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Glucose production and substrate cycle activity in a fasting adapted animal, the northern elephant seal

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Summary

During prolonged fasting physiological mechanisms defend lean tissue from catabolism. In the fasting state, glucose is derived solely from gluconeogenesis, requiring some catabolism of amino acids for gluconeogenic substrates. This creates a conflict in animals undergoing fasts concurrently with metabolically challenging activities. This study investigated glucose metabolism in fasting and developing neonatal elephant seals. Glucose production and glucose cycle activity were measured early (2 weeks) and late (6 weeks) in the postweaning fasting period. Additionally the role of regulatory hormones on glucose production and glucose cycle activity were investigated. Glucose cycle activity was highly variable throughout the study period, did not change over the fasting period, and was not correlated with insulin or glucagon level. Endogenous glucose production (EGP) was 2.80±0.65 mg kg^{-1} min^{-1} early and 2.21±0.12 during late fasting. Insulin to glucagon molar ratio decreased while cortisol levels increased over the fast (t=5.27, 2.84; P=0.003, 0.04; respectively). There was no relationship between EGP and hormone levels. The glucose production values measured in this study were high and exceeded the estimated gluconeogenic substrate available. These data suggest extensive glucose recycling *via* Cori cycle activity occurring in northern elephant seals, and we propose a possible justification for this recycling.

Key words: glucose metabolism, glucose cycle, fasting, elephant seal.

Introduction

Glucose is derived solely from gluconeogenesis during prolonged fasting, which can strain protein reserves through the commitment of amino acids to gluconeogenic pathways. This situation creates a conflict in animals that undergo long duration fasts in combination with other metabolically challenging processes, particularly those that demand the commitment of limited protein stores. For example, fasting in neonates is rare because of the high energy and material requirements of growth and development. The continued requirements of growth and development typically necessitate significant nutrient consumption by the neonate. Nevertheless, some species integrate fasting with postnatal development: king penguin chicks, *Aptenodytes patagonica*, undergo a fast of five months (Cherel et al., 1988) and many phocid seals (grey seals, *Halichoerus grypus*; harp seals, *Phoca groenlandica*; northern elephant seals, *Mirounga angustirostris*) undertake prolonged fasts of 2–12 weeks after weaning (Costa, 1991).

Northern elephant seals fast for 2–3 months, during which time metabolic requirements are met primarily through fatty acid oxidation (Ortiz et al., 1978). Postnatal development continues and the swimming and diving abilities necessary for foraging at sea are acquired during the fast (Reiter et al., 1978). This developmental period is evident in the increase in hematocrit, hemoglobin concentration, mass specific blood volume, and myoglobin concentration that occurs across the fast (Thorson and Le Boeuf, 1994). Based upon the observed developmental pattern, it may be expected that glucose requirements would increase due to a persistent requirement of some glucose-dependent tissues (e.g. CNS) and an increase in the requirement of others (e.g. red blood cells). Estimates of the contribution of glucose to metabolism during the fast are 1–5% of average metabolic rate (AMR), but it is not known whether this increases with time fasting (Keith and Ortiz, 1989). Protein catabolism contributes less than 4% to the AMR while fasting and declines with the progression of the fast (Adams and Costa, 1993; Houser and Costa, 2001; Pernia et al., 1980), suggesting that contributions of protein to gluconeogenesis may decline across the fast. However, blood glucose levels remain high throughout the fast (135–160 mg dl^{-1}; Costa and Ortiz, 1982; Ortiz et al., 2003a) complicating the interpretation of the contribution of glucose to the metabolism of the fasting and developing northern elephant seal weanling.

Monitoring glucose production across the postweaning fast can elucidate whether decreased protein catabolism is inversely correlated with increased glucose production, but accurate interpretation of how glucose is being utilized cannot be addressed without giving consideration to pathways that regulate glucose availability (e.g. substrate cycling; Fig. 1) or

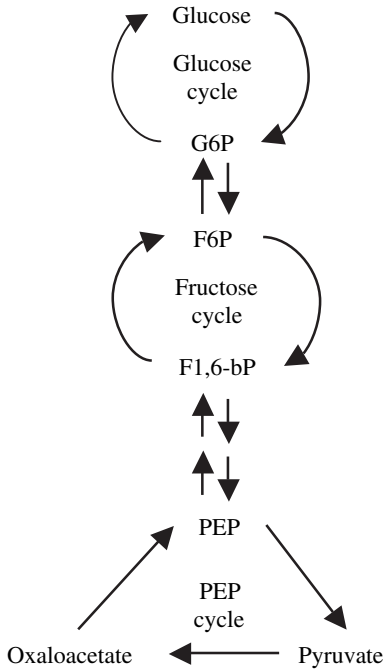

Fig. 1. Substrate cycles in glucose metabolism. Sites of ^{3}H loss: ^{3}H at C-2 of the glucose molecule is lost in the interconversion of glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P); ^{3}H at C-6 is retained through the glucose and fructose cycles and lost in the PEP and citric acid cycles. PEP, phosphoenolpyruvate; F1,6-bP, fructose-1,6 bisphosphate.

provide substitutive substrates for energy needs (e.g. ketone use by the CNS). Glucose cycling provides a mechanism by which the total flux of glucose can be varied in response to some regulating factor (Katz and Rongstad, 1976; Newsholme, 1980) or substrate concentration (Hue and Hers, 1974). The extent to which this variation can occur is related to the rate of cycling and the endogenous glucose production (EGP, the combined output of gluconeogenesis and glycogenolysis; Newsholme and Crabtree, 1976; Weber et al., 1990). More glucose can be made available for tissues if EGP increases without a concomitant change in cycling, or rates of glucose cycling decline without a concomitant reduction in endogenous glucose output (EGO; equivalent to gluconeogenesis + glycogenolysis + glucose cycle activity). Conversely, glucose availability can be reduced by increasing glucose cycle activity without a change in EGO.

