolpyruvate (PEP) (GNGPEP, v4), pyruvate cycling [from oxaloacetate (OAA) through pyruvate and back to OAA, v5], PEP carboxykinase (PEPCK) activity (OAA → PEP, v6), and tricarboxylic acid (TCA) cycle flux through citrate synthase (CS, v7). The sites of hydrogen exchange with body water are identified along with their position [e.g., hydrogen on carbon two of glucose is exchanged with body water between glucose-6-phosphate (G-6-P) and fructose-6-phosphate (F-6-P) and labeled “H2”]. Labeled propionate enters the TCA cycle via succinyl-CoA and labels carbons of all TCA cycle intermediates. Redrawn, with permission, from Burgess et al. (2005). DHAP, dihydroxyacetone phosphate; F-B-P, fructose-bisphosphate; G-3-P, glucose-3-phosphate.

AJP-Regul Integr Comp Physiol • doi:10.1152/ajpregu.00042.2012 • www.ajpregu.org
variability. At the end of the infusion, 200 ml blood were drawn from the initial catheter into chilled, heparinized vacutainers. Samples were transported back to the lab and centrifuged at 1,500 rpm and 4°C. Plasma and serum supernatant were collected and stored at −80°C until subsequent analysis.

Preparation of samples for NMR analysis. Plasma from the post-equilibration sample was thawed and deproteinated using barium hydroxide and zinc sulfate (both 0.3 N, from Sigma Aldrich, St. Louis, MO). The deproteinated supernatant was passed through an ion exchange column containing anion resin (AG1 X8 formate form) and cation resin (AG50W-X8 hydrogen form; both 200–400 mesh from Bio-Rad Laboratories, Hercules, CA) to remove any charged compounds. Each sample was then separated into two fractions, 1) a glucose fraction for the calculation of glucose production, and 2) a monoacetone glucose (MAG) fraction to determine relative sources of glucose and TCA flux rates. Glucose was extracted and converted to MAG using methods modified from Jones et al. (38). Both fractions were dried in a speed vacuum. The glucose fraction was reconstituted in several milliliters of water, and the glucose was extracted from the sample using Bio-Rad resin AG1-X8 in the boronate form. The sample was added to the column and washed with 5–6 bed volumes of water. Glucose was eluted from the column with 3 bed volumes of 0.5 M acetic acid. The collected glucose eluant was dried in a speed vacuum; when dry, the sample was ready for NMR analysis.

Glucose from the MAG fraction was converted to MAG by reconstituting the dried sample in 15 ml acetone and then slowly adding 600 μl H2SO4 and stirring for 6 h. Fifteen milliliters of water were then added to the sample, the pH was adjusted to 1.8 using Na2CO3, and the sample was stirred for an additional 24 h, completing the conversion of glucose to MAG. The glucose-to-MAG conversion reaction was stopped by increasing the pH to 8 using Na2CO3, and the sample was immediately dried on a speed vacuum with an ultracold trap. Once dry, the MAG was extracted into hot ethyl acetate solution and again evaporated to dryness. The sample was then reconstituted in several milliliters of water and passed through a final ion exchange column, containing the same two anion and cation resins as above, to remove remaining salts. The sample was dried a final time before NMR analysis.

NMR spectra collection. Spectra acquisition was performed by Acorn NMR, Livermore CA, using a JEOL ECX-400 NMR spectrometer and a 5-mm broadband probe. The [1,6-13C2]glucose enrichment was determined using 1H and 13C NMR spectra (Fig. 2). The dried glucose fraction was dissolved in 2H2O. 1H NMR spectra were collected at 30°C using a 45° pulse width, 0.9 s relaxation delay, and 4.3 s acquisition time. The 13C NMR spectra were collected at 30°C with a 30° pulse width, 0.5 s delay, and 2.08 s acquisition time. Both 2H and 13C NMR spectra were collected from the MAG sample fraction (example spectra are shown in Figs. 3 and 4). The MAG fraction was dissolved in several drops of water followed by acetonitrile. 2H NMR spectra were acquired at 50°C using a 90° pulse width, 5 s relaxation delay, and 1.8 s acquisition time. 13C NMR spectra were collected at 30°C with a 50° pulse width, 0.15 s relaxation delay, and 1.2 s acquisition time.

Positional isotope analysis. Pathway flux rates were calculated as described by Jones et al. (36–38) using both tracer dilution and tracer incorporation models and steady-state equations to calculate flux rates. The rate of glucose production was measured using the glucose sample fraction (not the MAG derivative) and calculated as:

\[ \text{EGP} = \frac{\text{infusion rate}}{13\text{C}-\text{sat}} - \frac{\text{infusion rate}}{13\text{C}-\text{sat}} \]

where EGP equals the rate of appearance of unlabeled glucose (90). The determination of [1,6-13C2]glucose enrichment is outlined in Fig. 2 following the methods of Jones et al. (36).

The fractional amount of glucose formed from PEP, glycerol, and glycogen was determined using the deuterated water method (46) and was obtained from the 2H NMR spectra of the MAG sample fraction (Fig. 3). 2H enrichment detection by NMR has been validated against mass spectrometry and found to accurately determine enrichment (9). The areas of the peaks resulting from 2H on carbons 2, 5, and 6 of glucose as a result of exchange with 2H2O from the labeled body water pool during gluconeogenesis were used to determine the fractional contribution from glycogen (v2/v1), glycerol (v3/v1), and PEP (v4/v1), relative to EGP (v1) (37). Fractional sources of EGP were converted to absolute fluxes by multiplication to calculate glycolysis (v2), gluconeogenesis from glycogen (GNGglycogen, v3), and from PEP (GNGPEP, v4).

