HIGHLIGHTS

- Endogenous glucocorticoids were assayed in harbour seal pups during rehabilitation
- Cortisol, Cortisone, Prednisolone, and Prednisone were measured in urine
- Median concentrations of Prednisolone and Prednisone were similar to Cortisol
- Levels of the four hormones correlated differently with rehabilitation variables
- Water access, pup mass and growth rate may influence glucocorticoid concentrations

1 **Urinary glucocorticoids in harbour seal (*Phoca vitulina*) pups during rehabilitation**

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ABSTRACT

The glucocorticoid (GC) hormone cortisol is often measured in seals to indicate their stress levels, although other endogenous GCs are usually overlooked. We investigated concentrations of four endogenous GCs in the urine of "orphan" harbour seal pups in rehabilitation. We hypothesised that the GC levels would be elevated if pups were socially isolated, without water access, and with low body mass. Ninety-six samples were collected from 32 pups at four different rehabilitation centres and were analysed by Ultra Performance Liquid Chromatography and Tandem Mass Spectrometry. Median urinary creatinine (Cr) concentrations of endogenous prednisolone (31.6 ng/mg/Cr) and prednisone (31.1 ng/mg/Cr) occurred in similar magnitude to cortisol (37.0 ng/mg/Cr), while median cortisone concentrations were higher (390 ng/mg/Cr). Prednisolone and prednisone concentrations were more strongly inversely related to pup growth rate and pup mass than cortisol and cortisone. Concentrations of all four GCs decreased with mass gain for pups with water access but did not decrease for pups without water; linear mixed models indicated the interaction between these trends was significant for cortisol and cortisone, but not for prednisolone or prednisone. These results indicate the potential value of measuring all four of these endogenous GC hormones in phocid seal pups.

Key words

Harbor seal pup, cortisol, glucocorticoids, rehabilitation, stress, rearing environment

1. Introduction

Rescue, captive care and subsequent release of stranded young phocid seals has become increasingly common and routine practice in North America and western Europe, most often of harbour seals, *Phoca vitulina*, grey seals, *Halichoerus grypus*, and northern elephant seals, *Mirounga angustirostris* with numbers now running into hundreds along some coastlines (e.g. Lander et al., 2002; Osinga and 't Hart 2010; Macrae et al., 2011). During the pupping seasons of these species, neonates that have been permanently separated from their mothers have been usually termed "orphans" (e.g. Riedman and Le Boeuf, 1982; Macrae et al., 2011; Wilson and Jones, 2021) or "healthy but abandoned" (Dailey et al., 2020). These orphan pups are typically taken to 'seal sanctuary' centres for mother-substitute care (usually termed 'rehabilitation') until they are considered well-enough grown to be released back into the sea, after a period usually varying between seven weeks to four months.

Harbour, grey and northern elephant seals are all currently listed by the International Union for the Conservation of Nature (IUCN) as "Least Concern", and rehabilitation (referred to henceforth as "rehab") of orphans of these species is not generally considered to have a species conservation benefit. The primary purpose of rescuing and rehab of these species at the present time is therefore individual welfare. Despite the large-scale rehab programmes tailored to the physical care of the pups, there has been very little research on the pups' physiological and psychological well-being during their first weeks in rehab (Oriel, 2010).

Rehab centres are only able to provide the pups with an artificial environment, which differs in many key aspects from the natural, physical and social environment of mother-dependent, free-ranging pups. Harbour seal pups are born at about 11 kg mass and, when nursing from their mothers, achieve a net mass gain of about 0.4 to 0.6 kg/day (Skinner, 2006; Muelbert and Bowen, 1993; Cottrell et al., 2002). They spend about 60% of their time in the water from birth, follow their mother closely in the water, and are continually mobile around the nursery site (Venables and Venables, 1955; Wilson, 1974; Bowen et al., 1999; Skinner, 2006; Wilson and Jones, 2018). The mothers attend their pups full-time for at least the first 10 days or so, after which they often leave their pups to make

offshore foraging trips for a few hours unencumbered by their pups (Wilson, 1978; Boness et al., 1994; Wilson and Jones, 2018), nevertheless leaving their mobile pups near other mothers and pups (Wilson and Jones, 2020). Thus reproducing the natural physical and social environment of free-ranging harbour seal pups for orphans in rehab is especially challenging. The average mass gain of orphan harbour seal pups in rehab centres is usually less than 0.3 kg/day, with a published range of 0.11 kg/day (MacRae et al., 2011), 0.10–0.16 (Richmond et al., 2010), 0.13 kg/day (Dailey et al., 2020), 0.21 kg/day (Trumble et al., 2013), and 0.30 kg/day (Wilson, 1999). Rehab facilities in the British Isles and North America are usually designed to maintain the pups in isolation and in dry pens for some weeks after admission, a principal reason for this being to prevent potential transmission of infections (Larmour 1989; Robinson, 1995; MacRae et al. 2011; Osinga and 't Hart, 2010). Less often, centres house the pups with free access to a pool, either in pairs (Wilson, 1999) or in small groups (Müller et al., 2003). Therefore in most cases of orphan harbour seal pup rehab, the social environment, time spent in the water, and weight gain may be very different from that of a free-living pup with its mother. These differences might result in stress factors specific to the rehab environment, and any consequent difference in the dynamics of the ("stress") hypothalamus-pituitary-adrenal (HPA) axis would affect the release of glucocorticoid (GC) stress hormones, such as cortisol (CL) (Atkinson et al., 2015).

When an infant mammal – such as a harbour seal, squirrel monkey (*Saimiri sciureus*) or guinea pig (*Cavia porcellus*) is initially separated from its mother, it usually emits distress calls and makes physical effort to regain contact (Perry and Renouf, 1988, Coe et al., 1983; Yusko et al., 2012). This has been termed the "acute" phase of maternal separation, when a dramatic increase in levels of CL has been recorded in squirrel monkeys (Coe et al., 1983), guinea pigs (Yusko et al., 2012) and harbour seals (di Poi et al., 2015).

CL is transported in plasma, with 80–90% bound to CL-binding globulin, 5–10% bound to albumin, while only 3–10% of CL is free and biologically active (Holst et al., 2004). At the target tissue, the free hormone diffuses across the cell membranes and binds to the glucocorticoid receptor; this complex migrates to the nucleus where it regulates gene expression (McWhinney et al., 2010). The principal effects of CL are to increase cardiac output and increase circulating glucose

concentrations (Sapolsky et al., 2000), thereby facilitating an appropriate energetic response by a separated infant attempting to reunite with its mother. However, if the infant is unable to reunite with its mother, the initial "acute" phase of the stress response will eventually give way to the next "depressive" phase of maternal separation, when the infant may be expected to display more passive behaviours (Spencer-Booth and Hinde, 1971), accompanied by reduced circulating cortisol (Yusko et al, 2012). By the time an "orphan" seal pup enters rehab, it may already be in the "depressive" phase of separation, as well as being in poor body condition. When harbour seal orphan pups enter rehab, therefore, circulating CL levels may be a dynamic function of time since maternal separation, time spent alone since separation, and other factors of an artificial environment. In pinnipeds, CL levels have been reported to increase during periods of fasting or nutritional stress (e.g. Ortiz et al., 2001; Guinet et al., 2004; du Dot et al., 2009; Bennett et al., 2013; Kershaw and Hall, 2016). Orphan pups will be experiencing nutritional stress when rescued, and this may not be alleviated fully in rehab due to the difficulty of achieving a species-typical growth rate.

CL levels in the blood of harbour seal pups in rehab have been measured to investigate possible stress factors specific to rehabilitation. Blood samples are taken from the extradural intervertebral vein following restraint of the pup (e.g. Gulland et al., 1999; Trumble et al, 2013; Dailey et al., 2020). However, blood sampling can measure the GC for just that moment in time, with the pup restraint possibly affecting circulating CL levels (e.g. Engelhard et al., 2002). From these studies thus far there is no consensus on whether or how circulating CL levels may reflect orphan pups' stress level during rehab. Gulland et al. (1999) found that baseline plasma CL levels decreased with increasing time in rehab up to eight weeks, and the authors suggested that the stress of the captive environment was reduced as the pups gradually adapted. However, Trumble et al., (2013) found that plasma CL levels *increased* in pups while they were being tube-fed formula up to about 8 weeks, stabilising at a lower level thereafter, and the authors suggested that the pups might have a stress response to the tube-feeding procedure. Dailey et al. (2020) found that plasma CL concentrations were highly variable and showed no trends with increasing time in rehab.

Urine samples may also be taken to assess circulating CL levels in seals (Constable et al., 2006). Because protein-bound steroids, present in blood, are excluded from the kidney filtrate, only free hormone is excreted in the urine. Urinary free CL concentration correlates well with the concentration of plasma free CL (Lupo et al., 2018) and therefore reflects concentrations of the free hormone in blood (Cook, 2012). Urinary GCs collect in the bladder since the previous voiding, and therefore reflect the stress response over a longer period than concentrations in a blood sample. Urine samples can also be collected from seal pups without requiring additional handling or restraint. A preliminary study of urinary CL in harbour seal pups in rehabilitation (Wilson et al., 2015) provided a first report of a range of urinary concentrations found in pups of this species.

Although there have been several studies of plasma CL levels in harbour seal pups in the field and in rehabilitation (Gulland et al., 1999; Trumble et al., 2013; Di Poi et al., 2015; Dailey et al., 2020), to date there have been no published studies in pinnipeds of the other endogenous GCs that are closely related to CL, i.e. cortisone (CN), prednisolone (PL) and prednisone (PN). However, naturally occurring concentrations of all of these GC hormones have been investigated in cattle (Pompa et al., 2011; Vincenti et al, 2012; Bertocchi et al., 2013; Ferranti et al., 2013; Chiesa et al., 2017) and equines (Fidani et al., 2012), although only small amounts of endogenous PL and PN relative to cortisol have so far been found in cattle, and have been thought to occur in conditions of extreme stress, such as transport and slaughterhouse.

The chemical structure of these four GCs is very similar. (e.g. Arioli et al., 2012; Vincenti et al., 2012; de Clercq et al., 2013; Chiesa et al., 2017; Supplementary file S1, Fig. S1.1). The oxidation of the HO group in the 3rd carbon ring of the biologically active CL and PL yields the inactive CN and PN respectively; these conversions occur endogenously to protect the tissues from excessive CL and PL action. However, the reverse conversion can occur when further CL or PL activity is required (McWhinney et al., 2010; Chiesa et al., 2017; Timmermans, 2019). Moreover, endogenous CL may theoretically be converted to PL by the introduction of a double bond between two carbon atoms in the 1st carbon ring (Fig.S1.1).

Because these four endogenous GCs appear to be interconnected within a dynamic system, it would seem desirable to measure all four simultaneously, as has been done in the cattle studies, rather

than CL alone. In the present study we measure concentrations of all four GCs in the urine of harbour seal pups during rehab with the aim of investigating how different rehab environments and pup body mass changes might be reflected in concentrations of the four GCs. A relationship between pup body mass and plasma CL levels has been sought in earlier studies (Trumble et al., 2013; Dailey et al., 2020), but the potential stressor effects of other variables of the rehab environment, as they occur in different rehab centres, has not so far been investigated. We hypothesised that the major factors in rehab centres that deviate most strongly from the pups' natural condition (i.e. social isolation, no free water access, low body mass or rate of mass gain), would lead to relatively elevated levels of CL or PL and their inactive forms CN and PN.

2. ANIMALS AND METHODS

2.1 Pup background

This study focused on 32 harbour seal pups, *Phoca vitulina vitulina*, that had been admitted to four participating rehabilitation centres, three in Ireland and one in N. Germany, having been found alone stranded on the shoreline during the pup birthing season in three separate years (2014, 2015 and 2016). The criteria, normally applied by Centres I and II in north-east Ireland, for identifying a pup as being permanently separated from its mother were: *if* the pup was found alone, i.e. it had been left behind at haul-out site on ebb or low tide *or* was found >200m from a haul-out site *and* was estimated visually to be new-born or around birth weight. Pups were additionally assessed on their behaviour (agitated or lethargic) and appearance (abnormal posture), while some pups were monitored over several hours or more than a day, according to circumstance (see Wilson and Jones 2021 for detailed description). Similar criteria were used by Centre III in south-east Ireland and by professional wardens in N. Germany, who were responsible for taking pups to Centre IV.

The average mass of the 32 pups on admission to the rehab centres was 9.04 kg (Table 1; Supplementary files S2, Table S2.1 and S1, Fig. S1.2). Thirty of the 32 pups were at or below average birth mass for harbour seals of around 11kg (Bowen et al., 1994; Cottrell et al., 2002) and only two of the pups (both from N. Germany) were over that average, at 12.2 kg and 13.3 kg (Supplementary file

S2, Table S2.1). One pup died on rehab day 1 ("Polly", see Table S2.1), but to our knowledge all other pups in this study survived to release.

2.2 Pup rehabilitation centres

There were differences in pup care procedures, captive environments, feeding methods, dietary constituents, and growth rates in the four centres (Table 1) during the sampling period. The care and conditions of pups at each centre could not, for ethical reasons, be controlled or manipulated for research purposes.

In Centres I and III the pups were usually in pairs or groups (i.e. kept socially) with free access to water, which was sufficient for the pups to submerge while socialising or sleeping (Fig. 1). In Centre II the pups were kept alone in dry pens, and in centre IV the pups were either alone or paired and in dry pens (Fig. 1; Table 1). These factors, i.e. access to water and social group or alone, together with pup sex and pup ID, were included in the analysis of GC concentrations. Other potentially confounding factors which differed between some centres were not measured in this study. These included potential disturbance from visitors viewing the outside of the pup pens at Centres II and IV (see Table 1), and the pups' diet and feeding method.

The feeding method and diet for individual pups was not changed during the sampling period. Liquid diet (milk matrix or herring soup; see Table I) was fed via a soft, flexible silicon tube (stomach gavage); pups in Centres I and II were fed only in this way throughout the sampling period (Supplementary file S2, Table S2.1). Handfeeding with solid fish for three pups in centre III and two pups in Centre IV (Table S2.1) was done in water; pups in IV were placed in the filled bath for fish feeding (Jeffers, 2019).

The rehab day and the estimated pup mass on sampling day were the factors against which the urinary GC concentrations were measured. Pups were weighed routinely by centre personnel on alternate days at Centre I and approximately once per week at the other centres. Average growth rates (kg/day) during the sampling period were calculated from the pups' mass at the beginning and end of the sampling period. Estimated pup mass on the sampling day was calculated from the pup mass on the nearest weighing dates before and after sampling and average growth rate during that period, e.g.

198 if a pup weighed 11.3 kg on one Saturday, 12.0 kg on the following Saturday, the average mass gain
199 that week would be 0.1kg/day. If the pup was sampled, for example, on the Wednesday between these
200 two Saturdays, the pup's mass on the sampling day would be estimated as 11.3 kg + 0.4 kg, = 11.7 kg.

201 Table 1. Number of pups, number of samples taken and rehab day (d) range of sampling at each of
202 four rehab centres, with summary of rehab environment and average growth rate (kg/day) at each
203 centre.

204

Centre	**I**	**II**	**III**	**IV**
No. pups sampled	4	7	13	6
Sex M/F	1M/3F	5M/2F	6M/7F	4M/2F d7–
Range of pup rehab days (d) during sampling period	d0–48	d0–12	d0–17	47
No. samples d 0–1 (with pup ID)	1	4	6	0
No. samples d2– (with pup ID)	24	8	31	15
No. samples (d2– (no pup ID	6	0	1	0
Pup average mass (kg) on entry to centre (range)	7.83 (7.1–8.3)	8.13 (6.5–10.3)	10.23 (6.9–13.1)	8.40 (7.0–10.2)
Est. Pup average mass (kg) on sample day (range)	12.5 (9.3–21.5)	9.4 (7.9–11.8)	11.6 (7.1–14.9)	11.4 (9.2–14.9)
Pups' food	*Pet Ag* milk matrix 30/55 + fish + mixed fish oil + digestive enzymes	herring soup + salmon oil	herring soup or whole herring	herring soup + salmon oil or whole herring
Feeding method	Tube feeding	Tube feeding	Tube feeding or hand-feeding fish	Tube feeding or hand-feeding fish
Growth rate av. (kg/d) during sampling period, d2– (range) (SD)	0.29 (0.27–0.30) 0.01	0.21 (0.1–0.26) 0.06	0.15 (0.01–0.26) 0.07	0.07 (-0.1–0.19) 0.10
Social groups at each centre	paired	alone	Usually in group, sometimes alone or paired at first	Alone at first, then paired
Free water access	Yes	No	Yes	No
Pup pens open to viewing by public	No	Yes	No	Yes

205

206 **2.3 Urine sampling**

207 Urine samples were collected opportunistically if the pups urinated spontaneously while
208 awakening and being handled during routine feeding, weighing and general care; pups were not
209 additionally disturbed, restrained or handled for the purpose of obtaining samples. The authors (SW
210 and SV) collected samples at Centre I in 2014 and 2016 whenever possible during the whole
211 rehabilitation period. Samples were collected during a week's visit by SV in 2015 to each of Centres
212 III and IV (Table 1); centre personnel collected samples at Centre II in 2014 and 2015 when they were
213 able to do so. Individual pup data and sampling from each pup are given in Supplementary file S2,
214 Table S2.1.