It is currently unknown how the demand for glucose varies with the progression of development and synthesis of glucose-demanding tissues in fasting elephant seal weanlings. Furthermore, it is unknown to what degree the glucose cycle plays a role in mediating glucose availability across the fast. The objectives of this study were to (1) assess glucose production and (2) investigate the glucose cycle as a possible regulatory mechanism for the availability of glucose in developing northern elephant seal pups during their postweaning fast. The study further investigated the relationship of glucose production and cycle activity to regulatory hormones and the relationship between glucose production and estimates of the contribution of glucose to the fasting metabolism of weanlings.

Materials and methods

Study site and subjects

The study was conducted at Año Nuevo state reserve, San Mateo County, California from February to May, 2003. Early fasting measurements were taken in ten pups, 2 weeks (12–20 days) postweaning. Late fasting measurements were repeated in six of these pups, 5–8 weeks postweaning. To facilitate identification of subjects late in the fast, each was given plastic flipper tags (jumbo roto-tags; Dalton Company, Oxon, UK) at or prior to weaning. Pups were marked with hair dye (Lady Clairol; Stamford, CT, USA) to further facilitate identification throughout the postweaning fast. During the study period weaned elephant seals become highly mobile and begin to spend considerable time in the water. Radio transmitters (Advanced Telemetry Systems, Isanti, MN, USA) were attached to the dorsal pelage of seals with 5-min epoxy (Devcon; Danvers, MA, USA) during the initial procedure to aid in relocation. Despite these efforts, four weanlings departed to sea prior to the later measurement. One pup was sampled early (37 days postweaning vice 49–60 days for all other study animals) because it was late season when most of the remaining weanlings were departing. All pups exhibited typical behavior of healthy elephant seal pups and departed to sea at the end of the fast.

Sample collection and processing

Using a bolus injection technique, a noncompartmental model was used to describe the glucose kinetics of each seal (Wolfe, 1992). Pups were immobilized using an initial intramuscular injection of telazol (teletamine/zolazepam HCl) at a dose of 1.0 mg kg^{-1} and administered intravenous doses of 50 mg ketamine and diazepam as needed to maintain immobilization (all drugs from Fort Dodge Labs, Fort Dodge, IA, USA). Mass was determined using a tripod and scale (MSI tension dynamometer, ±1.0 kg) in conjunction with a nylon restraint bag (Ortiz et al., 1978). Axillary girth and curved length measurements were taken to provide an index of body composition. Body composition index (BCI) was calculated as axillary girth divided by the curved length. Blood samples were collected in chilled heparinized vacutainers. Each animal was administered 0.1 mCi each of [2-^{3}H]glucose and [6-^{3}H]glucose *via* the extradural vein at the onset of the flux measurement. After injection, blood samples were drawn at 5 min intervals for 30 min, then every 15 min thereafter until 3 h post injection. Samples were stored on ice and transported to the laboratory, centrifuged for 15 min at 800 ***g*** and 4°C, and the plasma collected. Protein was precipitated from plasma by adding 1.5 ml of barium hydroxide and zinc sulfate (0.3 N; Sigma-Aldrich, St Louis, MO, USA) to 1.0 ml plasma, vortexing, and chilling for 20 min in an ice–water bath. Samples were then centrifuged at 1800 ***g*** for 20 min and

the supernatant decanted and stored at –80°C until further analysis.

To distinguish ^{3}H at the second carbon (C-2) from the sixth carbon (C-6) of the glucose molecule, an enzymatic detritiation, developed by Issekutz (1977) and modified by Rooney et al. (1992) was utilized to selectively remove the ^{3}H from C-2. Deproteinated samples were thawed and passed through an ion exchange column containing cation resin (AG 50W-X8 200-400 mesh hydrogen form) and anion resin (AG 1 X8 200-400 mesh formate form; both resins from Bio-Rad Laboratories, Hercules CA, USA; Wolfe, 1992). The eluate was collected and lyophilized for 36 h to remove any ^{3}H that had exchanged with the plasma water. Dried samples were reconstituted in 1.0 ml of 133 mmol l^{-1} phosphate buffer (pH 7.4) and divided into four 200 μl fractions. Two fractions were detritiated, two were non-detritiated, and the remaining portion was used to determine glucose concentration of the sample. To each fraction that was to be detritiated, 500 μl of a detritiation solution was added. The detritiation solution consisted of 133 mmol l^{-1} phosphate buffer, 8.4 mmol l^{-1} ATP, 9.0 mmol l^{-1} $MgCl_2$, 2.4 units ml^{-1} hexokinase, and 10 units ml^{-1} phosphoglucose isomerase (all reagents from Sigma, St Louis, MO, USA). The pH of the final solution was adjusted to 7.4 with 1 mol l^{-1} NaOH. To the non-detritiated aliquots, 500 μl of 133 mmol l^{-1} phosphate buffer was added. All samples were incubated in a shaker water bath at 37°C for 2 h and were subsequently lyophilized overnight. Samples were reconstituted in 500 μl of 1.0 mol l^{-1} H_2SO_4. Scintillation cocktail (6.5 ml; Econolite, Fisher, Pittsburg, PA, USA) was added to each sample and the sample then agitated for 1 min. Sample activity was determined by liquid scintillation counting on a Beckman LS 3801 scintillation spectrophotometer (Beckman, Fullerton, CA, USA) using standard scintillation technique. A quench correction factor was established for each sample based on a calculated H# using a series of ^{3}H standards with variable degrees of quench. Glucose concentration of each sample was measured in duplicate on an YSI 2300 glucose autoanalyzer (YSI inc., Yellow Springs, Ohio, USA) and the specific activity of counted samples determined. Since the non-detritiated fractions contain [2-^{3}H] and [6-^{3}H]glucose, and the detritiated fractions contain only [6-^{3}H]glucose, the specific activity of [2-^{3}H]glucose (SA_2) was determined by the equation:

$$SA_2 = SA_{total} - SA_6 ,$$

where SA_{total} is the sum of the specific activities resulting from dual label, and SA_6 is the specific activity attributed only to [6-^{3}H]glucose.

