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Effects of handling regime and sex on changes in cortisol, thyroid hormones and body mass in fasting grey seal pups

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ABSTRACT

Survival of seal pups may be affected by their ability to respond appropriately to stress. Chronic stress can adversely affect secretion of cortisol and thyroid hormones, which contribute to the control of fuel utilisation. Repeated handling could disrupt the endocrine response to stress and/or negatively impact upon mass changes during fasting. Here we investigated the effects of handling regime on cortisol and thyroid hormone levels, and body mass changes, in fasting male and female grey seal pups (*Halichoerus grypus*). Females had higher thyroid hormone levels than males throughout fasting and showed a reduction in cortisol midway through the fast that was not seen in males. This may reflect sex-specific fuel allocation or development. Neither handling frequency nor cumulative contact time affected plasma cortisol or thyroid hormone levels, the rate of increase in cortisol over the first five minutes of physical contact or the pattern of mass loss during fasting in either sex. The endocrine response to stress and the control of energy balance in grey seal pups appear to be robust to repeated, short periods of handling. Our results suggest that routine handling should have no additional impact on these animals than general disturbance caused by researchers moving around the colony.

1. Introduction

First year survivorship is a key component of population dynamics in marine mammals (Harwood and Prime, 1978; Sinclair, 1996), is often low and variable between years and may differ by up to three-fold between males and females (Le Boeuf et al., 1994; Burns, 1999; Hall et al., 2001; 2002; 2009). In birds and mammals, survival can crucially depend on the ability to mount an appropriate response to stress (Müllner et al., 2004; Blas et al., 2007; Busch and Hayward, 2009), the umbrella term applied to a vast array of psychological, physical or physiological challenges that activate the hypothalamic–pituitary–adrenal (HPA) axis. The generalised response to an acute stressor includes a rapid release of catecholamines followed immediately by a surge in glucocorticoid (primarily cortisol or corticosterone) secretion by the adrenal cortex. As a result, circulating glucocorticoid concentrations are elevated rapidly and dramatically within minutes of stress exposure (Sapolsky et al., 2000). At baseline levels, glucocorticoids maintain energy balance, but stress induced levels are a major component of the mechanism that allows an animal to respond to and cope with stress (Sapolsky et al., 2000; Busch and Hayward, 2009). They promote gluconeogenesis by facilitating the mobilisation of fat (Divertie et al., 1991; Samra et al., 1998; Djurhuus et al., 2002; 2004) and/or protein reserves (Simmons et al., 1984; Legaspi et al., 1985; Tataranni et al., 1996; Weiler et al., 1997; Mantha et al., 1999), and thus are instrumental in maintaining substrate supply in the face of a perceived increase in energy demand.

Baseline and stress-induced glucocorticoid levels can be indicative of background levels of stress and tend to be lower in healthier animals (Blas et al., 2007; Muehlenbein and Watts, 2010). Basal levels often progressively increase in response to chronic stress, such as fasting, repeated handling or disturbance (Bergendahl et al., 1996; Friedl et al., 2000; Creel et al., 2002). The acute stress-induced elevation in glucocorticoids is often blunted, but can also be increased, by chronic or repeated stress exposure (Harlow et al., 1992; Romero and Wikelski, 2002; Müllner et al., 2004; Rich and Romero, 2005; Walker et al., 2005; 2006; Busch et al., 2008). The acute and long term response to stress depends upon type, duration and frequency of stress exposure (Kioukia-Fougia et al., 2002), as well as developmental stage, sex

Abbreviations: %(B/B_0), percentage bound radioactivity relative to the 0 standard; % CV, co-efficient of variation of repeated sample measurements; %R, percentage recovery of standard from spiked serum; ACTH, adrenocorticotrophic hormone; AIC, Akaike's Information Criterion; CONTROL, group of pups sampled three times after weaning only; cpm, counts per minute; EIAs, enzyme immunoassays; HIGH, group of pups sampled twice during suckling and every three days after weaning; HPA, hypothalamic–pituitary–adrenal; LME, Linear Mixed effects Model; LOW, group of pups sampled twice during suckling and three times after weaning; RIA, radioimmunoassay; TT3, Total tri-iodothyronine; TT4, Total thyroxine.

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and physiological state or condition of the individual (Walker et al., 2005; 2006; Lovallo et al., 2006; Busch and Hayward, 2009; Padernera-Romano et al., 2010).

Changes to baseline glucocorticoid levels and the responsiveness of the HPA axis can be detrimental if they compromise the ability to cope with additional stress. Chronically elevated baseline cortisol levels in humans and animals can exacerbate pre-existing diseases and cause deterioration in overall health through immune suppression, abnormal fat deposition and protein wasting (Matalka, 2003; Padgett and Glaser, 2003; Busch and Hayward, 2009). Prognosis for patients with sepsis is reduced in those with higher baseline cortisol levels and reduced cortisol response to adrenocorticotrophic hormone (ACTH) (Bollaert et al., 2003). Long term corticosterone implants have been shown to reduce survival by 30% in black-legged kittiwakes (*Rissa tridactyla*; Goutte et al., 2010).