215 Sample collection was usually with a syringe from the ground beside the pup or directly from
216 the stream of urine, although one sample from Centre I was collected on a 'puppy pad', with plastic-
217 backed paper tissue layers. Samples were quickly transferred to standard plastic sample pots with a
218 label detailing the source pup, date and collection time and were then immediately transferred to a
219 freezer (at least -18°C) and stored until analysis. Only samples with at least 10 ml were used in the
220 analyses. Seven samples analysed were collected from a pair of pups (in Centres I and III), but the
221 donor pup was not identified (Table 1; Supplementary file S2, Table S2.1). These samples were
222 included in analyses of the overall range of GC concentrations and differences between centres in the
223 range of GC concentrations but were excluded from subsequent analyses. Since pups' GC
224 concentrations when they first arrive at the centre are likely to reflect their stranding conditions,
225 handling and transport to the centre, only samples from day 2 onwards for which the individual pup
226 from which the sample was taken was known were used in further analyses.

227 It has been suggested, in bovines, that neo-formation of the GCs PL and PN may occur due to
228 microbial action after urine collection if the samples are kept at a high temperature (e.g. 37°), or at
229 room temperature for more than a week, or if there is contamination (e.g. from faeces) (Arioli et al.
230 2012; De Clercq et al. 2013). The pups were not in a sterile environment, however, so we were
231 therefore careful to ensure that the samples used for analysis were free from visible faecal or other
232 contamination and were not subjected to inappropriate storage.

A study of plasma CL levels in captive harbour seals reported a diurnal pattern, peaking at 01:00 and waning around 13:00 (Gardiner and Hall, 1997). In this study, we therefore compared results between samples taken at different times of day to confirm that the time of day of our opportunistic sampling did not significantly affect the results. Samples from pups from rehab day 2 onwards (n=85) were assigned to time categories: 0700–12:59; 13:00–18:59; 19:00–00:59. No samples were collected during the overnight period (01:00–06:59).

2.4 Analysis of urinary glucocorticoid concentrations

2.4.1. *Simultaneous analysis for multiple steroids by UPLC/MS-MS.*

If the concentrations of several steroids in a sample are required, either separate kits for each steroid must be used (requiring splitting of the sample), or the steroids need to be separated by chromatography. Ultra-Performance Liquid Chromatography (UPLC) coupled with tandem mass spectrometry (UPLC–MS/MS) can quantify either one or multiple steroids at wide-ranging concentrations, while avoiding the use of antibody reagents and cross-reactivity issues (Koren et al., 2012; Antonelli et al., 2014). UPLC-MS/MS has been proven suitable for enabling sensitive detection of GCs in urine (e.g. McWhinney et al. 2010; Arioli et al., 2012; De Clercq, 2013; Fidani et al., 2013) and has been recommended for routine analysis of individual GCs from any tissue (McWhinney et al., 2010). The detailed UPLC-MS/MS methodology for analysing steroid concentrations has been described by McWhinney et al. (2010), De Clercq et al. (2013) and reviewed by Hawley and Keevil (2016).

2.4.2. Materials and methods for UPLC-MS/MS quantification of CL, CN., PL and PN concentrations in seal pup urine samples

For the present study, 96 samples, collected from Centres I, II, III, and IV in 2014–16, were analysed for four glucocorticoids (GCs), namely cortisol (CL), cortisone (CN), prednisolone (PL) and prednisone (PN) by UPLC-MS/MS at the Chemical and Immunodiagnostic Sciences Branch, Veterinary Sciences Division, Agri-Food and Biosciences Institute (AFBI), Belfast, N. Ireland. This laboratory routinely carries out this analysis on the liver of farm animals (cattle, sheep, and pigs) as

part of the routine statutory Veterinary Drug Residue testing programme, in accordance with EU guidelines (2002/657/EC).

For the seal pup urine analysis for these GCs, analytic standard powders for CL, CN, PL and PN and isotopically labelled internal standard powders for d6-prednisolone and d4-cortisol were used to prepare stock standard and internal stock standard solutions in methanol. These standards were used to prepare matrix matched calibration curves across the range 0.5–10 µg/kg (Supplementary file S3, Fig S3.1). Bovine urine was used as the control negative and for the matrix standard curve. On the day of analysis, the seal pup urine samples and control negatives were defrosted at room temperature and thoroughly mixed. Seal urine (5 ml) was pipetted into a centrifuge tube, while the bovine urine samples were set up for negative and positive controls and for matrix standards. All seal and bovine urine negative and positive control samples were spiked with the mixed internal standard solutions (Fig S3.2). Methanol and hydrochloric acid were added to each tube (samples, controls, and matrix standards), which was then vortexed, incubated in a 50° water bath, cooled to room temperature and centrifuged.

Solid phase extraction (SPE) was performed using Waters Oasis HLB SPE cartridges placed on a Vac-Elute manifold. Each cartridge was conditioned with methanol and water, and the sample extract was then applied and allowed to pass through under gravity. The cartridges were then washed with 0.02M sodium hydroxide/methanol (60/40 v/v), then water, dried under vacuum, and eluted with methanol into glass tubes. The matrix standard tubes were then spiked with the mixed standards and internal standards. All sample, control and matrix standard tubes were then evaporated under nitrogen, reconstituted in methanol, vortexed and water added. Extracts were transferred to 2 ml vials prior to UPLC-MS/MS analysis.

UPLC separations were carried out using an Agilent 1290 infinity LC system. Reverse phase gradient separation was achieved on an Agilent Eclipse Plus chromatographic column (Agilent Technologies, Inc. Santa Clara, USA).

An Agilent AG6490 triple-quadrupole connected to the UPLC system via an electrospray ionisation interface (ESI) source, was used for mass spectrometric analysis. The results (Fig S3.3)

were processed using Mass-Hunter quantitative software. Internal standard correction was applied for PN and PL (d6-prednisolone), and CN and CL (d4-cortisol).

Standard UPLC MS/MS method validation at this laboratory has been carried out for quantification of CL, CN, PL and PN in bovine, porcine and ovine liver. Three sets of six replicate blended negative liver samples were spiked at 1.0 ppb, 2.0 ppb and 3.0 ppb, then extracted and analysed against a matrix spiked calibration curve over the range 0.5–10 µg/kg. The extraction was repeated on three separate days and, after ensuring all relevant identification criteria were met, the collated data were used to determine recovery, within-day, between-day, intermediate precision, and – by using the intercept of the calibration curve method – the parameters decision limit and the detection capability, with the alpha-error set at 1%. However, specific UPLC-MS/MS method re-validation using harbour seal pup urine at this laboratory was not possible, due to cost and resource limitations. Full details of the UPLC-MS/MS reagents, equipment, and procedures for the pup urine analysis are therefore given in Supplementary file S4 to allow for comparison with any future analysis of seal urine by UPLC-MS/MS.

2.4.3. Measurement of the urinary creatinine (Cr) concentration of the samples and quality control

Creatinine (Cr) is commonly used as a correction factor to control for different urinary volumes as it is eliminated through urine at a constant rate and is reflective of excretion rates (Novak et al. 2013). Following the UPLC-MS/MS analysis of each sample, the remaining sample was analysed for Cr content (µmol/L). The urinary GC concentrations were then expressed as a ratio to the Cr concentration (ng GC/mg Cr) in each sample. Cr concentrations were analysed using a colorimetric method modified from the Jaffe reaction, in which Cr in an alkaline solution reacts with picric acid to form a coloured complex (Taussky and Kurrzmann 1954).

2.5 Statistical analyses

2.5.1. Non-parametric tests

The concentrations of all four GCs for all samples were not normally distributed. The correlations between concentrations of the four GCs (CL, CN, PL and PN) in all samples were therefore assessed using non-parametric Spearman correlation.

Because most pups entering rehab are likely to have suffered severe stress due to a combination of loss of mother, stranding, nutritional deficit, possible bacterial infection, transport to the centre and entering an unnatural environment, their stress levels due to some or all of these factors would be reflected in GC concentrations in samples collected in days 0–1 in rehab. concentrations in samples were therefore compared between pups in rehab days 0–1 (n=11) and day 2 onwards for samples from pups with known identity (n=78) using the Mann-Whitney U test. Because this study is investigating the effects of the rehab environment on GC concentrations rather than the effects of traumas and nutritional stress experienced by the pups prior to their arrival at the rehab centres, the continuing analyses omitted samples taken on days 0–1.

Either a single or an individual's median value for each GC was used to explore potential correlations between an individual's growth rate during the sample period. We therefore obtained as many samples as possible from both different and the same pups, with the median values of GC concentrations in several samples from an individual being the optimal measure. A median value rather than the mean was used to avoid undue skewing of the results by occasional high or low concentrations. Log transformations were used for CL and CN concentrations to include outliers in Figs. 3 and 4; Supplementary file S1.3).

To compare CL and PN concentrations among different time periods of the day at each of the centres, Kruskal-Wallis 3-sample tests and Mann-Whitney 2-sample tests were used. Kruskal-Wallis tests were also used to determine whether GC concentrations significantly differed among the four centres, as well as to determine whether pup growth rate differed among the four centres.

To investigate whether low body mass was associated with relatively high GCs, we compared GC concentrations in pups less than 11 kg (approx. average birth mass) with pups of mass ≥ 11 kg on the dates of urine sampling using the Mann-Whitney U test.

2.5.2. Linear mixed effects models

337 To determine which factors best explained GC concentrations in harbour seal pup urine, we ran
338 linear mixed effects models for each GC in R (version 3.6.0) (R Core Team 2020), using the R
339 package 'nlme' (Pinheiro et al. 2019). We used all the data from samples from pups with known ID
340 on rehab day 2 onwards (n=78), and we transformed the GC concentrations to achieve normal
341 distributions of data: CL and CN concentrations were log-transformed, and PL and PN concentrations
342 were square root transformed (denoted by $\sqrt{}$).

343 Predictor variables in candidate models included sex, estimated mass on sample day, access to
344 water, housing with or without other pups, as well as the interactions among these factors. These
345 factors as they occurred in the pups from which samples were taken were: samples from pups in social
346 group with free water access – n=47/54 (all in Centres I and III); pups in social group without water –
347 n=8/24 (all in Centre IV); pups alone with water – n=7/54 (all in Centre III); pups alone without water
348 – n=16/24 (in Centres II and IV).

349 We included individual pup as a random effect to account for variability among individuals. We
350 selected the best-fit model for each GC according to the lowest AIC. This not only minimises
351 information loss from the model, but also minimises the risk of selecting a bad model (e.g. Nayak,
352 2020). We could not include rehab day in this model as it was highly correlated with pup mass on
353 sample day (correlation = 0.81, variance inflation factor = 5.8). However, we also separately ran the
354 models with rehab day instead of pup mass.

355 **3. RESULTS**

356 **3.1 Ranges, medians, and correlation matrix of GC concentrations**

357 The ranges, mean and median values for the Cr and the four GCs concentrations in all the
358 samples analysed are given in Table 2 and Supplementary file S1, Fig. S1.3. The median values for
359 CN were about ten times higher than for CL, PN and PL. However, CL values were highly variable,
360 with three of the samples (all taken between rehab days 0–2) having values >2,000 ng/mg Cr. There
361 were six samples with undetectable concentrations (i.e. zero ng/mg) of both CL and PL, one sample

with zero PL and relatively low CL (6.0 ng/mg Cr), and one sample with zero CL and PN; however, six of these nine samples had relatively low Cr concentrations (ranging from 115–683 µM/L).

Table 2. Overall concentrations of Creatinine (Cr), Cortisol (CL), Cortisone (CN), Prednisolone (PL) and Prednisone (PN) in all 96 urine samples from all four centres

	Cr	**CL**	**CN**	**PL**	**PN**
	µM/L	ng/mg Cr			
Range	115–15,576	0–8,062	20.9–4,853	0–236.6	0–142.5
Mean	2,988	211.4	526	39.9	36.6
SD	2809	913	612	43.2	26.5
Median	2,155	37.0	390	31.6	31.1

The Spearman correlation matrix for concentrations of all four GCs in all samples indicates significant ($P < 0.001$) positive monotonal relationships. The strongest correlation was between CL and PL (rho = 0.75), while the weakest was between CN and PL (rho = 0.36), with the other correlations ranging between 0.50 to 0.59. Although CL and PL correlations were highly correlated, PL concentrations did not reach high concentrations in the same order of magnitude of the few extreme high CL concentrations (Table 2).

The ratios of median concentrations of CL to PL and CL to PN were close to parity (1.17:1 and 1.19:1 respectively), while the ratios of the mean concentrations were higher (5.3:1 for CL:PL and 5.8:1 for CL:PN). The ratios of median and mean concentrations of CL to CN were much lower, at only 0.1:1 and 0.4:1 respectively (Table 2).

3.2 Comparison of GC concentrations of newly admitted pups with those from rehab day 2 onwards

A total of 11 samples were obtained from seven newly admitted pups (days 0-1; Table 1; Supplementary file S2, Table S2.1). The mean and median CL concentrations were 1128 ng/mg Cr (S.D. 2552) and 124 ng/mg Cr, with CL concentrations for two pups on day 0 (Ula, from Centre I, suffering pneumonia at rescue, and Schultze from Centre III; Table S2.1) of 8062 and 4029 mg/mg Cr respectively, immediately after arrival at the centre (Table S2.2). The concentrations of CL, PN and PL were significantly higher in days 0-1 (n=11) than day 2 onwards (n=78); (2-tailed Mann-Whitney

U tests; 2-tailed, U expected = 429; CL - U=618P= 0.019; PL – U=641, P=0.008; PN – U=632, P=0.010), although CN concentrations did not differ significantly U=449, P=0.811). Only five pups were sampled at least once in both days 0–1 and day 2 onward; the GC concentrations for days 0–1 were higher than those for day 2 onward in 70% of these pups' samples (Supplementary file S2, Table S2.2).

3.3 No significant effect of time of day of sampling on GC concentrations

The distribution of the opportunistic urine sampling from rehab day 2 onwards indicated that most of the samples were taken in the morning (time period A, 0:700–12:59, n=26) and afternoon (time period B, 13:00–18:59, n=41), with fewer in the evening (time period C, 19:00–00:59, n=18). However, no significant differences between the time periods in GC concentrations were detected (Supplementary file S2, Table S2.3).

3.4 Differences in pup GC concentrations between the five centres

Concentrations of all four GCs (median or single value for each pup) differed significantly between centres (Kruskal-Wallis test $P < 0.0001$ for each GC). Centre III samples notably had the highest CL, CN and PL concentrations, while Centre II had the lowest CL concentrations and Centre I had the lowest PN concentrations. The Dunn's post-hoc test indicated where there were significant differences ($P < 0.05$) between each of the centres as indicated by letters a, b, c, and d (Fig. 2).

3.5 Pup growth rates, mass, and correlations with GC concentrations

3.5.1. Growth rates

There was a significant difference in pup growth rates between the four centres (Table 1; Kruskal-Wallis test, $P <0.001$; the differences between Centres I and III, I and IV, and II and IV were significant at the 5% level by the Dunn's post-hoc test). There was a significant negative correlation (Spearman's rho) between individual growth rate and concentrations of both PL and PN ($P = 0.001$ and 0.010 for PL and PN respectively; Fig. 3), but the correlation was not significant at the 5% level for CL and CN.

412 *3.5.2. GC concentrations in pups less than average birth mass vs. pups of birth mass or more*

413 A comparison of the range of concentrations of each GC for pups <11 kg (i.e. less than
414 average healthy birth mass) and pups ≥ 11kg suggests that the range of concentrations was generally
415 greater for the smaller pups (Supplementary file S1, Fig. S1.4). The PN concentrations were
416 significantly higher for pups <11 kg (n=27) than for pups ≥ 11kg (n=51) (U= 935; Expected 688.5;
417 P=0.009 Mann-Whitney U test) but there was no significant difference for the other GCs.

418 **3.6 Predictor variables influencing urinary GC concentrations**

419 Linear mixed effects models indicated that neither pup sex nor social group (alone or social)
420 had a significant influence on concentrations of any of the urinary GCs. The results of both the pup
421 mass and rehab day models are given in Table 3, and Fig. 4 shows the model for pup mass.

