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RESEARCH ARTICLE

Sex differences in fuel use and metabolism during development in fasting juvenile northern elephant seals

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SUMMARY

Many polygynous, capital breeders exhibit sexual dimorphism with respect to body size and composition. Sexual dimorphism is often facilitated by sex differences in foraging behavior, growth rates and patterns of nutrient deposition during development. In species that undergo extended fasts during development, metabolic strategies for fuel use have the potential to influence future reproductive success by directly impacting somatic growth and acquisition of traits required for successful breeding. We investigated sexual dimorphism associated with metabolic strategies for fasting in developing northern elephant seals. Thirty-one juvenile seals of both sexes were sampled over extended fasts during annual autumn haul-outs. Field metabolic rate (FMR) and the contribution of protein catabolism to energy expenditure were estimated from changes in mass and body composition over 23±5 days of fasting (mean ± s.d.). Protein catabolism was assessed directly in a subset of animals based on urea flux at the beginning and end of the fast. Regulatory hormones and blood metabolites measured included growth hormone, cortisol, thyroxine, triiodothyronine, insulin, glucagon, testosterone, estradiol, glucose, urea and β-hydroxybutyrate. Males exhibited higher rates of energy expenditure during the fast but spared body protein stores more effectively than females. Rates of protein catabolism and energy expenditure were significantly impacted by hormone levels, which varied between the sexes. These data suggest that sex differences in fuel metabolism and energy expenditure during fasting arise early in juvenile development and may play an important role in the development of adult traits associated with reproductive success.

Key words: sexual dimorphism, fasting, body composition, field metabolic rate, pinniped.

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INTRODUCTION

Energy used for reproduction comes from body reserves in ‘capital breeding’ animals. Body reserves available for breeding are influenced by lifetime patterns of growth and use of reserves during previous fasts. Many capital breeders are also polygynous, which increases the potential for variation in reproductive success and strategies between the sexes. While both sexes require extensive lipid and protein stores for fasting, differences in breeding strategies may create different priorities for body composition. Males may require larger size and muscle mass to effectively compete for access to mates while females may require larger adipose tissue reserves to provide the nutrients necessary for milk synthesis. Sexual dimorphism is associated with differences in foraging strategies (Ginnett and Demment, 1997; Le Boeuf et al., 2000), growth rates (Setchell et al., 2001) and body compositions (Berry and Shine, 1980; Schulte-Hostedde et al., 2001). Body composition differences in adults reflect different strategies for growth and energy storage, so it is possible that the sexes have different metabolic strategies for using stored reserves while undergoing natural fasts. These sex differences may be most crucial during development, and particularly during the period of adolescence.

Pinnipeds provide numerous examples of sexually dimorphic species that fast during development. Studies on sex differences in fasting metabolism in developing pinnipeds have yielded equivocal results. Arnould et al. found that female Antarctic fur seal (*Arctocephalus gazelle*) pups had greater proportional adipose reserves and higher field metabolic rates (FMR) than males (Arnould et al., 2001). Despite these differences, males and females met equivalent proportions of FMR with protein catabolism. Similarly, a different population of Antarctic fur seal exhibited higher rates of mass loss and FMR in fasting female pups than in fasting male pups (Guinet et al., 1999). By contrast, a study on sympatric subAntarctic fur seals (*Arctocephalus tropicalis*), with the longest intersuckling fasts found in otariids, reported no sex differences in FMR but found that leaner males spared protein more effectively than females (Beauplet et al., 2003). A more recent study on the same population suggested that male and female pups rapidly converged on an identical and low level of protein catabolism and found no sex differences in any aspect of fasting metabolism (Verrier et al., 2009).

Among the pinnipeds, the longest developmental fasts are found in phocid seals, many of which are capital breeders. Elephant seal pups can fast for up to 3 months before their first foraging migration (Reiter et al., 1981). Prior to reaching breeding ages, juvenile elephant seals haul out twice a year for approximately one month, during which they fast completely from food and water (Field et al., 2005). Northern elephant seals are capital breeders and exhibit extreme sexual dimorphism, with adult males as large as 10 times the mass of adult females (Deutsch et al., 1994). Two important features of the growth pattern of male elephant seals are the peak in growth rate, which coincides with the onset of puberty at 3–5 years of age, and the onset of sexual maturity at 8 years of age (Clinton,

1990). In contrast, female elephant seals reach maturity at 4 years of age and do not experience a similar growth spurt (Sydeman and Nur, 1994). Clinton and Le Boeuf suggested that male growth patterns may be associated with sexual selection for large body size in males and that the acceleration in growth rate around puberty may be connected to delayed maturity (Clinton and Le Boeuf, 1993).

Large muscle mass may be critical to the success of males in competitive dominance interactions that are required to gain access to breeding opportunities. Dominance interactions are frequently decided by combat although success is not driven solely by body mass (Haley et al., 1994). In contrast, the smaller females need to mobilize significant reserves of lipids to produce the energy-dense milk associated with abbreviated periods of parental investment (Crocker et al., 2001), and fat reserves directly impact protein sparing during lactation (Crocker et al., 1998). For these reasons, females may be selected to prioritize growth of adipose tissue reserves during development to breeding age.