In pinnipeds, background cortisol levels change with physiological state, such as breeding or moulting, and with the onset of sexual maturity (Gardiner and Hall, 1997; du Dot et al., 2009; Myers et al., 2010). Younger animals show more pronounced changes in cortisol levels. In Steller sea lions (*Eumatopias jubatus*) cortisol levels are negatively correlated with body mass changes during caloric restriction and may be used as an indicator of nutritional stress (Rosen and Kumegai, 2008; du Dot et al., 2009). Cortisol levels also increase during fasting in northern elephant seal (*Mirounga angustirostris*) pups (Ortiz et al., 2001a, b; Viscarra et al., 2010) and lactating female Antarctic fur seals (*Arctocephalus gazella*; Guinet et al., 2004), but do not seem to change in captive fasting grey (Halichoerus grypus) or harp (*Phoca groenlandica*) seal pups (Nordoy et al., 1990; 1992; 1993). Sex differences in hormone levels in wild phocid pups have not been reported.

The acute and chronic changes in cortisol in response to either handling stress or ACTH have been examined in suckling southern elephant seal (*Mirounga leonina*) pups and their mothers (Engelhard et al., 2002), adult male grey (Lidgard et al., 2008) and Weddell seals (*Leptonychotes weddelli*; Harcourt et al., 2010), rehabilitated Pacific harbour seals (*Phoca vitulina richardii*; Gulland et al., 1999), and wild California sea lion (*Zalophus californianus*) pups (Padernera-Romano et al., 2010), but not in fasting phocid pups. There may be a link between baseline and stress-induced cortisol levels and the ability to survive subsequent or ongoing stress exposure in seals. In Pacific harbour seals, baseline and ACTH-induced cortisol levels increased during rehabilitation in animals that subsequently died, and decreased in animals that survived. The animals that died showed a decline in magnitude of the cortisol response to ACTH (Gulland et al., 1999).

Although not considered part of the stress response itself, thyroid hormone levels in brain are increased in response an acute stressor in rats (Friedman et al., 1999), whereas circulating tri-iodothyronine (T3) levels decline 2 h after inescapable shock (Helmreich et al., 2006). Stress-induced alterations to the hypothalamic–pituitary–thyroid axis occur after repeated or chronic stress in marine as well as terrestrial mammals (Dohler et al., 1977; Bianco et al., 1987; St Aubin and Geraci, 1988; Schumacher et al., 1995; Cremaschi et al., 2000; Ortiz et al., 2000). This can impact on energy expenditure and fuel allocation in the longer term since thyroid hormones are intimately involved in long-term energy balance (Hadley, 1992; Harris et al., 1998; Laugero and Moberg, 2000) and enhance the rate of fat breakdown (Cheikh et al., 1994).

It is important to examine not only the endocrine response to stress caused by handling, which can be hard to interpret in isolation (Busch and Hayward, 2009), but also to investigate the downstream consequences for some parameter that has demonstrable effects on survival or fitness, such as body mass (Engelhard et al., 2001). First year survivorship increases with body mass and size of energy reserves at weaning in grey seal (Hall et al., 2001; Hall et al., 2002; Hall et al., 2009), southern elephant seal (McMahon et al., 2000), northern fur seal (*Callorhinus ursinus*; Baker and Fowler, 1992) and Weddell seal pups (Burns, 1999).

Phocid seal pups are fed on high fat milk during an intensive suckling period (15–18 days in grey seals), after which they are weaned abruptly when their mother returns to sea (Coulson, 1959; Fedak and Anderson, 1982; Anderson and Fedak, 1987; McCann et al., 1989; Fedak et al., 1996; Atkinson, 1997; Mellish et al., 1999). Most phocid pups undergo an extended land based fast after weaning, which can last up to two and a half months in elephant seals (Reiter et al., 1978) and 7 to 40 days in grey seals (Reilly, 1991; Noren et al., 2008) and is important for development of diving capabilities (Thorson and Le Boeuf, 1994; Lewis et al., 2001; Noren et al., 2005; Bennett et al., 2010; Burns et al., 2010). However, seal pups must terminate the fast and leave the colony with adequate fuel reserves to sustain them until they learn to forage.

Chronic or repeated acute stress, including that caused by handling and restraint, can change energy expenditure (Harris et al., 1998; Laugero and Moberg, 2000). Energy expenditure is likely to increase transiently in seal pups during handling if the animal struggles, and possibly also over the longer term, as a consequence of altered release of glucocorticoids and thyroid hormones. We might expect fasting animals to be more vulnerable to increased demand on energy supply because they have finite fuel reserves. Different handling regimes may contribute to variation in hormone levels and regulation of mass loss during fasting and could also complicate results and interpretation from different study protocols. Males and females may also differ in their hormone profiles due to sex-specific requirements for growth.