422 The predictor variables that best explained CL and CN concentrations were *either* pup mass
423 on sampling day *or* rehab day when sampled, whether the pup had access to water, and the interaction
424 between these factors (Table 3; Fig 4 a, b). As pup mass or rehab day increased, the model indicated
425 CL and CN concentrations declining for pups with water access but no change or slightly increasing
426 for pups without water access (Fig. 4 a, b).

427 The predictor variable that best explained PL and PN concentrations was pup mass on
428 sampling day (Table 3; Fig. 4 c, d). The model indicated PL concentrations declining for pups with or
429 without water access (Fig. 4c). Pup mass alone was the predictor variable that best explained PN
430 concentrations, with concentrations declining significantly as pups gained mass (Fig. 4d). However,
431 there was no significant interaction between water access and pup mass for PL and PN concentrations.
432 However, PL and PN concentrations declined with rehab day for pups with water access, and there
433 was significant interaction between water access and rehab day for both PL and PN concentrations
434 (Table 3).

435 For samples from pups with free water access, there was a significant negative correlation
436 between CL and PL and both pup mass (R^2 = 0.269 and 0.241 respectively) and rehab day (R^2 = 0.314
437 and 0.383), whereas for pups without water there was no significant decline between CL or PL and

either pup mass (R^2 = 0.060 and 0.024 respectively) or rehab day (R^2 = 0.052 and 0.008 respectively, and the correlation matrix was slightly positive (Table 4).

The correlations were examined separately for the four pups in Centre I (all pups were paired with water access) for which there were more data (n=24) over a greater period of mass gain and rehab day than at the other centres, and pup mass and rehab day were almost perfectly correlated (rho = 0.942; Table 4). For all four Centre I pups combined, the decline in GC concentrations with pup mass and rehab day was significant for CN, PL and PN, but not for CL (Table 4). When the data were examined for Centre I pup "Ula" alone (n=9), pup mass and rehab day were perfectly correlated (rho = 1.000); the decline in GCs with pup mass or rehab day was non-significant, but R^2 was much higher for PL and PN (R^2 = O.326 and 0.261 respectively) than for CL and CN (R^2 = 0.003 and 0.008 respectively; Table 4).

450 Table 3. Results of best-fit linear mixed models explaining the change in GC concentrations
451 in pup urine (cortisol (CL), cortisone (CN), prednisolone (PL), and prednisone (PN).

	PUP MASS & WATER ACCESS				REHAB DAY & WATER ACCESS		
BEST-FIT MODELS	**% variance**	**SE**	**P-value**		**% variance**	**SE**	**P value**
Log(CL + 1) df =45	**40.4%**				**37.1%**		
Water Access +		1.91	**0.001**	Water access +		0.55	**<0.001**
Pup mass +	30.3	0.16	0.165	Rehab day +	31.3	0.03	0.363
Interaction		0.17	**0.013**	Interaction		0.03	**0.008**
Pup ID	10.1			Pup ID	5.8		
Log(CN + 1) df=45	**45.4%**				**43.0%**		
Water Access +		0.96	**0.006**	Water access +		0.27	**0.003**
Pup Mass +	38.7	0.08	0.601	Rehab day +	40.3	0.01	0.648
Interaction		0.09	**0.016**	Interaction		0.02	**<0.001**
Pup ID	6.7			Pup ID	2.8		
√(PL) df=46	**48.7%**				**49.4%**		
Water Access +	8.7	0.73	0.059	Water access +		0.92	**0.015**
Pup Mass		0.09	**0.007**	Rehab day +	19.9	0.04	0.490
				Interaction		0.05	**0.026**
Pup ID	40.0			Pup ID	29.6		
√(PN) df=47	**43.9%**				**43.3%**		
				Water access +		0.68	0.094
Pup Mass	24.6	0.07	**<0.001**	Rehab day +	34.2	0.03	0.272
				Interaction		0.04	**0.001**
Pup ID	19.3			Pup ID	9.1		

452

453

Table 4. Pearson correlation matrix (R), correlation coefficient R^2 and P value for transformed GC values showing the change in each GC as pups gain mass or with increasing rehab day. Figures in bold are significant at the 5% level. Values with P < 0.05 are shown in bold type.

	Pup mass			**Rehab day**		
	R	R^2	P value	R	R^2	P value
ALL SAMPLES (n=78)						
mass vs rehab day Spearman rho = **0.388**						
Log CL+1	-0.139	0.019	0.226	**-0.280**	**0.078**	**0.013**
Log CN+1	**-0.531**	**0.282**	**<0.0001**	**-0.527**	**0.278**	**<0.0001**
√PL	**-0.229**	**0.052**	**0.044**	**-0.344**	**0.119**	**0.002**
√PN	**-0.503**	**0.253**	**<0.0001**	**-0.432**	**0.187**	**<0.0001**
SAMPLES - FREE WATER ACCESS (n=54)						
mass vs rehab day Spearman rho = **0.466**						
Log CL+1	**-0.519**	**0.269**	**<0.0001**	**-0.561**	**0.314**	**<0.0001**
Log CN+1	**-0.694**	**0.481**	**<0.0001**	**-0.703**	**0.495**	**<0.0001**
√PL	**-0.491**	**0.241**	**0.000**	**-0.619**	**0.383**	**<0.0001**
√PN	**-0.671**	**0.451**	**<0.0001**	**-0.728**	**0.529**	**<0.0001**
SAMPLES - WITHOUT WATER ACCESS (n=24)						
mass vs rehab day Spearman rho = **0.559**						
Log CL+1	0.245	0.060	0.249	0.228	0.052	0.283
Log CN+1	0.092	0.009	0.668	0.124	0.015	0.564
√PL	0.155	0.024	0.470	0.088	0.008	0.682
√PN	0.027	0.001	0.899	0.192	0.037	0.369
CENTRE I (4 pups) – FREE WATER ACCESS (n=24)						
mass vs rehab day Spearman rho = **0.942**						
Log CL+1	-0.388	0.151	0.061	-0.278	0.189	0.077
Log CN+1	**-0.592**	**0.350**	**0.002**	**-0.519**	**0.270**	**0.009**
√PL	**-0.474**	**0.225**	**0.019**	**-0.578**	**0.334**	**0.003**
√PN	**-0.622**	**0.387**	**0.001**	**-0.645**	**0.416**	**0.001**
CENTRE I (pup "Ula") – FREE WATER ACCESS (n=9)						
mass vs rehab day Spearman rho = **1.000**						
Log CL+1	-0.053	0.003	0.893	-0.051	0.003	0.896
Log CN+1	0.092	0.008	0.814	0.093	0.009	0.813
√PL	-0.571	0.326	0.108	-0.572	0.327	0.107
√PN	-0.511	0.261	0.160	-0.510	0.260	0.160

4. DISCUSSION

4.1 Endogenous PL and PN in the harbour seal pups of this study and in other species

The present study is the first (to our knowledge) to measure CL, CN, PL and PN simultaneously in phocid seal samples. This necessitated using ultra-performance liquid chromatography to separate the compounds, and tandem mass spectrometry to quantify the concentrations of each. We found detectable amounts of PL in almost all (90/96) of the seal pup samples analysed (median 30 ng/mg Cr; range 7–139 ng/mg Cr in the 90 samples with detectable concentrations).

So far, detectable levels of endogenous urinary PL have only been reported in very low concentrations in the calves of domestic cattle (CL:PL ratio of 64:1, Famele et al., 2015 or young cows (CL:PL ratio of 25:1, Chiesa et al., 2017), while PN has only been detected in samples from the slaughterhouse with the highest values of CL, CN and PL (Famele et al., 2015). However, in the seal pups the ratios of median CL:PL and CL:PN levels were both close to parity (~1.2:1). There was no indication that the PL levels in our study were the result of any extreme stressors, as has appeared to be the case in some cattle studies (e.g. Pompa et al., 2011; Ferranti et al., 2011; Bertocchi et al., 2013).

4.2 Urinary GC measurement in harbour seals and other species

The measured concentrations of urinary corticosteroids should quantitatively reflect adrenocortical activity (Cook, 2012). This condition is fulfilled by our study, because the concentrations of CL (and presumably also CN, PL and PN) in the urine are a function and direct reflection of adrenocortical output and hydration levels of the sample. The hydration levels in each urine sample in our study were corrected for by expressing the measurement as a ratio to urinary creatinine (Cook, 2012).

Our sampling method would not have caused additional stress to the pups beyond feeding and care routines, and the results are a measure of adrenocortical output over a period of few hours preceding the urine voiding. This may therefore give a more representative picture of ongoing GC levels than the more usual method of blood sampling from the extradural intervertebral vein following restraint of the pup.

Previous studies of urinary CL concentrations in other mammal species have suggested that a rise in urinary CL is likely to occur only following a severe stressor, such as cows being isolated from their herd (Higashiyama et al., 2009), anaesthesia of chimpanzees, *Pan troglodytes* (Anestis, 2009), or elevated corticosterone in laboratory albino mice, *Mus musculus*, when a submissive male was paired with a dominant and aggressive male (Fitchett et al., 2005). However, the pups in our study were not – so far as we were aware – subject to any known or measurable sudden stressor in the hours before the sample was collected.

4.3 Potentially reversible conversion between CL and CN, PL and PN, and CL and PL

. Naturally occurring enzymes known as 11β-HSDs in the tissues catalyse the conversion of CL to CN to protect tissues from excess CL, and CN is also converted back to CL to facilitate glucogenesis in tissues (see Supplementary file S1, Fig. S1.1) (e.g. Chiesa et al., 2017; Timmermans et al., 2019); conversion between PL and PN may also be subject to processing by the same enzyme (Timmermans et al., 2019).

The ratio of urinary CL:CN is considered to be a sensitive index of the conversion back to CL (McWhinney et al., 2010) and hence an index of the physiological need for CL activity. By the same argument, the ratio of urinary PL:PN should be an index of the physiological need for PL activity. Urinary CN has been reported to occur in similar urinary concentrations to CL in cattle (e.g. Keenan, 2015; Chiesa et al., 2017), but occurred in the seal pups in considerably greater concentrations, with mean and median CL:CN ratios of only 0.4:1 and 0.1:1 respectively. The low CL:CN ratio in our seal pups suggests the rate of conversion from CN back to CL is low. This may be because the pups in our four centres were believed to be in relatively stable and non-threatening environments. Our study found that CL levels did not decrease but apparently increased slightly for the 24/78 (31%) samples from pups without water access, but most of the pups in the study (69%) did have water access, and their CL levels declined significantly with time. This might be a reason why there may have been little physiological need for CN to CL conversion in most of the pups in the study. By contrast, the PL:PN ratio in our seal pups was close to parity, suggesting the likelihood that the inactive PN is frequently recycled back to PL as needed. Many of the pups in the study had either a low growth rate or low body mass at the time of sampling, which may have resulted in the need for glucogenesis to compensate for the nutritional deficit.

This still begs the question of why PL and CL might respond differentially to a stressor, or why CL might be transformed into PL to respond specifically to a chronic nutritional deficit. Redirection of the CL to CN transformation towards PL in some metabolic conditions has been suggested in cattle (Chiesa et al., 2017); these authors suggested that a decline in the CL:CN ratio is probably due to a rise in PL inhibiting the 11β-HSD enzyme activity normally converting CN back to CL. Possibly this could partly explain the low CL:CN ratio in our samples, coupled with the elevated PL levels relative

to that found in cattle. Our finding of a high positive correlation between CL and PL concentrations is consistent with the finding of Vincenti et al. (2012) and Chiesa et al. (2017) in cattle.

There is a suggestion that PL may be a much stronger glucocorticoid receptor (GR) activator than CL (Timmermans et al., 2019); since subtle chemical differences between endogenous and synthetic GCs (e.g., the difference between CL and dexamethasone) may result in different 11β-HSD enzyme processing ability (Timmermans et al., 2019), it may also be possible that CL and PL may influence enzyme activity differently.

4.4 Relation between GC levels and pup mass or rehab day

Although there was significant association between all four GCs and both body mass and rehab day for all pups with water access (Table 4), our results from the four Centre I pups, for which there was most longitudinal data, and which all had water access, indicated that the correlation between CL or CN and pup mass or rehab day was relatively weak compared to the correlation between PL or PN and mass or rehab day.

The daily mass gain (DMG) of free-living harbour seal pups is ~0.5 kg per day, giving a DMG to birth mass (BM) ratio of ~6%, while the DMG of the grey seal, *Halichoerus grypus*, is ~1.6 –2.8 kg/day (Fedak and Anderson, 1982; Spotte and Stake, 1982; Mellish et al., 1999) and a DMG/BM ratio of ~10%. By contrast, the DMG of otariid seal species is generally much lower than phocid seals, e.g. Antarctic fur seal pups, *Arctocephalus tropicalis* gain 0.05 kg/day giving a DMG/BM of ~1% (Guinet and Georges, 2000), while the DMG in domestic cattle calves is ~0.6 kg/day, with a DMG/BM ratio similarly of ~1% (Jasper and Weary, 2002). We would hypothesise that grey seal pups in rehab gaining only ~0.2 kg/day (O'Hara, 2019) would have relatively high urinary PL and PN concentrations, whereas an otariid pup's growth rate in rehab would likely be similar to that of the free-living pup, and consequently have relatively low PL and PN levels compared to phocid pups in rehab.

Because PL and PN appeared to be particularly associated negatively with body mass and growth rate in our pups, we hypothesise that the relatively high concentrations of PL and PN in our rehab

pups compared to those in cattle may be related to their failure to achieve the very rapid growth rate of free-living suckling harbour seal pups and other phocid species compared to cattle.

When the species-typical growth rate does not happen, and pups remain for a long period with low body mass, we suggest compensatory glucogenesis may be stimulated by PL/PN activity.

However, the positive correlations we found between the four GCs suggest that CL and PL release may have a common trigger. In conditions of nutritional deficit, possibly CL might be readily converted to PL by the introduction of a double bond in the first carbon ring (see Supplementary file S1, Fig. S1.1). GCs regulate the synthesis and release of growth hormone (GH), which also mobilises energy stores by lipolysis of adipose tissue and is highest in stranded harbour seal pups when they first arrive in rehab thereafter declining (Richmond et al., 2008, 2010; Dailey et al., 2020). However, serum GH and CL concentrations did not appear to be closely related over the first 10 weeks in rehab (Dailey et al., 2020); a future study might investigate whether there is a closer link between PL-PN and GH.

4.5 Relationship between GC levels and access to water

Our study found that CL and CN levels did not decline with body mass and days in rehab for pups without free access to water (Centres II and IV), although it did decline for pups with water access (Centres I and III). The studies of Trumble et al. (2013) and Dailey et al. (2020) also found that CL concentrations did not decline during the first 8–10 weeks in rehab, and they suggested this might be due to the possible stress of being fed by stomach gavage. However, the status of water access by these pups was not stated, and if they were without water during this period, our study suggests this might have been an alternative explanation for the continued elevated levels of CL.

There is some evidence that the normal post-natal development of the oxygen storage capability of the red blood cells and apnoea is dependent on the harbour seal pup being able to submerge during the early post-natal period, as occurs in rehab centres permitting free water access (Thomas and Ono, 2015). All previous field behaviour observations (e.g. Venables and Venables, 1955; Wilson, 1974; Skinner, 2006; Wilson and Jones, 2018) and a recent behavioural study of paired pups in rehab (Alger and Wilson, submitted) strongly suggest that the behaviour and physiology of the precocial harbour seal neonate is adapted to an amphibious existence, involving prolonged

immersion in sea water. A comparable study of trends in concentrations of the same four GCs in grey seal pups in rehab with and without water would be interesting, since, unlike harbour seals, grey seal pups do not normally enter the water during their first six weeks (e.g. Kovacs, 1987). The lack of water for young grey seal pups should therefore not be a predicted stressor.

A preliminary study (unpublished data), recording heart rate in a pair of pups at Centre I, found a significant decrease in heart rate (HR) when the pups were in the bath (Figure 1) as opposed to when they were dry. HR is inversely correlated with heart rate variability (HRV) in humans and other mammals (Kazmi et al., 2016), and HRV is inversely correlated with CL levels in humans (Johnsen et al., 2012), and therefore a decreased HR is likely to be accompanied by lowered CL. A separate study in which HR was recorded at Centre III in pups up to 7–10 weeks in rehab found that HR declined during the rehab period (Fonfara et al., 2015) – also possibly implying a decline in CL in these pups at this centre, where pups have free water access. This would be consistent with our model showing a decline in urinary CL and CN levels with rehab day for pups with free water access. The relatively high GC levels in the Centre III pups in our study may therefore be at least partly because they were relatively recently arrived in rehab (Table 1).