Flux rates related to the TCA cycle were measured using the [U-13C3]propionate tracer. 13C-carbon enrichments were assessed

![Fig. 2. Measurement of EGP. The enrichment of [1,6-13C2]glucose was determined using both 1H and 13C nuclear magnetic resonance (NMR) spectra of glucose. The enrichment (E) of 13C at carbon-1 of glucose was measured using the 1H NMR of glucose and calculated as the area of the 13C satellites relative to the total area: E1,13Cglucose = \sum(\text{13C-sat})/\sum(\text{12C} + \text{13C-sat}). The fraction of [1,6-13C2]glucose also labeled at C-6 (fC-6) was measured using the 13C NMR spectra of glucose and calculated as the area of D16 relative to the total: fC-6 = \sum(D16)/\sum(S + D12 + D16 + D126). The enrichment of [1,6-13C2]glucose was calculated as: E1,6-13C2glucose = E1,13Cglucose·fC-6.](http://ajpregu.physiology.org/doi/10.1152/ajpregu.00042.2012)
from the C-2 region of the $^{13}$C NMR spectra of MAG to calculate flux rates from the TCA cycle [primarily through phosphoenolpyruvate carboxykinase (PEPCK), v6/v7], pyruvate cycling (v5/v7) back to oxaloacetate (OAA) through either pyruvate kinase (PK) or malic enzyme (ME), and a second measure of GNGPEP (v4/v7) relative to citrate synthase (CS) flux (v7) (Fig. 4) (36). The value of GNGPEP, measured by $[\text{U}-^{13}\text{C}]$propionate, was used to convert the relative fluxes to absolute flux rates as previously described (38). These pathway fluxes represent substrate exchange rather than activity of specific enzymes and may result from the activity of multiple enzymes. We use “PEPCK” (the primary enzyme responsible for removal of intermediates from the TCA cycle) to describe v6, and “pyruvate cycling” to describe pyruvate cycled back into the TCA cycle by either PK or ME (diagrammed in Fig. 1 but see Ref. 7 for details).

**Hepatic ATP flux.** The energy required to support the rates of glucose production and the energy made available from hepatic energy-producing reactions were calculated using established stoichiometry of the reactions and their flux rates measured in this study, similar to Browning et al. (6). For the purposes of this estimation, we assumed that all glucose production was derived from lactate, and we combined the ATP and GTP into “ATP equivalents.” The energy-consuming reactions required for glucose production were the following: OAA $\rightarrow$ PEP (v6), pyruvate $\rightarrow$ OAA (assumed equal to v6), and PEP $\rightarrow$ glucose (v4). ATP-producing reactions were PEP $\rightarrow$ pyruvate (v5), TCA cycle flux (v7), and $\beta$-oxidation. Flux from $\beta$-oxidation was estimated by assuming all acetyl-CoA entering the TCA cycle was derived from fatty-acetyl CoA, thus $\beta$-oxidation flux was set equal to v7. This underestimates total $\beta$-oxidation flux as some acetyl-CoA derived from fatty-acetyl CoA is directed toward ketone production and not detected in the v7 flux rate. Ketone levels in elephant seals, however, are low until at least the last few weeks of fasting (12, 15), therefore, this error is probably small relative to the rate of fatty acid oxidation. The ATP production rate by gluconeogenic tissues (“hepatic” for simplicity) was further converted to a rate of oxygen use assuming a free energy of hydrolysis of ATP $\rightarrow$ ADP + P$_i$ $=-46.7 \text{ J/mmol}$ (at $37^\circ\text{C}$ and typical rat hepatocyte concentrations for ATP, ADP, and P$_i$ of 3.38, 1.32, and 4.8 mM, respectively). We used the equation from Noren (59) to estimate whole animal metabolic rate from body mass: $\text{MR} = 140.7 + 3.0 \text{ M}_b$ where MR is metabolic rate (in ml O$_2$/min) and $\text{M}_b$ is body mass (in kg).

**Hormones and metabolites.** Glucose and lactate concentrations were measured in the initial blood samples using a YSI 2300 analyzer (Yellow Springs Instruments, Yellow Springs, OH). $\beta$-Hydroxybutyrate ($\beta$-HBA) was assayed using a colorimetric assay (cat no. 700190, Cayman Chemical, Ann Arbor, MI). Insulin, glucagon, and cortisol were measured using commercially available radioimmunoassay (RIA) kits. Insulin was measured using a sensitive rat insulin kit (cat no. SRI-13K and glucagon using a glucagon kit (cat no. GL-32K, both from Linco Research, St Charles, MO). Cortisol was measured using a cortisol RIA kit (cat no. TKCO2, Diagnostic Products, Los Angeles, CA). These assays have all been previously validated in this species (15, 66). Intra-assay coefficient of variations for insulin, glucagon, and cortisol were 2.6, 1.0, and 4.7%, respectively.

**Statistical analysis.** Of the 16 turnover procedures performed, glucose turnover measurements were successfully collected in 15 procedures; the late fasting infusion procedure of one seal failed, probably due to catheter displacement during the infusion. Thus metabolic turnover measurements were collected for eight seals early and seven late in the postweaning fast, whereas other metrics were collected for all eight study animals in both fasting states. Turnover rates for this seal were removed from paired statistical analyses.

To detect changes across fasting in whole animal metabolic flux rates, the absolute rates of v1-v7, we used ANCOVA to account for body mass loss across the fast in a mixed-model analysis with fasting state and mass as fixed effects and seal as a random effect (67). Fasting state by mass interactions were not significant in any test and were removed from the models. Statistical analyses were performed using R (version 2.11.1, R Development Core Team, www.R-project.org) and JMP ver 9 (SAS Institute, Cary NC).

