The role of glucocorticoids in naturally fasting grey seal (*Halichoerus grypus*) pups: dexamethasone stimulates mass loss and protein utilisation, but not departure from the colony

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Summary

Seals must manage their energy reserves carefully while they fast on land to ensure they go to sea with sufficient fuel to sustain them until they find food. Glucocorticoids (GC) have been implicated in the control of fuel metabolism and termination of fasting in pinnipeds. Here we tested the hypothesis that dexamethasone, an artificial GC, increases fat and protein catabolism, and induces departure from the breeding colony in wild, fasting grey seal pups. A single intramuscular dose of dexamethasone completely suppressed cortisol production for 24-72 hours, demonstrating activation of GC receptors. In experiment 1, we compared the effects of a single dose of dexamethasone or saline administered ten days after weaning on fasting mass and body composition changes, cortisol, blood urea nitrogen (BUN) and glucose levels, and timing of departure from the colony. In experiment 2, we investigated the effects of dexamethasone on short-term (5 days) changes in mass loss, body composition and BUN. In experiment 1, dexamethasone induced a short-lived increase in mass loss, but there was no difference in timing of departure between dexamethasone and saline treated pups (n = 10). In experiment 2, dexamethasone increased protein and water loss and prevented a decrease in BUN levels (n = 11). Our data suggest changes in cortisol contribute to regulation of protein catabolism in fasting seal pups, irrespective of the sex of the animal, but do not terminate fasting. By affecting the rate of protein depletion, lasting changes in cortisol levels could influence the amount of time seal pups have to find food, and thus may have important consequences for their survival.

Introduction:

The mechanisms that regulate fuel use and the onset of foraging behaviour in fasting seal pups are poorly understood. Most phocid pups fast on land after weaning during which time they undergo physiological changes that prepare them for diving (Burns et al., 2007; Lewis et al., 2001; Noren et al., 2005; Soňanez-Organis et al., 2012; Thorson and Le Boeuf, 1994; Vásquez-Medina et al., 2010; 2011). The duration of the postweaning fast has a positive effect on diving capabilities when pups first go to sea (Bennett et al., 2010). Larger pups are also better divers (Bennett et al., 2010; Burns et al., 1997; Burns and Castellini, 1996; Hindell et al., 1999; Irvine et al., 2000) and have an increased probability of survival (Hall et al., 2001; 2002; 2009; Harding et al., 2005; Hindell, 1991; Le Boeuf et al., 1994; McMahon et al., 2000). While larger pups can undergo a long fast and leave the colony with sufficient reserves (Arnbom et al., 1993; Noren et al., 2008; Noren and Mangel, 2004), smaller pups face a trade-off between the need to develop whilst fasting on land, and the need to learn to forage successfully at sea before energy stores become critically reduced (McConnell et al., 2002). This requires careful management of endogenous fuel reserves during the postweaning fast, and appropriate timing of departure from the colony well in advance of fuel depletion (McConnell et al., 2002). The duration of fasting can be dictated by the size and rate of utilisation of their fat and protein depots (Bennett et al., 2007; Noren et al., 2008; Noren and Mangel, 2004; Reilly, 1991). Although pups have substantial fat reserves, the availability of expendable protein depots is limited (Bennett et al., 2007; Caloin, 2004). Fuel allocation during fasting is thus likely to impact upon survival.

Effective fuel management and appropriate timing of departure require a mechanism whereby information about the state of fuel depots is relayed to the central nervous system and periphery to effect appropriate changes in energy use and behaviour patterns. The co-ordination of an integrated behavioural and physiological response to changes in fuel availability is achieved in other mammals through the action of hormonal intermediaries. Glucocorticoids (GC) are responsive to changes in fuel supply and metabolism and effect changes in energy acquisition and utilisation in other mammals, thereby regulating long term energy balance in conjunction with other metabolic and endocrine signals. GC enhance the gluconeogenic capacity of the liver, increasing blood glucose levels. They facilitate the mobilisation of fat (Divertie et al., 1991; Djurhuus et al., 2002; 2004; Samra et al., 1998) and/ or protein reserves as gluconeogenic

precursors (Darmaun et al., 1988; Legaspi et al., 1985; Simmons et al., 1984; Weiler et al., 1997). Increased GC direct fuel utilisation towards an increased reliance on protein catabolism, and promote food-seeking behaviour through appetite centres in the brain in rodents, humans, horses (*Equus ferus caballus*) penguins and bottlenose dolphins (*Tursiops truncatus*) (Challet et al., 1995; Chen and Romsos, 1996; Debons et al., 1986; Groscolas and Robin, 2001; Koubi et al., 1994; Reidarson and McBain, 1999; Robin et al., 1998). If GC act in a similar way in grey seals (*Halichoerus grypus*; Fabricius) as they do in other animals, they may be involved in the control of fuel use during fasting and timing of departure from the colony. Cortisol, the major GC in pinnipeds, has been implicated in both these roles in pinnipeds (Crocker et al., 2012; Guinet et al., 2004; Ortiz et al., 2001a and b; Verrier et al., 2012). Cortisol levels have been measured in fasting pinnipeds, and tend to be stable, for example in fasting grey and harp seal (*Pagophilus groenlandicus*) pups, Subantarctic fur seal pups (*Arctocephalus tropicalis*) and juvenile and breeding male northern elephant seals (*Mirounga angustirostris*) (Bennett et al., 2012; Crocker et al., 2012; Kelso et al., 2012; Nordøy et al., 1990; 1993; Verrier et al., 2012). In some studies, fasting individuals show an increase in cortisol, such as fasting elephant seals pups and lactating female Subantarctic fur seals (Guinet et al., 2004; Ortiz et al., 2001a and b). There is no clear relationship between cortisol and glucose in the blood in fasting pinnipeds (Crocker et al., 2012; Kelso et al., 2012; Verrier et al., 2012), making it difficult to make inferences about its role in fuel allocation. However, handling increases cortisol, glucose levels and gluconeogenesis in physically restrained elephant seal pups (Champagne et al., 2012). Crucially, there is no experimental evidence for the proposed roles of cortisol in either fuel allocation or initiation of foraging behaviour in pinnipeds.