Here we examined (i) changes in circulating cortisol and thyroid hormone levels during fasting in male and female pups, which have implications for the control of fasting fuel use and key developmental processes, (ii) whether handling regime affects the levels of these hormones or impacts upon the rate at which cortisol increases as a result of acute handling, which has implications for the ability of the animals to respond to subsequent stress, and (iii) whether body mass changes are affected by handling frequency, which may impact on the time available for them to find food.

2. Materials and methods

We examined the effects of handling regime in 21 grey seal pups from the Isle of May, Firth of Forth, Scotland (56° 11′N, 2° 33′W) in October to December 2001. All capture and handling procedures were performed under Home Office project licence #60/2589 and conformed to the UK Animals (Scientific Procedures) Act, 1986.

2.1. Handling regime

Fourteen pups were captured early (4.7 ± 1.2 (SD) days after birth) and late (16.18 ± 0.98 (SD) days after birth) in the suckling period. Pup sex was recorded and flipper tags (Rototag; Dalton ID Systems, Henley on Thames, Oxon, UK) were attached at first capture. Weaning date was determined from daily observations of mother–pup pairs and occurred 2.09 ± 1.38 days after the late suckling mass measurement.

Pups were penned in a large (~115 m × 80 m) outdoor enclosure within two days after weaning (Bennett et al., 2007) and were assigned to one of two handling regimes: either every three days, to obtain regular mass and hormone measurements (HIGH), or three times throughout the fast (LOW): at early (2.2 ± 1.1 days after weaning), mid (12.53 ± 2 days after weaning) and late (20.6 ± 4 days after weaning) in the postweaning fast. Seven pups that had not been handled during suckling (CONTROLs) were also penned in the same enclosure within two days after weaning and handled with the same frequency as the LOW group.

All pups remained in the pen until they reached 70% of their weaning mass or 30 kg, whichever happened first (Bennett et al.,

2007; 2010). On release, pups were painted with large symbols on their backs, and their presence/absence on the colony was noted daily. Although they were free to do so, only five pups left the colony on the day of release. Pups present after release were re-weighed every three days until all animals had left. Date of departure was assumed to be the day after the last sighting of the animal (Bennett et al., 2010) and occurred 0 to 8 days (mean = 3. 08 ± 2.66) days after release from the pen.

2.2. Blood samples

At early, mid and late postweaning a serum sample (time 0) was taken as quickly as possible from each pup from the extradural vein into a sterile 10 mL plain (serum) vacutainer (Becton Dickinson, Cowley, Oxon, UK) using either 19 gauge, 2 in. or spinal needles, whichever was most appropriate for the size of the animal. All samples were taken in the morning between 09:00 and 12:00 h, to minimise the effects of circadian rhythms on hormone measurements. The number of minutes to obtain the sample from first contact with the animal was recorded. A second serum sample was taken five minutes later (time 5) to determine whether the cortisol response to stress changed throughout the fast and/or in response to repeated handling. All puncture sites were disinfected with Savlon before sampling, and sprayed with topical terramycin™ (oxytetracycline: Pfizer Ltd) immediately afterwards. The amount of time the researchers were in direct physical contact with the pup was recorded each time it was captured.

To investigate the stability of cortisol prior to storage (effect of processing time on cortisol measurement), serum samples were drawn from three female grey seals in the captive facility at SMRU (one juvenile and two adults). These animals were anaesthetized using a pressurised dart to deliver an intramuscular dose of zoletil (Virbac, France) (Pomeroy et al., 1999). Five vacutainers of blood were taken from each female on a single occasion. One tube from each animal was processed at 0, 2, 4, 8 and 12 h after obtaining the sample, to reflect the maximum possible range of time taken to store samples under field conditions. The samples were kept in laboratory light and temperature conditions until they were centrifuged, aliquotted and stored prior to assay.

Serum was centrifuged in a swing-out bench top centrifuge at 2000 g for 15 min, as soon as possible, and within 10 h, after sample collection. Aliquots were transferred to 500 μL microtubes using glass Pasteur pipettes, and stored at −20 °C until analysis.

2.3. Hormone measurements

All serum samples were analysed within six months of collection. Cortisol concentrations were measured using Spectria ^{125}I-cortisol radioimmunoassay (RIA) (Orion Diagnostica, Espoo, Finland) in accordance with the manufacturers' instructions. Total serum T3 (TT3) and thyroxine (TT4) concentrations were determined using Serozyme magnetic solid phase enzyme immunoassays (EIAs) (BioChem ImmunoSystems (UK) Ltd., Woking, Surrey). Assay procedures for TT3 and TT4 measurement were adapted from the kit protocols for use in 96 well flat-bottom microtitre plates (Dynex technologies). Quarter volumes of all reagents were used and incubation times extended accordingly. For any given sample, the serum required for both thyroid hormone assays was drawn from the same aliquot of serum and the assays were performed within 24 h of defrosting the aliquot to minimise any effects of sample degradation. Serum was stored at 4–8 °C between assays.