**RESULTS**

**Body mass, metabolite, and hormone concentrations.** Mass, metabolite, and hormone concentrations are summarized in Table 1. Mass decreased during the postweaning fast (paired $t = 7.4, P < 0.001$) as pups lost 0.7 (SD 0.3) kg/day across the sampling interval. Neither circulating glucose nor lactate concentrations changed between early and late fasting (paired $t$-tests, $P > 0.15$), whereas $\beta$-HBA increased with fasting
Circulating insulin concentration decreased (paired t = 2.3, P < 0.01), while glucagon concentration did not change (P = 0.19) across the fast. The molar ratio of insulin to glucagon (I:G) also decreased across the fast (paired t = 4.2, P = 0.01).

EGP and the sources of glucose production. Relative and absolute flux rates measured in this study are shown in Table 2. Decreasing body mass across the fasting interval confounds comparisons of whole animal metabolism. When accounting for decreasing body mass, EGP decreased with fasting (ANCOVA, F1,13 = 5.7, P = 0.035) while body mass did not have a significant effect in the model (F1,13 = 0.48, P = 0.5).

The fractional contributions of glycogen, glycerol, and PEP to glucose production did not significantly change across the fasting period (paired t < 0.5 and P > 0.4 in all cases). PEP was the main precursor for glucose produced in northern elephant seals and accounted for 95% of glucose produced during the postweaning fast; glycerol made a minor contribution to EGP while no contribution from glycogen was detected (Table 2). The absolute rates of glycogenolysis (v2) and GNGPEP (v4) did not change with fasting (ANCOVA, P > 0.1), but GNGglycerol (v3) did show a statistically significant decrease with fasting, when accounting for body mass loss (ANCOVA, F1,11.9 = 4.8, P = 0.048).

Table 1. Mass, metabolite, and hormone concentrations early and late in the postweaning fast

<table>
<thead>
<tr>
<th>Fasting State</th>
<th>Mass, kg</th>
<th>Glucose, mM</th>
<th>Lactate, mM</th>
<th>β-HBA, μM</th>
<th>Insulin, pM</th>
<th>Glucagon, pM</th>
<th>I:G</th>
<th>Cortisol, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early</td>
<td>122 (12)</td>
<td>9.73 (0.79)</td>
<td>2.95 (0.82)</td>
<td>56.6 (10.4)</td>
<td>10.6 (2.3)</td>
<td>12.8 (2.6)</td>
<td>0.87 (0.29)</td>
<td>564 (238)</td>
</tr>
<tr>
<td>Late</td>
<td>99 (11)</td>
<td>10.28 (0.82)</td>
<td>2.21 (0.81)</td>
<td>128.6 (19.3)</td>
<td>6.1 (2.6)</td>
<td>16.8 (5.5)</td>
<td>0.35 (0.12)</td>
<td>788 (419)</td>
</tr>
</tbody>
</table>

Values are reported as mean (SD), and the results from paired t-tests are shown in the final row; n = 8 for both early and late fasting states. I:G is the molar ratio of insulin to glucagon; β-HBA, β-hydroxybutyrate. nsd, No significant difference.
The fractional or relative contribution and absolute flux rates of each pathway measured are shown in either hexose (6-carbon) or triose (3-carbon) units. Values are means (SD); shown as a fraction of endogenous glucose production (EGP, fractional contribution), relative to citrate synthase (CS, relative flux rate), and absolute rates (in mmol/min). EGP and the absolute flux rates of glycogenolysis (GNG), GNGglycerol, and GNGPEP are reported in hexose units, whereas pathways relative to the TCA cycle are reported in triose units. See text and Fig. 1 for further description of the pathways investigated; codes v1–v7 match those in Fig. 1. The mean rates of glycogenolysis were negative both early and late in the fast. These negative values do not represent glycogen synthesis but show the level of accuracy in the measurement when the rate of glycogenolysis approaches zero and deuterium enrichment of H2 and H5 are nearly equivalent. *Rates measured with the [U-13C3]propionate tracer are defined as relative to citrate synthase activity. PEP, phosphoenolpyruvate, PEPCK, phosphoenolpyruvate carboxykinase.

**TCA cycle fluxes.** Flux rates of PEPCK and pyruvate cycling, relative to CS, did not change across the fast (P = 0.9 and 0.7, respectively; Table 2). The absolute flux rates of both PEPCK and pyruvate cycling decreased across the fast (paired t = 3.7 and 3.3, respectively; P < 0.02), but this occurred simultaneously with decreases in EGP and body mass. When accounting for body mass, however, there were no significant changes in the absolute rates of PEPCK, pyruvate cycling, or CS flux (ANCOVA analyses, F1,15 < 2.5, P > 0.14 in all cases).

Calculated hepatic ATP production and use are shown in Table 3. When accounting for body size, rates of hepatic ATP consumption and production did not change across the fast (ANCOVA analysis, F1,11,9 = 1.81, 2.3, respectively; P > 0.1).

**Relationships among flux rates, metabolites, and hormone levels.** Several flux measurements were correlated within early and late fasting periods (Fig. 5). There were no strong correlations between EGP and hormone concentrations (insulin, glucagon, IGF ratio, or cortisol) within early or late fasting (P > 0.05). Similarly, circulating plasma glucose and lactate concentrations were not correlated with gluconeoregulatory hormones (P > 0.05). There was, however, a negative correlation between glucose and lactate concentrations early in the fast (r = −0.77, P = 0.02), but this relationship was not present late in the postweaning fast (P > 0.1). Hepatic ATP production and use were tightly correlated both early and late and the postweaning fast (r = 0.99, P < 0.01, for both fasting states, Fig. 6).