Here we tested the hypotheses that (i) GC induce long or short term mass loss through increased fat and/ or protein catabolism and (ii) that GC induce departure from the colony in grey seal pups. We performed an initial study to determine the effect and time course of a single intramuscular dose of dexamethasone, a potent and long-acting artificial cortisol analogue, on cortisol levels in captive grey seal pups-of-the-year (10 months of age). We then investigated the effect of dexamethasone on body composition changes, metabolite and cortisol levels and timing of departure from the colony in wild, fasting pups.

Materials and Methods:

Capture and handling procedures were performed under Home Office project licence #60/2589 and conformed to the UK Animals (Scientific Procedures) Act, 1986.

Time course study: Two 10-month old grey seal pups in the captive facility at the Sea Mammal Research Unit (SMRU), St. Andrews, Scotland, were used to investigate the safety, efficacy and time-course of the effect of a single intramuscular dose (50 μg kg^{-1}) of dexamethasone (Dexadreson®: Intervet, Milton Keynes, UK; 2mg/ml dexamethasone sodium phosphate), on serum levels of cortisol. The physiological effects of dexamethasone are mediated by glucocorticoid receptors (GR). Through activation of GR in the hypothalamus and pituitary, dexamethasone, like endogenous GC, reduces cortisol concentrations by down regulating secretion of adrenocorticotrophic hormone (ACTH) and corticotropin releasing hormone (CRH), which form the negative feedback loop that controls cortisol secretion. In this study, its ability to reduce cortisol secretion was used as an indication that the dose of dexamethasone was sufficient to activate GR and thus induce other GR-mediated physiological effects of GC.

The pups were held together with access to a small pool and a dry area for the duration of the experiment. Both pups had been trained to station on a specific focus shape with fish as a food reward to minimize stress while moving the animals to the small dry area used during manual restraint and blood sampling. The pups were left in the dry area for 20 min prior to restraint and sampling to dissociate the response to the focus shapes and feeding from the experience of being handled. They were allowed to return to the pool area immediately after each sampling period.

A plasma sample was taken from the extradural vein into a heparin-coated vacutainer (Becton Dickenson, Oxford, UK) at 09:00 on day 1 (0 h), followed by an intramuscular injection of 0.1 ml kg^{-1} Terramycin® (Pfizer, Maidenhead, UK) to provide antibiotic cover. The animals were then injected on the opposite side of the body with either 0.025 ml kg^{-1} Dexadreson® or the equivalent volume of sterile saline solution (Aqupharm, York, UK). Blood samples were then taken 4, 8, 12, 24, 48 and 72 h after injection. The saline trial was performed first in each case, and the animals were given 24 hours recovery between the two trials.

As described previously (Bennett et al., 2012), plasma was centrifuged in a swing-out bench top centrifuge at 2000g for 15 minutes, as soon as possible, and within ten hours, after

sample collection. Aliquots were transferred to 500µl microtubes using glass pasteur pipettes, and stored at –20°C until analysis, which occurred within 8 months of sample collection.

Impact of dexamethasone on wild, fasting grey seal pups: We examined the effects of dexamethasone on plasma cortisol, blood urea nitrogen (BUN) and glucose levels (indices of increased proteolysis), mass and body composition changes and timing of departure from the colony in thirty grey seal pups born on the Isle of May, in the Firth of Forth, Scotland (56º 11' N, 2º 33' W) in October and November of 2002 (experiment 1). All pups were captured early (age ~ four days) and late (~age 15 days) in the suckling period to obtain mass transfer information as part of a long term study. Weaning, determined from daily observations of mother-pup pairs, occurred 2.4 ± 1.9 days after the late suckling capture. Pups were penned in a large outdoor enclosure within two days of weaning to allow them to be located easily without disturbing other animals on the colony (Bennett et al., 2007). On entry to the pen, each animal was assigned to one of three treatment groups (CONTROL, SAL_1 or DEX_1 (subscripts to distinguish groups from experiment 2) based on its weaning mass and sex, such that, as far as possible, each group contained ten animals of a range of sizes and a similar number of males and females (Table 1). Body mass was measured using a 50 kg (± 0.2 kg) or, where the pups were > 50 kg, a100 kg (± 0.5 kg) Salter spring balance and blood samples were taken every three days. Blood samples were obtained as quickly as possible (1.84 ± 1.27 min; range = 1-8 min) after first contact with the animal, before the pup was weighed, and between 09:00 and 12:00, to minimise the effects of stress and circadian rhythms on cortisol and metabolite measurements. At ten days postweaning pups were given intramuscular Terramycin® to provide antibiotic cover, and either no additional injection (CONTROL), 0.025 ml kg^{-1} sterile saline (SAL_1) or 0.025 ml kg^{-1} Dexadreson ® (DEX_1). A blood sample was taken 24 hours later and pups were released from the pen and allowed to range freely for the remainder of the fast. They were given a unique painted letter mark on the back and their presence/absence on the colony was noted daily. Pups still present on the colony after release were re-measured and blood sampled every three days until departure of the animal or 34 days after weaning, whichever happened sooner. The date of departure was assumed to be the day after the last sighting of the animal. One pup from the CONTROL group was excluded from the study because it developed an infection.