Mean absorbance was calculated for duplicate wells in the thyroid hormone EIAs. Mean counts per minute (cpm) was calculated for duplicate tubes in the cortisol RIA, and expressed as a percentage of radiolabel bound to the antibody compared to the zero standard (%(B/B_0)). For each assay run, a standard curve was constructed from the standard absorbance or %(B/B_0) values using Curve Expert 1.34 and the sample hormone concentration was calculated from the equation of the line. All samples, standards and quality controls were assayed in duplicate.

2.4. Mass measurements

All pups were weighed (±0.2 kg) at each capture. Weaning mass was determined by extrapolation using rates of change during suckling (Bennett et al., 2007). Mass on the day of departure was extrapolated from the rate of change between the final measurement and the day of departure (Bennett et al., 2007).

2.5. Statistical analysis

Statistical analyses were performed using MINITAB (Minitab 13.32, Minitab Inc, 2000) or R (R 2.13.1, R Development Core Team, 2003) (Ihaka and Gentleman, 1996). Linear mixed-effects models (LMEs) that included blood sample as a random effect and dilution as a fixed effect (Chatfield, 1989; Crawley, 2002) were used to determine the limit of linearity for each hormone assay. LMEs that included individual as a random term and time since sampling as a fixed effect were used to determine whether cortisol measurements were influenced by time taken to process the blood sample. LMEs were also used to investigate sex and handling regime differences in cortisol levels, the magnitude of the acute increase in cortisol during the first five minutes of handling, TT3 and TT4 and the daily rate of mass loss during the postweaning fast. The models were fitted using a maximum likelihood estimate. Handling regime, sex and timepoint (early, mid and late fast) were included as fixed effects in the complete models. Stepwise model selection was performed and models were compared by ANOVA. We investigated whether there was a difference in weaning mass or departure mass between groups using ANOVA, and differences in the timing of blood sampling/weighing using ANOVA or Kruskal–Wallis tests. In all cases p was considered significant at <0.05.

3. Results

3.1. Cortisol

%(B/B_0) values for serum sample dilutions were parallel to the standard curve and measured cortisol values were independent of volume to a dilution factor of 8 (Fig. 1). Inter and intra assay coefficients of variation (%CV) were <11% and <10%, respectively. Percentage recovery (%R) of cortisol from spiked samples ranged from 82.75 to 91.64%. The lower %R values in this study corresponded to samples

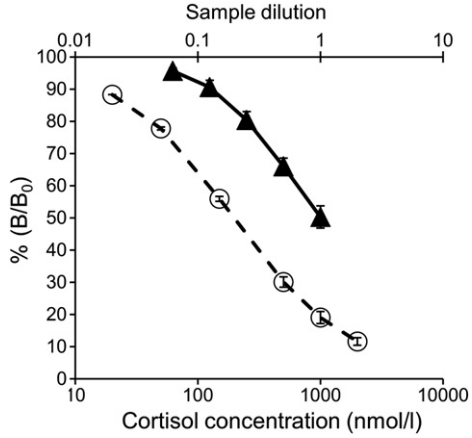

Fig. 1. Parallelism of mean ± SD %(B/B_0) values of serum sample dilutions (filled triangles; n = 7) with the cortisol RIA standard curve (open circles; n = 5).

containing low cortisol. Measured cortisol in grey seal serum samples did not change significantly in serum samples stored at laboratory light and temperature conditions for up to 12 h after sampling before they were processed and frozen (LME: $t = 1.331$; $p = 0.21$; n (observations) = 15; n (individuals) = 3; AIC = 140.164).

Males and females had a significantly different pattern of change in time 0 cortisol levels (LME: $t = 3.410$; $p = 0.0016$; n (observations) = 61; n (individuals) = 21; AIC = 557.295; Fig. 2). In males, there was no difference between any of the time points ($p > 0.05$). In females, time 0 cortisol at the mid fast measurement was lower than the measurements early in the fast (LME: $t = 2.944$; $p = 0.0056$) and late in the fast (LME: $t = 2.434$; $p = 0.020$), and was lower than the mid fast measurement in males ($t = 2.303$; $p = 0.0328$). There was no difference between sexes in time 0 cortisol at early ($t = 0.869$; $p = 0.396$) or late fast measurements ($t = 0.543$; $p = 0.5931$). Handling regime did not improve the model describing changes in time 0 cortisol levels during the postweaning fast (ANOVA: $L = 9.898$; $p = 0.6249$).