**DISCUSSION**

**EGP and the sources of glucose production.** The primary finding of this work is that PEP was the dominant gluconeogenic precursor in northern elephant seals usually responsible for over 95% of EGP throughout the prolonged postweaning fast. Whole animal EGP decreased during fasting even when

### Table 2. Flux rates of hepatic carbohydrate metabolism in the northern elephant seal

<table>
<thead>
<tr>
<th>Tracer Pathway</th>
<th>Sources of glucose</th>
<th>Fractional contribution</th>
<th>Absolute flux rate, mmol hexose/min</th>
<th>Fractional contribution</th>
<th>Absolute flux rate, mmol hexose/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1,6-13C2]Glucose v1 EGP</td>
<td>1.75 (0.28) mmol/min</td>
<td>1.21 (0.20) mmol/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[U-13C3]Propionate v4 GNG</td>
<td>1.62 (1.12) mmol/min</td>
<td>1.65 (0.21) mmol/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2H2O v2 Glycogenolysis</td>
<td>3.31 (0.56) mmol/min</td>
<td>1.18 (0.21) mmol/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>v3 GNGglycerol</td>
<td>1.18 (0.21) mmol/min</td>
<td>2.37 (0.36) mmol/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>v4 GNGPEP</td>
<td>1.18 (0.21) mmol/min</td>
<td>9.55 (2.89) mmol/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCA cycle Relative flux rate</td>
<td>1.18 (0.21) mmol/min</td>
<td>3.31 (0.56) mmol/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mmol triose/min</td>
<td>1.18 (0.21) mmol/min</td>
<td>3.31 (0.56) mmol/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>v5 Pyruvate cycling</td>
<td>11.95 (4.76) mmol/min</td>
<td>7.18 (2.79) mmol/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>v6 PEPCK</td>
<td>15.26 (5.21) mmol/min</td>
<td>9.55 (2.89) mmol/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>v7 CS</td>
<td>3.39 (1.04) mmol/min</td>
<td>2.37 (0.36) mmol/min</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The fractional or relative contribution and absolute flux rates of each pathway measured are shown in either hexose (6-carbon) or triose (3-carbon) units. Values are means (SD); shown as a fraction of endogenous glucose production (EGP, fractional contribution), relative to citrate synthase (CS, relative flux rate), and absolute flux rates (in mmol/min). EGP and the absolute flux rates of glycogenolysis (GNG), GNGglycerol, and GNGPEP are reported in hexose units, whereas pathways relative to the TCA cycle are reported in triose units. See text and Fig. 1 for further description of the pathways investigated; codes v1–v7 match those in Fig. 1. The mean rates of glycogenolysis were negative both early and late in the fast. These negative values do not represent glycogen synthesis but show the level of accuracy in the measurement when the rate of glycogenolysis approaches zero and deuterium enrichment of H2 and H5 are nearly equivalent. *Rates measured with the [U-13C3]propionate tracer are defined as relative to citrate synthase activity. PEP, phosphoenolpyruvate, PEPCK, phosphoenolpyruvate carboxykinase.

### Table 3. Hepatic energy use during fasting

<table>
<thead>
<tr>
<th>Energy consuming reactions</th>
<th>Reaction Rate</th>
<th>ATP Equivalents</th>
<th>Early</th>
<th>Late</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate → OAA v6</td>
<td>1</td>
<td>15.3 (5.2)</td>
<td>9.5 (2.9)</td>
<td></td>
</tr>
<tr>
<td>OAA → PEP v6</td>
<td>1</td>
<td>15.3 (5.2)</td>
<td>9.5 (2.9)</td>
<td></td>
</tr>
<tr>
<td>GNGPEP v4</td>
<td>1</td>
<td>3.3 (0.6)</td>
<td>2.4 (0.4)</td>
<td></td>
</tr>
<tr>
<td>ATP required</td>
<td>33.8 (10.9)</td>
<td>21.5 (5.9)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Energy producing reactions</th>
<th>Reaction Rate</th>
<th>ATP Equivalents</th>
<th>Early</th>
<th>Late</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate cycling v5</td>
<td>1</td>
<td>11.9 (4.8)</td>
<td>7.2 (2.8)</td>
<td></td>
</tr>
<tr>
<td>β-oxidation v7</td>
<td>4</td>
<td>13.6 (4.2)</td>
<td>8.2 (1.7)</td>
<td></td>
</tr>
<tr>
<td>TCA cycle v7</td>
<td>10</td>
<td>33.9 (10.4)</td>
<td>20.6 (4.3)</td>
<td></td>
</tr>
<tr>
<td>ATP produced</td>
<td>59.4 (19.3)</td>
<td>36.0 (8.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatic O2 requirement, ml O2/min</td>
<td>140.2 (45.5)</td>
<td>89.9 (32.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estimated metabolic rate, ml O2/min</td>
<td>505.6 (36.3)</td>
<td>439.4 (35.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction hepatic metabolism</td>
<td>0.28 (0.08)</td>
<td>0.19 (0.04)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values reported are average mmoles ATP per min (SD); ATP equivalents were calculated using the stoichiometry of each pathway: 1GTP = 1ATP, 1NADH = 2.5ATP, IFADH2 = 1.5ATP. Calculated hepatic ATP consumption and production early and late in the postweaning fast. Total hepatic energy production was always greater than required for EGP. When accounting for decreased body mass, there was no significant change in hepatic ATP production and use with fasting (ANCOVA, P > 0.1). We assumed that the rate of β-oxidation is equal to the rate of acetyl-CoA entrance into the tricarboxylic acid (TCA) cycle (v7). This underestimates the actual rate of β-oxidation by the rate of hepatic ketogenesis. Hepatic O2 requirement was calculated from the ATP production rate assuming 46.7 J/mmol ATP produced and 1 liter O2 required per 19.8 kJ (fat-based metabolism). The metabolic rates of scales were estimated from Noren (59). Fraction hepatic metabolism is the hepatic O2 requirement per estimated metabolic rate.
accounting for the decrease in body size across the fast. This agrees with previous findings in weaned elephant seal (15) and grey seal pups (58) and is consistent with the typical mammalian response to fasting (28, 39, 80).