In 2004 (experiment 2), thirty suckling stage IV (Woldstad and Jenssen, 1999), partially moulted pups were given individual identification marks using yellow paint and monitored daily to determine date of weaning. Pups were brought into the pen 1- 4 days after weaning (mean = 1.47 ± 0.97 days) and after they had completely moulted. They were assigned to either DEX_2 or SAL_2 groups using the same criteria as in 2002 to give 15 animals in each group (Table 1) and were given an intramuscular dose of either dexamethasone or saline, as described above. Body mass measurement and blood sampling was performed on entry into the pen and again five days later, when they were released.

Body composition measurements: In experiment 1, body composition of 22 pups (CONTROL: n = 5; SAL_1 n = 7; DEX_1 n = 8) was measured at each capture during suckling, and again in 11 pups (CONTROL and SAL_1 pups combined: n = 6 with 3 males and 3 females; DEX_1: n = 5 with 2 males and 3 females) 6.79 ± 2.42 days after dexamethasone or saline injection (17.5 ± 3.1 (range = 14-22) days postweaning). In experiment 2, body composition measurement was performed in 10 of the SAL_2 and 11 of the DEX_2 pups at the same time as mass measurements and blood sampling on entry to the pen and five days later. Body composition was measured using deuterium oxide ($^2[H]_2O$) dilution (Reilly and Fedak, 1990) as described previously (Bennett et al., 2007; 2010). Briefly, after the animal was weighed, a blood sample was collected from the extradural vein, both before and 3-4.5 h (Bennett et al., 2007; Costa et al., 1986; Reilly, 1991) after intravenous injection of a pre-weighed dose of 3-5 ml $^2[H]_2O$ (99.9%; Sigma-Aldrich Chemicals, Gillingham, Dorset, UK). $^2[H]_2O$ enrichment in parts per million in two sub-samples of the background and enriched plasma samples and standards was measured in duplicate in a Micromass isoprime pyrolysis inlet mass spectrometer (Speakman and Krol, 2005; Speakman and Racey, 1987: method D). Dilution space was calculated (Krol and Speakman, 1999) and percentage and absolute mass of fat, protein, water and ash were determined from body water content, using equations derived by comparison of $^2[H]_2O$ dilution with chemical composition of grey seal carcasses (Reilly and Fedak, 1990). Mass and body composition at weaning in experiment 1 were determined by extrapolation using rates of change in mass and body components during suckling (Bennett et al., 2007; 2010).

Blood sample analysis: Serum cortisol concentrations in captive pups and wild pups from experiment 1 were quantified in duplicate using a Spectria ^{125}I -cortisol radioimmunoassay

(Orion Diagnostica, Espoo, Finland), previously validated for use in grey seal serum (Bennett et al., 2012). Inter and intra assay coefficients of variation (% CV) for seal serum are <11% and <10%, respectively, and percentage recovery is 82.75-91.64% for this assay (Bennett et al., 2012). BUN for all samples was measured in duplicate using Randox kit # UR107 (Randox Laboratories Ltd., Crumlin, Co. Antrim, UK) according to the manufacturers' instructions. Glucose was measured in duplicate in plasma from the captive pups and 23 of the 30 pups throughout the fast in experiment 1 using Sigma kit # 510 adapted for use in 96 well plates.

Statistical analysis: All statistical analyses were performed in Minitab 15 or R (R 1.9.1, R Development Core Team, 2003; Ihaka and Gentleman, 1996). Anderson-Darling tests were used to check that continuous data had a normal distribution, and values were log transformed where appropriate. F tests and Bartlett's tests were used to determine whether variance between categories was equal. Changes in cortisol, glucose and mass loss over time in experiment 1, and BUN from both experiments, were analysed using linear mixed effects models (LMEs), which included a random term for each individual (Chatfield, 1989; Crawley, 2002). Fixed effects included day postweaning, sex and treatment group. Models were fitted using maximum likelihood estimates and model selection was performed using ANOVA. Weaning mass and mass loss rate in experiment 2 were investigated using ANOVA.

Since body composition data is derived from body mass and water, MANOVA was used to investigate whether these two variables were different between treatment groups (Bennett et al., 2007). Where MANOVA indicated a significant treatment effect, the univariate analyses were examined, and where there was an effect on body water, protein and fat mass differences were explored. We had insufficient power to investigate the effects of sex on body composition. However, the number of males and females in each group was similar in each experiment.

Results:

Time course in captive animals: The effect of dexamethasone on cortisol in the two captive pups is shown in Fig. 1. Cortisol was elevated by 31% (male) and 58% (female) before injection in the dexamethasone trial compared with the start of the saline trial (Fig. 1). In both trials, cortisol levels fell within 4 h of treatment, but were substantially more reduced after dexamethasone treatment (cortisol = 0 - 14% of initial values) than after saline injection (cortisol

= 58 - 67% of initial values). Lowest cortisol levels occurred 8 - 12 h after dexamethasone injection. Cortisol recovered by 8-24 h after saline treatment and by 72 h after dexamethasone treatment.

Cortisol in wild pups: Cortisol changed significantly throughout the fast and the changes were different between groups (LME: AIC = 1594. 408; BIC = 1655. 911; Log Lik = -777. 2039, n (observations) = 160; n (individuals) = 29; Fig. 2A). SAL_1 animals showed a significant reduction in cortisol from days 1 and 4 to lower levels on days 7 and 10 ($p < 0.04$). Cortisol then returned to levels similar to those at the start of the fast by day 11 and this increase approached significance ($T = 1.803$; $p = 0.074$). A similar, but smaller change was observed in the CONTROL animals ($p < 0.08$). In this group, cortisol was also lower on day 14 than on day 1 of the fast ($T = 2.055$; $p = 0.0422$). DEX_1 animals showed a highly significant drop in cortisol 24 hours after dexamethasone injection (day 11: $T = 3.475$; $p = 0.0007$), which recovered to pre-injection levels by day 14. The interaction between sex, treatment and day was not significant (ANOVA: L ratio = 8.582, $p = 0.5721$). The differences in cortisol between treatment groups were not influenced by sex (ANOVA: L ratio =2.648, $p = 0.2661$), the changes in cortisol over time were not affected by sex (ANOVA: L ratio = 3.499, $p = 0.6235$), and cortisol did not differ between the sexes (ANOVA: L ratio = 17.745, $p = 0.473$).

