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

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Short title: harbour seal pup urinary glucocorticoids in rehabilitation

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ABSTRACT

The aim of this descriptive study was to investigate whether aspects of the care and conditions of harbour seal (*Phoca vitulina*) pups in rehabilitation centres may be reflected in urinary concentrations of naturally-occurring glucocorticoid hormones (GCs). Urine samples were collected non-invasively from pups at five different rehabilitation centres, three on the Irish Sea and two in the southern North Sea. Concentrations of urinary cortisol, cortisone, prednisolone and prednisone were analysed by mass spectrometry. Urinary concentrations of prednisolone and prednisone were found, for the first time in any mammal species, to occur in similar magnitude to cortisol. Cortisol, cortisone and prednisolone concentrations were reciprocally correlated, but prednisone was not correlated with cortisol and only weakly correlated with the other three GCs. The strongest relationships between GC concentrations and pup conditions and procedures were negative correlations of prednisone with growth rate of individual pups and of prednisolone with body weight. GC concentrations were not significantly influenced by social groupings (pups alone, paired or in groups of more than two) or free access to water. Our results suggest that endogenous prednisone and prednisolone may be elevated in harbour seal pups during rehabilitation in response to slow growth rates relative to the very rapid growth of free-living nursing pups. Since the physical and social captive environment of the pups was not significantly reflected in urinary GC concentrations in our study, we suggest that multimodal indices of pup welfare should be used to assess design of existing rehab environments. Based only on the results of our study of urinary GCs in our sample of pups, we recommend feeding a diet, from first entry to a rescue centre, which maximises weight gain; this should not only help to prevent chronically elevated GC levels during the rehabilitation period, but would also minimise time spent in captivity.

Key words

Seal pups, glucocorticoids, rehabilitation, growth, welfare, captive environment

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INTRODUCTION

Harbour seal pups (*Phoca vitulina*) are highly precocial, and free-living pups spend up to 60% of their time in the water from birth [1, 2], constantly in close proximity to their mother while they move in and out of the water [3], and the importance of water in the daily activity pattern of free-living harbour seal pups has been highlighted in many behavioural studies [1, 3, 4, 5, 6, 7, 8, 9].

The average birth weight of healthy pups is around 11 kg [10]. The fat content of the milk increases from about 41% at birth to 50% by day 7 until weaning [11] with pups typically achieving an average net weight gain of 0.4 to 0.6 kg/day until weaning at 3.5–6 weeks of age, depending on the population [2, 10, 12]. After about 10 days of age, by which time most pups will have gained 4–6 kg, mothers sometimes leave their pups in the haul-out group of seals for a few hours while they make foraging trips offshore [6, 13], but such healthy pups without their mothers in attendance are rarely more than a few metres from other conspecifics.

New-born harbour seal pups found stranded without their mother on the shoreline, away from any seal group, are usually considered to be ‘orphan’ pups, permanently separated from their mother [14, 15], and are typically taken to seal rehabilitation (‘rehab’) centres for mother-substitute care until they are considered well-enough grown to be released back into the sea, after a period varying between about six weeks to 5 months [14, 15, 16, 17].

Newly-admitted orphan pups are often held in isolation in dry pens [15, 18, 19], with the objective ‘quarantine’ to prevent cross-infection of potential disease organisms [15, 20], although some centres usually house orphan pups that appear to be clinically healthy in pairs or groups with free water access [16, 21]. Recently it has been queried whether the traditional ‘quarantine’ in a dry pen of orphan pups can represent good welfare since it prevents the expression of natural behaviours of a pup with its mother [3].

Because orphan harbour seal pups in rehab will seldom suckle from a bottle, they are sometimes force-fed on pieces of whole fish [18, 22], but more usually fed a liquid formulation through a gastric tube [14, 19, 16, 23, 24]. The liquid formulation has varied from liquidised fish ‘soup’ [14, 15, 19, 25,

26] to complex high-fat, high-protein liquid formulations [14, 16]. Feeding early post-natal pups only on fish pieces or fish soup results in little or no weight gain by pups at this stage [14, 16, 26], but the high fat /protein formulations can achieve as much as 0.3 kg/day [16], which is nevertheless still only a fraction of the growth of a free-living pup. It may therefore also be questioned whether a slow growth rate during the early rehab weeks can be compatible with good welfare.