However, a keystone part of our initial hypothesis, i.e. that housing orphan pups alone might lead to elevated CL levels, was not supported by our study – although the factors of social housing and water access were largely confounded in the study (46/54 samples from pups with water access were also housed with at least one other pup, whereas 16/24 samples from pups without water were alone). The benefits to a pup of having a companion pup in the absence of water are limited, because most social interaction and all play by harbour seal pups requires water (e.g. Wilson, 1974; Wilson and Kleiman, 1974; Alger and Wilson, submitted). A further factor to be considered is that orphan pups housed alone may be in the depressive state of separation, when CL levels may fall (Yusko et al., 2012). Thus CL levels in rehab pups may be the outcome of complex interplay between social housing, water access – and possibly also confounding factors (such as disturbance levels) that were not part of the present analysis.

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4.6 Differences in GC levels between centres, between individuals, and confounding factors

603 The comparison of concentrations of the four GCs between the four centres found significant
604 differences, particularly between Centres I and III and between II and III (Fig. 2). However, the
605 differences between Centres I and II for all four GCs were non-significant, despite these two centres
606 contrasting in water access, social group, and rehab day. Seemingly paradoxically, Centre I had overall
607 lower levels of all four GCs than Centre III, although both centres had free water access and social
608 group (for most of the Centre III pups). There may be multiple reasons for these results for centre
609 differences, including population of origin (Centre III pups were all from the German Wadden Sea,
610 while Centres I, II and IV pups were from the Irish Sea). Furthermore, the age of pups and stage of
611 rehabilitation was unequal in the centres, with Centre II and III pups being generally younger when
612 sampled than Centre I and IV pups (Table 1). This study was therefore not intended to investigate the
613 differences between centres *per se*, but instead to look at specified measurable conditions of the rehab
614 environment, days in rehab and pup mass as they occurred across the centres.
615 We found a high level of variation in GC concentrations in samples among pups from the same
616 centre and from the same pup. Individual differences in adrenocortical responsiveness may contribute
617 to the high level of variation among individual pups, as indicated by our results, particularly for PL,
618 for which individual ID contributed 30–40% of the variability (Table 3). Individual differences in
619 growth rate and body mass may have contributed to this individual variability, since these factors
620 were correlated with prednisolone concentrations (see Figs. 3 and 4, and Table 4).
621 High variation in serum CL (measured by radio immunoassay) was also reported by Dailey et al
622 (2020). Variation in sample concentrations in our study from the same pup may be due to the episodic
623 nature of GC production and release in a relatively stable rehab environment with no known sudden
624 stressor. For pups in Centres I and III, with one or more companions and free water access, the pups'
625 cycle of activity and rest in relation to the timing of sampling might be a factor. For pups in all centres
626 the cycle of feeding, cleaning, and weighing might also be factors, although urine collected in the
627 bladder over a period of a few hours would potentially dilute any sudden release of GCs due to a
628 stressor caused by human activity. We did not detect any relation between each six-hour timeframe of
629 sampling and GC concentrations, likely because any diurnal pattern of GC release was obscured by
630 the collection of urine in the bladder over a few hours.

631 The linear mixed effects models indicated that the rehab centre factors (pup mass or rehab day,
632 free water access and pup ID) accounted for 37–49% of the variation in the GC concentrations
633 amongst the samples (rehab day 2 onwards). This leaves about 50–60% of the variation unaccounted
634 for, likely by factors that could not be incorporated into the model or could not readily be quantified.
635 These factors could include varying number of pups in the enclosure in socially housed pups in Centre
636 III, health fluctuations, disturbance outside feeding times (such as for veterinary visits, and noise from
637 visitors to the centres).

638 Tube (stomach gavage) feeding has been suggested to cause a stress response with increased CL,
639 resulting in highly variable serum concentrations and to be possibly responsible for an increase in CL
640 concentrations during the first 8–10 weeks in rehab (Trumble et al., 2013; Dailey et al., 2020).
641 However, tube feeding was used exclusively throughout the sampling period in Centres I and II
642 (Supplementary file S2, Table S2.1), both of which had relatively low overall urinary CL.
643 Measurements of HR before, during and after tube-feeding at Centre I found no elevation of HR
644 during feeding, and pups at Centres I and III were observed to accept tube feeding willingly – although
645 a study measuring HR at Centre IV did record an increase in heart rate, with an average 4-min
646 recovery time (Jeffers, 2019).

647 Periodic acoustic disturbance by visitors to the outside of the pens in Centres II and IV was a
648 potential confounding factor with the variable of no free water access at these two centres. A study of
649 visitor numbers at a captive harbour seal facility found that more seals submerged underwater with
650 increasing visitor numbers (Stevens et al., 2013), and when free-ranging seals enter the water due to
651 human disturbance, their HR reduces (Karpovich et al., 2015). HR measured after tube-feeding at
652 Centre IV (in a separate study) found an additional recovery time of nearly 2 min during visitor
653 opening hours (Jeffers et al., 2019). Thus visitor presence outside the pup pens might compound the
654 stress of no water access since the pups cannot submerge in response to disturbance.
655

656 **4.7 Conclusions**

This is the first study to our knowledge to investigate concentrations of endogenous PL and PN as well as CL and CN in non-human mammals other than bovines and equines. Since only trace amounts have so far been documented in bovine urine, our finding of more substantial concentrations in the urine of almost all the 96 rehab seal pups of our study suggests that further investigations into the potential occurrence and role of PL in different species may be worthwhile.

The present study has provided some evidence suggesting a working hypothesis that PL and PN activity may be triggered by nutritional stress in harbour seal pups in rehab, whereas CL and CN activity may be increased by lack of water access or other stressors. This provides some support to our more general prediction, with which this study began, that GCs concentrations would be elevated where aspects of the rehab pups' environment deviate substantially from the most fundamental aspects of the natural environment of free-living pups of the same age.

Further studies would be advantageous to collect more data at all stages of the harbour seal pup rehab process, to support or modify our findings. A future study of these four GCs in rehab "orphan" pups of other phocid species with different natural developmental parameters – such as the grey seal – could help to elucidate the response of cortisol and prednisolone to different stressors.

6. Acknowledgements

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CONFLICTS OF INTEREST

The authors have no conflicts of interest

FUNDING

This study has not received any grant from any funding agency.

ETHICS STATEMENT

The urine sampling in this study was carried out with the permission and collaboration of each of the four seal centres. Three of the four centres (II, III and IV) were in the public domain and acting within relevant government guidelines. Centre I was a voluntary private centre operating with local government (N. Ireland) permission. The sampling did not cause additional disturbance to the pups beyond their routine care and handling and did not impact their welfare. No specific license for this study was therefore required.

697 **REFERENCES**

698 Alger, R., Wilson, S.C. Submitted. Behavior of harbor seal pups in rehabilitation with reference to
699 free-living pup behavior.

700 Anestis, S.F., 2007. Urinary cortisol responses to unusual events in captive chimpanzees (*Pan*
701 *troglodytes*). Stress 12, 49–57, doi: 10.1080/10253890802041308

702 Antonelli, G., Artusi, C., Marinova, M., Brugnolo, L., Zaninotto, M., Scaroni, C., Gatti, R., Mantero,
703 F., Plebani, M., 2014. Cortisol and cortisone ratio in urine: LC-MS/MS method validation and
704 preliminary clinical application. Clin. Chem. Lab. Med. 52, 213–220, DOI: 10.1515/cclm-2013-0471

705 Arioli, F., Casati, A., Fidani, M., Silvestri, M., Pompa, G., 2012. Prednisolone and prednisone neo-
706 formation in bovine urine after sampling. Animal 6, 1023–1029.
707 https://doi:10.1017/S1751731111002497

708 Atkinson, S., Crocker, D., Houser, D., Mashburn, K. 2015. Stress physiology in marine mammals:
709 how well do they fit the terrestrial model? J. Comp. Physiol. B 185(5), 463–486. DOI 10.1007/s00360-
710 015-0901-0

711 Bennett, K.A., Fedak, M.A., Moss, S.E.W., Pomeroy, P.P., Speakman, J.R., Hall, A.J. 2013. 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. J. Exp. Biol. 216, 984–991.
doi:10.1242/jeb.077438.

712 Bertocchi, L., Dusi, G., Ghidelli, V., Hathaway, T., Nassuato, C., Casati, A., Fidani, M., Pompa, G.,
713 Arioli, F. 2013. Investigation on the origin of prednisolone in urine and adrenal glands of cows. Food
714 Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment
715 30, 1055–1062.

716 Boness, D.J., Bowen, W.D., Oftedal, O.T., 1994. Evidence of a maternal foraging cycle resembling
717 that of otariid seals in a small phocid, the harbour seal. Behav. Ecol. Sociobiol. 34, 95–104.

718 Bowen, W.D., Oftedal, O.T., Boness, D.J., Iverson, S.J., 1994. The effect of maternal age and other
719 factors on birth mass in the harbour seal. Can. J. Zool. 72**,** 8–14.

720 Bowen W.D., Boness, D.J., Iverson, S.J., 1999. Diving behaviour of lactating harbour seals and their

pups during maternal foraging trips. Can. J. Zool. 77, 978–988.

Chiesa, L., Pavone. S., Pasquale. E., Pavlovic, R., Panseri, S., Valiani A., Arioli, F., Manuali E., 2017. Study on cortisol, cortisone and prednisolone presence in urine of Chianina cattle breed. J. Anim. Physiol. Anim. Nutr. (Berl) 101, 893–903.

Coe, C.L., Glass, J.C., Wiener, S.G., Levine, S., 1983. Behavioral, but not physiological, adaptation to repeated separation in mother and infant primates. Psychoneuroendocrinology 8, 401–409.

Constable, S., Parslow, A., Dutton, G., Rogers, T., Hogg, C., 2006. Urinary cortisol sampling: a non-invasive technique for examining cortisol concentrations in the Weddell seal, *Leptonychotes weddellii*. Zoo Biol. 25, 137–144.

Cook, N.J., 2012. Review: Minimally invasive sampling media and the measurement of corticosteroids as biomarkers of stress in animals. Can. J. Anim. Sci. 92, 227–259.

Cottrell, P.E., Jeffries, S., Beck, B., Ross, P.S., 2002. Growth and development in free-ranging harbour seal (*Phoca vitulina*) pups from southern British Columbia, Canada. Mar. Mam. Sci. 18, 721–733.

Dailey, R.E., Smith, K., Fontaine, C., Jia, Y., Avery, J.P., 2020. Response of metabolic hormones and blood metabolites to realimentation in rehabilitated harbor seal (*Phoca vitulina*) pups. J. Comp. Physiol. B 190, 629–640.

De Clercq, N., Van den Bussche, J., Croubels, S., De la Haut, P., Van Haecke, L., 2013. A validated analytical method to study the long-term stability of natural and synthetic glucocorticoids in livestock urine using ultra-high performance liquid chromatography coupled to Orbitrap-high resolution mass spectrometry. J. Chromatogr. A 1301, 111–121.

Di Poi, C., Atkinson, S., Hoover-Miller, A., Blundell, G., 2015. Maternal buffering of stress response in free-ranging Pacific harbour seal pups in Alaska. Mar. Mam. Sci. 31, 1098–1117.

Du Dot, T.J., Rosen, D.A.S., Richmond, J.P., Kitaysky, A.S., Zinn, S.A., Trites, A.W., 2009. Changes in glucocorticoids, IGF-I and thyroid hormones as indicators of nutritional stress and

746 subsequent refeeding in Steller sea lions (*Eumetopias jubatus*). Comp. Biochem. Physiol. A 152,
747 524–534.

748 Engelhard, G.H., Brasseur, S.M.J.M., Hall, A.J., Burton, H.R., Reijnders, P.J.H. 2002. Adrenocortical
749 responsiveness in southern elephant seal mothers and pups during lactation and the effect of scientific
750 handling. J. Comp. Physiol. B 172, 315–328. DOI 10.1007/s00360-002-0257-0

751 Famele, M., Ferranti, C., Palleschi, L., Abenavoli, C., Fidente, R.M., Pezzolato, M., Botta, M.,
752 Bozzetta, E., Draisci, R., 2015. Quantification of natural and synthetic glucocorticoids in calf urine
753 following different growth-promoting prednisolone treatments. Steroids 104, 196–202.

754 Fedak, M.A., Anderson, S.S., 1982. The energetics of lactation: accurate measurements from a large
755 wild mammal, the grey seal (*Halichoerus grypus*). J. Zool. Lond. 198: 473–479.

756 Ferranti, C., Famele, M., Palleschi, L., Bozzetta, E., Pezzolato, M., Draisci, R. 2013. Excretion profile
757 of corticosteroids in bovine urine compared with tissue residues after therapeutic and growth-
758 promoting administration of dexamethasone. Steroids 78, 803–812.

759 Fidani, M., Pompa, G., Mungiguerra, F., Casati, A., Fracchiolla, M. L., Arioli, F., 2012. Investigation
760 of the presence of endogenous prednisolone in equine urine by high-performance liquid
761 chromatography mass spectrometry and high-resolution mass spectrometry. Rapid Communications
762 in Mass Spectrometry 26, 879–886.

763 Fidani, M., Gamberini, M.C., Pompa, G., Mungiguerra, F., Casati, A., Arioli, F., 2013. Presence of
764 endogenous prednisolone in human urine. Steroids 78, 121–126.

765 Fitchett, A.E., Collins, S.A., Mason, H., Barnard, C.J., Cassaday, H.J., 2005. Urinary corticosterone
766 measures: effect of strain and social rank in BKW and CD-1 mice. Behavioural Processes 70, 168–
767 176. doi:10.1016/j.beproc.2005.06.006

768 Fonfara, S., Casamian-Sorrosal, D., Sundermayer, J., Rosenberger, T., 2015. Variations in heart rate
769 and rhythm of harbor seal pups during rehabilitation. Marine Mammal Science 31(3), 998–1013.
770 https://doi.org/10.1111/mms.12201

771 Gardiner, K.J., Hall, A.J., 1997. Diel and annual variation in plasma cortisol concentrations among
772 wild and captive harbour seals (*Phoca vitulina*). Can. J. Zool. 75, 1773–1779.

773 Guinet, C., Georges, J-Y., 2000. Growth in pups of the subantarctic fur seal (*Arctocephalus*
774 *tropicalis*) on Amsterdam Island. J. Zool. Lond. 251, 289–296.

775 Guinet, C., Servera, N., Mangin, S., Georges, J.-Y., Lacroix, A., 2004. Change in plasma cortisol and
776 metabolites during the attendance period ashore in fasting lactating subantarctic fur seals. Comp.
777 Biochem. Physiol. A 137, 523–531.

778 Gulland, F.M.D., Haulena, M., Lowenstine, L.J., Munro, C., Graham, P.A., Bauman, J., Harvey, J.,
779 1999. Adrenal function in wild and rehabilitated Pacific harbour seals (*Phoca vitulina richardii*) and
780 in seals with phocine herpesvirus-associated adrenal necrosis. Mar. Mam. Sci. 15, 810–827.

781 Hawley, J.M., Keevil, B.G., 2016. Endogenous glucocorticoid analysis by liquid chromatography-
782 tandem mass spectrometry in routine clinical laboratories. J. Steroid Biochem. Mol. Biol. 162, 27–40.
783 https://doi.org/10.1016/j.jsbmb.2016.05.014

784 Higashiyama, Y., Nashiki, M., Narita, H., 2009. Urinary catecholamine and cortisol responses of
785 Japanese shorthorn cows to social isolation. Asian-Aust. J. Anim. Sci. 22, 1437–1440.

786 Holst, J.P., Soldin, O.P., Guo, T., Soldin, S.J., 2004. Steroid hormones: relevance and measurement
787 in the clinical laboratory. Clin. Lab. Med. 24: 105–118.

788 Jasper, J., Weary, D.M., 2002. Effects of ad libitum milk intake on dairy calves. J. Dairy Sci. 85,
789 3054–3058.

790 Jeffers, B., 2019. Use of heart rates to assess stress levels in orphaned common seal pups (*Phoca*
791 *vitulina*) during multiple feeding methods in rehabilitation. MSc dissertation, University of York, UK.

792 Johnstone, C.P., Reina, R.D., Lill, A. 2012. Interpreting indices of physiological stress in free-living
vertebrates. J. Comp. Physiol. B 182, 861–879.

793 Karpovich, S.A., Skinner, J.P., Mondragon, J.E., Blundell, G.M. 2015. Combined physiological and
behavioral observations to assess the influence of vessel encounters on harbor seals in glacial fjords of
southeast Alaska. J. Exp. Biol. 473, 110–120. http://dx.doi.org/10.1016/j.jembe.2015.07.016.

794 Kazmi, S.Z.H., Zhang, H., Aziz, W., Monfredi, O., Abbas, S.A., Shah, S.A., Kazmi, S.S.H., Butt, W.H.

2016. Inverse correlation between heart rate variability and heart rate demonstrated by linear and nonlinear analysis. PLoS ONE 11(6): e0157557. doi:10.1371/journal.pone.0157557.