Previous measures of protein catabolism in elephant seals yielded highly variable estimates that were potentially impacted by the method of estimation and the life history stage of the seal. Studies using urine collection or urea flux report averages of 1–3% of the average metabolic rate being derived from protein catabolism in weaned pups (Adams and Costa, 1993; Houser and Costa, 2001; Pernia et al., 1980). In contrast, use of mass balance approaches has yielded higher estimates of the contribution of protein catabolism to energy expenditure – in excess of 15% in individual weaned pups and juveniles (Carlini et al., 2001; Field et al., 2005; Noren et al., 2003). During the post-weaning fast, comparisons of protein catabolism among sexes suggest no differences in fuel use (Carlini et al., 2001; Noren et al., 2003), but there is strong evidence for enhanced protein sparing in male juvenile southern elephant seals (Field et al., 2005). Estimates of protein contributions to energy metabolism in breeding adult animals are slightly higher in females than in males (10% *vs* 7% of FMR) (Crocker et al., 1998; Crocker et al., 2001). The disparity between estimates of protein catabolism using the two methodologies suggests that the mass balance approach may overestimate protein catabolism relative to measures of urea flux. Additionally, failure to account for protein loss as pelage during the molt may increase estimates of protein catabolism during this period (Carlini et al., 2001; Field et al., 2005). Applications of the mass balance method to other phocid species have yielded similar estimates, with protein providing ~10% of energy metabolism (Bennett et al., 2007).

In the present study, we investigated sex differences in metabolism and fuel use over the autumn juvenile northern elephant seal (*Mirounga angustirostris* Gill 1866) haul-out; investigation during this period avoids potential errors associated with molting pelage loss. We examined FMR, body composition changes, protein catabolism, blood metabolites and regulatory hormones for evidence of sex differences in metabolic strategies for fasting. Additionally, we simultaneously compared estimates of protein catabolism in fasting animals derived from the mass balance method with measurements of urea flux.

MATERIALS AND METHODS

Study site and subjects

Work was conducted under NMFS Marine Mammal Permit # 786-1463, and all procedures were approved by the Sonoma State University Institutional Animal Care and Use Committee. This study was carried out at Año Nuevo State Reserve, San Mateo County, CA, USA during the autumn juvenile haul-out (August–November) in 2008 and 2009. Daily censuses of the rookery were carried out to estimate arrival dates of the animals. Known age, 1.8-year-old seals were identified *via* flipper tags and marked with hair dye (Lady Clairol, Stamford, CT, USA) upon arrival at the rookery to facilitate identification throughout the study period. Early fasting samples were collected within six days of arrival at the rookery. An average of 22.6±4.6 days (s.d.) later, animals were recaptured and sampled once more. Body composition was measured using the isotopic dilution method (Iverson et al., 1993). A total of 41 seals was sampled early in the fasting period (19 males and 22 females). Of these seals, 31 were sampled again late in the fasting period (15 males and 16 females). Only the 31 seals sampled twice were used in the subsequent analysis. In 11 of these animals, protein catabolism was measured by urea flux early in the fasting period. The process was repeated in nine of these seals late in the fasting period for a total of 20 urea flux measurements.

Mass and body composition measurements

Seals 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 100 mg ketamine as needed to maintain immobilization (all drugs from Fort Dodge Labs, Fort Dodge, IA, USA). Animals were weighed using a digital scale (MSI tension dynamometer, Seattle, WA, USA; ±1.0 kg) suspended from an aluminum tripod. A pre-injection blood sample was collected *via* the extradural vein, and a bolus injection of 37.0 MBq of tritiated water (HTO) was administered in 2 ml of sterile injectable water. Serial blood sampling demonstrated that HTO equilibration in the total body pool occurs within 90 min of intravenous injection in northern elephant seal pups (Houser and Costa, 2001). To confirm equilibration time in juveniles, serial blood samples were collected at 30, 45, 60, 75, 90 and 100 min post-injection in a subset of six animals. All animals were equilibrated by 90 min. Equilibration was confirmed for all measurements by comparing two blood samples taken at least 10 min apart after 90 min. Blood samples were collected in chilled vacutainers, stored on ice, transported to the laboratory and centrifuged for 20 min at 2000 ***g***. and 4°C. Serum and plasma samples were immediately frozen at –80°C for later analysis. This procedure was repeated late in the fasting period, 22.6±4.6 days following the first procedure, using a bolus injection of 11.1 MBq of HTO in 3 ml of sterile water to re-measure the total body water (TBW) pool.

Water was collected from samples of serum (~250 μl) into scintillation vials by way of dry-ice distillation (Ortiz et al., 1978). Betaphase scintillation cocktail (7 ml; Westchem, San Diego, CA, USA) was added to each scintillation vial, and the plasma activity of each sample determined using a Beckmann model LS 6500 liquid scintillation counter (Beckmann, Orange County, CA, USA). All samples were analyzed in triplicate. The absolute amount of tracer injected was determined by gravimetric calibration of the syringes used for isotope administration.

Total body water (TBW) was calculated as the total amount of radioactivity injected divided by the radioactivity of the post-equilibration sample. The activity of pre-injection blood samples collected during the second sampling period was subtracted from equilibration values in order to account for residual tritium activity from body water measurements made early in the fasting period. TBW determinations were decreased by four percent, as the tritium dilution method slightly overestimates TBW volume (Nagy and Costa, 1980; Reilly and Fedak, 1991; Bowen and Iverson, 1998).

Body composition was calculated from measurements of TBW estimated by the tritium dilution method (Iverson et al., 1993),

assuming that lipid has no free water and that fat-free mass has a hydration state of 73.3% free water (Iverson et al., 1993; Worthy et al., 1992). Lipid mass was calculated as:

$$M_{\text{lipid}} = (M_{\text{total}}) - 1.37 \times (\text{TBW}), \quad (1)$$

where M_{lipid} is lipid mass and M_{total} is total body mass. Lean mass was calculated as the difference between total and lipid mass.

Field metabolic rate (FMR) was estimated using the mass balance method (Crocker et al., 2001; Noren et al., 2003). The amount of energy obtained from lipids was calculated as the changes in lipid mass over the duration of the fast $\times$ 39.33 kJ g^{-1}. Lean mass loss was assumed to be 73% water and 27% protein (Noren et al., 2003), and the amount of energy obtained from protein was calculated as 17.99 kJ g^{-1} protein. The sum of energy calculated from the changes in lipid mass and protein was then divided by the days elapsed between measurements to estimate the daily field metabolic rate of the animal.