Cortisol levels increased (paired t-test: $t = 12.14$; $p < 0.001$; n = 61) by ~1.6 fold, from 60.71 ± 25.09 nmol/L to 99.63 ± 36.01 nmol/L in all animals during acute handling (from time 0 to time 5). The magnitude of change was not different between handling regimes or sexes at any timepoint (LME: $p > 0.05$; n (observations) = 62; n (individuals) = 21; AIC = 581.8443).

3.2. Thyroid hormones

Absorbance values for serum sample dilutions were parallel to the standard curve and measured TT3 and TT4 were independent of volume to a dilution factor of 1.33 and 4, respectively (Fig. 3). Inter and intra %CV for TT3 were <20% and <22%, respectively and %R ranged from 71.94 to 153.97%. This range was observed in samples containing low TT3. Inter and intra %CV for TT4 were <15% and <20%, respectively and %R ranged from 80.62 to 144.62%.

Males and females had a significantly different pattern of change in TT4 during the postweaning fast (Fig. 4), and there was no effect of handing regime (ANOVA: $L = 8.801$; $p = 0.720$). TT4 did not change in males throughout the fasting period, whereas females had higher TT4 early in the fast than at the mid fast (LME: $t = 2.515$; $p = 0.0164$; n (observations) = 62; n (individuals) = 21; AIC = 553.000) or late fast time points LPW (LME: $t = 2.860$; $p = 0.0069$). Females had higher TT4 than males only at the early time point (LME: $t = 2.568$; $p = 0.0188$).

TT3 was log transformed before analysis and was higher in females than in males throughout fasting (LME: $t = 4.095$; $p < 0.001$; n (observations) = 62; n (individuals) = 21; AIC = 46.039). The inclusion of either treatment group (ANOVA: $L = 19.137$; $p = 0.085$) or timepoint (ANOVA: $L = 15.056$; $p = 0.238$) did not improve the model.

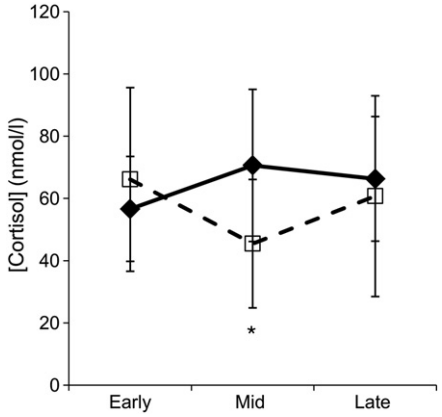

Fig. 2. Cortisol concentration ± SD within four minutes of first contact in males (filled diamonds; solid line) and females (open squares; dashed line) from early, mid and late in the postweaning fast. * indicates the mid fast timepoint in females when cortisol was significantly ($p < 0.05$) lower than at all other timepoints in both sexes.

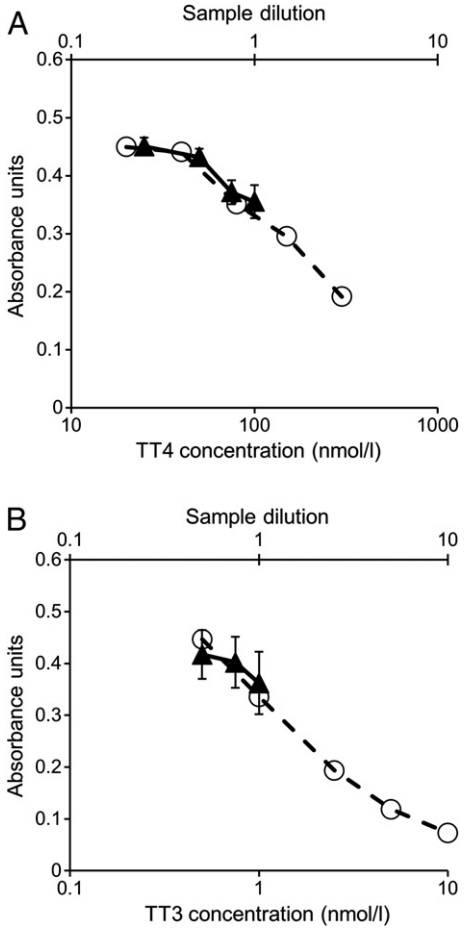

Fig. 3. Parallelism of mean ± SD absorbance values of serum sample dilutions (filled triangles; solid line; n = 5) with A. the TT4 standard curve (open circles; dashed line) and B. the TT3 standard curve (open circles; dashed line).

The TT3:TT4 ratio was arcsine transformed before analysis. It was higher in females throughout the fasting period (LME: $t = 2.610$; $p = 0.0172$; n (observations) = 62; n (individuals) = 21; AIC = 232.097), irrespective of handling regime (ANOVA: $L = 18.411$; $p = 0.104$), at 0.029 ± 0.008 compared with 0.024 ± 0.010 in males (raw values).