Single label tritiated glucose standards were run in parallel with samples to determine degree of detritiation of each isotope and correct for detritiation efficiency. Average detritiation of [2-^{3}H]glucose was 96.3±2.9%. Within each assay detritiation efficiency was corrected for by multiplying sample SA_6 by [2-^{3}H]glucose standard detritiation efficiency. Average detritiation of [6-^{3}H]glucose was less than 1.0%. Detritiation of [6-^{3}H]glucose standards ranged from –3.0 to 7.0%; therefore, detritiation efficiency of less than |3|% was not corrected for, while efficiencies of 3–7% were adjusted for as above.

Hormone and metabolite analysis

Plasma samples drawn prior to tracer injection were thawed for use in assays of insulin, glucagon, cortisol and glucose. Insulin was assayed using a Sensitive Rat Insulin RIA kit (cat. no. SRI-13K, Linco Research Inc, St Charles, MO, USA). This kit's stated specificity is 100% for rat, porcine, sheep, hamster, and mouse. Glucagon was assayed with a Glucagon RIA kit (cat. no. GL-32K, Linco Research Inc). Cortisol levels were measured using a Cortisol RIA kit (cat. no. TKCO2, Diagnostic Products Corporation, Los Angeles, CA, USA). All kits used for this study were validated by comparing results from serially diluted samples of pooled elephant seal plasma to the assay standard curve. Serially diluted pooled elephant seal plasma samples displayed significant parallelism with the standard curves. The cortisol kit has also been validated previously for this species (Ortiz et al., 2001). Despite the use of a sensitive insulin RIA kit, insulin values were frequently measured near the lower detection limits of the assay and the mean intra-assay coefficient of variation was 14.6%. Mean intra assay coefficient of variation for glucagon was 5.9% and 5.5% for cortisol. Since insulin and glucagon are antagonistic and it is believed that their molar ratios determine their metabolic effect rather than absolute plasma concentrations (Kraus-Friedman, 1984), values of insulin and glucagon were used to calculate the insulin:glucagon molar ratio.

Glucose concentration of plasma samples collected prior to isotope administration was processed in triplicate using a glucose autoanalyzer as above. β-hydroxybutyrate (βHBA) was assayed using a GM-7 Micro-Stat autoanalyzer (Analox Instruments Inc, Lunenburg, MA, USA).

Kinetic analysis

The rate of appearance of glucose was determined as:

$$Ra = Dose_{dpm} / y(t)dt ,$$

where Ra is the rate of appearance of unlabeled glucose, $Dose_{dpm}$ is the radioactivity of the injected tracer in disintegrations per minute, and $y(t)$ is the exponential function describing the decay of tracer specific activity with respect to time (Wolfe, 1992). Two exponential functions were fit to the clearance curve by maximizing the r^2 value for each curve (Fig. 2A,B). Curve-fitting and integration were performed using the software program *Mathematica!* (Wolfram Research, Champaign, IL, USA). The volume of administered tracer was determined by gravimetric calibration of the injection syringe.

In vivo, hydrogen at C-2 of glucose is removed early in the conversion of glucose-6-phosphate to fructose-6-phosphate (Fig. 1). Therefore, endogenous glucose output (EGO= gluconeogenesis + glycogenolysis + glucose cycle activity) was measured as the rate of appearance of glucose with respect to [2-^{3}H]glucose (Ra_2). Hydrogen at C-6 is not removed until the phosphoenolpyruvate (PEP) and citric acid cycles, so

endogenous glucose production (EGP=gluconeogenesis + glycogenolysis) was measured as the rate of appearance with respect to [6-^{3}H]glucose (Ra_6). Glucose cycle activity (GCA) was calculated as the difference between EGO and EGP ($GCA=Ra_2–Ra_6$).

Statistical analysis

Early fasting and late fasting measurements were compared using paired *t*-tests; only matched early and late fasting measurements were used (seals 7–10 were removed for these comparisons) but reported means (± S.E.M.) are calculated from all samples unless otherwise stated. Paired longitudinal measurements of body condition and plasma hormone concentration were used to examine relationships between changes in body condition and regulatory hormones with changes in plasma glucose concentration.

Results

Fasting duration, mass change, glucose production and glucose cycle activity are reported in Table 1. Average mass during early fasting was 103.7±17.2 kg, and late fasting was 93.2±13.0 kg. Mass significantly decreased over the fast (t=16.2, P=0.001). Study animals lost an average of 0.60±0.14 kg day^{-1} over the sampled fasting duration resulting in a 17.7±1.0% loss of initial body mass at the end of the study period. BCI during early fasting was 0.78±0.06 and during late fasting was 0.70±0.03; BCI decreased with fasting (t=4.16, P=0.009).

EGO during early fasting was 3.16±0.87 mg glucose kg^{-1} min^{-1} and during late fasting was 2.43±0.24 mg glucose kg^{-1} min^{-1}. There was no significant change in EGO over fasting duration (t=1.87, P=0.12). EGP during early fasting was 2.80±0.65 mg glucose kg^{-1} min^{-1} and during late fasting was 2.21±0.12 mg glucose kg^{-1} min^{-1}. There was a strong trend towards decreased EGP with fasting duration (t=2.46, P=0.057).