One indicator of poor welfare has historically been considered to be the ‘stress’ level, often assessed by measuring concentrations of the glucocorticoid (GC) hormone cortisol in blood serum, with relatively high cortisol levels believed to reflect greater ‘stress’ and therefore poor welfare. However, it is recognised that serum cortisol offers only a ‘snapshot’ hormone level and may not truly monitor the ‘stressed’ state of the animal [27, 28, 29]. Faecal corticosterone has sometimes been used instead to monitor potential stressors, and has the advantage of being non-invasive [27, 30, 31].

Caveats to a simplistic ‘stress’ interpretation of cortisol levels include feedback mechanisms resulting in reduced cortisol output in chronic or sustained stress situations [31, 32, 33]. An immediate ‘stress’ situation to a free-living harbour seal pups occurs when it loses contact with its mother, and then typically gives the distress call [34] accompanied by agitated movement. Serum cortisol levels have been used as a measure of stress in seal pups when being captured in the field [35, 36]. Pups captured in the field while separated from their mothers have higher cortisol levels than pups captured with their mothers [36]. Elevated cortisol levels trigger the distress call and the energy release necessary to fuel the agitated activity shown by a pup trying to reconnect with its mother. This is considered (in domestic guinea-pigs, *Cavia porcellus*) to be the initial “acute” stage of separation [37], which in most cases would already be over by the time orphan seal pups enter rehab centres. Cortisol levels are expected to reduce during the next “depressive” stage of separation, when the pup is likely to show reduced locomotion and other more passive behaviours [37, 38, 39]. Orphan pups in rehab centres may therefore be particularly prone to be in the “depressive” stage of separation if they are held in isolation.

Elevated serum cortisol found in newborn harp seal (*Phoca groenlandica*) pups and in both harp seal and grey seal (*Halichoerus grypus*) pups in the immediate postweaning period are thought to be involved in releasing glucose from body tissues for immediate energy [40]. Increased cortisol production by post-weaning grey seal pups is also thought to contribute to the regulation of protein catabolism in the post-weaning, fasting period [41]. For harbour seal pups in rehab, serum cortisol levels rose while pups were being tube-fed a milk formula, but stabilised when they were weaned on to fish, even though the average growth rate remained similar (0.21–0.22 kg/day) [24].

These studies suggest that GC release in seal pups is likely to be related to regulating energy expenditure, growth and fat storage in the rapidly growing and physically active free-living neonate harbour seal pup. It would therefore not be surprising if GC release would respond to regulating energy, growth and fat storage in the much slower-growing and physically less active pup in a rehab environment, but it is not yet understood whether the widely varying conditions in different rehab centres of feeding and captive environment may be reflected in levels of GCs.

The levels of other GCs have not so far been assessed in phocid seals. Cortisone (CN) is a downstream oxidised metabolite of CL [42], with less than half of CL's biological activity [43, 44]. Endogenous levels of urinary CN have been investigated in cattle in both natural conditions [45] and conditions assumed to be stressful [46]. Urinary prednisolone (PL) and prednisone (PN) have often been considered to be synthetic GCs, but may also be naturally-occurring in cattle in trace amounts, becoming elevated in stressful situations [47, 48]. Significant levels of endogenous PL and PN may occur in other mammals, but have not yet been reported.

In order to detect and distinguish between the four GCs (CL, CN, PL and PN) in a single sample, mass spectrometry for multiple steroids is essential [49]. Thus far, CL levels in seal species have been investigated by immunoassay techniques, which cannot simultaneously distinguish CN, PL and PN which may be present in the same sample, and which – especially PL – may have significant cross-reactivity with CL [50] and therefore result in a potentially elevated reading for CL [24, 36, 51].