795 Keenan, J.B., 2015. Metabolomic approaches to assessing pre-slaughter stress in relation to beef
796 quality. PhD Thesis, University of Reading, UK. uk.bl.ethos.679250

797 Kershaw, J.L., Hall, A.J., 2016. Seasonal variation in harbour seal (*Phoca vitulina*) blubber cortisol –
798 a novel indicator of physiological state? Sci. Rep 6:21889. https://DOI:10.1038/srep2188

799 Koren, L., Ng, E.S.M., Soma, K.K., Wynne-Edwards, K.E., 2012. Sample preparation and liquid
800 chromatography –tandem mass spectrometry for multiple steroids in mammalian and avian
801 circulation. PLoS ONE 7(2): e32496. https://doi:10.1371/journal.pone.0032496

802 Lander, M.E., Harvey, J.T., Hanni, K.D., Morgan, L.E. 2002. Behavior, movements, and apparent survival of rehabilitated and free-ranging harbor seal pups. J. Wildl. Manag. 66(1), 19–28.

803 Larmour, L.J., 1989. Hand-rearing and rehabilitation of common seal pups *Phoca vitulina* at the
804 Oban Sea Life Centre. Int Zoo Yearb. 28: 272–279. doi.org/10.1111/j.1748-1090.1989.tb03299.x

805 Lupo, S., Carroll, F., Liang, S.-H., Alagandula, R., Kahler., T., 2018. Analysis of urinary free cortisol
806 and cortisone by LC-MS/MS. Restek Corporation. www.restek.com.

807 MacRae, A.M., Haulena, M., Fraser, D., 2011. The effect of diet and feeding level on survival and
808 weight gain of hand-raised harbour seal pups (*Phoca vitulina*). Zoo Biol. 30, 532–541.

809 McWhinney, B.C., Briscoe, S.E., Ungerer, J.P.J., Pretorius, C.J., 2010. Measurement of cortisol,
810 cortisone, prednisolone, dexamethasone and 11-deoxycortisol with ultra high performance liquid
811 chromatography–tandem mass spectrometry: application for plasma, plasma ultrafiltrate, urine and
812 saliva in a routine laboratory. J. Chromatogr. B. 878, 2863–2869.

813 Mellish, J.E., Iverson, S.J., Bowen, W.D., 1999. Variation in milk production and lactation
814 performance in grey seals and consequences for pup growth and weaning characteristics. Physiol.
815 Biochem. Zool. 72:677–690.

816 Muelbert, M.M.C., Bowen, W.D., 1993. Duration of lactation and postweaning changes in mass and
817 body composition of harbour seal, *Phoca vitulina*, pups. Can. J. Zool. 71, 1405–1414.

818 Müller, G., Gröters, S., Siebert, U., Rosenberger, T., Driver, J., König, M., Becher, P., Hetzel, U.,
819 Baumgärtner, W., 2003. Parapox infection in harbour seals (*Phoca vitulina*) from the German North

820 Sea. Vet. Pathol. 40, 445–454.

821 Nayak, A., 2020. Akaike information theory. https://towardsdatascience.com/akaike-information-
822 criteria-942d1f554537

823 Novak, M.A., Hamel, A.F., Kelly, B.J., Dettmer, A.M., Zeyer J.S., 2013. Stress, the HPA axis, and
824 non-human primate well-being: a review. Appl. Anim. Behav. Sci. 143, 135-149.

825 O'Hara, A.D., 2019. Does rehabilitation work? Investigating sex- and species-differences in daily
826 weight gain of harbour and grey seal pups in rehabilitation. B.Sc. dissertation, Bangor University,
827 UK. https://doi:10.13140/RG.2.2.36591.07843

828 Ortiz, R.M., Wade, C., Ortiz, C.L., 2001. Effects of prolonged fasting on plasma cortisol and TH in
829 postweaned northern elephant seal pups. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 280,
830 R790–R795.

831 Osinga, N., 't Hart, P., 2010. Harbour seals (*Phoca vitulina*) and rehabilitation. NAMMCO Sci Publ.
832 8, 355–372.

833 Perry, E.A., Renouf, D., 1988. Further studies of the role of harbour seal (*Phoca vitulina*) pup
834 vocalizations in preventing separation of mother-pup pairs. Can. J. Zool. 66, 934–938.

835 Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., 2019. nlme: Linear and Nonlinear Mixed Effects
836 Models. R package version 3.1-139. https://CRAN.

837 Pompa, G., Arioli, F., Casati, A., Fidani, M., Bertocchi, L., Dusi, G., 2011. Investigation of the origin
838 of prednisolone in cow urine. Steroids 76, 104–110.

839 R Core Team., 2020. R: A language and environment for statistical computing. R Foundation for
840 Statistical Computing, Vienna, Austria. https://www.R-project.org

841 Richmond, J.P., Skinner, J.G., Mazzaro, L.M. Zinn, S.A. 2008. Comparison of the somatotropic axis
842 in free-ranging and rehabilitated harbor seal pups (*Phoca vitulina*). J. Zoo Wildl Med., 39(3), 342–
843 348. https://doi.org/10.1638/2007-0076.1

844 Richmond, J.P., Norris, T., Zinn, S.A. 2010. Re-alimentation in harbor seal pups: effects on the
845 somatotropic axis and growth rate. Gen. Comp. Endocrinol., 165, 286–292.
846 https://doi:10.1016/j.ygcen.2009.07.007

847 Riedman, M.L., LeBoeuf, BJ. 1982. Mother-pup separation and adoption in Northern elephant seals.
Behav. Ecol. Sociobiol., 11, 203–215.

848 Robinson I., 1995. The rehabilitation of seals at the RSPCA Norfolk Wildlife Hospital. J. Br. Vet.
849 Zool. Soc. 1, 13-17.

850 Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress
851 responses? Integrating permissive, suppressive, stimulatory and preparative actions. Endocr. Rev. 21,
852 55–89.

853 Skinner, J.P., 2006. Physical and behavioural development of nursing harbour seal (*Phoca vitulina*)
854 pups in Maine. MSc thesis, University of Maine. Available from:
855 https://digitalcommons.library.umaine.edu/cgi/viewcontent.cgi?article=1655&context=etd

856 Spencer-Booth, Y., Hinde, R.A., 1971. Effects of brief separations from mothers during infancy on
857 behaviour of rhesus monkeys 6–24 months later. J. Child Psychol. Psychiatry 12, 157–172.

858 Spotte, S. Stake, P.E. 1982. Hand-rearing of twin gray seals (*Halichoerus grypus*) from birth to
859 weaning. Mar. Ecol. Progr. Ser. 9, 181–189.

860 Stevens, J.M.G., Thyssen, A., Laevens, H. Vervaecke, H. 2013. The influence of zoo visitor numbers
861 on the behaviour of harbour seals (*Phoca vitulina*). JZAR 1(1), 31–34.

862 Taussky, H.H., Kurzmann, R., 1954. A microcolorimetric determination of creatinine in urine by the
863 Jaffe reaction. J. Biol. Chem. 208, 853–862.

864 Thomas, A., Ono, K., 2015. Diving related changes in the blood oxygen stores of rehabilitating
865 harbor seal pups (*Phoca vitulina*). PLoS ONE 10(6): e0128930. https://doi:10.1371/journal

866 Timmermans, S., Souffriau, J., Libert, C., 2019. A general introduction to glucocorticoid biology.
867 Frontiers in Immunology 10, 1545. doi: 10.3389/fimmu.2019.01545

868 Trumble, S.J., O'Neill, D.O., Cornick, L.A., Gulland, F.M.D., Castellini, M.A., Atkinson, S., 2013.
869 Endocrine changes in harbour seal (*Phoca vitulina*) pups undergoing rehabilitation. Zoo Biol. 32,
870 134–141.

871 Venables, U.M., Venables, L.S.V., 1955. Observations on a breeding colony of the seal *Phoca*

vitulina in Shetland. Proc. Zool. Soc. Lond. 125, 521–532. https://doi:10.1111/j.1096-642.1955.tb00614.x

Vincenti, M., Leporati, M., Capra, P., Gatto, S., Attucci, A., Barbarino, G., Nebbia, C., 2012. A field survey on the presence of prednisolone and prednisone in urine samples from untreated cows. Food Addit. Contam: Part A. 29:12, 1893–1900. http://dx.doi.org/10.1080/19440049.2012.719645

Wilson, S., 1974. Mother-young interactions in the common seal *Phoca vitulina vitulina*. Behaviour 48, 23–26.

Wilson, S.C., 1978. Social organization and behavior of harbor seals, *Phoca vitulina concolor*, in Maine. Report to the Marine Mammal Commission PB-280 188. Contract no. MM6AC013, April 1978. xi + 103pp.

Wilson, S.C., 1999. Radiotelemetry study of two rehabilitated harbour seal pups released close to the time of weaning in the wild. J. Wildlife Rehabil. 22, 5–11.

Wilson, S.C., Kleiman, D.G., 1974. Eliciting play: a comparative study (*Octodon*, *Octodontomys*, *Pediolagus*, *Phoca*, *Choeropsis*, *Ailuropoda*). Amer. Zool. 14, 341–370.

Wilson, S., Matthews, W., Keenan, J., 2015. Do harbour seal 'orphans' need a companion during their first weeks in rehabilitation? In Marine Mammals of the Holarctic, 8(2), 236–241. St. Petersburg, Russia, September 22–27, 2014.

Riedman, M.L., LeBoeuf, BJ. 1982. Mother-pup separation and adoption in Northern elephant seals. Behav. Ecol. Sociobiol., 11, 203–215.

Wilson, S.C., Jones KA., 2018. Behaviour of harbour seal (*Phoca vitulina vitulina*) mother-pup pairs in Irish Sea intertidal habitats. Biol. Environ. 118, 1–15. https://doi:10.3318/BIOE.2018.02

Wilson, S.C., Jones, K.A., 2020. Behaviour of harbour seal (*Phoca vitulina vitulina*) pups in Dundrum Bay, north-east Ireland, during transition from filial dependency to weaning. Biol. Environ. 120, 187–202. https://doi.org/10.3318/BIOE.2020.17

Wilson, S.C., Jones, K.A., 2021. Body mass and behaviour of stranded harbour seal (*Phoca vitulina vitulina*) pups during the peak pupping season in Co. Down, north-east Ireland. Biol. Environ. 121, 9–20. https://doi.org/10.3318/BIOE.2021.03

Yusko, B., Hawk, K., Schiml, P.A., Deak, T., Hennessy, M.B., 2012. Sensitization of depressive-like behaviour during repeated maternal separation is associated with more-rapid increase in core body temperature and reduced plasma cortisol levels. Physiol. Behav. 105, 861–867.

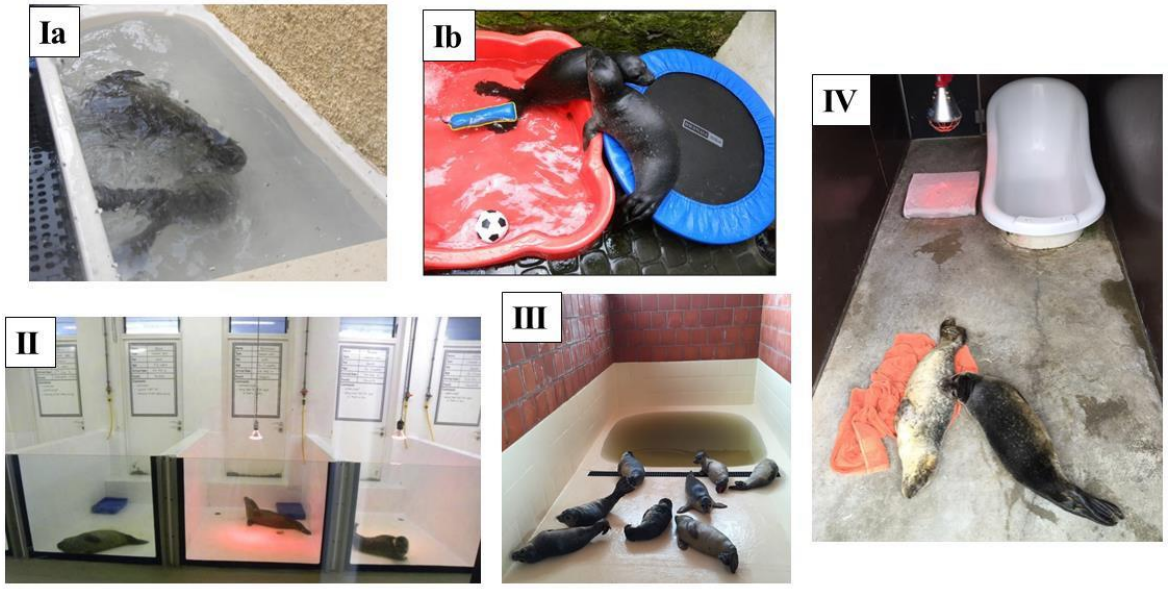

Fig. 1. Pups at each of the four centres. **Ia and Ib** – outdoor open enclosure ~130x600 cm including pool, bath and two trampolines; **II** – indoor pens ~100x200 cm; **III** – outdoor covered enclosures, varying dimensions, with pool; **IV** – outdoor covered enclosure ~ 120x320 cm, bath filled only for fish-feeding.

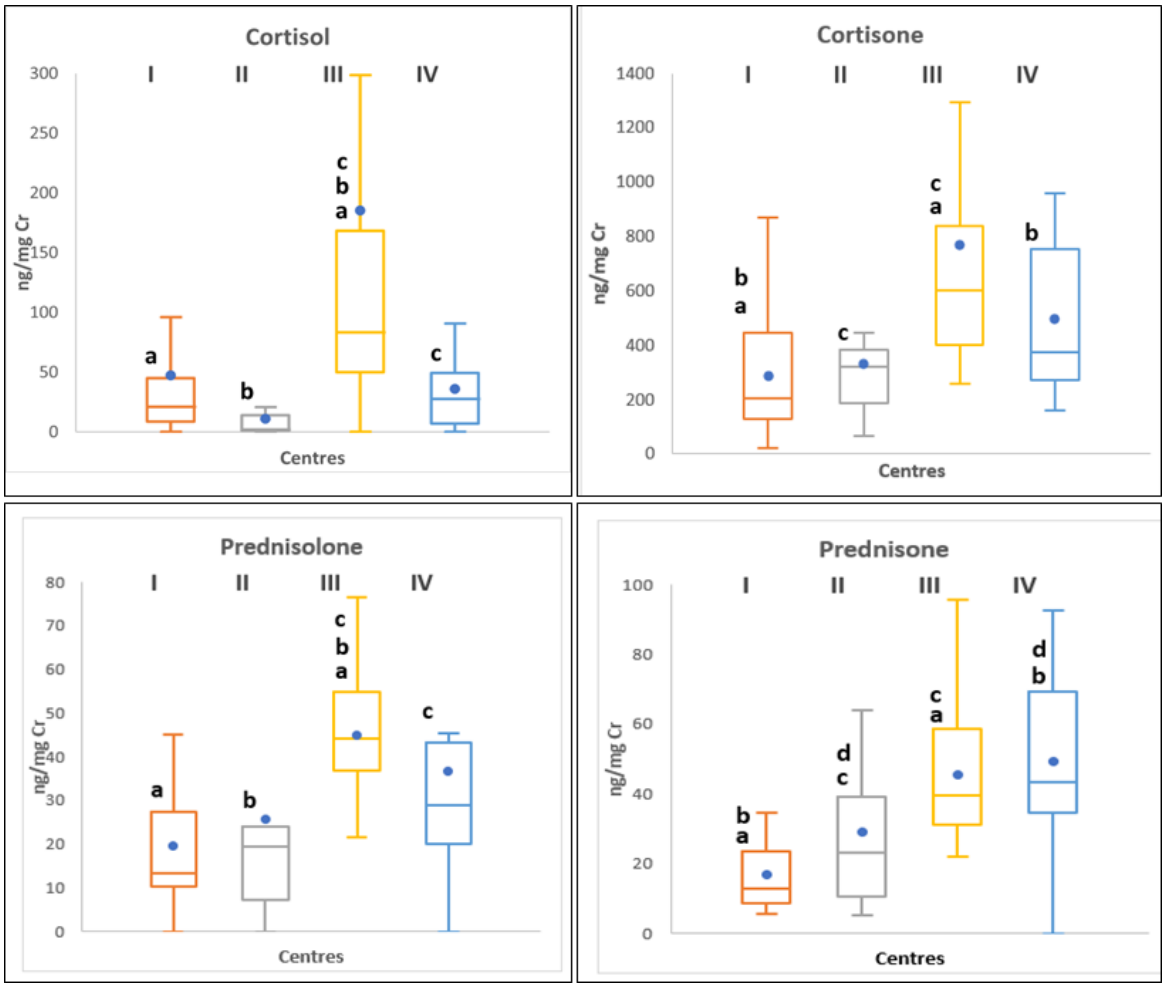

Fig. 2. Boxplots of urinary concentrations of four GCs at each rehab centre I–IV (day2 onwards), using individual and median values of GC concentrations for each pup as data points. Horizontal lines indicate 1st quartile, median and 3rd quartile and dot markers indicate means. Letters a, b, c and d indicate significant differences ($P < 0.05$) between centres by the Kruskal-Wallis post-hoc Dunn's test.