There was a large degree of variation in GCA during both sampling periods. Early fasting GCA ranged from –0.045 to 0.764 mg glucose kg^{-1} min^{-1}, mean 0.361 mg glucose kg^{-1} min^{-1}. Late fasting GCA ranged from 0 to 0.475 mg glucose kg^{-1} min^{-1}, mean 0.221 mg glucose kg^{-1} min^{-1}. Mean GCA was 12.2±7.6% of EGP during early fasting and 10.1±10.4% during late fasting. Proportional GCA (pGCA=GCA/EGP) changed by less than 5% in four animals (seals 1, 3, 4 and 5), increased by 13.2% in seal 2, and decreased dramatically in seal 6, going from the highest proportional GCA, 21.4% to one of the lowest, 0.1%.

There was no significant change in plasma glucose over the study period (t=2.25, P=0.075); mean plasma glucose was 154.4±15.5 mg dl^{-1} during early fasting and 143.5±8.5 mg dl^{-1} during late fasting. βHBA increased across the postweaning fast from 0.77±0.20 mmol l^{-1} during early fasting to 1.65±0.39 mmol l^{-1} during late fasting (t=5.54, P=0.004). Hormone levels measured in all study animals are presented in Table 2. Mean plasma insulin did not significantly change throughout the fast; mean concentration during early fasting was 67.6±10.5 pg ml^{-1} while during late fasting it was 57.1±13.6 pg ml^{-1}. Mean plasma glucagon concentration significantly increased (t=2.96, P=0.031) between early fasting (51.6±14.0 pg ml^{-1}) and late fasting (69.4±17.0 pg ml^{-1}). Insulin:glucagon (I:G) molar ratio during early fasting was 0.83±0.22 and during late fasting was 0.49±0.16 and the ratio significantly decreased across the fast (t=5.27, P=0.003). Mean cortisol level during early fasting was 5.33±2.61 μg ml^{-1} and 6.13±2.27 μg ml^{-1} during late fasting. Cortisol levels showed a small but significant increase over the postweaning fast (t=2.84, P=0.037).

The magnitude of change in EGP decreased with the reductions in BCI (Fig. 3, $\Delta EGP=–1.25–7.13\Delta BCI$; r^2=0.85, P=0.01). The magnitude of change in plasma glucose (ΔPG) varied inversely with that of glucagon ($\Delta PG=2.10–0.85\Delta glucagon$; r^2=0.75, P=0.02) and directly with that of I:G (Fig. 4, $\Delta PG=6.13+66.64\Delta I:G$; r^2=0.78, P=0.02). Decreased I:G ratio was associated with decreased plasma glucose. The change in insulin was positively correlated with the rise in cortisol levels (r=0.82, P=0.048). There was no

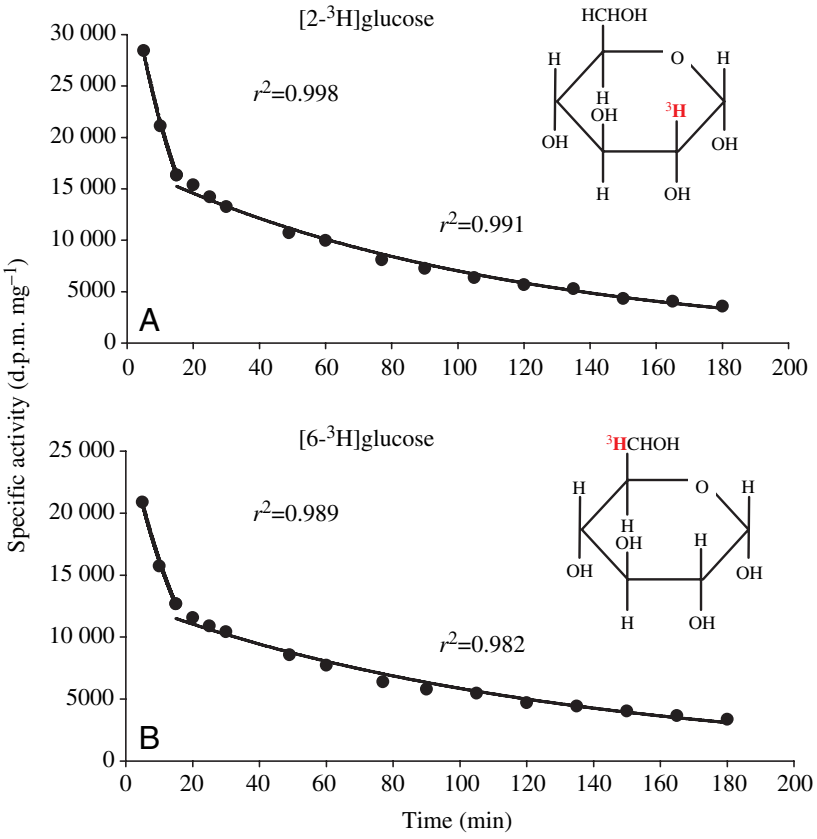

Fig. 2. Representative clearance curves for (A) [2-^{3}H]glucose and (B) [6-^{3}H]glucose, and the corresponding labeled glucose structures.