Because restraining animals to obtain blood samples for cortisol (CL) assays is stressful *per se*, urine sampling has been suggested as being a less invasive procedure [52, 28] and has been trialled successfully in free-living Weddell seals [53]. GC measurement in urine has the advantage that protein-bound GCs are excluded from the kidney filtrate and so urinary GCs should reflect the free hormone circulating in blood over the time that the urine collects between voiding [28, 29].

Because the extent to which the rehab environment of 'orphan' pups mimics aspects of the free-living nursing pup's natural environment – in terms of its close physical relationship with its mother, large amount of time spent active in the water and rapid growth rate – varies among different rehab centres, we hypothesised that the pups' GC levels, might be relatively high if held in isolation, without water and with slow growth rate. The present study is a preliminary report to explore these hypotheses by investigating naturally-occurring urinary GC concentrations in harbour seal pups in five rehab centres with different feeding and care systems. This study is purely descriptive, since aspects of the care and conditions of orphan pups at each centre could not, for ethical reasons, be manipulated experimentally for research purposes.

ANIMALS AND METHODS

Pup background

This study involved harbour seal pups which had been admitted to five participating rehabilitation centres, three on the Irish Sea coast of Ireland and two in the southern North Sea (Lincolnshire, UK and northern Germany). All the pups had been found stranded on the shoreline during the pup birthing season. On admittance to the centres, the pups were found to be mostly at or below average birth weight of approximately 11kg for harbour seals [2, 10, 12] and were all therefore believed to have been permanently separated from their mothers in the neonatal or early post-natal period, which we consider to be an operational definition of 'orphans'. None of the pups were of sufficient size to have been healthy pups aged 10 days or more, mistakenly taken into captivity when their mothers had left them onshore temporarily while foraging offshore [Boness et al. 1994].

None of the pups in the study received treatment with synthetic steroids.

Pup rehabilitation centres

Five seal pup rehabilitation centres participated in this study. Two were on the southern North Sea coast: centre I in Lincolnshire, UK and centre IV in northern Germany. The other three centres (II, III and V) were located on the Irish Sea coast of Ireland. There were therefore differences between centres in the population of the pups' birth in addition to differences in pup care procedures and the rehab captive environment (Table 1) during the sampling period. In centres I and III, the pups were kept alone in dry pens, in centres II and IV the pups were usually in pairs or groups with free access to water, and in centre V the pups were either alone or paired and in dry pens (Table 1, Supporting file S1 Photos).

Table 1. Number of pups at each of five rehab centres urine-sampled in the study, with summary of rehab environment and average growth rate (kg/day) at each centre

centre	**I**	**II**	**III**	**IV**	**V**
No. pups sampled	4	6	7	13	6
Sex M/F	2M	2M/4F	5M/2F	6M/7F	4M/2F
No. samples analysed by MS	11	39	13	37	15
Analysed by ELISA	17	NA	NA	NA	NA
variables					
Social groups at each centre	1	2	1	1, 2, 3	1, 2
Free water access	No	Yes	No	Yes	No
x sampling *rehab day* (range)	13 (4-21)	25 (8–44)	6 (2–12)	6 (2–17)	19 (7–40)
x growth rate (kg/day)	0.06	0.29	0.21	0.15	0.07
(range)	(0.03–0.08)	(0.27–0.30)	(0.1–0.26)	(0.01–0.26)	(-0.1–0.19)
(SD)	0.04	0.01	0.06	0.07	0.10
x weight (kg) on sample day	9.7	15.6	9.4	11.6	11.4
(range)	(9.5–10.3)	(9.3–21.5)	(7.9–11.8)	(7.1–14.9)	(9.2–14.9)
(SD)	0.31	4.14	1.48	1.74	1.82

In this study we considered *pup growth rate* (kg/day) recorded by the centre personnel during our sampling period. We also recorded three categories of each pup's *social group* environment at the time of sampling, i.e. (1) alone, (2) paired or (3) more than two pups, and whether the pups had *free access to water* (Table 1).