Urea flux measurements

In a subset of 11 seals, the dilution of [^{14}C]urea was used to calculate urea pool size and rate of flux. Clearance of urea tracer was used to independently estimate the level of protein catabolism throughout the fasting period (Crocker et al., 1998; Houser and Costa, 2001). An initial blood sample was taken *via* the extradural vein, and a bolus injection of 7.4 MBq of [^{14}C]urea in a 4 ml volume was administered in the extradural vein. Beginning at 30 min, blood samples were taken every 15 min for the next 90 min to confirm equilibration of the isotope in the urea pool (Crocker et al., 1998). Seals were briefly recaptured 1.9±0.6 days later, and a blood sample was taken to measure clearance of the labeled urea. Nine animals were recaptured 21.7±2.8 days after the initial procedure, and the entire procedure was repeated.

The [^{14}C] activity of the serum was determined in triplicate. 200 µl samples were combined with 7 ml of Betaphase scintillation cocktail and counted with a Beckman model LS 6500 liquid scintillation counter (Beckmann, Orange County, CA, USA).

The pool volume was calculated from the dilution of the injectate at equilibrium. Urea pool size was calculated from the dilution volume and blood urea nitrogen (BUN) concentration. Mean urea flux was calculated based on the flux constant derived from ^{14}C flux curves and a measure of the urea pool size. This urea flux constant (K_{u}) was calculated as:

$$K_{\text{u}} = -\Delta \ln (A) \times t^{-1}, \quad (2)$$

where A is the activity of the sample and t is time in days. Thus, urea flux constant is the negative slope of a semilog plot of the clearance curve. Urea pool size (N_{u}) in grams was calculated as:

$$N_{\text{u}} = [\text{BUN}]_{(t-0)} \times DPM_{\text{i}} / A_{(t-0)}, \quad (3)$$

where DPM_{i} is the total injected activity of ^{14}C, $A_{(t-0)}$ is the equilibration activity, and $[\text{BUN}]_{(t-0)}$ is serum urea concentration at equilibrium. Assuming amino acids are the only significant source of nitrogen for urea formation, the daily rate of protein catabolism (r_{p}) was calculated as:

$$r_{\text{p}} = r_{\text{u}} \times 2.92 \text{ g protein g}^{-1} \text{ urea}, \quad (4)$$

where r_{u} is the mean urea flux.

Metabolite and hormone analysis

Serum samples drawn prior to tracer injections were used to measure cortisol, insulin, total thyroxine (tT4), total triiodothyronine (tT3), growth hormone (GH), testosterone and estradiol. Plasma samples drawn prior to tracer injections were used in the measurement of glucagon, glucose, β-hydroxybutyrate (β-HBA) and blood urea nitrogen (BUN). Cortisol, insulin, tT3, tT4, glucagon, total testosterone, estradiol and GH were analyzed using commercially available RIA Kits (all except GH, Siemens, New York, NY, USA; GH, Millipore, Billerica, MA, USA). All kits have been previously validated for use in elephant seals (Ortiz et al., 2003a; Ortiz et al., 2003b; Champagne et al., 2005; Champagne et al., 2006). The mean intra-assay coefficient of variation was between 1.9 and 3.1 for cortisol, insulin, glucagon, tT3, tT4, testosterone and estradiol and 5.8 for GH. Plasma glucose was measured in duplicate using an YSI 2300 glucose autoanalyzer (YSI, Yellow Springs, OH, USA). β-HBA was measured in duplicate using colorimetric assay (Cayman Chemical Co., Ann Arbor, MI, USA). BUN was measured in duplicate using an enzymatic colorimetric assay (Stanbio Laboratory, Boerne, TX, USA).

Statistical analysis

Changes across the fast were evaluated using linear mixed-effects models with individual seal as a random effect subject term (SAS 9.2, Cary, NC, USA). The initial model that was run for all animals included sex, fasting status (early *vs* late) and their interaction. This interaction term was removed from the model if not significant (P>0.05). Relationships between variables within fasting periods or across the sampling period were evaluated using simple linear regression. Model residuals were evaluated to assess approximate normality. Means are presented as ± 1 s.d.

RESULTS

Mass and body composition and FMR

Initial body mass did not vary significantly between the sexes ($F_{1,30}$=0.47, P=0.50; Table 1). Seals lost 13.9±3.6% of body mass over the measurement period and this value did not vary between the sexes ($F_{1,30}$=0.47, P=0.50; Table 1). Initial body composition did not vary between the sexes ($F_{1,29}$=0.92, P=0.3). However, males depleted greater proportions of initial body fat than females (19.6±5.9% *vs* 14.5±3.7%; $F_{1,29}$=7.45, P=0.01; Table 1).

When controlling for mass, FMR was greater in males than in females ($F_{2,28}$=7.2, P=0.01; Fig. 1, Table 1). Females obtained a greater percentage of FMR from protein than males ($F_{2,28}$=11.0, P<0.01; Table 1). However, the absolute rate of protein loss was only marginally different ($F_{2,28}$=3.00, P=0.07). Initial body fat

Table 1. Mean (±s.d.) initial body mass, body composition, percentage of total and fat mass loss, daily rate of protein loss (r_{p}), field metabolic rate (FMR), and proportion of FMR from protein catabolism, measured early and late in the fasting period

Sex	Initial mass (kg)	Mass loss (%)	Initial fat (%)	Fat loss (%)	r_{p} (g day^{-1})	FMR (MJ day^{-1})	FMR from protein (%)
Male	189.4±24.8	14.3±3.4	35.1±1.2	19.6±5.9*	68.8±20.0	34.1±5.4*	3.6±1.3*
Female	184.8±22.6	13.5±3.9	35.1±2.0	14.5±3.7*	84.1±28.2	30.6±5.9*	5.2±2.0*
All	187.3±23.2	13.9±3.6	35.0±1.6	17.0±15.0	76.7±25.4	32.3±5.9	4.5±1.9

Asterisks denote significant differences between the sexes (P<0.05).