Table 1. *Weanling sex, fasting duration, mass, endogenous glucose production and glucose cycle activity % change in mass for all seals during early and late fasting periods*

		Early fasting						Late fasting						
Seal	Sex	Fasting duration	Mass	Plasma [glucose]	EGP	GCA	pGCA	Fasting duration	Mass	Plasma [glucose]	EGP	GCA	pGCA	Change in mass (%)
1	M	20	120	181.0	2.14	–0.045	–2.1	60	98	152.0	2.14	0.060	2.8	18.3
2	F	20	105	144.0	2.42	0.205	8.5	56	88	154.7	2.19	0.475	21.7	16.2
3	M	17	127	164.3	2.83	0.111	3.9	51	106	135.8	2.09	0	0	16.5
4	M	16	87	143.0	3.42	0.360	10.5	52	71	135.0	2.25	0.317	14.1	18.4
5	M	16	129	159.3	2.16	0.372	17.3	49	105	138.0	2.16	0.471	21.8	18.6
6	M	12	111	153.8	3.18	0.680	21.4	37	91	145.8	2.43	0.002	0.1	18.0
7	F	14	83	137.0	3.75	0.764	20.4							
8	M	14	91	131.5	3.56	0.662	18.6							
9	F	19	95	168.7	1.91	0.270	14.2							
10	M	19	89	161.7	2.62	0.234	8.9							
Mean		17	104	154.4	2.80	0.361	12.2	51	93	143.5	2.21	0.221	10.1	17.7
S.E.M.		2.8	17.2	15.5	0.65	0.265	7.6	7.8	13.0	8.5	0.12	0.228	10.4	1.0

Fasting duration, days; mass, kg; plasma [glucose] (mg dl^{-1}); endogenous glucose production (EGP), mg glucose kg^{-1} min^{-1}; glucose cycle activity (GCA), mg glucose kg^{-1} min^{-1}; pGCA, glucose cycle activity relative to glucose production (%).

relationship between the changes in EGP and plasma glucose, insulin, glucagon, I:G, or cortisol (P>0.5).

Considering only the early fasting samples, EGP decreased with plasma glucose (EGP=7.96–0.0335PG, r^2=0.63, P=0.006) and with Δ_HBA (EGP=4.58–2.31Δ_HBA, r^2=0.52, P=0.018). Proportional glucose cycle activity decreased with days fasting (pGCA=0.464–0.0203 days fasting, r^2=0.63, F=13.4, P=0.006). There was no correlation between insulin, glucagon, I:G ratio or cortisol level with plasma glucose or EGP (P>0.05). None of these relationships were significant in the late fasting measurements (P>0.05), although, glucagon concentration was negatively correlated with EGO (r=–0.84, P=0.036).

Discussion

The contribution of glycogenolysis to glucose release during a prolonged fast is negligible since glycogen stores are rapidly used during the first days of fasting. Thus, by removing glycogenolysis from the definitions of EGO and EGP presented earlier, EGO can be redefined for the measurement periods in this study as gluconeogenesis plus glucose cycle activity, while EGP will represent exclusively gluconeogenesis. This leads to the conclusion that gluconeogenesis decreases with time fasting in weaned elephant seal pups, a finding that agrees with previous studies in dogs (Cowan et al., 1969) and humans (Streja et al., 1977; Katz and Tayek, 1998) and is consistent with prior estimates of a reduced contribution of protein to metabolic processes across the fast (Houser and Costa, 2001). Findings from grey seal pups submitted to fasts beyond their natural duration also demonstrated a variable but notable decline in glucose production with time fasting (Nordøy and Blix, 1991). The rate of appearance of glucose here fell within the values observed by Nordøy and Blix across the 52-day forced fast, but the magnitude of the mean change in this study was much smaller.

It is unlikely that there is any significant change across the measurement interval in the mass of the CNS or renal medulla that accompanies an increase in mass-specific red blood cell volume (Thorson and Le Boeuf, 1994), suggesting that an overall increase in mass-specific glucose-dependent tissues occurs with the progression of the fast. This is compounded by an apparent increase in physical activity with time fasting that is related to the development of diving. Despite this, gluconeogenesis decreased over the fast while plasma glucose levels essentially remained the same. This suggests that the mass-specific utilization of glucose becomes more efficient with the progression of the fast, possibly due to a reduction in the utilization of glucose by tissues that can exploit substrates other than glucose for energy. This compensation would make more glucose available to glucose-dependent tissues, and if it proportionally matched the consumption requirements of the *de novo* synthesized glucose-dependent tissues, could result in the stasis of plasma glucose levels observed across the fast.

Contrary to expectations in a fasting animal, glucose production values in fasting northern elephant seal weanlings

Table 2. *Hormone levels of each study animal*

	Early fasting				Late fasting			
Seal	Insulin (pg ml^{-1})	Glucagon (pg ml^{-1})	I:G	Cortisol (μg dl^{-1})	Insulin (pg ml^{-1})	Glucagon (pg ml^{-1})	I:G	Cortisol (μg dl^{-1})
1	56.5	42.3	0.80	7.86	50.9	85.4	0.40	9.54
2	62.6	50.4	0.75	5.00	71.2	55.1	0.50	5.53
3	80.9	61.8	0.79	3.15	66.9	94.0	0.73	4.69
4	56.4	50.8	0.67	4.17	38.7	57.2	0.25	5.06
5	80.6	50.8	0.95	8.20	46.2	70.0	0.48	8.29
6	75.5	46.3	0.98	3.60	68.4	54.4	0.59	3.69
7	66.2	42.3	0.94	4.19				
8	52.5	86.8	0.36	3.60				
9	68.7	46.8	0.88	2.98				
10	76.4	38.1	1.20	10.57				
Mean	67.6	51.6	0.83	5.33	57.1	69.4	0.49	6.13
S.E.M.	10.5	14.0	0.22	2.61	13.6	17.0	0.16	2.27

I:G, insulin to glucagon molar ratio.