Glycerol was only a minor contributor, accounting for less than 10% of total EGP. This small contribution was similar to a previous study using different methodology in adult elephant seals fasting while lactating (31). Thus it appears that glycerol does not substantially contribute to total glucose production at multiple life stages in elephant seals. Although, it is likely that \( \text{GNG}_{\text{glycerol}} \) is sufficient to replace glucose carbons lost to \( \text{CO}_2 \) (complete oxidation) and, therefore, is the primary precursor of de novo gluconeogenesis since glucose recycling through lactate (i.e., Cori cycle activity) cannot contribute net glucose to whole animal carbohydrate stores.

Glycogen made negligible contributions to EGP. Very low rates of glycogenolysis have been reported in one rat strain fasting for 24 h, but in obese rats (\( \text{fa/}\text{fa} \)) hepatic glycogen was maintained and contributed 10–15% of EGP (35). We did not measure hepatic glycogen concentration, but it seems that glycogen stores were depleted early in fasting. In fact, calculated rates of glycogenolysis were slightly negative in many cases (Table 2) due to the similarity in \( ^{2}\text{H} \) enrichment at \( H_2 \) and \( H_5 \) (see Fig. 2 for example); the similarity in enrichment at these two positions indicates that the contribution from glycogenolysis was small and \( \text{GNG}_{\text{PEP}} \) was the dominant gluconeogenic pathway (8). The high fractional GNG reported here is similar to that measured in prolonged (24 h) fasted rats (9). The small negative values of \( v_4 \) do not represent glycogen synthesis, which is not measured by the methods used here; rather, they reflect the error present in this methodology at these very low/negligible rates of glycogenolysis.

The rates of EGP measured in the present study using stable isotopes and positional isotope analysis were similar to those reported previously in this species using a single dose of [6-\(^{3}\text{H}\)]glucose (15). In fact, when the rates of EGP measured using positional isotope analysis were compared with this earlier study, there was no detectable difference between the two methods (\( P = 0.69 \)). The similarity in measured rates of EGP between the studies indicate that [6-\(^{3}\text{H}\)]glucose and [1,6-\(^{13}\text{C}_2\)]glucose yield similar rates of EGP and are comparable tracers of glucose production in elephant seal weanlings; this may be true of the species in general although the impact of other metabolic factors (e.g., lactation) is unknown.

For comparison with other species, reported mass-specific rates of carbohydrate metabolism from domestic cats (42) and humans (38) are shown alongside those from northern elephant seals in Table 4. Although we did detect a statistically significant decrease in whole animal EGP with fasting, the mass-specific rates remain similar to other species undertaking an overnight fast, despite complete abstinence from food and water for nearly 2 mo. No other studies to date have used this methodology to investigate prolonged fasting; thus, the contribution of glycogenolysis is noticeably greater after an overnight fast in cats and humans compared with the glycogen-depleted state found in elephant seals fasting for 3–7 wk. The relationship between prolonged fasting and the contribution from \( \text{GNG}_{\text{PEP}} \) has not been well studied in other species, although one study in mice did find a moderate increase in fractional \( \text{GNG}_{\text{PEP}} \) among several mice strains fasted for 24 h (8). During prolonged fasting in humans, however, EGP de-

---

**Fig. 5.** Relationships among flux rates. A correlation matrix of absolute flux rates during the early and late fasting periods. In the bottom left of the matrix are correlations among early fasting (\( n = 8 \)), whereas values in the top right are among late fasting values (\( n = 7 \)). Statistically significant correlations are shown in bold (\( P < 0.05 \)). There were 11 significant correlations among flux rates early in the postweaning fast and only five at the end of the fast.

<table>
<thead>
<tr>
<th></th>
<th>EGP</th>
<th>( \text{GNG}_{\text{PEP}} )</th>
<th>( \text{GNG}_{\text{glycerol}} )</th>
<th>Glycogenolysis</th>
<th>( \text{PEPCK} )</th>
<th>Pyruvate cycling</th>
<th>CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGP</td>
<td>1</td>
<td>0.93</td>
<td>0.20</td>
<td>0.67</td>
<td>0.50</td>
<td>0.40</td>
<td>0.66</td>
</tr>
<tr>
<td>( \text{GNG}_{\text{PEP}} )</td>
<td>0.72</td>
<td>1</td>
<td>-0.16</td>
<td>0.85</td>
<td>0.35</td>
<td>0.24</td>
<td>0.58</td>
</tr>
<tr>
<td>( \text{GNG}_{\text{glycerol}} )</td>
<td>-0.05</td>
<td>-0.69</td>
<td>1</td>
<td>-0.52</td>
<td>0.51</td>
<td>0.55</td>
<td>0.32</td>
</tr>
<tr>
<td>Glycogenolysis</td>
<td>0.71</td>
<td>0.10</td>
<td>0.42</td>
<td>1</td>
<td>-0.18</td>
<td>-0.29</td>
<td>0.10</td>
</tr>
<tr>
<td>( \text{PEPCK} )</td>
<td>0.84</td>
<td>0.82</td>
<td>-0.32</td>
<td>0.42</td>
<td>1</td>
<td>0.99</td>
<td>0.94</td>
</tr>
<tr>
<td>Pyruvate cycling</td>
<td>0.84</td>
<td>0.78</td>
<td>-0.27</td>
<td>0.44</td>
<td>1.00</td>
<td>1</td>
<td>0.90</td>
</tr>
<tr>
<td>CS</td>
<td>0.88</td>
<td>0.78</td>
<td>-0.22</td>
<td>0.46</td>
<td>0.98</td>
<td>0.98</td>
<td>1</td>
</tr>
</tbody>
</table>

**Fig. 6.** Hepatic ATP production and use were tightly correlated both early and late in the postweaning fast; values are in mmoles ATP per min. Correlation coefficient (\( r \)) was 0.99, \( P < 0.001 \), in both fasting states.
creases with fasting [to <5 μmol·kg⁻¹·min⁻¹ in one study of obese subjects (80)] reducing the overall flux of GNGPEP. The contribution of GNGglycerol appears similar between seals and humans, whereas cats showed higher contributions; other studies in both humans (6) and mice (8), however, have shown GNGglycerol contributions of 10–20%, similar to those observed in cats.