Mass loss in wild pups: Weaning (Kruskal-Wallis: $H_{(2)} = 1.16$, $p = 0.559$; Table 1) and departure mass (32.9 ± 4.9kg) were not significantly different between the three groups in experiment 1 (MANOVA: $F_{(4,52)} = 0.365$, $p = 0.832$). The changes in rate of mass loss over three-day intervals were significantly different between groups (Fig. 2B) and this difference persisted when body mass was included as a covariate. All groups in experiment 1 showed a progressive decline in the rate of mass loss over the first ten days postweaning to a lower level. This did not change substantially thereafter in the CONTROL and SAL_1 (LME: AIC= -10.163; BIC = 59. 234; Log Lik = 28. 082, n (observations) = 151; n (individuals) = 29). However, in the DEX_1 group there was an increase in the rate of mass loss between one and three days after treatment (day 11-14) to levels comparable with those at the start of the fasting period. Rate of mass loss then declined to previous levels by day 17, and this reduction approached significance. There was no significant interaction between the effects of day, sex and treatment (ANOVA: L ratio = 10.613, $p = 0.2246$). The differences in mass loss rate between treatment groups were not

influenced by sex (ANOVA: L ratio = 0.053, p = 0.9738). However, the changes in mass loss rate over time were different between males and females, irrespective of treatment (ANOVA: L ratio = 10.280, p = 0.036). Males had a significantly lower rate of mass loss over the first three days after weaning than females (LME: T = 2.247; p = 0.0336) and the rate of mass loss did not differ significantly between sexes thereafter (p > 0.05). As a result, mass loss rate in females was significantly higher during the first three days of the fast compared with the remainder of the fast (P <0.01). In males, the rate of mass loss was lower at the start and declined less steeply. The rate of mass loss in male pups was not significantly reduced compared with values at the start of the fast until day 10 (p <0.05).

In experiment 2, there was no significant difference in initial body mass between groups (Table 1: ANOVA: $F_{(1,28)} = 0.39$, p = 0.537). Pups lost body mass at 0.55 ± 0.1 kg d^{-1} and there were no differences between groups (ANOVA: $F_{(1,28)} = 1.56$, p = 0.224) in mass loss rate over the five days of the experiment.

Body composition changes in wild pups: In experiment 1, there was no significant difference in body composition (mass and body water combined) between the three treatment groups (MANOVA (Pillai's trace): $F_{(4,32)} = 0.563$; p = 0.691) at weaning (mean ± S.D: body mass = 41.19 ± 6.89 kg; water = 18.36 ± 2.35 kg; fat = 20.40 ± 4.08 kg; protein = 5.57 ± 0.68 kg; n = 22), and no significant difference in body composition between the SAL_1 treated and CONTROL pups combined (n = 10) and the DEX_1 group (n =11) at departure (MANOVA (Pillai's trace): $F_{(2,8)} = 0.531$; p = 0.607; mean ± S.D: body mass = 30.24 ± 4.20 kg; water = 11.45 ± 1.67 kg; fat = 14.94 ± 2.58kg; protein = 3.37 ± 0.54kg).

In experiment 2, there was no significant difference at the start of the experiment in body mass and water content between groups (MANOVA: Pillai's trace = 0.005; $F_{(2,17)} = 0.047$; p = 0.955). There was a significant difference in daily rate of mass and water loss between groups (MANOVA: Pillai's trace = 0.307; $F_{(2,17)} = 3.767$; p = 0.044). Both mass loss rate (ANOVA: $F_{(1,20)} = 5.07$, p = 0.037) and water loss rate (ANOVA: $F_{(1,20)} = 6.80$, p = 0.018) were higher in DEX_2 pups compared with SAL_2 (Table 2). Fat and protein loss responded significantly differently to dexamethasone treatment (MANOVA: Pillai's trace = 0.317; $F_{(2,18)} = 4.172$; p = 0.032); whereas the rate of fat loss was not different between groups (ANOVA: $F_{(1,20)} = 0.22$, p =

0.645), the rate of protein loss was significantly higher in DEX_2 pups (ANOVA: $F_{(1,20)}$= 6.62, p = 0.019).

Metabolites in wild pups: In experiment 1, plasma BUN levels did not change significantly over the first seven days of the fast (p > 0.05; mean = 14.28 ± 3.85 (s.d.) mM) and showed a significant elevation on days 10 (LME: T = 2.414; p = 0.0172) and 11 (LME: T = 3.705; p = 0.003) compared with day 1 (mean = 16.73 ± 5.22 (s.d.) mM; LME: AIC = 920.807, n observations = 160; n individuals = 30). BUN on day 14 returned to levels that were not significantly different from those on day 1 (LME: T = 1.505 p = 0.1346; mean = 14.97 ± 4.34 (s.d.) mM). This change in BUN did not differ significantly between groups (ANOVA: L ratio = 5.98, p = 0.917) or between sexes (ANOVA: L ratio = 17.067, p = 0.519) and there was no interaction between effects of sex and group on the change in BUN over time (ANOVA: L ratio = 4.086, p = 0.9433).