were higher than non-fasting adapted species undertaking similar duration fasts (Table 3). During the early fasting measurement, average glucose production was 2.8 mg kg^{-1} min^{-1} and mean mass was 104 kg, resulting in an average of 2.3 moles of glucose produced per day. To explore the energetic demands of glucose-dependent tissues, the glucose consumption of the brain and red blood cells were estimated using measurements made in elephant seals and closely related phocid species. Murphy et al. (1980) and Hochachka (1981) estimated that, for a 500 kg Weddell seal *Leptonychotes weddellii* with 0.5 kg brain, 0.3–0.4 μmol g^{-1} min^{-1} of glucose was required to support a CNS metabolic rate of 6 μmol ATP g^{-1} min^{-1}. Assuming a 250 g brain for a 100 kg elephant seal pup, and using the same mass-specific energy requirements as estimated for the Weddell seal, glucose utilization for the brain amounts to 0.126 mol d^{-1}. Plasma ketone levels more than doubled over the fasting period, and values were similar to those reported in Castellini and Costa (1990), but levels remain much lower than required to induce CNS utilization of ketone as a fuel source in non-fasting adapted mammals (Felts et al., 1964; Nehlig and de Vasconcelos, 1993). Since blood glucose is elevated in weanlings, it seems likely that most of the energy for nervous system metabolism is supplied by glucose. If the CNS of fasting elephant seals is capable of utilizing ketones at low plasma concentrations (e.g. high flux but low hemococentration), the demand for glucose would be further reduced. Nordøy and Blix (1991) measured ketone turnover rates in fasting grey seals of 9.3–13.8 μmol kg^{-1} min^{-1} and argued that ketone utilization facilitates protein sparing in this species. For the calculations made here, no ketone utilization

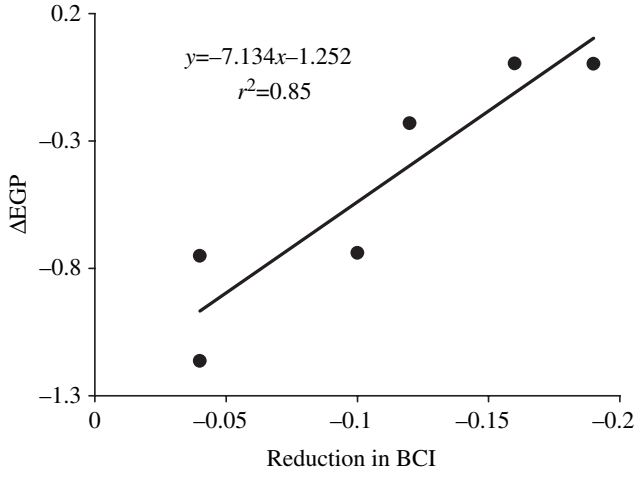

Fig. 3. The change in endogenous glucose production (ΔEGP) in mg glucose kg^{-1} min^{-1} over the decrease in body composition index (BCI; P=0.01). Note that the values of the x axis decrease from left to right.

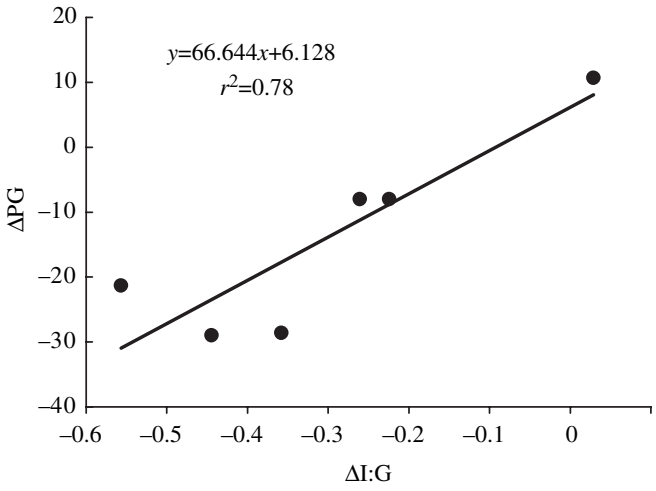

Fig. 4. The change in plasma glucose (ΔPG) in mg glucose dl^{-1} over the change in the insulin:glucagon molar ratio (ΔI:G; P=0.02).

Table 3. *Endogenous glucose production (EGP) for several species in various metabolic states*

Species	State	EGP (mg glucose kg^{-1} min^{-1})	Reference
Northern elephant seal	Fasting 2 weeks	2.80	Present study
	Fasting 6 weeks	2.21	
Grey seal	Fasting 9 days	3.62	Nordøy and Blix, 1991
	Fasting 37 days	2.03	
Dog	Post-absorptive	3.57	Issekutz, 1977; Cowan et al., 1969
	Fasting 3 days	2.08	
	Fasting 4 weeks	1.39	
Rabbit	Post-absorptive	3.13	Dunn et al., 1976
Human	Post-absorptive	1.8–2.5	Streja et al., 1977; Weber et al., 1990; Heaney et al., 1997
	Fasting 3–5 weeks	0.81	
	During exercise	6.75	

by glucose-dependent tissues was assumed. Thus our estimates are potentially biased upwards, and actual glucose utilization may be less than estimated here if elephant seals are adapted to ketone utilization at lower plasma concentrations.