3.3. Time taken to obtain blood samples and cumulative contact time

Blood samples were obtained within four and in most cases within two minutes (mean = 1.49 ± 0.70 min) from initial contact with the animals. There was no difference in the time taken to obtain the time 0 blood sample between males and females at the three postweaning timepoints or between treatment groups (LME: $p < 0.05$; n (observations) = 61; n (individuals) = 21; AIC = 125.619). The time 5 blood sample was obtained within 11 min after first contact with the animal. It took longer to obtain it in females late in the fast than at any other time in any group and in either sex (7.6 ± 1.51 min vs 6.47 ± 0.67 min; LME: $t = 3.520$; $p = 0.0012$; n (observations) = 61; n (groups) = 21; AIC = 160.859).

There was no difference between groups in the median number of days postweaning of the early (Kruskal–Wallis: $H = 4.25$; $p = 0.119$; $df = 2$), mid (Kruskal–Wallis: $H = 1.06$; $p = 0.589$; $df = 2$) or late (Kruskal–Wallis: $H = 0.65$; $p = 0.723$; $df = 2$) sampling timepoints. As expected, at the early fast time point the CONTROL group had experienced less cumulative contact time than the other two groups,

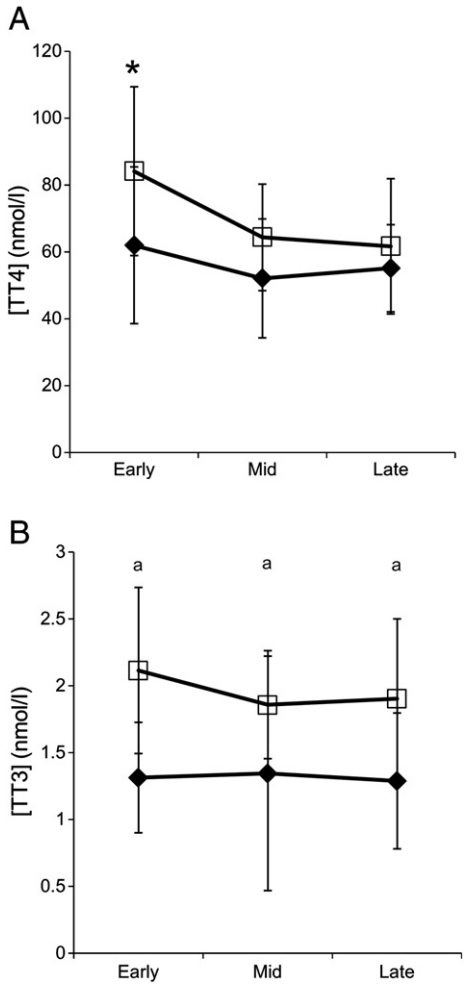

Fig. 4. Mean ± SD A. TT4 and B. TT3 in males (filled diamonds) and females (open squares) from early, mid and late in the postweaning fast. * indicates the early timepoint in females, when TT4 was significantly higher than at all other timepoints in both sexes. a indicates a significant difference between males and females.

but there was no difference in cumulative contact time between LOW and HIGH groups (Kruskal–Wallis: $H = 13.10$, $df = 2$, $p = 0.001$). There was a greater degree of variance in cumulative contact time in the LOW group at the mid (Bartlett's test: $F = 17.30$; $p < 0.001$) and late fast time points (Bartlett's test: $F = 18.20$; $p < 0.001$) than in the other two groups. Median cumulative contact time was different between all three groups both in the middle (Kruskal–Wallis: $H = 16.03$; $p < 0.001$) and late in the fast (Kruskal–Wallis: $H = 15.24$; $p < 0.001$). There was no difference between males and females in cumulative contact time at early (t-test: $T = 0.96$; $p = 0.347$; $df = 19$), mid (Mann–Whitney: $W = 104.0$; $p = 0.245$) or late fast time points (Mann–Whitney: $W = 94.0$; $p = 0.653$).

3.4. Body mass

Pups weaned at 45.08 ± 5.79 (SD) kg and there was no difference in weaning mass between the three groups (ANOVA: $F_{(2,20)} = 0.21$; $p = 0.813$). Daily mass loss rate declined from 0.45 ± 0.16 kg day^{-1} to 0.34 ± 0.13 kg day^{-1} (LME: $t = 2.332$; $p = 0.0309$; n (observations) = 41; n (individuals) = 21; AIC = 35.012) Neither sex (ANOVA: $L = 8.064$; $p = 0.233$) nor treatment (ANOVA: $L = 2.503$; $p = 0.6442$) improved the model, showing there were no differences in mass loss rate between males and females or handling regimes. The fall in mass loss rate was due to the concomitant decline in body mass, because there was no difference in mass specific mass loss rates between timepoints (paired t test: $t = 1.19$; $p = 0.249$; $df = 20$). Pups left the colony at 32.74 ± 4.24 (SD) kg and there was no difference in departure mass between groups (ANOVA: $F_{(2,17)} = 0.14$; $p = 0.872$).