Glucose levels in experiment 1 showed a small but significant decline between day 1 (mean = 7.14 ± 0.91 (s.d.) mM and day 7 postweaning (LME: T = 3.544; p = 0.0006; n observations = 127; n individuals = 23), which did not change between days 7-11 (mean = 6.30 ± 0.93 (s.d.) mM; p > 0.05). By day 14, glucose returned to levels that were not significantly different from those at the start of the fast (LME: T = 1.132; p = 0.2602) and this change was not significantly different between groups (ANOVA: L ratio = 13.60, p = 0.628). There were too few females in the control group to investigate sex effects at the same time as treatment and day. However, there was no overall difference between males and females in glucose levels in the control group, where there was an imbalance in the sex ratio of the group (LME: T = 0.769; p = 0.4765; n observations = 37; n individuals = 7; AIC = 104.249) and there was no interaction between the effects of sex and day on glucose levels (ANOVA: L ratio = 4.963; p = 0.6644), indicating no difference in pattern of change in glucose over time between the sexes.

In experiment 2, there was a small but significant decline in BUN levels in the SAL_2 group (before injection = 13.27 ± 3.37mM vs after injection = 11.34 mM ± 4.40 (s.d.) mM; LME: T = 2.484; p = 0.0192), which did not occur in the DEX_2 group (before injection = 11.73 ± 2.85 (s.d.) mM vs after injection = 12.32 ± 1.97 (s.d.) mM; LME: T = 0.761; p = 0.453). There was no interaction between the effects of sex, group and day on BUN (ANOVA: L ratio = 1.250; p = 0.2635), there was no difference in the response of BUN to treatment between the sexes

(ANOVA: L ratio = 0.432; p = 0.5112), and no difference in the change in BUN over time between the sexes (ANOVA: L ratio = 0.424; p = 0.5148).

Departure from the colony: In experiment 1, there was no significant difference in log fast duration between groups (ANOVA: $F_{(1, 28)} = 0.12$, p = 0.891). Pups remained on the colony 8.5 ± 5 (s.d.) days after treatment (range = 2 - 23 days) and fasted for an average of 19 ± 5 (s.d.) days. Fast duration was not recorded in experiment 2.

Discussion: We were able to produce near maximal inhibition of endogenous cortisol production for an appropriate duration to investigate the effects of high GC levels on fuel use and timing of departure in wild grey seal pups. Cortisol levels were reduced relative to pre-injection levels by 86% - 90% within four hours of treatment and remained suppressed for 48-72 hours. This occurred in the face of higher circulating cortisol levels prior to dexamethasone treatment compared to the same time on the previous day, which was likely a result of repeated handling (Sapolsky et al., 2000; Bennett et al., 2012). In bottlenose dolphins, humans and horses a similar or slightly higher mass-specific dose of dexamethasone causes suppression of circulating cortisol to 0-30% of initial levels within 24 hours and has observable effects on food seeking behaviour (Barton et al., 2002; Froin et al., 1998; Reidarson and McBain, 1999). The rapid, dramatic and sustained impact of the dose of dexamethasone used here indicated that it mimicked the negative feedback effect of high levels of endogenous cortisol over a period of 1-2 days. We therefore assumed that it had also reached GR targets in all parts of the body to induce other GR-mediated effects of the drug over a similar time frame.

GC can cause mass loss through their impact on gluconeogenesis, lipolysis and proteolysis in other animals (Darmaun et al., 1988; Divertie et al., 1991; Djurhuus et al., 2002; 2004; Weiler et al., 1997). It has been proposed that cortisol promotes high rates of lipolysis to maintain a largely fat-based metabolism in fasting northern elephant seal pups (Ortiz et al., 2001 a and b). It has also previously been suggested that cortisol could increase protein catabolism in fasting, lactating female Subantarctic fur seals (Guinet et al., 2004). In fasting male elephant seals, cortisol was negatively related to body mass and the lack of an increase in cortisol during fasting in these animals and fasting Subantarctic fur seal pups has been implicated in protein sparing (Crocker et al., 2012; Verrier et al., 2012). Consistent with this suggestion, here we

present the first direct experimental evidence that high GC levels can alter fuel allocation and mass loss rate, specifically by increasing protein break-down in fasting grey seal pups.

Mass loss rates were elevated 1-3 days after dexamethasone treatment relative to saline treated and untreated controls in experiment 1. Interestingly, in control and saline treated pups, changes in mass loss rate mirrored changes in cortisol levels, and this is consistent with findings in male elephant seals, which show a negative relationship between body mass and cortisol levels (Crocker et al., 2012). Here, although there were sex differences in the change in mass loss over time, the response to dexamethasone treatment was similar for males and females. These findings suggest that male and female grey seal pups have a similar response to GC. Short term (5 day) mass, water and protein loss were higher, and BUN levels failed to show a reduction, in dexamethasone treated animals compared with saline treated controls in experiment 2. Dexamethasone treated pups lost, on average, 0.45kg more body mass and 0.1kg more protein over the five day period than saline treated pups. These differences, which were ~2.5 times the precision of the measurement in each case, represent an 18% higher daily mass loss rate and a 22% higher daily rate of protein loss in dexamethasone treated animals. These small but significant differences between dexamethasone and saline treated pups suggest that natural changes in cortisol during fasting could affect fuel use in grey seal pups. Specifically, higher GC levels increase the rate of mass and protein loss, but not fat utilisation. Acute changes in cortisol as a result of physical restraint are accompanied by higher rates of gluconeogenesis in elephant seal pups (Champagne et al., 2012). The decline in cortisol at the start of the postweaning fast could contribute to the early reduction in the rate of mass loss in fasting grey seal pups (present study; Nordøy et al., 1990), through effects on gluconeogenesis and protein metabolism.