Pups were weighed by centre personnel on alternate days at centre II and approximately once a week at the other centres. Average *growth rate* (kg/day) during the sampling period was calculated from the first and last weighings. Estimated pup *weight* on sampling day was calculated as the mid-point between two adjacent weighings in centre II and in the other centres from the nearest pup weighing and average growth rate during the sampling period. The average growth rate during the sampling period for pups at each centre is given in Table 1. Individual pup *sex* and *ID* were also included as variables for the analyses.

Urine sampling

This study used urine rather than blood sampling in order for the sampling to be completely non-invasive. The urine samples were collected opportunistically if the pups urinated while being handled during routine feeding or weighing. The authors (SW and SV) collected samples at centres II, IV and V while centre personnel collected samples at centres I and III. Collection was usually with a syringe from the ground beside the pup or directly from the stream of urine, although one sample from centre II (TSR-14-18; Supporting file S2-dataset) was collected on a 'puppy pad' (plastic-backed tissue layers). Some samples at centre I were collected from a tray under the slatted floor of the pen during morning cleaning (representing urine voided at any time since the previous evening). Samples were transferred to standard plastic sample pots with a record for each sample of the source pup, date and collection time. Samples were immediately transferred to a freezer (at least -18°C) and stored until analysis. Only samples with at least 10 ml were used in the analyses.

Since it has been suggested that neo-formation of the GC prednisolone due to microbial action may occur in bovine urine after collection if the samples are kept at a high temperature (e.g. 37°) or if there is contamination (e.g. from faeces) [54]. We were therefore careful to ensure that the samples used for analysis were free from visible faecal contamination, were not subjected to high temperatures.

Since a study of plasma cortisol levels in captive harbour seals found a diurnal pattern, peaking at 01:00 and waning around 13:00 [51], the time of day of each sample collection was recorded and samples from pup rehab day 2 onwards used in the analyses (n=112) were assigned to time categories:

0700–12:59; 13:00–18:59; 19:00–00:59 (Fig 1); no samples were collected during the overnight period.

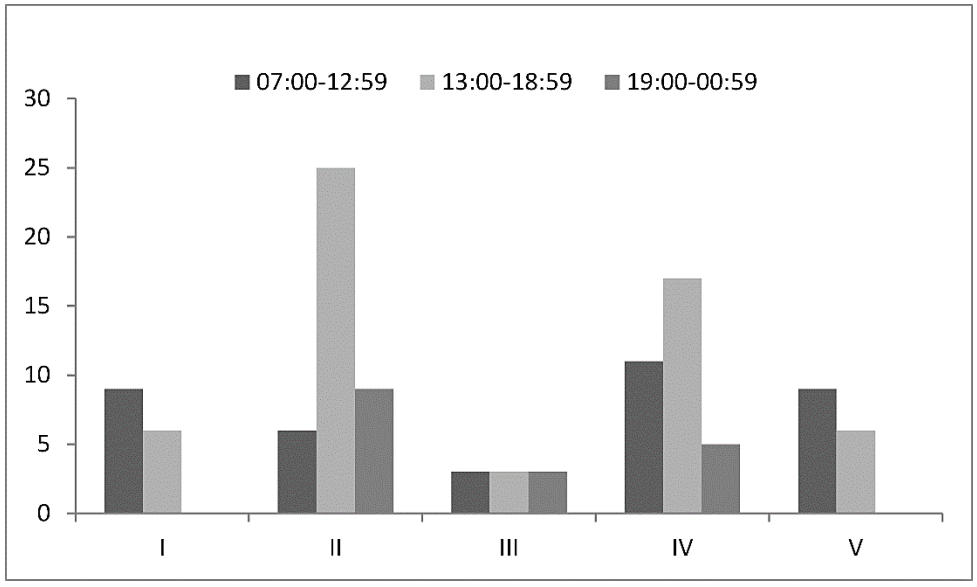

Fig 1. Distribution of urine sample collection between three 6-h periods at each centre (from all samples day 2 onwards).