4. Discussion

4.1. Hormone levels during the postweaning fast

Male and female grey seal pups showed differences in their hormone profiles during the postweaning fast. Females showed a small reduction in cortisol concentrations midway through the fast that was not apparent in the males. The significance of this small reduction is unclear. In other pinnipeds, higher cortisol levels have been reported in females than in males (Padernera-Romano et al., 2010). Females also had consistently higher TT3 levels, a higher TT3: TT4 ratio than males throughout fasting, and higher TT4 levels early in the fast. The difference in the pattern of change in cortisol and thyroid hormones between males and females cannot be ascribed to differences in cumulative contact time or time taken to obtain the blood samples. Instead they may be a result of sex differences in the prioritisation of development of particular tissue types and allocation of fat and protein reserves for metabolism and growth. Adult male grey seals are up to two times and 250 kg larger than adult females, but adult females tend to be proportionally fatter (Reilly and Fedak, 1990; Beck et al., 2003). Although we saw no sex differences in the rates of mass loss associated with the differences in hormone profiles, sex specific requirements for growth and metabolism may begin early in life in grey seals as a result of hormonal differences such as those we have reported here. The role of these hormones in fuel metabolism and development in male and female fasting seal pups requires further investigation.

Low, stable cortisol levels (absolute change in cortisol ~20 nmol l^{-1}) were characteristic of the fasting period, which is consistent with findings from captive grey and harp (*P. groenlandica*) seal pups (Nordoy et al., 1990; 1992; 1993). This contrasts with the progressive increase in cortisol observed throughout the first eight weeks of the postweaning fast in northern elephant seal pups (Ortiz et al., 2001a, b; Viscarra et al., 2010), and the rise in cortisol in conjunction with declining body condition index and time spent ashore in fasting, lactating subantarctic fur seal females and in food restricted Steller sea lions (Guinet et al., 2004; Rosen and Kumegai, 2008; du Dot et al., 2009). It has been proposed that cortisol helps to maintain high levels of lipolysis for the fat-based metabolism in fasting northern elephant seal pups (Ortiz et al., 2001a, b). However, the grey seal pups here and previously (Nordoy et al., 1990; 1993; Bennett et al., 2007) relied predominantly on fat as their major metabolic fuel without needing to increase basal cortisol. There may thus be species differences in the role or requirements for cortisol during fasting, even in related species at a comparable level of development.

It has been suggested that cortisol contributes to the eventual signal to depart from the colony in fasting pinnipeds (Ortiz et al., 2001a, b; Guinet et al., 2004). Increased basal glucocorticoid concentrations are associated with an increase in the motivation to seek food in other animals (Green et al., 1992; Ponsalle et al., 1992). High glucocorticoid levels drive a marked increase in food seeking behaviour in humans (Tataranni et al., 1996), rodents (Challet et al., 1995; Arvaniti et al., 1998) and bottlenose dolphins (*Tursiops truncatus*; Reidarson and McBain, 1999) and prompt fledging and the onset of foraging in many bird species (Heath, 1997; Belthoff and Dufty, 1998; Kern et al., 2001). A four-fold increase in corticosterone has been implicated as the signal that terminates the moulting and breeding fasting periods in Emperor (*Aptenodytes forsteri*) and king (*Aptenodytes patagonica*) penguins (Cherel et al., 1988a, b, c; Robin et al., 1998). Here, most animals left the colony without a change in cortisol, suggesting that elevated cortisol is not required to trigger departure from the colony in fasting grey seal pups.

Thyroid hormone levels control basal metabolic rate and high levels, particularly of the more bioactive TT3, result in enhanced tissue breakdown (Hadley, 1992). Thyroid hormone levels normally decline in non-fast adapted species in response to food deprivation to reduce energy utilisation (Spencer et al., 1983; Blake et al., 1991). This was observed only in the TT4 levels of the females in this study and is not seen in fasting northern elephant seals (Ortiz et al., 2001a, b), food restricted manatees (*Trichechus manatus*; Ortiz et al., 2000) or Steller sea lions (du Dot et al., 2009). It is possible that the lack of a drop, particularly in TT3 titre, in fasting seal pups is due to a conflict between the need to conserve fuel for an extended fast and the requirement for active development and high levels of lipolysis to sustain fat-based metabolism. The way that seal pups are able to deal with this dichotomy is unknown.

4.2. Handling effects on hormone dynamics

The increase in cortisol over the first five minutes of handling seen here in fasting grey seal pups is typical of the mammalian response to stress (Gardiner and Hall, 1997; Sapolsky et al., 2000) and contrasts with findings from California sea lion pups, which showed no change in cortisol levels between two samples taken five minutes apart, similar to this study (Padernera-Romano et al., 2010). The disparity may be due to time taken to obtain the blood sample, and the use of anaesthesia in the sea lion study.