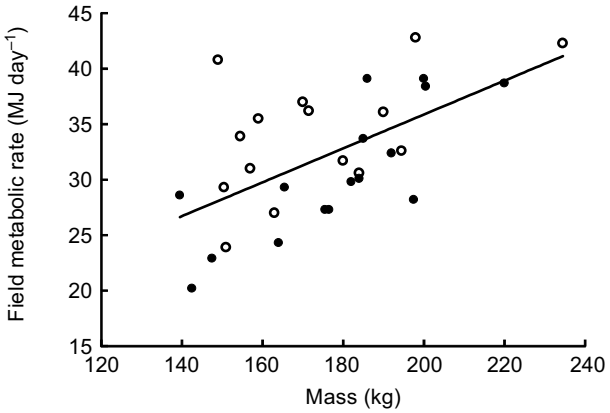

Fig. 1. Relationship between mean body mass and field metabolic rate (MJ day^{-1}). The equation for the regression line is y=5.4+0.15x (r^2=0.35, $F_{1,29}$=15.61, P<0.0001). Field metabolic rate was calculated based on the mass balance method. Closed circles represent females; open circles are males.

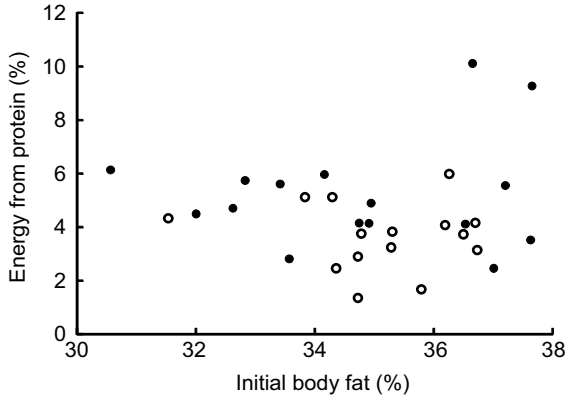

Fig. 2. There was no relationship between early body composition and percent energy from protein derived from the mass balance method. Closed circles represent females; open circles represent males.

proportion was not a significant predictor of the percentage of FMR obtained from protein ($F_{1,29}$=0.02, P=0.90; Fig. 2).

Metabolites and hormones

Sexes did not vary in plasma glucose ($F_{1,30}$=0.18, P=0.67; Table 2), β-HBA ($F_{1,29}$=0.06, P=0.80; Table 2) or BUN ($F_{1,30}$=0.01, P=0.91; Table 2) concentrations. There was no change in the concentration of glucose ($F_{1,30}$=0.40, P=0.53; Table 2) or β-HBA ($F_{1,29}$=0.02, P=0.88; Table 2) across the fasting period. Mean BUN concentrations decreased significantly across the fasting period ($F_{1,30}$=99.2, P<0.0001, Table 2). With one exception, there were no significant relationships between any of the measured hormones and measured metabolites in the early or late samples (P>0.05). In the early fast sample of females, plasma β-HBA concentrations were affected by estradiol levels (Fig. 3).

Neither glucagon ($F_{1,30}$=0.02, P=0.88; Table 3) nor insulin ($F_{1,30}$=2.71, P=0.11; Table 3) concentrations varied significantly between the sexes. Glucagon increased 34% between the early sampling periods ($F_{1,30}$=7.13, P=0.01; Table 3); however, insulin did not change across the measurement period ($F_{1,30}$=1.15, P=0.29) and remained relatively low (Table 3). Despite the increase in glucagon, insulin to glucagon molar ratio (I/G) did not decrease significantly across the fasting period ($F_{1,30}$=0.01, P=0.91; Table 3). There were no sex differences detected in cortisol concentrations ($F_{1,29}$=0.06, P=0.80; Table 3), and cortisol concentrations did not change across the fasting period ($F_{1,29}$=0.44, P=0.51; Table 3). T3 concentrations varied between the sexes ($F_{1,30}$=18.30, P<0.001; Table 4) but did not change across the fasting period ($F_{1,30}$=0.58, P=0.45). T4 concentrations varied between the sexes ($F_{1,30}$=22.34, P<0.0001; Table 4) and declined across the fasting period ($F_{1,30}$=27.85, P<0.0001; Table 4).

Growth hormone concentrations varied between the sexes ($F_{1,29}$=10.5, P<0.01; Table 4), and concentrations decreased significantly across the fast ($F_{1,29}$=24.0, P<0.0001; Table 4). Male and female GH concentrations declined 68 and 36% respectively, indicating a significant sex difference in the way GH changes in juvenile elephant seals with time fasting ($F_{1,29}$=24.0, P<0.0001; Table 4).

No significant changes were detected in male testosterone concentrations between early and late samples (45.3±26.9 *vs* 40.3±37.0 ng dl^{-1}; $F_{1,14}$=0.5, P=0.48). Initial body fat proportions declined with increasing testosterone levels in males (Fig. 4). Female estradiol concentrations declined significantly across the fasting period (105.6±22.3 *vs* 82.0±37.0 pg ml^{-1}; $F_{1,15}$=5.1, P=0.03). β-HBA concentrations increased with estradiol concentrations in the early sample females (Fig. 3). No significant changes were detected in male testosterone concentrations between early and late samples (45.3±26.9 *vs* 40.3±37.0 ng dl^{-1}; $F_{1,14}$=0.5, P=0.48). Initial body fat proportions declined with increasing testosterone levels in males (Fig. 4).