Seven samples were collected from a pair of pups (in Centres II and IV), but the donor pup was not identified (Supporting file S2). These samples were included in analyses of the overall range of GC concentrations recorded and of the possible effect of the time of day of sampling, but were excluded from subsequent analyses.

Glucocorticoid analysis

Seventeen samples from Centre I (2012) were analysed by ELISA (enzyme-linked immunosorbent assay) for cortisol (CL) at Riseholme Park, University of Lincoln (Lab 1). Eleven duplicate samples from Centre 1 and a further 8 from centre II (2013) were analysed for CL at the Institute of Global Food Research, Queens University, Belfast (Lab 2) by ultra-performance liquid chromatography – tandem mass spectrometry (UPLC–MS/MS) [55, 56]. Ninety-six further samples collected from centres II, III, IV and V in 2014–15 were analysed at the Chemical and Immunodiagnostic Sciences Branch, Veterinary Sciences Division, Agri-Food & Biosciences Institute (AFBI), Belfast (Lab 3) for CL, cortisone (CN), prednisolone (PL) and prednisone (PN), also by UPLC–MS/MS. The full list of

samples for each of the three analytical laboratories and methods are given in Supporting file S2-Dataset.

Urinary creatinine concentrations.

In order to control for the water content of the sample, urinary GC concentrations are expressed as a ratio to the creatinine (Cr) concentration (ng GC mg/Cr) in each sample [24]. Creatinine concentrations at all three laboratories 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 [57]. The detailed methods of Cr determination and quality control procedures followed by each laboratory are given in Supporting files S3 and S4 Protocols.

Analysis of urinary cortisol concentrations by ELISA

Urinary CL concentrations in 17 samples from centre I in 2012 were determined at Lab 1 using a cortisol ELISA kit (Cat No ADI-900-071) for colorimetric detection from Enzo Life Sciences (UK) Ltd. The principle of the ELISA assay is the binding of a specific antibody to an enzyme-labelled target antigen (CL in this case). The enzyme activity is measured using a solid substrate that changes colour when modified by the enzyme. The intensity of the colour depends on the CL concentration and is read on a microplate. The details of the procedures undertaken following the kit instructions are given in Supporting file S3 Protocol. This commercial kit is available having already been validated for determining CL concentrations in plasma, saliva, serum or urine from any species. However, because the concentration range of CL in harbour seal urine was not known, a test ELISA run was initially carried out to estimate the urine sample dilutions needed for the middle calibrator of the ELISA kit. The method (S3 Protocol) followed the kit instructions.

The cross-reactivity with other steroid hormones is stated by the manufacturer of this ELISA kit to be Cortisol (100%), Prednisolone (122.35%), Corticosterone (27.68%), 11-deoxycortisol (4%), Progesterone (3.64%), Prednisone (0.85%), Testosterone (0.12%) and ≤ 0.10%: Androstenedione, Cortisone and Estradiol. Because of the high cross-reactivity with prednisolone, which was one of the four GCs later analysed in this study by UPLC-MS/MS, the limitations of the ELISA method are

considered in the detailed protocol (Supporting file S3). Duplicates of eight of the samples from centre I were later also analysed by UPLC-MS/MS to enable a comparison of the results from the same samples by both methods.

Analysis of urinary glucocorticoid concentrations by Ultra Performance Liquid Chromatography–tandem mass spectrometry (UPLC–MS/MS)

Immunoassays – as the ELISA described above – may be used to determine the concentration of a single steroid hormone (assuming cross-reactivity is not considered to be high), but if the concentrations of several steroids 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. Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) can quantify either one or multiple steroids at wide-ranging concentrations, while avoiding the issue of antibody cross-reactivity [49], as discussed in relation to the ELISA kit above for high cross-reactivity between CL and PL.

For this study of seal pup urine glucocorticoid steroids, ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC–MS/MS) was employed by Lab 2 to determine urinary CL concentrations of 19 samples collected in 2012–13 and by Lab 3 to determine concentrations of the four GCs, CL, CN, PL and PN in 96 samples collected in 2014–2016.