Sources of PEP and glucose carbon recycling. PEP was directed toward hepatic gluconeogenesis at a rate of 3.3 and 2.4 mmol/min early and late in the postweaning fast, respectively (Table 2). The PEP directed to EGP must be replaced at an equal rate. Sources of PEP for gluconeogenesis may derive from amino acids or the pyruvate-lactate pool. All measures of protein catabolism in elephant seals during the postweaning fast have been low (1, 32, 60) as is expected for fasting-adapted animals (24, 84). For example, one study reported protein catabolism in weaned pups of 14.4 and 9.2 g per day early and late in the postweaning fast (32). Using these rates of protein catabolism, if we assume that all of this protein was directed toward hepatic gluconeogenesis at a rate of 3.3 and 2.4 mmol triose/min, early and late in the fast, from Table 2 and substituting pool sizes from above, we calculate that k = 0.05 min⁻¹ both early and late in the fast. Thus only 5% of the lactate pool each minute is needed to completely replace the PEP directed to gluconeogenesis. Because lactate rapidly equilibrates with pyruvate in vivo, the kinetics of these metabolites are linked and problematic to study with metabolic tracer methods (5, 45, 89, 90). The precise relations between lactate and pyruvate are, however, not of great importance in this example and we may consider “lactate turnover” as the sum of pyruvate and lactate turnover. Nonetheless, the estimates provided here are reasonable rates of lactate turnover, comparable to those reported in humans at rest (54) and quite similar to lactate kinetics reported by Davis (23) in harbor seals.

Hypoxia may influence various metabolic pathways, including carbohydrate metabolism (50, 73, 91), and one of the consequences is increased glucose conversion to lactate (55). Pinnipeds, especially phocid seals, experience repeated hypoxia and tissue ischemia both during diving (44, 53) and in periodic apneas on land (72, 79). The hypoxia-inducible transcription factor (HIF) is responsive to oxygen availability with wide-ranging effects on whole animal metabolism (3). In conditions of low oxygen availability, the oxygen sensors prolyl hydroxylase domain-containing enzymes (PHDs) and factor-inhibiting HIF (FIH) cease to target HIF for enzymatic degradation. HIF then dimerizes and has extensive effects on whole animal metabolism (3). In conditions of low oxygen availability, the oxygen sensors prolyl hydroxylase domain-containing enzymes (PHDs) and factor-inhibiting HIF (FIH) cease to target HIF for enzymatic degradation. HIF then dimerizes and has extensive effects on whole animal metabolism (3). In conditions of low oxygen availability, the oxygen sensors prolyl hydroxylase domain-containing enzymes (PHDs) and factor-inhibiting HIF (FIH) cease to target HIF for enzymatic degradation. HIF then dimerizes and has extensive effects on whole animal metabolism (3). In conditions of low oxygen availability, the oxygen sensors prolyl hydroxylase domain-containing enzymes (PHDs) and factor-inhibiting HIF (FIH) cease to target HIF for enzymatic degradation. HIF then dimerizes and has extensive effects on whole animal metabolism (3). In conditions of low oxygen availability, the oxygen sensors prolyl hydroxylase domain-containing enzymes (PHDs) and factor-inhibiting HIF (FIH) cease to target HIF for enzymatic degradation. HIF then dimerizes and has extensive effects on whole animal metabolism (3).
of glucose carbon recycling are consistent with upregulation of HIF. In select species, like seals, some aspects of fasting metabolism may be related to adaptation to hypoxia. Investigations between animals of varying degrees of diving and fasting abilities may clarify this relationship.

**GNGglycerol.** The rate of GNGglycerol is probably sufficient to replace completely oxidized glucose and adequate to meet the energetic needs of the central nervous system. We calculate that 2.0 and 1.4 μmol-min⁻¹·kg⁻¹ glycerol was directed toward gluconeogenesis early and late in the postweaning fast, respectively (calculated from Table 2). Given elephant seals’ reliance on fat catabolism, there is ample glycerol available for GNGglycerol. Castellini and coauthors (10) measured fatty acid turnover in elephant seal pups after 2 mo fasting; they found the average rate of palmitate and oleate turnover was 11 μmol-min⁻¹·kg⁻¹. Assuming a simple model of fatty acid kinetics and ignoring any interaction with the lipoprotein pool, this rate of fatty acid turnover yields a glycerol turnover rate of 3.7 μmol-min⁻¹·kg⁻¹ that may be available for GNGglycerol. Additionally, Houser and others (31) directly measured glycerol Rₖ in adult female northern elephant seals. The average rate of glycerol turnover in nonlactating adults was 2.9 μmol-min⁻¹·kg⁻¹, whereas during lactation glycerol Rₖ was substantially higher. Either of these measures of glycerol turnover are sufficient to support the rates of GNGglycerol measured in this study. The rates of GNGglycerol are consistent with low rates of glucose oxidation measured in elephant seals (43) and pinnipeds in general (4, 84). It appears that the contribution of GNGglycerol to glucose synthesis is sufficient to replace the glucose irreversibly lost to oxidation. We calculated that 120 and 70 μmol of glucose was produced from glycerol each minute, early and late in the postweaning fast, respectively. To completely oxidize an equivalent amount of glucose (i.e., 120–70 μmol) would require 16.1 and 9.4 ml oxygen each minute (see Table 3). Assuming the same metabolic rate as previously discussed, these rates of glucose oxidation comprise only 2–3% of metabolic rate, which agree with previous estimates (40).