Relationship of hormones and metabolites to protein loss and FMR

Mean BUN was a not a significant predictor of the percentage of FMR obtained from protein catabolism ($F_{1,29}$=0.48, P=0.49) or the absolute rate of protein loss ($F_{1,29}$=0.51, P=0.48). The percentage of FMR obtained from protein catabolism varied negatively with mean GH concentrations over the fast (Fig. 5). The percentage FMR obtained from protein catabolism varied negatively with mean estradiol concentrations in females ($F_{1,14}$=6.55, P=0.02).

In order to look for synergistic or additive effects of hormones, we evaluated general linear models (GLM) for each sex that included either 'average, early and late' or 'concentration' changes across the study period in GH, cortisol, thyroid and sex

Table 2. Mean (±s.d.) blood urea nitrogen, glucose and β-hydroxybutyrate (β-HBA), measured early and late in the fasting period

	BUN (mg dl^{-1})		Glucose (mg dl^{-1})		β-HBA (mmol l^{-1})	
Sex	Early	Late	Early	Late	Early	Late
Male	26.2±6.9	18.0±4.3	137.1±15.0	137.0±19.0	279.1±224.0	225.1±110.0
Female	27.0±5.0	16.2±3.7	137.2±13.0	134.8±9.4	260.8±92.1	257.8±78.6
All	26.5±5.8*	17.1±4.1*	41.7±16.0	55.8±32.6	258.1±154.5	251.7±92.6

Asterisks denote significant differences across the fast (linear mixed effects model P<0.05).

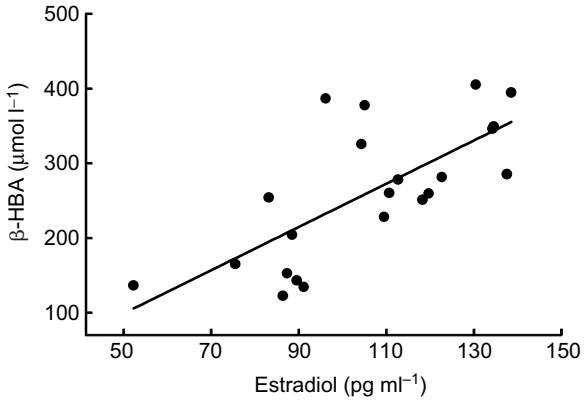

Fig. 3. Relationship between early female estradiol and β-hydroxybutyrate (β-HBA) concentrations (y=–46+2.89x; r^2=0.51, $F_{1,20}$=21.1, P<0.001).

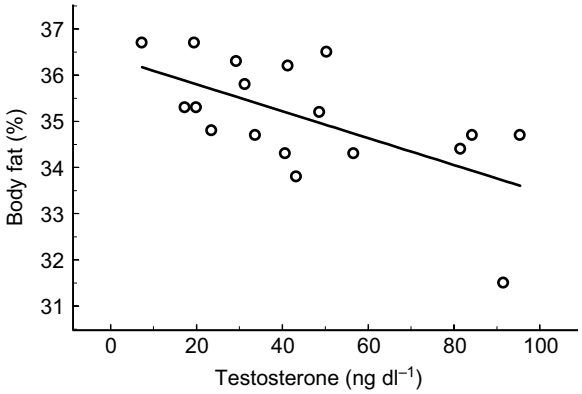

Fig. 4. Relationship between male testosterone and early body fat. The equation for the regression line is y=0.4–2.9x10^{-4}x (r^2=0.38, $F_{1,16}$=9.8, P<0.05).

hormones (testosterone for males and estradiol for females) and looked for effects on the proportion of FMR met by protein catabolism. The model with the lowest Akaike information criteria (AIC_c) value for each sex was evaluated. This analysis yielded different results for the sexes. In females, estradiol levels early in the fast negatively impacted the contribution of protein catabolism to FMR ($F_{1,12}$=8.4, P=0.01), and cortisol levels late in the fast had strong positive impacts on protein catabolism ($F_{1,12}$=19.6, P<0.001; Fig. 6). In males, only hormone concentrations late in the fast were significant predictors of proportional contribution of protein catabolism to FMR. Late values of GH ($F_{1,11}$=6.75, P=0.02) and testosterone ($F_{1,11}$=5.33, P=0.04) had negative effects on proportional contributions of protein to FMR, and late values of cortisol ($F_{1,11}$=6.17, P=0.03) had significant positive effects on protein catabolism. A similar result was found when the sexes were combined and testosterone and estradiol were removed from the analysis. Early GH values ($F_{1,28}$=7.3, P=0.01) had negative impacts on protein catabolism, and late cortisol values ($F_{1,28}$=6.5, P=0.02) had positive impacts on protein catabolism. Thyroid hormone levels had no impact on protein catabolism in any model (P>0.05).

Both mean tT3 values and mean tT4 values had significant impacts on FMR. Mean tT3 values accounted for 35% of the variation in the residuals of mean mass *vs* FMR (r^2=0.35, $F_{1,29}$=16.0, P<0.01). Mean tT4 values accounted for 50% of the variation in the residuals of mean mass *vs* FMR (r^2=0.50, $F_{1,29}$=28.8, P<0.0001; Fig. 7).

Urea flux

Urea flux rate decreased significantly across the fasting period ($F_{1,7}$=47.6, P<0.001; Table 5). While the ability to directly compare the two methods was limited owing to differing sample intervals, we compared the mean of the early and late rates of protein loss estimated from urea flux measurements to those estimated from mass loss. The rate of protein loss was similar when the mean of the two urea flux measurements was compared with estimates from mass loss (t=1.31, d.f.=7, P=0.23). Despite this lack of difference in the mean values, there was no correlation between the mean values of protein loss from the two urea flux measurements and the estimate over the entire haul-out from mass balance (P=0.63).