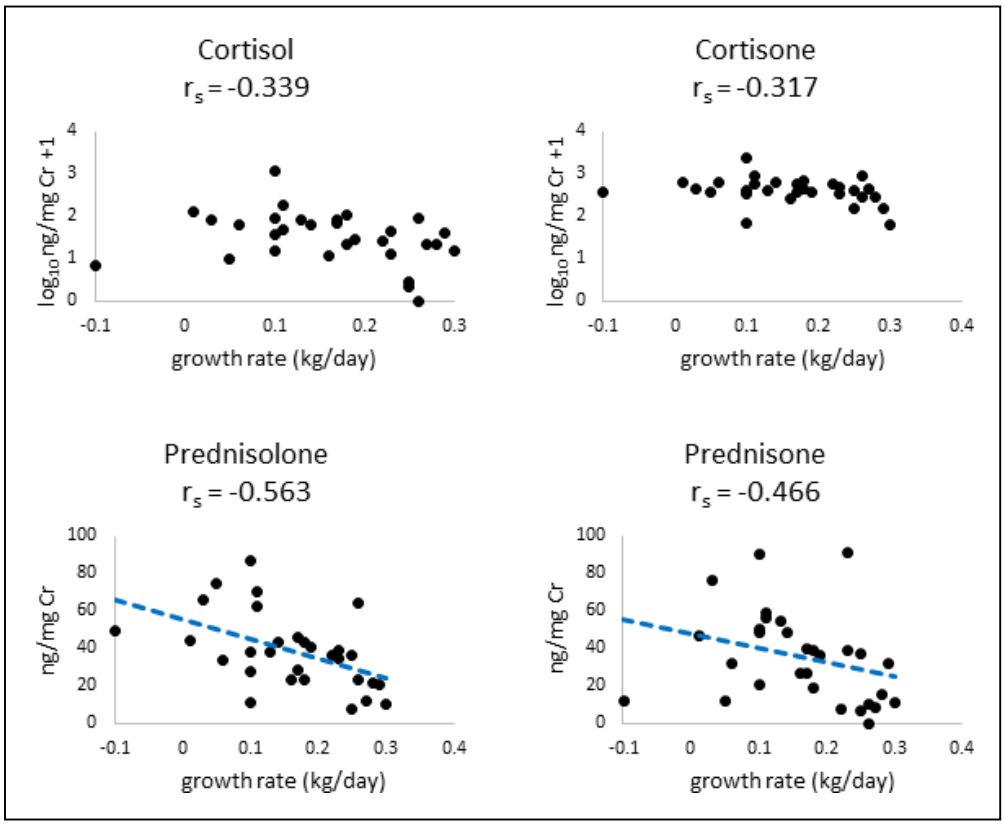

Fig. 3. Scatter plots showing monotonal relationship, with Spearman correlation r_s between median GC concentrations (ng/mg Cr) and average growth rate (during the sample period) for each pup sampled from rehab day 2 onwards. Plots for cortisol and cortisone are given as log10 of the concentrations +1, owing to some zero concentrations for cortisol and relatively high concentrations of cortisone.

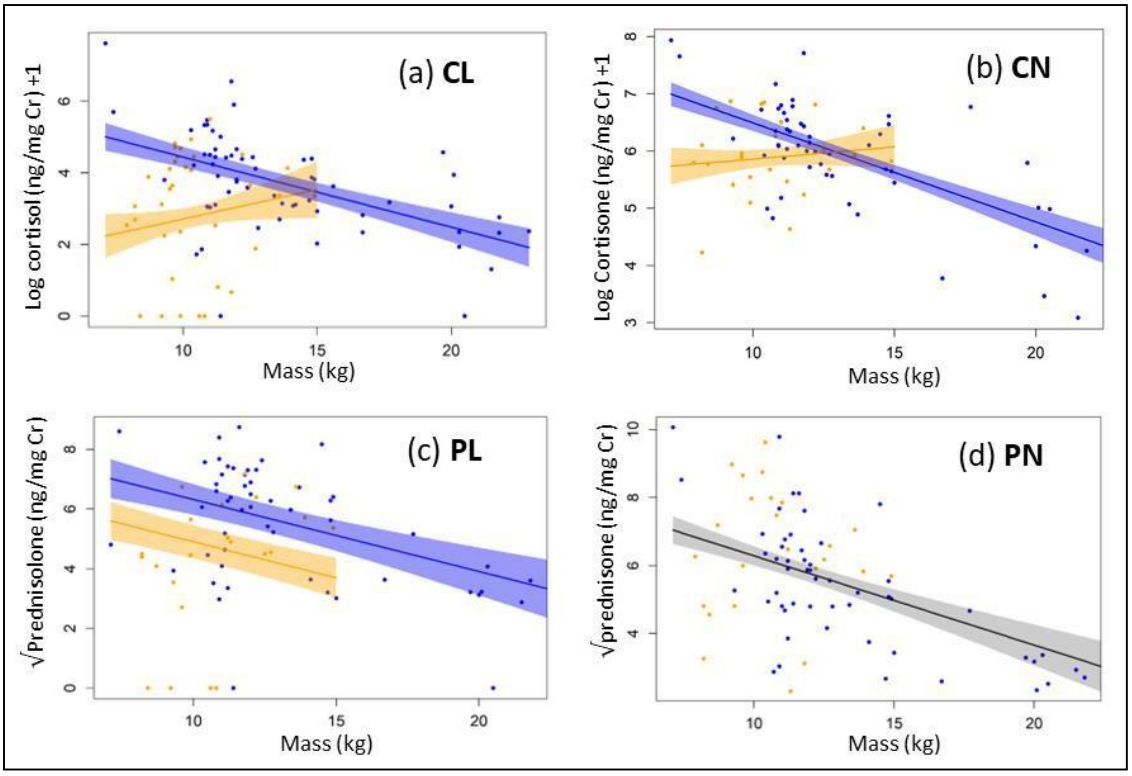

Fig. 4. Best-fit linear mixed effects models showing the change in each GC as pups gain mass (from day 2 onwards), from pups of known ID provided with water (blue) and without water (orange). CL = cortisol, CN = cortisone, PL = prednisolone, PN = prednisone. Points are GC concentrations, lines are fitted trends, and shaded areas show standard error. n= 78 samples for each GC.

Figure

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Figure

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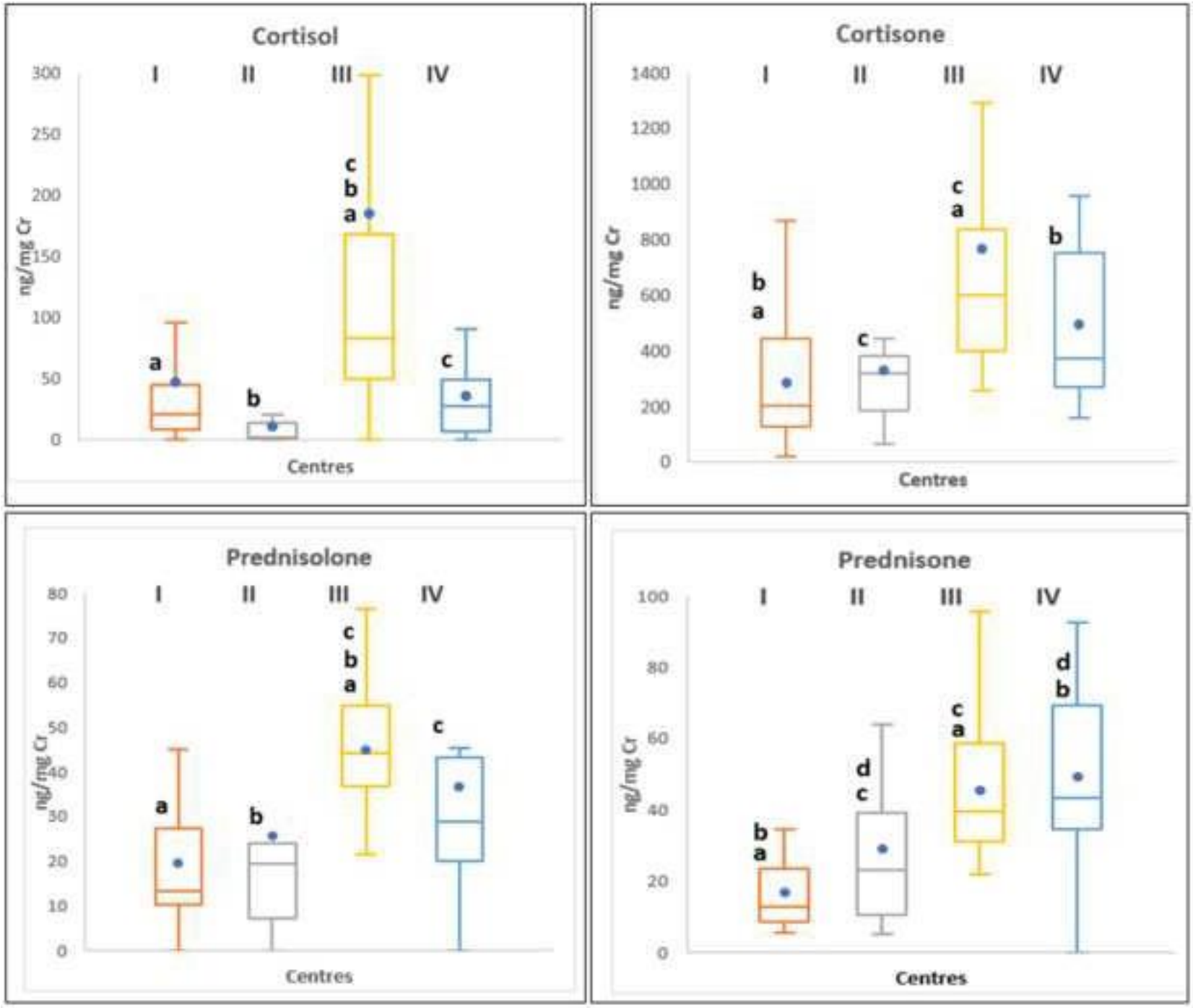

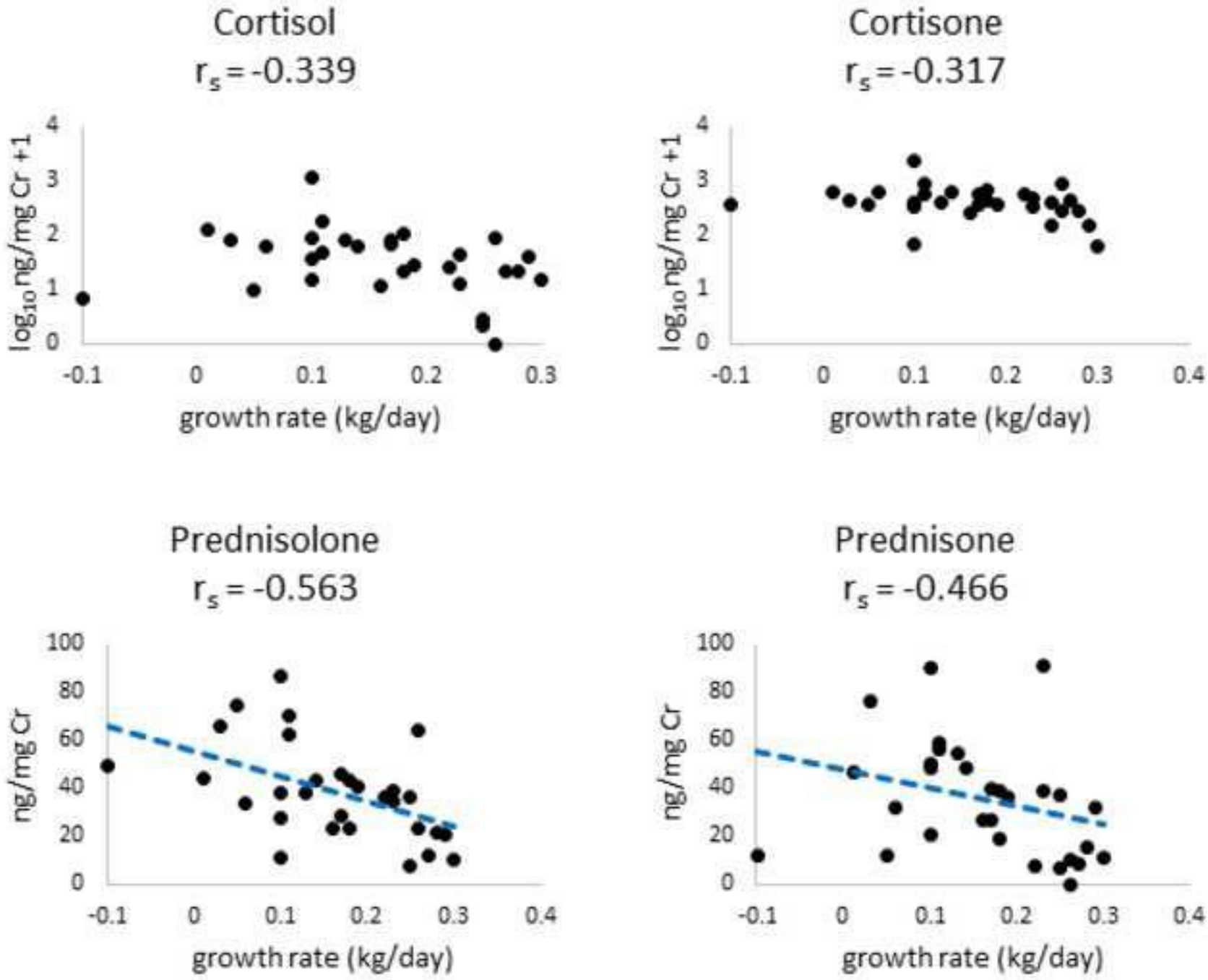

Figure

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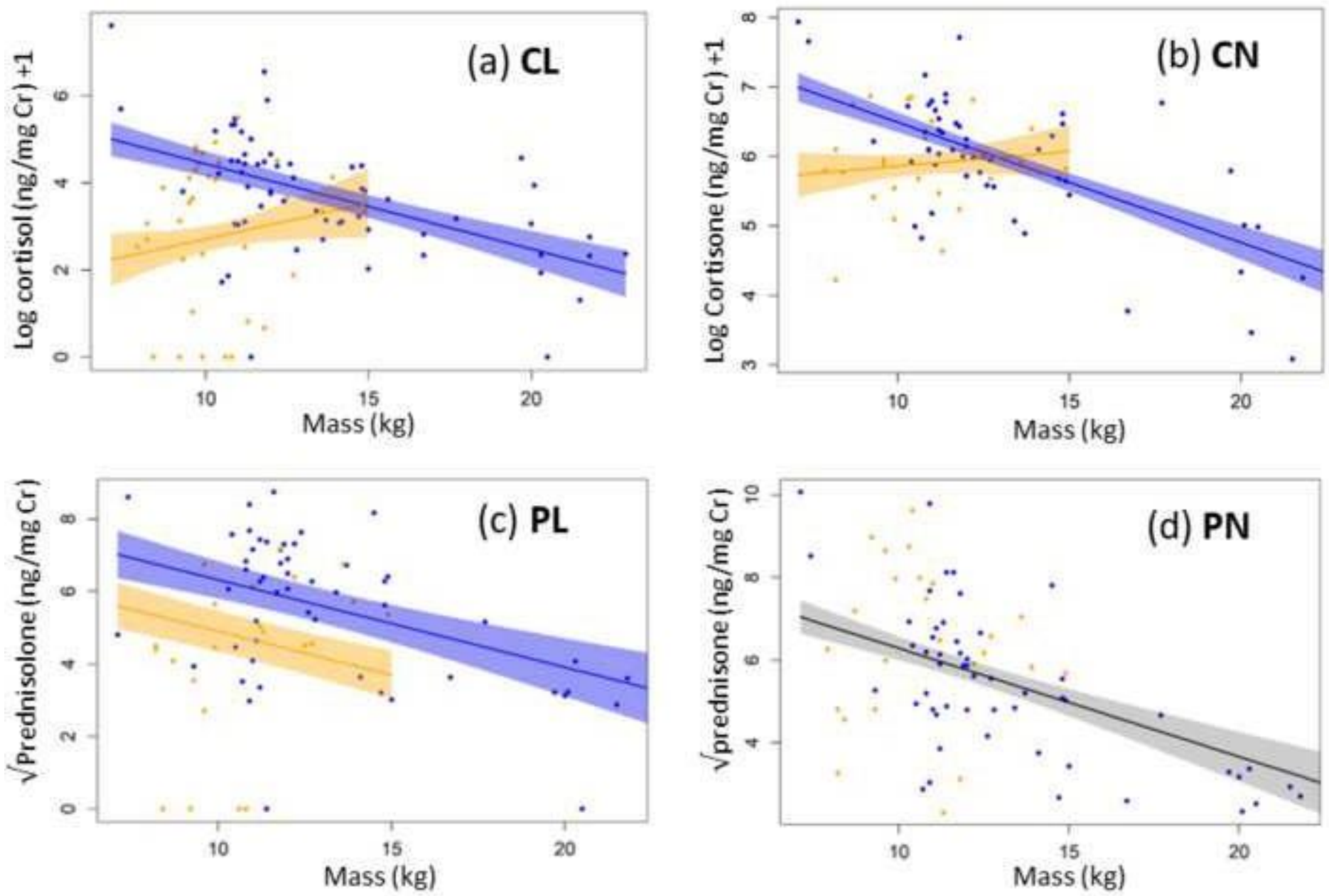