It appears that the rate of GNGglycerol is sufficient to meet the energetic needs of the central nervous system (CNS). Whereas no direct measures of the glucose requirements of the elephant seal CNS have been made, estimates are available from Weddell seals *Leptonychotes weddellii*. Murphy et al. (56) and Hochachka (29) estimated that 0.3–0.4 μmol glucose-min⁻¹·g⁻¹ were required to support Weddell seal brain tissue, although 20–25% was not completely oxidized but released as lactate. If we assume 250 g for the brain size of an elephant seal pup, ∼75–100 μmol glucose is required to support brain metabolism each minute. Despite the large number of assumptions contributing to this estimate, it is quite similar to the measured rate of GNGglycerol (120–70 μmol glucose derived from glycerol each minute early and late in the postweaning fast). While fat metabolism dictates glycerol Rₖ, GNGglycerol may be closely tied with the glucose demands of the CNS.

**Glycerol and triacylglycerol reesterification.** The measures of lipolysis from Castellini et al. (10) used above yield a glycerol Rₖ of at least 3.7 μmol-min⁻¹·kg⁻¹ during the postweaning fast. This study measured GNGglycerol rates of 1.4–2.0 μmol glycerol-min⁻¹·kg⁻¹. The fate of the remaining glycerol; i.e., the difference between glycerol Rₖ and GNGglycerol (∼2 μmol-min⁻¹·kg⁻¹) remains to be studied. High rates of triacylglycerol (TAG), fatty acid recycling, are common in mammals; for example, Jensen et al. reported 60% of fatty acid released from lipolysis was reesterified in humans fasting 60 h (34). From the measurements of this study as well as Houser et al. (31), northern elephant seals appear to fit this pattern of excess lipolysis followed by reesterification. Glycerol and fatty acids from lipolysis are taken up from circulation by the liver; fatty acids not directed to β-oxidation for use in the TCA cycle or ketone production are reesterified. Glycerol kinase, expressed in hepatocytes but not in adipose tissue, converts glycerol to 3-phosphoglycerol (3-PG) for reesterification; TAG is released as VLDL for uptake by peripheral tissues (2). There has, however, been a growing realization that gluconeogenesis (the production of 3-PG from precursors other than glycerol or glucose) may play a significant role in TAG reesterification and may be active in liver and adipose (27, 61). Nye et al. (62) proposed a two-cell model for gluconeogenesis in adipose where one glycolytic cell produces lactate while another mature adipocyte converts lactate to 3-PG for TAG synthesis. Thus the glucose-lactate pools and TAG-fatty acid cycles may be linked via gluconeogenesis, and this may be an important pathway influencing both fat and carbohydrate metabolism that has been largely overlooked in fasting animals.

Carnivores eat a largely carbohydrate-free diet. Among pinnipeds, even suckling pups consume carbohydrate-free milk (63, 78). Thus, during feeding, amino acids may be an important precursor for 3-PG necessary for TAG synthesis and storage, while in the fasting state lactate may become the dominant precursor for 3-PG synthesis necessary for fatty acid reesterification. Conversely, the differential between glycerol Rₖ and GNGglycerol suggests that a substantial proportion of glycerol released into circulation from lipolysis is not directed toward gluconeogenesis. Both fasting and the availability of circulating fatty acid activate peroxisome proliferator-activated receptor (PPARα), an important regulator of hepatic metabolism, and it may be chronically activated in obesity (41, 48). PPARα stimulates, among other processes, hepatic uptake of glycerol released from lipolysis by increasing expression of aquaglyceroporins (49, 68). The physiological state of fasting elephant seals suggests that glycerol uptake should be high but much of it is not converted to glucose. A portion of 3-PG derived from glycerol may be used to reesterify fatty acids instead of contributing to gluconeogenesis. This pathway was not investigated in the current study and may account for a substantial fraction of glycerol turnover.

**TCA cycle fluxes.** When accounting for decreased body size (or relative to EGP, data not shown), there was no change in PEPCK, pyruvate cycling, or CS flux rates with fasting. Thus it does not appear that the duration of fasting had a substantial influence on the TCA cycle and related flux rates within hepatocytes. As discussed above, the mass-specific rates of EGP in fasting elephant seals were similar to or slightly greater than those observed in other species. The fluxes of PEPCK, pyruvate cycling, and CS, on the other hand, were substantially higher in prolonged fasting seals than in cats and humans undergoing an overnight fast, often two or three times greater (Table 4). The high rates of TCA cycle activity may be expected in fasting northern elephant seals; their rates of lipolysis and levels of circulating fatty acids are elevated to support nearly all of their energetic needs through fat catabolism (10, 31). The high TCA cycle fluxes support both the ATP and substrates required for gluconeogenesis throughout the
fast. PEPCK expression increases with fasting (77) and in animals eating carbohydrate-free diets (69), both of which describe fasting northern elephant seals, so we may expect high rates of PEPCK activity. During fasting, excess carbon loss from the TCA cycle to gluconeogenesis would be detrimental since these carbons require replacement from limited gluconeogenic precursors. Carbon loss from the TCA cycle, however, appeared to be mitigated by active pyruvate cycling; most (~75%) of the carbon leaving the TCA cycle through PEPCK (v6) was recycled by pyruvate cycling (v5) back into the TCA cycle.