At 2 weeks postweaning, elephant seal pups have approximately 6.05 l of red blood cells (Thorson and Le Boeuf, 1994). Using rates of glucose consumption measured in red blood cells by Castellini et al. (1992), and correcting for the temperature dependence of enzymatic activity, it is estimated that red blood cells require approximately 700 nmol glucose (ml RBC)$^{-1}$ h^{-1}. This comes to 0.102 moles glucose consumed by RBC per day. Therefore, total glucose demand by CNS and erythrocytes is 0.228 moles glucose per day; this represents only 10% of the glucose produced each day. Renal glucose utilization accounts for 10–25% of glucose turnover in humans (Cersosimo et al., 1999) and as much as 30% in dogs (Cersosimo et al., 1994) in the post absorptive state. As reliable data on renal glucose consumption during long duration fasting or in phocid seals are unavailable at this time, we have not included estimates of glucose utilization by the renal medulla in our calculations. Castellini et al. (1987) estimated that an elephant seal pup of 98 kg had 686 μmoles glycerol min^{-1} available for gluconeogenesis. If all available glycerol were converted to glucose, it would account for 0.49 moles of glucose each day; leaving ~1.8 moles of glucose production unaccounted for, but meeting the glucose requirement of the glucose-dependent tissues. It is not practical for a fasting adapted animal to make up the balance catabolizing lean tissue, and previous research has demonstrated lean tissue loss to be low. Houser and Costa (2001) estimated that over 6 weeks of fasting, pups lose only 1.8 kg of lean tissue. Assuming that all of the lost lean mass produced amino acids in proportion to those observed during the late fasting period in weanlings (Houser and Crocker, 2004), and that the gluconeogenic precursors that make up the majority of the amino acid pool (i.e., alanine, glutamine and glycine) are converted to glucose, it is crudely estimated that ~3.9 moles of glucose would be formed. This value is certainly an overestimate but provides a theoretical ceiling to which experimental results can be compared. Given this theoretical ceiling and assuming a constant rate of production of 2.3 mol glucose d^{-1}, the contribution of amino acids would only constitute ~4% of the glucose produced over a 6-week period. Keith (1984), after injection of U[^{14}C]alanine, found only minor amounts of labeled carbon incorporated into glucose, which agrees with estimates of a minimal contribution of lean tissue to gluconeogenesis.

The apparent disparity between EGP and the estimated contribution of glycerol and amino acids to gluconeogenesis suggests that other remaining potential precursors (namely lactate and pyruvate) are utilized. Erythrocytes represent a significant glucose-dependent tissue in this species. In adult elephant seals 21% of their body mass is blood. In pups the value is closer to 11%, with hematocrit levels greater than 50% (Thorson and Le Boeuf, 1994). The primary mechanism by which erythrocytes meet metabolic costs is the breakdown of glucose to lactate. Produced lactate may subsequently act as a gluconeogenic precursor with resulting glucose released back into circulation. This form of glucose cycling was first hypothesized by Cori (1931) as a process of importance in the regulation of blood glucose. Owen et al. (1969) proposed that Cori cycle activity may be important in fasting humans and glucose oxidation findings of Keith and Ortiz (1989; <2.5% of EGP) in elephant seals led them to suggest that the Cori cycle was the primary mechanism of recycling of radioactive carbon in fasting elephant seal pups. Tayek and Katz (1997) determined that Cori cycle activity accounted for 20% of gluconeogenesis after an overnight fast in normal man and over 33% in some cancer patients.

In the fasting state, the energy to fuel gluconeogenesis is likely supplied through fatty-acid oxidation. Glucose that is released following hepatic gluconeogenesis could be utilized by erythrocytes and kidney medulla *via* the Cori cycle. This would allow glucose to act as a shuttle for ATP between fat oxidation in liver and glycolysis in glucose-dependent tissue. By recycling glucose in this manner, elephant seals may provision erythrocytes and the renal medulla using the energy from fat. However, liberally estimating that erythrocyte and

renal medulla glucose consumption are met via the Cori cycle, the CNS meets its energetic needs through the complete oxidation of glucose, and that the contributions to gluconeogenesis from glycerol and amino acids liberated via lean mass catabolism are complete and irreversible, less than 20% of EGP can be accounted for. This presents a conundrum if the rest of the EGP is equated to Cori cycling since it is unknown which tissues would be contributing to the remaining 80% of net glucose flux. Regardless, the rates of gluconeogenesis as well as glucose recycling appear higher in elephant seals than other fasting animals studied (Katz et al., 1974). This degree of futile cycling suggested by the glucose flux measurements made here implies an energetically inefficient system and suggests that protection of lean tissue is more important than energetic efficiency. Goodman et al. (1980) as well as Henry et al. (1988) suggested that suppression of metabolic rate during long-term fasting is essential to facilitate protein sparing. Sparing of amino acid precursors may take priority over energy efficiency during extended fasts provided extensive lipid energy reserves are available. This study found that weanlings maintaining body composition over the fasting duration reduced glucose production to a greater extent than weanlings greatly decreasing BCI (Fig. 3). However, using our methods we are unable to differentiate between the gluconeogenic substrates that accounted for the decrease in glucose production. It remains unclear whether the change in body composition impairs weanlings ability to spare protein or if the loss in lipid reserves is associated with increased Cori cycle activity.

The regulatory role of the glucose cycle is best viewed as a proportion of EGP. Variation in proportional glucose cycle activity was high between individuals, but taken as a whole, the proportion was nearly consistent between early and late fasting periods. Values of pGCA found in this study are similar to those reported in other species measured: 12.6% in post-absorptive dog (Issekutz, 1977) and from 11–25% in post-absorptive humans (Shulman et al., 1985; Neely et al., 1992; Rooney et al., 1992; Heaney et al., 1997). There is no evidence to suggest that the glucose cycle varies the regulation of glucose availability across the fast; rather, it changes in parallel with EGP. It is worth noting that the high plasma glucose concentration after extended fasting observed in elephant seals is near that of post-absorptive carnivores (Opazo et al., 2004). A change in GCA may be expected if the demand for glucose changes over the fast, provided there was a premium on glucose availability. In contrast, the northern elephant seal weanling may stabilize glucose utilization by making glucose available for consumption in excess, but by reducing the sensitivity of non-target tissues to glucose uptake. It has been suggested that elephant seals are insensitive to insulin and there was no relationship between GCA, hormone levels or I:G, suggesting an insensitivity of regulatory processes to hormonal variation. This contrasts with previous studies in non-fasting adapted mammals which demonstrated that both insulin (Rooney et al., 1992) and glucagon increase GCA (Issekutz, 1977; Miyoshi et al., 1988). However, Wolfe (1992) has argued that alterations in GCA in response to hormonal changes are not due to a direct affect on GCA, but that GCA changes in proportion to total flux; i.e. glucose cycling is a passive consequence of total glucose production and not under hormonal control. The results of this study agree with this assertion.