The reduction in cortisol levels followed by low stable levels seen here is comparable with previous work in the same species (Bennett et al., 2012) and consistent with previous findings from captive grey and harp seal pups and wild fasting Subantarctic fur seal pups and juvenile and breeding male northern elephant seals (Bennett et al., 2012; Crocker et al., 2012; Kelso et al., 2012; Nordøy et al., 1990; 1993; Verrier et al., 2012). Low cortisol may help to minimise rates of mass loss and promote protein sparing that is characteristic of fasting seals (Houser and Costa, 2001; Crocker et al, 2012; Nordøy and Blix, 1985; Kelso et al., 2012; Nordøy et al, 1990; 1993; Reilly, 1991). These findings contrast with the rise seen in cortisol in

fasting northern elephant seal pups in previous studies (Ortiz et al., 2001a and b). The difference between saline and control groups in the size of the drop in cortisol at the start of the fast may be due to the imbalance in the sex ratio in the two groups, although we found no evidence of a sex difference in cortisol levels in this study. In a previous study, females showed a decline in cortisol mid way through the fast that did not occur in males (Bennett et al., 2012). The greater reduction in cortisol in the saline treated group here may thus have resulted from the higher number of female pups in that group compared with the control group. However, despite sex differences in changes in mass loss over time, here we showed changes in mass loss in response to dexamethasone that were similar between male and female pups. Interestingly, in contrast to our findings, data from juvenile elephant seals suggest a synergistic impact of cortisol levels and sex hormones on fuel allocation during fasting that may contribute to sex differences in body composition (Kelso et al., 2012). The consequences of changes in cortisol levels on fuel metabolism during fasting thus require further investigation, particularly between species, sexes and age categories. As in other animals, the effects of GC on fuel metabolism are likely to depend on levels of and sensitivity to other simultaneous metabolic and hormonal signals, which may change throughout the fasting period and vary between individuals and species. Certainly studies in other pinniped species have demonstrated sex differences in hormone levels and fuel metabolism that may be causally linked, at least in older animals (e.g. Kelso et al., 2012). Manipulation of hormone levels, similar to that performed here, would allow these relationships to be tested experimentally in more detail.

The impact of GC on protein utilisation may have important consequences for the trade-offs grey seal pups face during the postweaning fast. Pups are predicted to starve to death from protein depletion well in advance of the significant loss of fat depots, and within up to two weeks of departure from the colony if they do not encounter food (Bennett et al., 2007). We predict that pups that maintain lower cortisol levels will reduce protein utilisation to a greater extent than those that maintain higher cortisol levels. They will therefore have the possibility of either fasting longer on land, which is associated with better developed diving abilities by departure (Bennett et al., 2010; Noren et al., 2008), or leaving the colony with a greater margin in protein reserves, which will provide more time in which to find food and learn to forage and/ or greater muscle mass that may increase muscle power and swimming ability. Higher rates of protein catabolism caused by increased GC may thus impact on survival in grey seals by indirectly

394 influencing diving abilities and time available to find food. Higher cortisol levels, for example
395 as a result of infection (eg. Sures et al., 2006), or social encounters, such as aggression (eg.
396 Abbot et al., 2003), may have a greater impact on time available to find food in pups that
397 already face a trade-off between fast duration and diving capability due to their smaller size
398 (Bennett et al., 2010).

399 A single dose of dexamethasone administered once after ten days of fasting did not alter
400 the overall fuel utilisation and body composition changes measured at the end of the postweaning
401 fast. Glucose levels in experiment 1 remained high, relative to fasting levels in dogs and humans
402 (Steele et al., 1968; Umminger, 1975), and stable throughout the fast, as in other studies (Costa
403 and Ortiz, 1982; Crocker et al., 2012; Kelso et al., 2012; Nordøy and Blix, 1991; Sakamoto et al.,
404 2009; Schweigert, 1993). All groups in experiment 1 showed increased BUN levels, an index of
405 protein catabolism, 24 hours after treatment. This could reflect a short lived increase in
406 proteolysis as a result of acute natural increases in cortisol that were not measured here, but
407 likely follow a handling episode (Bennett et al., 2012; Champagne et al., 2012; Engelhard et al.,
408 2002; Sapolsky et al., 2000). Indeed, Champagne, et al. (2012) found that handling- induced
409 elevations in cortisol were accompanied by increased gluconeogenesis in weaned elephant seal
410 pups. Together our data demonstrate that the effects of the single dose of dexamethasone
411 administered here on mass loss and fuel use were small and short lived relative to the whole post
412 weaning fast.

413 Dexamethasone treatment did not prompt departure from the colony. In addition, control
414 and saline treated pups left the colony without exhibiting a natural increase in cortisol levels, as
415 was seen in a previous study (Bennett et al., 2012). Together these results suggest that, under
416 normal circumstances, a sustained elevation in cortisol is not required to trigger departure from
417 the colony in fasting grey seal pups. This is in agreement with the absence of an increase in
418 cortisol even after more than 38 days of fasting in captive grey and harp seal pups (Nordøy et al.,
419 1990; 1993). It does not support the suggested role of increasing cortisol levels as a signal that
420 prompts departure from the colony in lactating Subantarctic fur seal females (Guinet et al., 2004)
421 and northern elephant seal pups (Ortiz et al., 2001 a and b). In rats, humans and penguins
422 (*Aptenodytes* sp.), circulating GC increase abruptly and dramatically and stimulate food seeking
423 behaviour at the onset of phase III of fasting, when fat reserves reach a low critical threshold and
424 protein catabolism increases to meet metabolic costs (Challet et al., 1995; Cherel et al., 1988 a, b

and c; 1992; Friedl et al., 2000; Groscolas and Robin, 2001; Robin et al., 1998). A cue to initiate foraging that occurs when fat reserves are already depleted would likely occur too late for seal pups to reach foraging grounds and learn to feed before the onset of terminal starvation due to compromised tissue structure and function. Indeed, as in other pinnipeds, there is no evidence that healthy grey seal pups enter phase III during the normal course of the postweaning fast (Nordøy et al., 1990).