Pyruvate cycling, PEPCK, and CS flux were all closely correlated both early and late in the fast, much more so than has been observed in other species (6, 30, 42). Such close correlation suggests coordinated regulation of these pathways during the postweaning fast (Table 5). Pyruvate cycling acts as a substrate or “futile” cycle in concert with the TCA cycle (35). One substrate cycle, the glucose cycle, was previously investigated in this species (14, 15); glucose cycle flux was determined to be low relative to EGP and, thus, unlikely to play an important role in carbohydrate metabolism. Conversely, the active pyruvate cycling relative to overall flux of GNGPEP measured using stable isotopes fits with the model of substrate cycles potentially regulating pathway flux (57). This potential has been recognized in similar studies in rats (35) and cats (42). Jin et al. suggested that the high rate of pyruvate cycling observed in obese rats was due to continued glycogen availability permitting reduced gluconeogenesis from TCA cycle intermediates while maintaining the rate of EGP (35). This work in elephant seals demonstrates that high rates of pyruvate cycling are possible without glycolysis. One pathway of pyruvate cycling, malate → pyruvate catalyzed by malic enzyme, produces cytosolic NADPH required for fatty acid synthesis. All phocid seals, and especially elephant seals, store large amounts of energy as fat during foraging trips. Animals were fasting during this study but seals may have active malic enzyme to provide reducing equivalents in the form of NADPH for hepatic fatty acid synthesis and subsequent storage.

**Energetics of gluconeogenesis.** According to conventional thought, gluconeogenesis is expensive, requiring 6 moles of ATP equivalents for each mole of glucose produced from pyruvate. In fasting elephant seals, however, ATP available from hepatic fatty acid oxidation was nearly twice that required for EGP (Table 3). Furthermore, most of the ATP required in these energy-consuming reactions was used in PEPCK and pyruvate cycling reactions. Only a fraction of the energy was required for GNGPEP (v4) per se. Thus maintaining rates of EGP near those of postabdominal measurements.

Estimates of whole animal metabolic rate from body mass and hepatic metabolic rate calculated from pathway fluxes measured in this study are shown in Table 3. Gluconeogenic tissues, primarily the liver, appear to account for approximately one-quarter of whole animal metabolic rate. This rough estimate is similar to that in humans, approximately one-fifth of total oxygen consumption (47) but somewhat larger than the 15% predicted using organ-specific scaling relationships estimated by Wang and coauthors (85), although active liver metabolism may be expected during prolonged fasting.

**Regulation of EGP.** Patterns in gluoregulatory hormones measured in this study were similar to those previously reported in this species. Here, the variability in insulin and glucagon resulted in a marked reduction in I:G ratio, from 0.87 to 0.35, across the postweaning fast. This is similar to the trend reported previously in weaned pups (15, 65). We did not detect any significant relationships between circulating metabolite concentrations or metabolic fluxes with their regulatory hormones: insulin, glucagon, or cortisol. This was not necessarily surprising as previous studies in this species have also failed to detect relationships between metabolites, flux rates, and putative regulatory hormones (14, 15, 31). Cortisol concentration was noticeably higher in this study than previously reported baseline values in this species. In this study, hormone concentrations were measured immediately before flux measurements, but this occurred after 480 ml of 2H2O were administered and the seal was subsequently held in a pen overnight. It has been shown that handling and transport influence cortisol release in this species (13), and it appears that this handling before the hormone and carbohydrate flux measurement caused a cortisol release that was still detected the following morning. The rates of EGP measured, however, are similar to those previously reported (15), and cortisol release does not have a detectable influence on EGP in elephant seals (13).

Daily variation in metabolism is widely reported among mammals, and many endotherms reduce energy expenditure by entering torpor during periods of food deprivation (51). Diel variability in energy use, if present, would influence some of the estimates of fasting energetics we have discussed above. However, many pinnipeds, including elephant seals, remain active throughout fasting and do not exhibit strong diel behavioral changes on land or at sea. In this instance it is unlikely that daily variability had an important influence on our measurements.

**Perspectives and Significance**

This study used positional isotope analysis to investigate the metabolic pathways involved in carbohydrate metabolism in free-ranging northern elephant seals during prolonged fasting. The contributions of glycogenolysis, glycerol, and PEP to glucose production were quantified. These findings confirm previous measures of EGP and low rates of glycerol gluconeogenesis in this species. We estimate that the small contribution of GNGglycerol is sufficient to replace glucose lost to complete oxidation and support the energetic requirements of the CNS. GNGPEP accounted for the majority of endogenous glucose production. Given the low rate of protein catabolism in fasting elephant seals, these rates of GNGPEP indicate high rates of glucose recycling through three-carbon intermediates, most likely the lactate-pyruvate pool. Potentially, elephant seals’ adaptation to repeated tissue hypoxia may upregulate HIF and promote high rates of glucose → lactate flux, but this metabolic feature remains to be explored. We found high rates of TCA cycle flux and related pathways (PEPCK and pyruvate cycling)
FASTING CARBOHYDRATE METABOLISM IN NORTHERN ELEPHANT SEALS

compared with observations in cats and humans (6, 42). The detailed examination of carbohydrate metabolism allowed estimates of hepatic energy requirements, of which gluconeogenesis did not appear to be a significant energetic cost; rather, PEPCK and pyruvate cycling were far more energetically costly. These pathways, however, may play important roles in fasting carbohydrate metabolism; the high pyruvate cycling replaces TCA cycle intermediates without requiring anaeroperoxidase from protein-derived amino acids while simultaneous PEPCK and pyruvate cycle activity may provide a regulatory point for glucose production.

ACKNOWLEDGMENTS

We extend special thanks to the faculty and staff at the University of Texas, Southwestern Medical Center and the Rogers NMR Center for providing invaluable training on the tracer technique used in this study and Acorn NMR for their work collecting the NMR spectra. Cambridge Isotope Laboratories generously provided an in-kind grant of stable isotopes, and the Friends of Long Marine Lab provided travel funds for CDC to attend training at the Rogers NMR Center. The depictions of NMR spectra and corresponding metabolite formations were based on comments by T. Williams and three anonymous reviewers.

GRANTS

This work was performed under National Marine Fisheries Service permit 87-1743. The research was supported by National Science Foundation (NSF) Grant 0818018. Any opinions and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the NSF.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


Fasting Carbohydrate Metabolism in Northern Elephant Seals


AJP-Regul Integr Comp Physiol • doi:10.1152/ajpregu.00042.2012 • www.ajpregu.org