A small but significant increase in cortisol was measured across the study period. Increased cortisol with fasting is common in seals (Guinet et al., 2004; Ortiz et al., 2003b). Cortisol has been shown to increase gluconeogenesis (Friedmann et al., 1967; Issekutz and Allen, 1972) as well as increasing glucose recycling (Goldstein et al., 1993). However, we found no relationship between cortisol levels and glucose production or cycle activity. Ortiz et al. (2003b) measured a much larger increase in cortisol, 5.8 $\mu g\ dl^{-1}$ early in the postweaning fast and 14.1 $\mu g\ dl^{-1}$ at the end of the fast.

Previous studies have shown a reduction in plasma insulin concentration with the progression of fasting, but the effect of fasting on glucagon is less clear. Glucagon did not change over the fasting duration in rat (Goodman et al., 1980), while in man glucagon levels rose early in the fast and then returned to baseline values (Marliss et al., 1970). In contrast, glucagon increased across the fast in penguin (Cherel et al., 1988). Previous research in elephant seals has found decreases in insulin and increases in glucagon over the postweaning fast (Ortiz et al., 2003a). Ortiz et al. (2003a) measured insulin levels of 3–5 $\mu U\ ml^{-1}$ and glucagon levels of ~80 $pg\ ml^{-1}$ in pups fasting less than 1 week; and 2–3 $\mu U\ ml^{-1}$ insulin and ~190 $pg\ ml^{-1}$ glucagon in pups fasting 6–8 weeks. The study by Ortiz et al. involved a much greater sample size (N=40) and values measured were higher than those of this study, especially glucagon during late fasting. Although the pattern of variation in hormone levels is similar between the two studies, the large disparities in glucagon and cortisol values can only be speculated upon until more data are collected on the levels of these hormones at similar intervals. Some possible explanations for these discrepancies include the smaller sample size of the current study and the possibility that there were sampling differences within the 6–8 week late fasting sampling range.

In this study, as expected, the insulin-glucagon ratio decreased over the fast. A similar trend is found in fasting penguins (Cherel et al., 1988) and humans undergoing a long duration fast (Streja et al., 1977), although absolute hormone levels were much higher in both penguins and humans. Decreasing I:G ratio is indicative of an upregulation of catabolic processes and stimulates glucose release by the liver and renal cortex. Contrary to expectations, decreased I:G ratio was accompanied by decreased levels of plasma glucose (Fig. 4). Increased levels of glucagon should lead to phosphorylation and inactivation of pyruvate kinase and stimulation of phosphoenolpuyruvate carboxykinase transcription and activity, allowing for an increase in gluconeogenesis (Jiang and Zhang, 2003). Previous studies in this species have not detected an insulin response to glucose injection (Kirby and Ortiz, 1994) and it has been proposed that

elephant seals do not closely regulate blood glucose by the conventional insulin-glucagon push pull model. In this study we found no relationship between insulin, glucagon, or I:G ratio to EGP or GCA. This lack of correlation between hormone levels and glucose production support the conclusions of Kirby and Ortiz (1994). However, this may also be due to the high degree of individual variation in EGP and GCA. More controlled longitudinal studies involving hormonal manipulations may be needed to reveal regulatory effects of hormones that are masked by individual variability.

Placing the results of the glucose flux experiment and hormonal measurements into proper context cannot be done without some consideration being given to potential confounding factors. One such factor is the use of immobilizing drugs during the measurement period. The impact of ketamine and tiletamine/zolazepam on glucose kinetics is unknown and there was a large degree of variation in the total amount of drugs required to maintain immobilization during the measurement period, 2.0–7.5 ml of ketamine; initial immobilization with tiletamine/zolazepam was standardized to animal mass. Despite this variation, there was no relationship between the amount of drugs administered with EGO, EGP or GCA (F=0.6-1.8, r^2=0.04–0.10, P=0.23–0.45) suggesting that there was no effect of the maintenance schedule on glucose production or cycle activity. Another potential source of variation may be the incomplete removal of tritium from the administered tracers via the predicted pathways, or loss via unaccounted for pathways. Errors due to these processes may cause EGP to be underestimated by 3% and EGO by as much as 20% (Landau, 1993). There are no data on *in vivo* loss of label in seals and we report uncorrected data in this study with the understanding that these values represent conservative estimates of both GCA and EGP. This underestimation is probably the cause of zero and slightly negative GCA values that were occasionally observed.

In summary, we measured unexpectedly high levels of EGP in weaned elephant seals that decreased across the duration of the fast. GCA did not appear to play an important role in regulating EGP across the fast. Proportional glucose cycle activity was highly variable between individuals, but, overall, was nearly consistent between early and late fasting periods and was similar to those reported in other species in which measurements have been made. High levels of EGP relative to estimated glucose utilization and gluconeogenic substrate availability suggest extensive recycling of glucose and a potential increased importance of Cori cycle activity in fasting elephant seals.

List of abbreviations

AMR	average metabolic rate
BCI	body composition index
C-2	second carbon of glucose
C-6	sixth carbon of glucose
CNS	central nervous system
cv	coefficient of variation
EGO	endogenous glucose output; EGP endogenous glucose production
F6P	fructose-6-phosphate
G6P	glucose-6-phosphate
GCA	glucose cycle activity
I:G	molar ratio of insulin to glucagon
PEP	phosphoenolpyruvate
PG	plasma glucose
pGCA	proportional glucose cycle activity
Ra	rate of appearance
RBC	red blood cells
SA	specific activity
βHBA	β-hydroxybutyrate
ΔN	change in parameter N

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