The CL analyses of the 2012-2013 samples by Lab 2 were carried out alongside a separate study of CL and CN concentrations in cattle [46], while the analyses of CL, CN, PL and PN in the 2014–2016 samples by Lab 3 were carried out according to the procedures for analysis of bovine liver as part of the routine statutory Veterinary Drug Residue testing programme in N. Ireland. The UPLC–MS/MS methods employed by both Labs 2 and 3 are described in full in Supporting file S4 Protocol.

Method validation. The seal pup urine samples were analysed by UPLC-MS/MS at laboratories where the analytical method validation was previously undertaken for analysis accuracy/recovery, intra- and inter-day repeatability and linearity for bovine urine in Lab 2 [46] and bovine, porcine and ovine liver

in Lab 3, in accordance with EU guidelines (2002/657/EC). The method validation procedures followed at both laboratories are described in Supporting file S4 Protocol.

Statistical analyses

Statistical analyses were carried out in the XLSTAT-pro statistical package [58]. intercorrelation between concentrations of the four GCs (CL, CN, PL and PN), and comparison of the CL results between ELISA and UPLC-MS/MS methods used correlation (Pearson coefficient). Comparison of CL and PN concentrations during different time periods of the day used Kruskal-Wallis 3-sample test or Mann-Whitney 2-sample test. Analysis of variance (ANOVA) was used to describe the variation in GC concentrations and in pup *growth rate* among the five centres.

Because multiple samples were taken on different days from many pups, a median value of GC concentrations from each pup was used to investigate the potential influence of variables that were constant for each pup during the sampling period, i.e. pup sex, average *growth rate* and *free access to water*.

Regression analyses were used (a) to investigate the potential effect of pup weight on the day of entry to the centre and subsequent growth rate, (b) the potential influence of growth rate on GC concentrations and (c) the potential influence of pup weight on sample day on GC concentrations.

Analyses of covariance (ANCOVA) were used to describe the combined influence of the variables in pup care on each of the GCs analysed by UPLC/MS-MS. The ‘Type III’ ANCOVA results were used throughout; these are computed by removing one variable of the model at a time to evaluate its impact on the quality of the model. This means that the order in which the variables are selected will not have any effect on the values in the type III sum of squares, and this is the best method to use to interpret results when interaction between variables is part of the model [58]. The concentrations in all samples were used to investigate the potential influence of variables which changed for all pups (*rehab day*, *weight* on the day of sampling) or for some pups (*social group*) during the sampling period; where all samples were used in the analysis, pup ID was also an explanatory variable.

RESULTS

Range and median concentrations and correlation matrix of four glucocorticoid hormones (GCs) by UPLC-MS/MS

The range of creatinine (Cr) concentrations in the 115 samples analysed by MS/MS was 115–15576 µM/L, (mean 3219, median 2273 µM/L). The range of GC concentrations (ng/mg Cr) was greatest for cortisol (CL) (0–8062 ng/mg; next for cortisone (CN) (0–4853 ng/mg), with a lower range for prednisolone (PL) (0–236.6 ng/mg) and prednisone (PN) (0–142.5ng/mg). The complete set of raw data is given in Supporting file S2 Dataset and the range of MS/MS results on a $\log_{10}$ scale is presented in Fig 2.