Author statement

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Susan Wilson: Conceptualisation, Methodology, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review and editing, Visualisation, Supervision, Project administration

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Lilia Dmitrieva: Formal analysis, Writing – review and editing

Wesley Smyth: Methodology, Validation, Investigation, Resources, Writing – original draft; Writing – review and editing, Project administration

URINARY GLUCOCORTICOIDS IN HARBOUR SEAL PUPS

Supplementary file S1

Additional Figures S1.1–1.4

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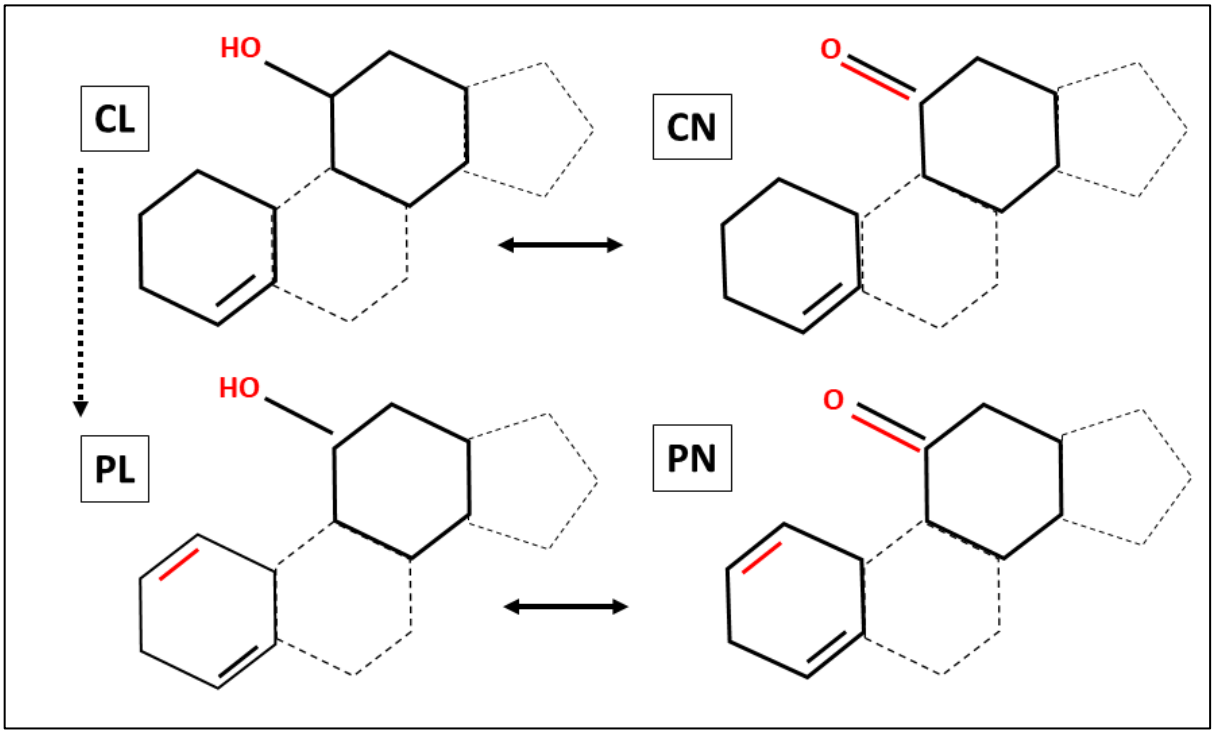

Fig. S1.1. Schematic representation of structural changes believed to occur endogenously between Cortisol (CL) and Cortisone (CN) and between Prednisolone (PL) and Prednisone (PN). In both cases the single bonded HO group is replaced with a double-bonded O. PL and PN are distinguished from CL and CN only by a single bond in the first carbon ring being replaced by a double bond. The relevant carbon rings are shown in bold with the structural changes between the GCs shown in red (Figure adapted from Vincenti et al., 2012).

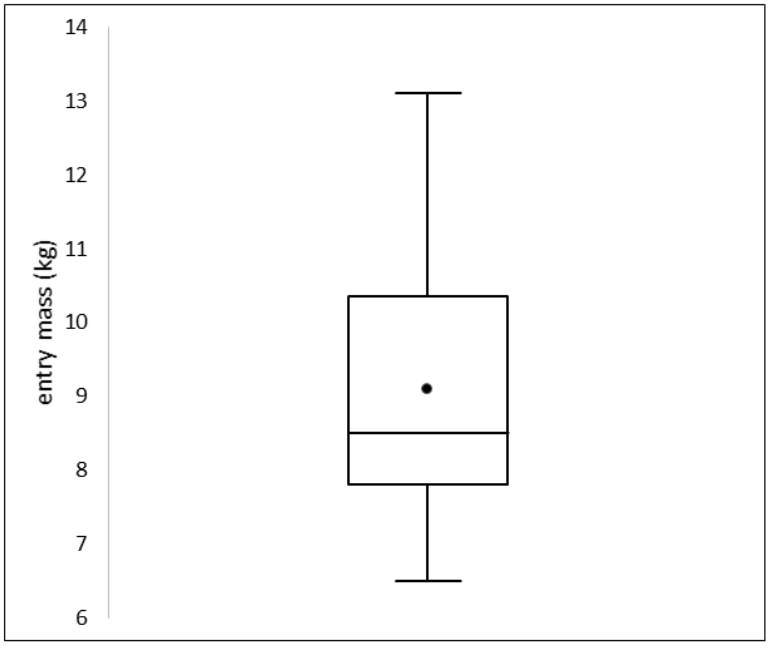

Figure S1.2. Box plot of pup mass on rescue and entry into rehab centres (kg) for all pups in the study. Dot = mean; horizontal lines are 1st quartile, median and 3rd quartile.

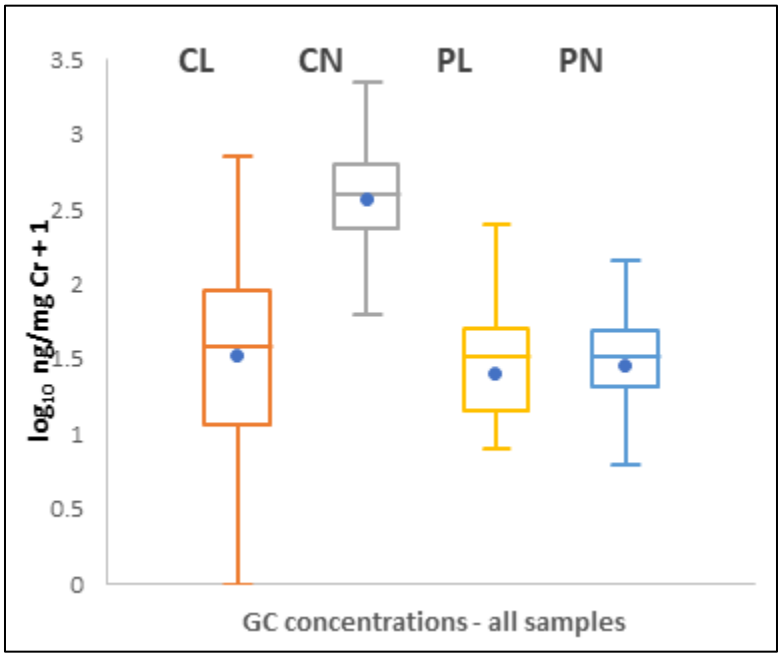

Figure S1.3. Box plots of urinary concentrations of four GCs from all samples (n=96). CL=cortisol, CN=cortisone, PL=prednisolone, PN=prednisone. Concentrations in ng/mg Cr on $\log_{10}$ scale. Horizontal lines indicate 1st quartile, median and 3rd quartile and spot markers indicate means.

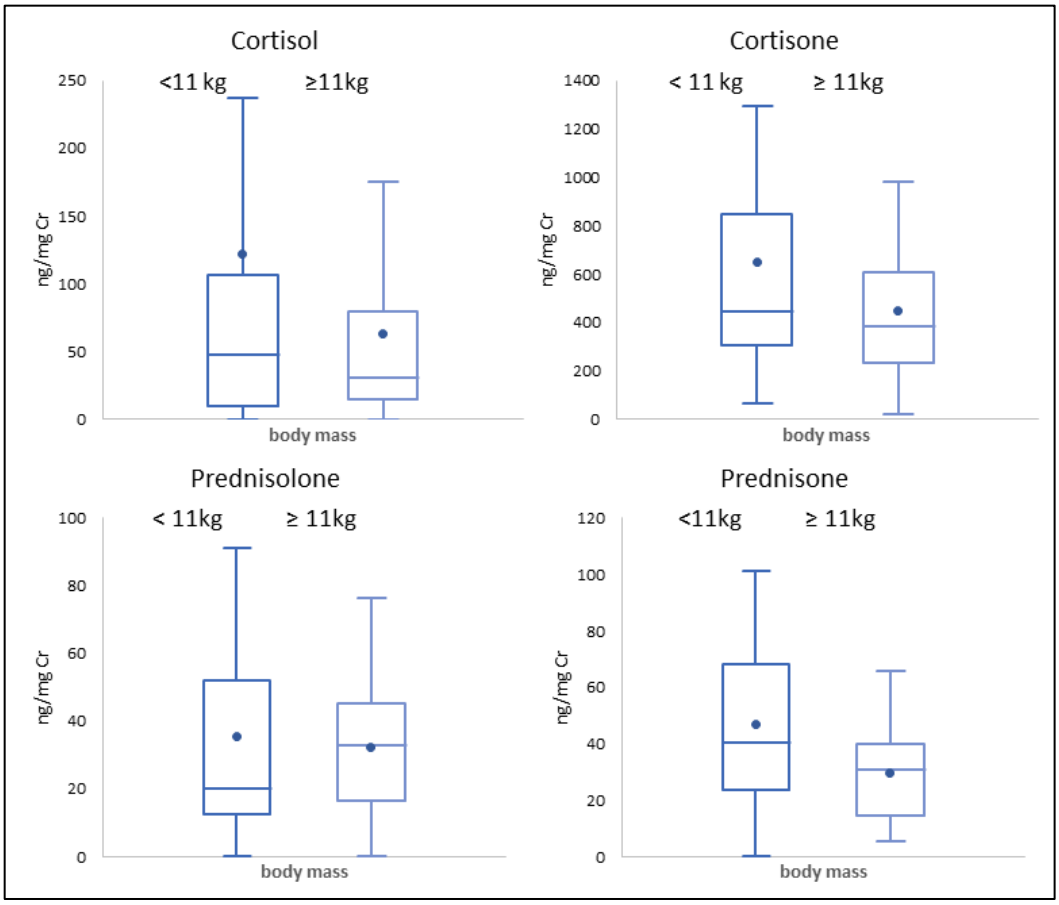

Fig. S1.4. Urinary GC concentrations (ng/mg Cr) for pups (rehab day 2 onwards) weighing < 11 kg and ≥ 11 kg on date of urine sampling (outliers are not shown). Horizontal lines indicate 1st quartile, median and 3rd quartile, dot indicates mean. For pups < 11 kg, n= 27 samples were taken from 15 individual pups. For pups ≥11 kg, n= 51 samples were taken from 20 pups.

URINARY GLUCOCORTICOIDS IN HARBOUR SEAL PUPS

Procedures for analysing seal pup urine for concentrations of cortisol (CL), cortisone (CN), prednisolone (PL), and prednisone (PN) at the Chemical and Immunodiagnostic Sciences Branch, Veterinary Sciences Division, Agri-Food and Biosciences Institute (AFBI), Belfast, N. Ireland, 2014–2016.

Additional tables S2.1 – S2.3

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Table S2.1. Individual pup data

Centre	Pup	Sex	Entry date (D/M/Y)	Entry mass (kg)	Feeding method sampling period	No. samples day 2 onwards	No. samples day 0-1
I	Ula	F	05/07/14	7.1	Tube	9	1
I	Earendil	M	07/07/14	7.4	Tube	3	0
I	Coral	F	03/07/16	8.5	Tube	6	0
I	Pearl	F	05/07/16	8.3	Tube	6	0
II	Elm	M	20/06/14	8.0	Tube	2	0
II	Rowan	M	24/06/14	7.8	Tube	1	2
II	Birch	M	13/06/14	7.5	Tube	1	0
II	Fern	F	04/07/14	7.8	Tube	1	0
II	Beech	M	06/07/14	10.3	Tube	0	1
II	Finn	M	03/07/15	8.5	Tube	2	0
II	Isla	F	16/07/15	8.0	Tube	1	0
II	Polly*	F	18/07/15	6.5	Tube	0	1
III	Klamath	M	30/06/15	7.0	Tube	1	0
III	Bärchen	M	29/06/15	12.2	Fish HF	1	0
III	Beate	F	03/07/15	10.6	Tube	2	0
III	Chigoma	M	03/07/15	10.3	Tube	2	0
III	Daggi	F	25/06/15	10.7	Fish HF	1	0
III	Eddi	M	01/07/15	13.1	Tube	4	0
III	Henry	M	03/07/15	10.1	Tube	3	0
III	Jens	M	26/06/15	8.8	Fish HF	3	0
III	Julio	M	08/07/15	11.5	Tube	1	3
III	Kawamba	F	06/07/15	10.4	Tube	1	0
III	Lisi	F	01/07/15	11.0	Tube	4	0
III	Schultze	F	08/07/15	6.9	Tube	2	1
III	Urmel	F	05/07/15	10.6	Tube	4	2
III	Yukari	F	01/07/15	10.0	Tube	2	0
IV	Aquaman	M	07/07/15	10.2	Tube	3	0
IV	Blackbird	M	04/07/15	7.4	Tube	3	0
IV	Hawkeye	M	09/07/15	8.4	Tube	4	0
IV	Medusa	F	27/06/15	7.0	Tube	2	0
IV	Mystique	F	21/06/15	9.6	Fish HF	1	0
IV	Wolverine	M	06/06/15	7.8	Fish HF	2	0
I	Ula or Earendil			See above		6	0
III	Daggi or Jens			See above		1	0

Mean entry mass	9.04 kg
Standard deviation	1.72 kg
Median entry mass	8.5 kg
Total no. samples day 0–1	11
Total no samples day 2– (with pup ID)	78
Total no. samples day 2– (no pup ID)	7

*Polly died the day after entry (day 1). Tube = feeding by tube (stomach gavage); HF = handfeeding in water

Table S2.2. Comparison of median (or single) GC concentrations (ng/mg Cr, to nearest whole number) from five individuals on rehab days 0–1 and subsequent days (day 2 onwards). Shaded cells indicate lower values on day 2 onwards relative to days 0–1.

Centre	Pup name	No. samples		CL		CN		PL		PN	
		d0–1	d2–	d0–1	d2–	d0–1	d2–	d0–1	d2–	d0–1	d2–
I	Ula	1	9	8062	21	4853	293	249	15	40	22
II	Rowan	2	1	11	2	412	385	13	7	48	36
III	Urmel	2	4	96	178	181	862	99	57	116	62
III	Julio	3	1	245	82	484	442	53	76	57	66
III	Schultze	1	2	4029	1165	460	2448	206	49	25	87
II	Beech	1	0	14	NA	407	NA	14	NA	29	NA
II	Polly*	1	0	487	NA	455	NA	237	NA	115	NA

*Polly died on rehab day 1

Table S2.3. Median concentrations of four urinary GCs (CL, CN, PL and PN) from rehab day 2 onwards and significance of differences between each of three 6-hour time periods: A=07:00–12:59, B= 13:00–18:59, and C=19:00–00:59. P values from Kruskal-Wallis (K-W) 3-sample test or Mann-Whitney M-W) 2-sample test.