DISCUSSION

Our data suggest several important sex differences in metabolism, substrate utilization and hormonal regulation of fasting metabolism in juvenile elephant seals. Although there were no sex differences detected in mass or body composition at the start of the fast, males had higher FMR and spared protein more effectively than females. It is notable that these differences are present prior to the rapid growth spurt and onset of dimorphic traits in males.

Energy expenditure for male and females, respectively, averaged 2.3 and 2.0 times the predicted standard metabolic rate (SMR) as predicted from allometric scaling (Kleiber, 1975), or a 15% higher FMR in males. While juvenile elephant seals spend a significant portion of the haul-out sleeping, they also spend a substantial amount of time swimming, terrestrially locomoting and in agonistic interactions with conspecifics. Males spend considerable time in mock dominance interactions that mimic the adult breeding behaviors. While we made no attempt to quantify behavior in this study, the increased FMR of males may reflect different activity budgets or intrinsically higher resting metabolic rates. The strong sex difference in thyroid hormone levels and their impacts on FMR suggest that these differences are largely mediated by thyroid hormones. We found significant suppression of tT4 across the fast but not tT3 in both sexes, suggesting alteration in the rates of deiodination across the fast. Suppression of the thyroid axis in response to fasting is common in numerous species including those

Table 3. Mean (±s.d.) cortisol, insulin, glucagon and insulin to glucagon molar ratio (I/G), measured early and late in the fasting period

	Cortisol (μg dl^{-1})		Insulin (pg ml^{-1})		Glucagon (pg ml^{-1})		I/G	
Sex	Early	Late	Early	Late	Early	Late	Early	Late
Male	7.9±5.7	9.9±6.1	82.3±44.4	101.7±71.5	42.8±19.6	53.4±37.0	1.4±0.9	1.4±1.2
Female	9.0±7.3	8.9±6.6	90.4±43.9	102.5±37.2	40.9±13.0	58.0±28.9	1.6±1.4	1.5±1.4
All	8.1±6.0	9.4±6.3	86.8±43.7	102.1±55.5	41.7±16.0*	55.8±32.6*	1.5±1.2	1.5±1.3

Asterisks denote significant differences across the fast (linear mixed effects model P<0.05).

Table 4. Mean (±s.d.) growth hormone (GH), total triiodothyronine (tT3) and total thyroxine (tT4), measured early and late in the fasting period for male and female juvenile elephant seals

	GH (mg ml^{-1})†		tT3 (ng dl^{-1})†		tT4 (μg dl^{-1})†	
Sex	Early	Late	Early	Late	Early	Late
Male	4.7±2.6	1.5±0.9	108.0±25.7	102.2±20.3	7.7±2.4	4.8±2.2
Female	2.2±1.7	1.4±0.6	76.4±27.3	76.8±26.3	4.5±1.2	3.5±1.0
All	3.3±2.5*	1.4±0.8*	90.6±30.7	89.1±26.5	5.9±2.5*	4.1±1.8*

Asterisks denote significant differences across the fast. Daggers (†) denote significant differences between the sexes. Linear mixed model (P<0.05).

with metabolic adaptations to fasting (e.g. Cherel et al., 1988). Despite strong impacts on FMR, thyroid hormones had no influence on protein utilization in the present study. This is surprising given the strong evidence for impacts of thyroid hormones on protein degradation in other animal systems (e.g. Carter et al., 1975). Ortiz and colleagues found that thyroid hormones increased across the fast in developing weaned pups and suggested that these changes were associated with mobilization of protein and synthesis of respiratory pigments (Ortiz et al., 2001). However, other studies (Adams and Costa, 1993; Houser et al., 2001) found reductions in protein catabolism and nitrogen excretion over the same period. The uncoupling of thyroid hormones from protein catabolism in weaned pups is consistent with that found in the present study.

Animals that have greater adipose reserves are generally able to spare protein more efficiently while fasting. This relationship is thought to be driven by a differential ability to mobilize lipid stores and has been reported in a wide variety of species (Goodman et al., 1980) including lactating female northern elephant seals (Crocker et al., 2001) and weaned pups (Noren et al., 2003). However, our results show that males spared lean tissue more effectively than females despite the fact that both sexes had similar adipose reserves at the beginning of the fast. The lack of relationship between adipose reserves and protein sparing in juvenile animals is unexpected and suggests sex differences in regulation of fuel utilization that are independent of effects of the depot available for mobilization.

Similar to the findings in weanling northern elephant seals (Houser et al., 2001), urea flux, rates of protein loss and BUN declined across the fast in juveniles. In contrast, BUN does not change across the fasting period in adult northern elephant seals (Crocker et al., 1998). In lactating adults, increases in glomerular filtration rate (GFR) late in lactation uncoupled static BUN values from rates of urea flux in adult females, so that BUN declined as urea flux increased. In contrast, weaned pups maintained a more consistent GFR across the fast (Houser et al., 2001) such that BUN values better reflected reductions in urea flux. In the current study, BUN was not correlated with rates of urea flux. This suggests potential variation in GFR in fasting juveniles and provides further evidence that standard clinical proxies for metabolite flux need to be interpreted with caution in wildlife systems.