Handling frequency and cumulative handling time did not alter baseline or stress induced cortisol, or thyroid hormones in suckling and fasting grey seal pups. These findings corroborate work by Engelhard et al. (2002), which showed no effect of previous handling during suckling on cortisol secretion in suckling southern elephant seal pups, and by Harcourt et al. (2010), which showed a similar magnitude of cortisol response in male Weddell seals early and late in the breeding season, despite repeated handling. Our data extend the work on the effect of handling on seals to include fasting pups. The lack of a change in baseline or handling stress-induced cortisol in response to short periods of repeated handling suggests that our study pups did not habituate to handling, nor were they chronically stressed by it. The handling regimes used here therefore are unlikely to have compromised the ability of pups to respond to future stressors. This is of key importance because the ability to mount an appropriate stress response can impact on survival (Müllner et al., 2004; Blas et al., 2007; Busch and Hayward, 2009).

The absence of a handling regime effect on baseline and stress induced cortisol levels differs markedly from the changes seen in regularly handled rehabilitated harbour seals (Gulland et al., 1999). The seal pups here were healthy, experienced minimal confinement and had relatively little contact with humans compared with the animals in the rehabilitation study. While we cannot exclude the possibility that all the animals in our study were chronically stressed as a result of penning, irrespective of handling regime, the pen was large enough for pups to be able to move freely over a wide area, they had access to a pool, and no animals escaped, even when the fencing was destroyed by an adult male moving through the enclosure.

Accurate measurement of circulating glucocorticoid levels can be hampered by the stress inherent in obtaining the blood sample. While some studies on pinnipeds have attempted to avoid this problem by using faecal samples (Hunt et al., 2004; Mashburn and Atkinson, 2004, 2007), faecal levels do not correlate well with blood levels in phocid seals (Gulland et al., 1999), and faecal samples are hard to obtain from specific fasting animals in the wild. While we could not account for any increase in cortisol levels prior to or during our approach of the animals, the time taken to obtain initial (time 0) cortisol levels was highly consistent between animals and in most cases within two minutes of first physical contact. This contrasts with studies on northern elephant seals in which samples took up to 16 min to obtain (mean = 6.5 ± 0.4 min; Ortiz et al., 2001b). The rapidity with which we were able to obtain the blood samples may partially explain differences between our findings and those in other pinnipeds (e.g. Ortiz et al., 2001b; Padernera-Romano et al., 2010). In birds and rats, samples obtained within the first three minutes are considered to be near baseline (Dallman and Bhatnagar, 2001; Romero and Reed, 2005). We also saw no effect of the time to process samples prior to storage on measured cortisol. We therefore assumed that our time 0 samples were as close as practicable to baseline and were comparable within and between animals.

4.3. Handling regime effects on body mass

The rates of mass loss seen here were comparable to those found previously in grey seal pups (Coulson, 1959; Nordoy and Blix, 1985; Worthy and Lavigne, 1987; Reilly, 1991; Mellish et al., 1999; Noren et al., 2008) and were not affected by handling regime. Any temporary increase in energy expenditure during and after the short handling periods therefore did not have long term negative effects on body mass. The handling frequencies after weaning were of much lower intensity than those that cause lasting changes in metabolism in other animals (Zhou et al., 1999). In growing mice, repeated episodes of stress are energetically costly, but compensatory mechanisms prevent long term changes in body mass once the stress has been removed (Laugero and Moberg, 2000). Similar mechanisms could be operating here in grey seal pups.

In southern elephant seals, both mothers and pups were heavier in areas that did not experience disturbance by humans compared with areas that were frequented by researchers, although these differences could not be ascribed specifically to disturbance and may have arisen for other, unknown, reasons (Engelhard et al., 2001). Southern elephant seal pups showed no differences in survival between groups handled at different intensities during suckling and postweaning (McMahon et al., 2005). Here, the lack of a difference in body mass and daily mass loss rates between pups handled at different frequencies could be due to an overall similarity in general disturbance by humans. Our results suggest that short bouts of handling do not cause any additional impact on pups than exposure to researchers moving around the colony.

In summary, male and female grey seal pups showed differences in cortisol and thyroid hormone changes throughout fasting. These hormonal differences may lead to differences in allocation of fat and protein to growth and development that are not apparent in mass loss rates. Body mass and hormone changes and the ability of pups to respond appropriately to stress appear to be unaltered by handling during the key developmental stages of suckling and fasting in grey seals. Although this study does not rule out possible negative impacts of general disturbance it suggests that pups handled repeatedly for short periods are not compromised to a greater degree than other pups on the colony. The impact of handling on long term survival, over and above that caused by disturbance, is therefore likely to be minimal.

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