Healthy grey seal pups may not respond to artificially high GC levels if other key hormonal and metabolic cues are not also present. For example, a dramatic change in fatty acid oxidation and BUN, prolactin and glucagon concentrations occur at the same time as elevated GC levels in animals on entry into phase III (Bernard et al., 2002a and b; Cherel et al., 1988 a, b and c; Groscolas and Robin, 2001; Le Maho et al., 1981; Robin et al., 1998). Our findings do not exclude the possibility that cortisol provides a cue to forage in seals when fat reserves are very low, such as in starvelings. However, our data suggest that elevated GC alone are not sufficient to prompt departure in healthy grey seal pups. It is therefore necessary to look elsewhere for endocrine cues that ordinarily terminate fasting and initiate food seeking behaviour in healthy phocid seal pups.

Symbols and Abbreviations:

ACTH = adrenocorticotrophic hormone; BUN = blood urea nitrogen; CONTROL = pups in experiment 1 that received no treatment; CRH = corticotropin releasing hormone; DEX_1 = pups in experiment 1 that received dexamethasone; DEX_2 = pups in experiment 2 that received dexamethasone; GC = glucocorticoids; GR = glucocorticoid receptor; ^2H_2O = deuterium oxide; LME = linear mixed effect model; SAL_1 = pups in experiment 1 that received saline; SAL_2 = pups in experiment 2 that received saline; SMRU = Sea Mammal Research Unit

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Figure legends:

Figure 1: Changes in cortisol in plasma from two captive 10 month old grey seal pups (diamonds = male; circles = female) in response to saline (open symbols) and dexamethasone (closed symbols) injection. Inter and intra assay coefficients of variation are <11% and <10%, respectively, and percentage recovery is 82.75-91.64% for this assay (Bennett et al., 2012).

Figure 2: Changes in mean ± s.d. a. plasma cortisol and b. daily rate of mass loss in control (squares), saline (triangles) and dexamethasone (circles) treated pups in 2002 up to 14 days postweaning. A black arrow indicates the time of injection of either saline or dexamethasone. Points with the same letter do not differ from each other, either within a treatment between days, or between treatments on a given day postweaning ($p < 0.05$). Underlined letters represent CONTROL pups, lower case letters represent saline treated (SAL_1) and italics represent dexamethasone treated (DEX_1) pups.