Cortisol, GH and sex hormones (testosterone and estradiol) all had significant impacts on protein's contribution to energy metabolism and contributed to sex differences in protein catabolism. When the sexes were analyzed together, the strong sex differences in initial GH values were the strongest driver of protein catabolism. When considered separately, the smaller variation in GH levels in females had no impact on protein use while the level of suppression of GH was the best predictor of protein use within males. The metabolic effects of GH include increased lipolysis and stimulation of insulin-like growth factor 1 (IGF-1) activity, which promotes reuptake of mobilized amino acids by muscle (Nørrelund et al., 2001). Juvenile male seals had significantly higher GH concentrations than females, suppressed GH levels less across the fast, and had lower contributions of protein catabolism to FMR. However, the association of the decline in GH across the fast in both sexes with reduced rates of protein loss was puzzling. Growth hormone increases in response to fasting in most species and is important in the conservation of protein during fasting (Eigenmann et al., 1985; Ho et al., 1988; Webster et al., 1999). Thus, despite evidence that increased suppression of GH levels negatively influences rates of protein catabolism in individuals, suppression of mean GH levels across the fast was associated with reduced rates of protein loss as measured from urea turnover. Taken together, these data suggest that other hormones, including cortisol,

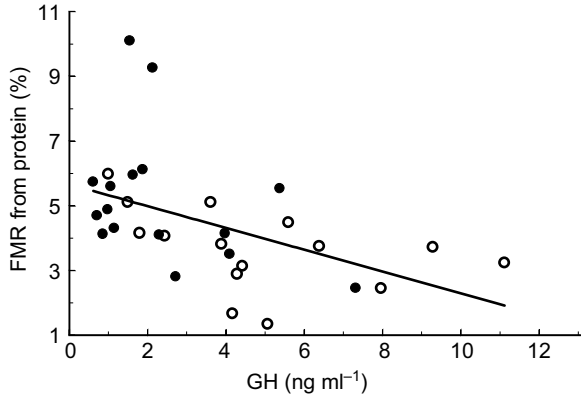

Fig. 5. Relationship between early sample growth hormone (GH) and percent energy from protein (y=0.057–0.003x; r^2=0.25, $F_{1,29}$=8.91, P<0.01). Open circles represent males; closed circles represent females.

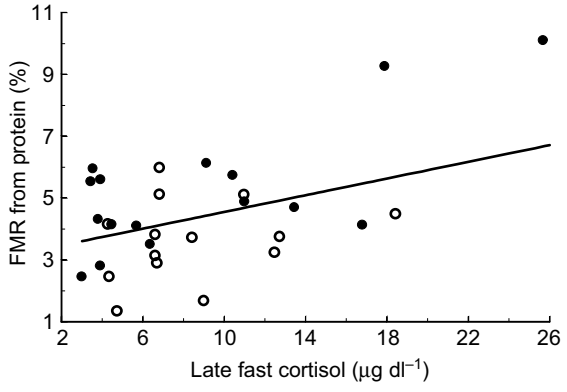

Fig. 6. The contribution of protein catabolism to field metabolic rate (FMR) varied with late fast cortisol levels in juvenile elephant seals (y=3.2+0.22x; r^2=0.22, $F_{1,29}$=7.8, P<0.01). Open circles represent males; closed circles represent females.

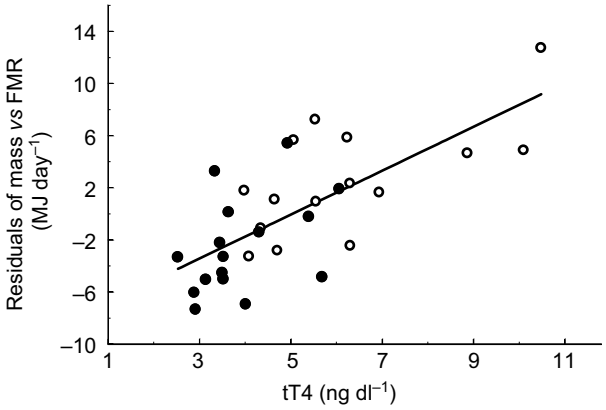

Fig. 7. Impact of mean total thyroxine (tT4) on the residuals of field metabolic rate (FMR) *vs* mass in juvenile elephant seals. Closed circles represent females; open circles represent males (y=–8.5+1.68x; r^2=0.50, $F_{1,29}$=28.8, P<0.0001).

Table 5. Urea flux constant (K_u), urea flux (r_u) and rate of protein loss from urea flux (r_{pu}) per day, determined from the urea flux method

	K_u		r_{pu} (g day^{-1})		r_u (g day^{-1})	
Seal I.D.	Early	Late	Early	Late	Early	Late
♀U544	0.89	0.86	84.1	59.3	28.8	20.3
♀U883	0.98	0.68	112.7	35.0	38.6	12.0
♀4019	0.84	0.79	71.0	28.0	24.3	9.6
♀4021	0.73	0.46	63.7	28.9	21.8	9.9
♀X109	0.87	0.73	105.4	46.1	36.1	15.8
♂T850	1.02	0.83	111.8	54.9	38.3	18.8
♂U35	0.82	0.67	77.1	50.8	26.4	17.4
♂4020	0.72	0.70	69.2	32.4	23.7	11.1
♂X545	0.83	–	62.8	–	21.5	–
♂U244	0.74	–	82.1	–	28.1	–
♀4016	0.91	–	64.5	–	22.1	–
♂V621	–	0.91	–	64.5	–	22.1
Mean	0.85±0.10	0.74±0.10	82.2±19.3*	44.4±13.8*	28.1±6.6*	15.2±4.7*

Asterisks denote significant differences between early and late measures (Student's *t*-test, P<0.001).

testosterone and estradiol, may also be important in regulating the level of protein catabolism across the fast.