Centre	Time period	No. samples	CL	CN	PL	PN
			Median values ng/mg Cr			
I	A	4	28.47	700.30	29.48	26.88
	B	18	16.57	147.47	13.22	11.55
	C	8	15.44	233.25	11.28	8.72
	P value (M-W)		***0.849***	***0.160***	***0.644***	***0.495***
II	A	2	23.86	570.56	8.35	57.73
	B	4	1.10	144.53	22.08	10.17
	C	3	11.73	384.96	19.31	39.92
	P value	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*
III	A	10	78.84	441.44	48.11	34.18
	B	14	119.19	836.36	42.53	46.17
	C	7	87.17	515.52	45.90	34.85
	P value (K-W)		***0.511***	***0.057***	***0.762***	***0.186***
IV	A	10	32.49	390.67	30.72	40.03
	B	5	8.38	291.10	20.65	55.83
	C	0	N/A	N/A	N/A	N/A
	P value (M-W)		***0.370***	***0.310***	***0.539***	***0.679***

URINARY GLUCOCORTICOIDS IN HARBOUR SEAL PUPS

Supplementary file S3

Figs. S3.1–3.3. Examples of chromatograms for GC quantification in harbour seal pup urine by UPLC-MS/MS

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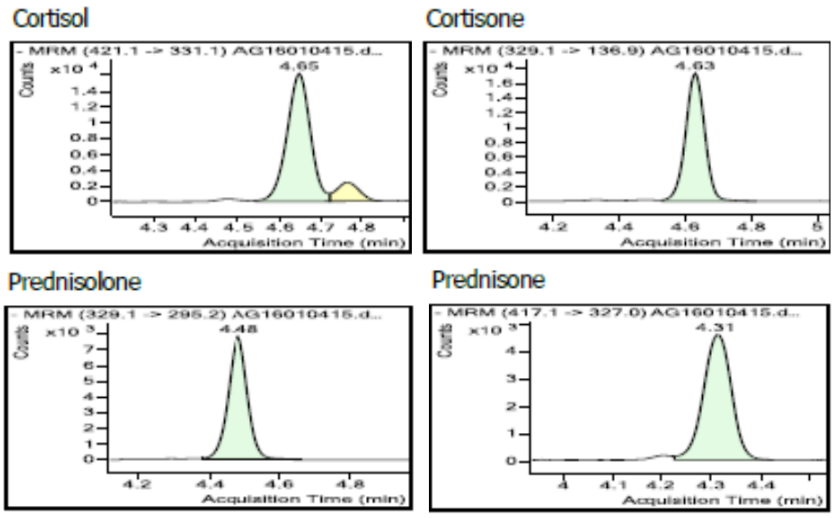

Fig. S3.1. Chromatograms for the lowest calibration standard (0.5 µg/kg)

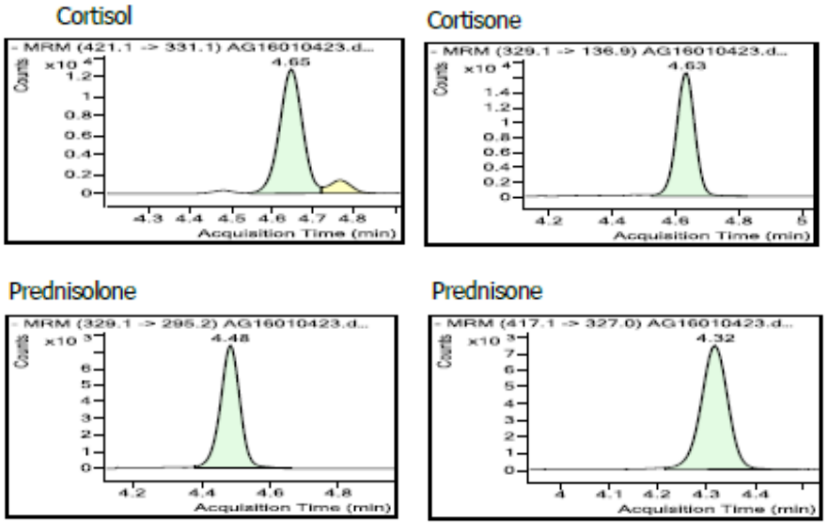

Fig. S3.2. Examples of chromatograms for positive controls (spiked with a mixed standard containing all the GCs in the method)

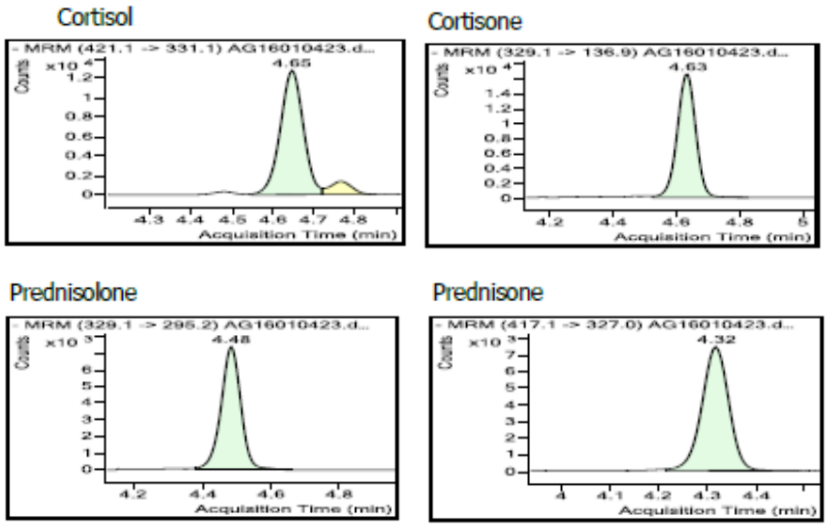

Fig S3.3. Example of detection peaks from seal pup urine sample 1600272

URINARY GLUCOCORTICOIDS IN HARBOUR SEAL PUPS

Supplementary file S3

Procedures for analysing seal pup urine for concentrations of cortisol (CL), cortisone (CN), prednisolone (PL), and prednisone (PN) at the Chemical and Immunodiagnostic Sciences Branch, Veterinary Sciences Division, Agri-Food and Biosciences Institute (AFBI), Belfast, N. Ireland, 2014–2016.

S2.1. Reagents and Materials for UPLC-MS/MS analysis of seal pup urine samples

Analytical grade reagents and HPLC grade solvents were used throughout. Methanol was obtained from Romil (Cambridge UK), acetonitrile, propan-2-ol and sodium hydroxide (NaOH) pellets from Fisher Scientific (Leicestershire, UK), 1M HCl from BDH (Dorset, UK), and ammonium acetate from Sigma Aldrich (Dorset, UK). Grade 1 (18MΩ.cm) water was obtained from an in-house Milli-Q system (Millipore corp., Livingston, UK). Solid-phase extraction was performed on Oasis HLB SPE columns (Waters Chromatography Ireland Limited). Screw capped polypropylene centrifuge tubes were used for hydrolysis. Analytical standard powders for CL, CN, PL and PN were obtained from Sigma-Aldrich. Isotopically labelled internal standard powders for d6-prednisolone and d4-cortisol were obtained from CDN isotopes (Quebec, Canada).

Individual stock standard solutions were prepared at 1 mg/ml in methanol. Mixed working standard solutions for CL, CN, PL and PN were prepared at 1µg/ml and 100 ng/ml by dilution of the stock standard with methanol, and a mixed internal standard spiking solution for d6-PL and d4-CL at 1 µg/ ml in methanol. Standards were stored in 25ml amber glass vials at 4ºC for up to 12 months. The mixed 100 ng/ml and 1 µg/ml working standards were used to prepare matrix matched calibration curves across the range 0.5–10.0 µg/kg (ng/ml equivalent).

Additional reagent solutions were: 0.2 M NaOH (prepared fresh weekly and stored at room temperature), 0.02 M NaOH/methanol (60/40 v/v) and 5mmol ammonium acetate solution in methanol (prepared fresh weekly and refrigerated), UPLC mobile phase A: 0.05 mM ammonium acetate in 10% methanol (prepared fresh daily), UPLC mobile phase B: acetonitrile (fresh for each analysis), and UPLC needle wash: methanol (30%), acetonitrile (30%), propan-2-ol (30%) and water (10%) (prepared fresh for each analysis).

S2.2. Sample preparation and extraction of urine for UPLC-MS/MS

Bovine urine was used as the control negative, and for the matrix standard curve. On the day of analysis, the seal urine samples and control negatives were defrosted at room temperature and mixed thoroughly prior to dispensing of aliquots.

Seal urine (5 ml) was pipetted into a 50 ml screw-cap polypropylene centrifuge tube, while

bovine urine samples were set up for negative and positive controls and for matrix standards. All seal urine, and all positive/negative controls were spiked with 20 µl of the 1 µg/ml mixed internal standard. The selected bovine urine blank samples for recovery evaluation were fortified with 100 µl of the mixed 100 ng/ml standard and allowed to equilibrate for 10 minutes before proceeding. Methanol (10ml) and 1M Hydrochloric acid (15 ml) were added to each tube, which was then capped and vortexed for 20 sec. All tubes were incubated in a water bath for 4 hours at 50ºC. After incubation tubes were allowed to cool to room temperature and centrifuged at 3500 rpm for 15 min.

Solid phase extraction (SPE) was performed using Oasis HLB 3cc, 60 mg SPE cartridges, placed on a Vac-Elute manifold. Each cartridge was conditioned with methanol (3 ml) followed by water (3 ml) (cartridges were not allowed to dry out at any stage until the final wash). The sample extract was applied and allowed to pass through under gravity. The cartridges were washed with water (3 ml) and allowed to dry by passing air through under vacuum for 5 min. The samples were eluted into 13*100 mm disposable glass tubes with methanol (3 ml). At this stage the matrix standard tubes were spiked with the mixed 100 ng/ml and 1 µg/ml standards, and with the 1µg/ml mixed standard (Table S2-1).

Table S2-1. Volumes of standard solution used to spike the matrix standard tubes at each concentration

Standard concentration (ng/ml equivalent)	Volume (µl) of 100 ng/ml mixed quaternary std	Volume (µl) of 1 µg/ml mixed tertiary std	Volume (µl) of 1 µg/ml mixed internal tertiary std
0.5	25	-	20
1.0	50	-	20
2.0	100	-	20
5.0	-	25	20
10.0	-	50	20

All sample, control and matrix standard tubes were evaporated on a TurboVap, under nitrogen, at 55 ºC, and reconstituted in methanol (250 µl), vortexed for 20 seconds, followed by water (250 µl). Extracts were transferred to a 2 ml vial with tapered insert prior to UPLC-MS/MS analysis.

S2.3. Instrumentation and conditions

UPLC separations were carried out using an Agilent 1290 infinity LC system comprising a G4220A Binary Pump, G1330B Thermostat, G4226A Sampler, and a G1316C Thermo-stated Column Compartment (Agilent Technologies, Inc. Santa Clara, USA). A reverse phase gradient separation was achieved on an Agilent EclipsePlusC18, RRHD 1.8 µm, 2.1*100mm chromatographic column (Agilent Technologies, Inc. Santa Clara, USA) with an in-line filter assembly (0.5 µm porosity) (Waters, Milford, USA). The sample compartment was set at 10ºC, column temperature at 40ºC, and an injection volume of 1 µl used. The mobile phases were (A) 0.05 mM ammonium acetate in 10% methanol and (B) acetonitrile, with a flow rate of 0.4 ml/min. The flow was diverted to waste during sample injection cycles at 0.0 – 2.5 min and again from 9.4 min until the end of an injection cycle. Linear gradient steps were used with initial conditions set at 80% A, decreasing to 68.5% A after 2 min, with a 1 min hold at 68.5% A, and returning to 80% A at 9.0 min. A re-equilibration period of 2.6 min was used. Total analysis time was 11.6 min per sample.

An Agilent AG6490 triple-quadrupole controlled by Mass-Hunter acquisition software was used for mass spectrometric analysis; this was connected to the UPLC system via an electrospray ionisation (ESI) interface source. All compounds were analysed in negative ESI mode with Jet Stream. The following source parameter conditions were used: Gas Temp 200 ºC, Gas Flow 12 L/min, Nebulizer 50 psi, Sheath Gas 350ºC, and Capillary and Nozzle voltages 4000V and 2000V respectively in in negative mode. Nitrogen was used for nebulizing, sheath, drying and collision gas. Detection was performed in dynamic multiple reaction monitoring mode (DYN-MRM) at unit mass resolution with a Cycle Time of 300 msec. The MRM experiment is summarised in Table S2-2. The most intense MRM was used as the quantitative ion, with the remaining MRMs being used for ion ratio confirmation.

Table S2-2. Summary of multiple reaction monitoring (MRM) experiment

Compound Name	**Precursor Ion**	**Product Ion**	**Collision Energy (V)**	**Ref. Time (min)**	**Ref. Window**	**Polarity**
Prednisone	417.1	327	8	4.23	0.59	Negative
Prednisone	327.1	285.1	20	4.23	0.59	Negative
Prednisone	327.1	148.8	36	4.23	0.59	Negative

d6 Prednisolone	333.1	284	36	4.31	0.51	Negative
Prednisolone	419.1	329.1	24	4.39	0.46	Negative
Prednisolone	328.1	295.2	20	4.39	0.46	Negative
Prednisolone	329.1	280	36	4.39	0.46	Negative
Cortisone	419.1	329.1	12	4.53	0.82	Negative
Cortisone	329.1	301.2	8	4.53	0.82	Negative
Cortisone	329.1	136.9	28	4.53	0.82	Negative
d4 Cortisol	335.1	301.2	20	4.53	0.6	Negative
Cortisol	421.1	331.1	12	4.56	0.71	Negative
Cortisol	421.1	297.1	36	4.56	0.71	Negative
Cortisol	331.1	297.2	20	4.56	0.71	Negative
Cortisol	331.1	282.1	28	4.56	0.71	Negative

The results were processed using Mass-Hunter quantitative software. Internal standard correction was applied for PN and PL (d6-prednisolone), and CN and CL (d4-cortisol).

S 2.4. UPLC-MS/MS method laboratory validation for quantification of CL, CN, PL and PN in bovine liver samples.

For the bovine liver validation at this laboratory, three sets of six replicate blended negative liver samples were spiked at 1.0 ppb, 2.0 ppb and 3.0 ppb and extracted and analysed, as in the procedure described above, against a matrix spiked calibration curve over the range 0.5 µg/kg –10 µg/kg. Liver samples were homogenised for 30 seconds using a Silverson SL2 laboratory homogeniser.

The extraction was repeated on three separate days, and after ensuring all relevant identification criteria were met, the collated data were used to determine recovery, within-day, between-day and intermediate precision, and - by using the intercept of the calibration curve method - the parameters CCα (decision limit) and CCβ (detection capability). As corticosteroids are not authorised for use in the EU, the alpha-error was set at 1%. To determine CCα a calibration line is produced based on all the contributing recovery responses at the three levels 1.0 ppb, 2.0 ppb and 3.0 ppb over the three days. This is extrapolated to determine the signal (y response) at concentration x equal to zero; the Standard Error of the intercept is determined and multiplied by 2.33 (1%, one sided) to give a response equivalent to a concentration CCα. The detection capability is equivalent to the concentration obtained from a signal at CCα plus 1.64 times the Within-

Laboratory reproducibility at CCα.

S2.5. Measurement of the urinary creatinine (Cr) concentration of the samples and quality control

Following the UPLC-MS/MS analysis of each sample, the remaining sample was analysed for Cr content (µmol/L). This analysis used an Audit Diagnostics Creatinine Jaffe Reaction kit and an Audit Diagnostics Sapphire 800 analyser. The automated sampler takes up 15 µl of undiluted urine and transfers it to a reaction cell. 250 µl of reagent 1 (Alkaline buffer) is added and mixed. 50 µl of reagent 2 (picric acid) is added and mixed. The reaction time is 10 minutes and measurements recorded '2-point rate' with main wavelength 505 nm and second wavelength 570 nm.

The analyser is calibrated daily using the Audit Diagnostics General Chemistry calibrator. Calibration is 2-point linearity. The analyser performance is quality controlled using the Audit Diagnostics General Chemistry level 1 (low) and level 2 (high) QC controls. These have assigned values based on each lot number of the kit used. The QC values are taken each day and recorded in the lab database. For an analysis to be valid, QC must fall within 1.0 standard deviation of the expected value.

The urinary GC concentrations were then expressed as a ratio to the Cr concentration (ng GC/mg Cr) in each sample.

Supplementary Material