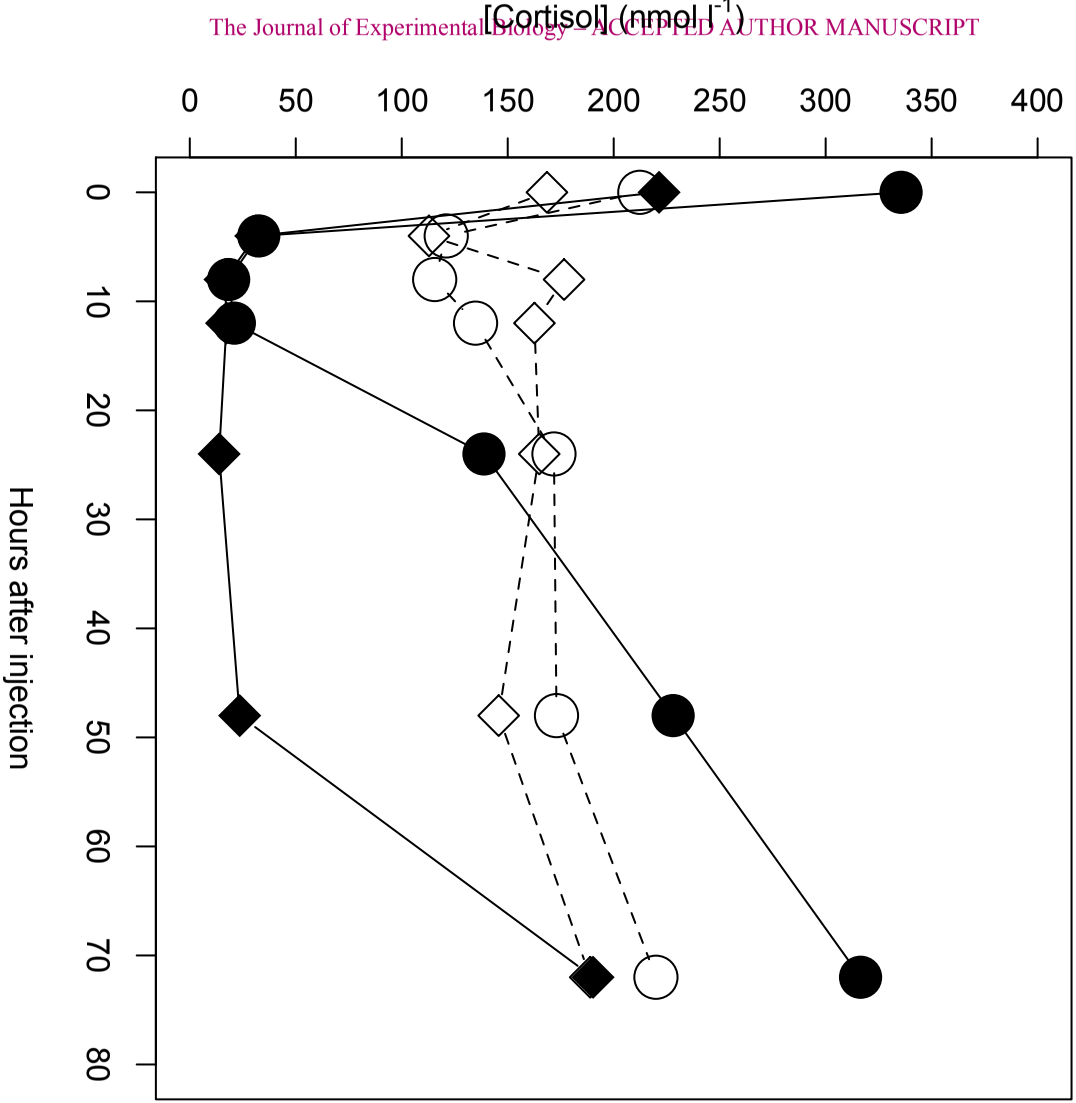

Figure 2

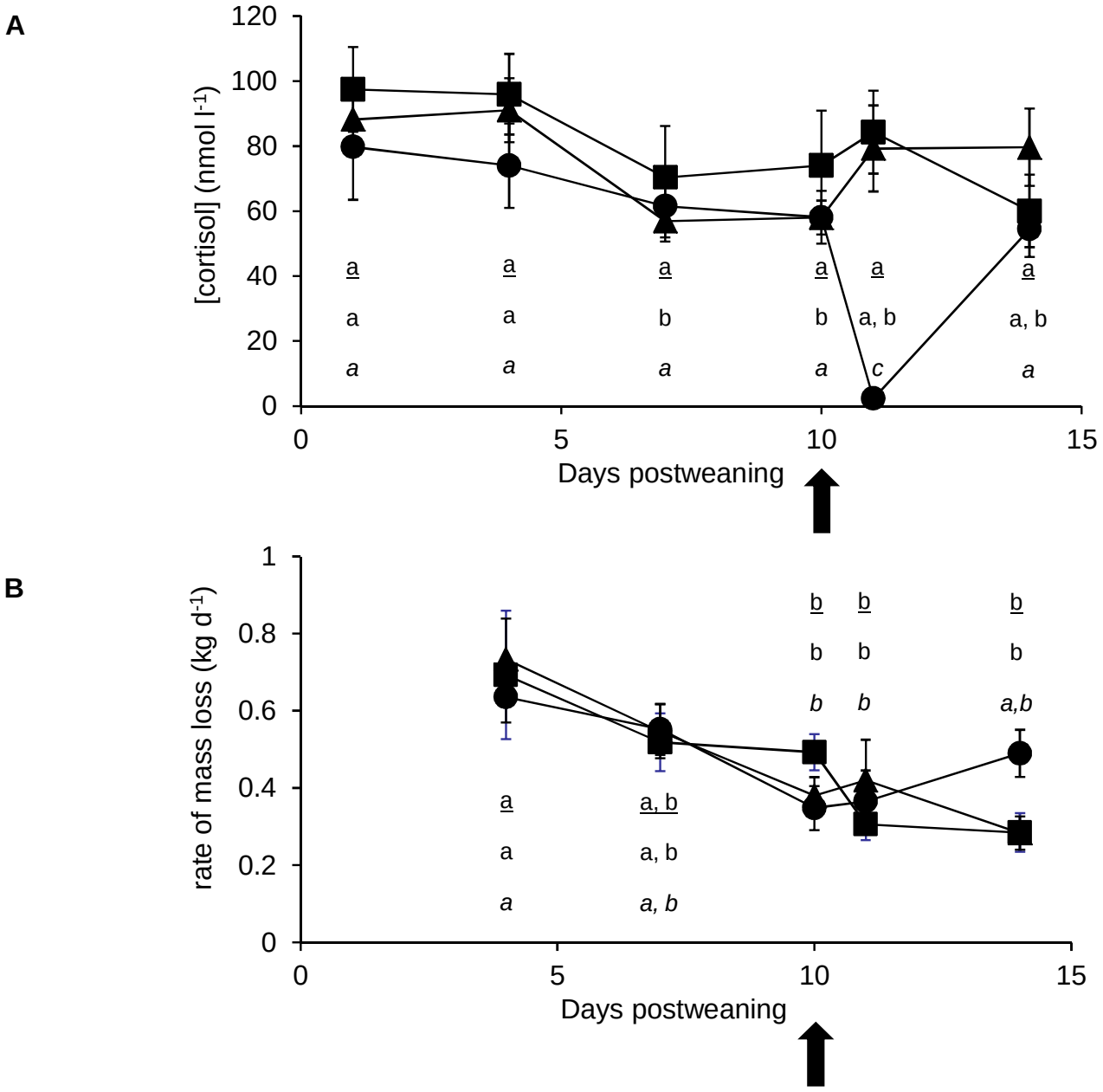

Table 1: Number of male and female pups and mean ± s.d. mass for each treatment group

Group	Weaning mass (2002) or mass at first capture (2004) (kg)			
	Males	n	Females	n
$CONTROL_{02}$	42.87 ± 6.63	6	40.99 ± 2.98	3
SAL_{02}	46.27 ± 4.93	5	41.12 ± 5.23	5
DEX_{02}	43.54 ± 6.79	5	41.67 ± 7.93	5
SAL_{04}	40.89 ± 5.04	8	37.11 ± 5.16	7
DEX_{04}	41.93 ± 6.31	7	35.94 ± 3.09	8

Table 2: Mean ± s.d. change in body composition variables during the five days after saline or dexamethasone treatment in fasting grey seal pups in experiment 2. Bold highlights significant differences ($p < 0.05$) between groups.

	SAL_{04}	n	DEX_{04}	n
Δ Mass (kg)	**0.50 ± 0.07**		**0.59 ± 0.10**	
Δ Water (kg)	**0.26 ± 0.06**		**0.34 ± 0.07**	
Δ Fat (kg)	0.14 ± 0.10	10	0.12 ± 0.09	11
Δ Protein (kg)	**0.09 ± 0.02**		**0.11 ± 0.03**	
Δ Ash (kg)	0.01 ± 0.002		0.01 ± 0.003	