Despite no evident changes across the fast or consistent sex differences, variation in individual cortisol levels late in the fast significantly impacted protein catabolism. This is consistent with the well-established impact of cortisol on protein mobilization (Brillon et al., 1995). Previous studies on fasting lactating females (Champagne et al., 2006) and weaned pups (Ortiz et al., 2001) revealed increased cortisol levels over the fasts, suggesting that impacts of cortisol on lipid mobilization predominated over protein wasting effects. Lactating females mobilize significant amounts of protein for milk synthesis, and weaned pups mobilize proteins for synthesis of respiratory pigments. Without these constraints, cortisol levels do not increase across the fast in juveniles, and the animals that maintain lower cortisol levels across the fast are more effective at sparing protein.

Morrow and colleagues reported that doses of estradiol and progesterone administered to female rats accelerated ketogenesis during fasting (Morrow et al., 1981a). In a subsequent study, the same group found that chronic exposure to oral birth-control pills in pre-menopausal women promotes an amplification of ketone bodies in the blood early in the fasting period (Morrow et al., 1981b). The effect of sex steroids on ketosis in both studies was limited to the initial part of the fast, and this relationship was no longer detected later in the fasting period. Likewise, the females in the present study only exhibited a relationship between β-HBA and estradiol early in the fasting period. High estradiol early in the fast was also associated with reduced protein catabolism. Together, these effects suggest that estradiol potentially mediates the mobilization of fats for oxidation early in the fast in females, which results in a reduced need for protein oxidation. Similarly, high testosterone negatively impacted protein catabolism in males. Testosterone has been shown to interact positively with GH to regulate energy expenditure, fat metabolism and protein anabolism by modifying GH responsiveness (Yu et al., 1996). Males who exhibited lower suppression of GH and testosterone across the fast and minimized cortisol increases were the most effective at sparing lean tissue from catabolism.

Glucose homeostasis while fasting is predominantly regulated by insulin and glucagon interactions in birds and mammals. Additionally, increased substrate mobilization and stimulation of gluconeogenesis are correlated with declining I/G ratios (Cuendet et al., 1975; Hazelwood, 1984). Investigations in adult male (Crocker et al., 2012) and weanling elephant seals show declining I/G ratios while fasting that result from increased glucagon levels and stable insulin levels (Champagne et al., 2005). Juvenile elephant seals show a similar pattern to adult males and weanlings with increasing glucagon levels and stable insulin levels. Lactating, female elephant seals reduce I/G ratios *via* suppression of insulin alone, which also leads to declining I/G ratios across the fasting period (Champagne et al., 2006; Houser et al., 2007). In all age classes, the reduction of the I/G ratio across the fast occurred without impacting circulating glucose levels. In contrast, there was no significant change in the juvenile I/G ratios across the fasting period, although there was significant variation in the insulin measures within males. Plasma glucagon concentrations were low compared with those in fasting adapted birds (Cherel et al., 1988) and non-fasting adapted mammals (Unger and Orci, 1976) and were less than or similar to those recorded in other fasting adapted mammals.

Extended fasting will markedly increase the concentration of β-HBA in most fasting species. However, despite a predominantly fat-based metabolism, the accumulation of this ketone was negligible in this study. This suggests elevated rates of ketoacid usage independent of plasma levels or an ability of the citric acid cycle to maintain processing capacity of acetyl-CoA despite high rates of beta oxidation of fatty acids. No change was found in the levels of β-HBA for either sex, and the concentration remained exceptionally low when compared with other fasting adapted species (Cherel and Le, 1988; LeBlanc et al., 2001). The static β-HBA levels found in this study differ from reported patterns at other life stages where β-HBA levels increased in weaned pups (Champagne et al., 2005). Comparisons are equivocal in lactating females as one study found increases in β-HBA with time lactating (Champagne et al., 2006) and another did not (Houser et al., 2007). Juvenile levels were similar to the early fasting measurements made in breeding adults and were considerably lower than levels in weanlings. The higher rates of energy expenditure and longer fasting durations found in other groups may contribute to this difference.

Estimates of protein catabolism

Although measured over different time periods, our study allowed some comparison of protein catabolism estimates derived from the

mass balance method with direct tracer measurements of urea flux. The two methods gave similar estimates of the rate of protein catabolism and the percentage of energy derived from protein. Despite the fact that this study demonstrates a reasonable agreement in the estimates from both methods, the values determined from the mass balance method were significantly lower than other estimates from this method published for elephant seals (Carlini et al., 2001; Field et al., 2005; Noren et al., 2003). The most likely explanation for some of these differences is the error associated with pelage loss in molting periods. Alternatively, the urea flux method assumes that all nitrogenous waste from protein catabolism results in urea production. Significant loss of urinary nitrogen as ammonia, uric acid or creatinine would result in underestimates of protein catabolism. Adams and Costa examined urinary nitrogen output in elephant seal weanlings and found that ~20% of urinary nitrogen was in the form of ammonia, uric acid or creatinine (Adams and Costa, 1993). This would result in a commensurate underestimate of protein loss. If the average of the two urea flux estimates is corrected for contributions from other nitrogenous end products, the r_p was a mean of 20% higher than that from mass loss for males and a mean of 10% lower than that from mass loss for females. Regardless, both methods resulted in low estimates of the contribution of protein to metabolism (<5%) that seem reasonable in a fasting adapted species and in prior estimates of neonates within this species.

Conclusions

In summary, juvenile northern elephant seals exhibited sex differences in metabolic strategies for fasting, including differences in FMR, the contribution of protein catabolism to FMR, and the hormonal regulation of metabolism. More effective protein sparing in males during biannual fasts throughout development may contribute to body composition differences in adults that are associated with breeding success. Conversely, maintenance of higher adipose tissue stores may be crucial to preparing for the eventual mobilization of lipids for synthesis of energy-dense milk in primiparous females. Our findings suggest that sex differences arise early in development, prior to visual evidence of sexual dimorphism, and that for species that undergo frequent extended natural fasts, sex differences in metabolism may contribute to the development of dimorphic adult phenotypes.

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