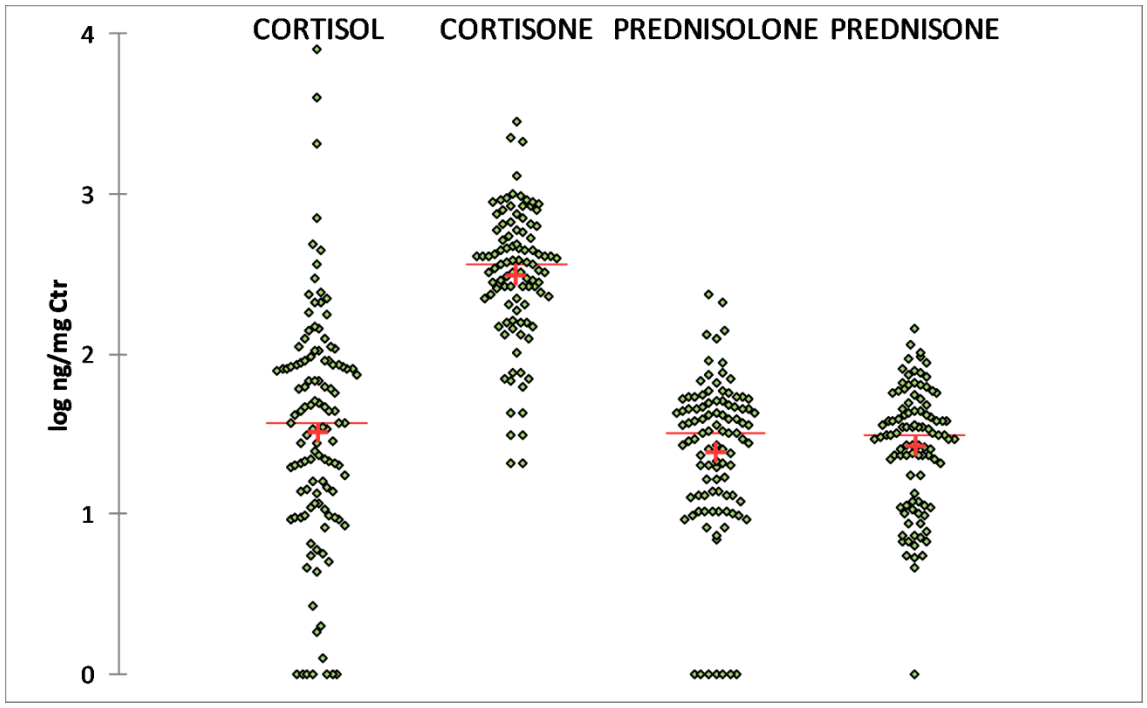

Fig 2. Scattergram of urinary concentrations of four GCs from all samples analysed by UPLC MS/MS. (n=CL–115; CN, PL, PN – 96). Concentrations ng/mg creatinine on $\log_{10}$ scale. Horizontal lines indicate medians and + markers indicate means.

The median values for CL, PN and PL were similar to each other (36.9, 31.1 and 31.57 ng/mg Cr respectively), while median CN concentrations were an order of magnitude higher (390.4 ng/mg Cr). There were seven samples with CL and PL concentrations below the detection limit (Fig 2). Six of these samples had low Cr concentrations (range 115–683 µM/L). One sample which gave a very high

CL concentration (2030 ng/mg Cr) also had a low Cr concentration (444 µM/L). Because some very high or very low results for concentrations came from dilute samples, the median concentrations rather than the means are used in continuing analysis of the data.

The correlation matrix (Table 2) for all four GCs for the samples measured by UPLC MS/MS indicates that CL, CN and PL were significantly correlated with each other. PN was not correlated with CL and correlated relatively weakly with PL and CN.

Table 2. Correlation (Pearson coefficient) between concentrations of four GCs (ng/mg creatinine) by UPLC MS/MS (n=96 samples)

	Cortisol	**Cortisone**	**Prednisolone**	**Prednisone**
Cortisol	1	0.751	0.726	0.070
Prednisone	0.070	0.262	0.362	1
Cortisone	0.751	1	0.504	0.262
Prednisolone	0.726	0.504	1	0.362

The ratios of mean and median CL concentrations to the other three GCs from all samples from which all four GCs were analysed are given in Table 3.

Table 3. Ratios of mean and median concentrations (ng/mg Cr) of Cortisol (CL) to each of the other GCs in all samples for which all four GCs were analysed (n=96 samples).

	Cortisol	**Cortisone**	**Prednisolone**	**Prednisone**
Means	214.37	526.19	39.91	36.68
SD	922.39	617.28	43.62	26.73
Median	35.96	390.38	30.42	31.72
Ratio of CL: other GC (mean)		0.41	5.37	5.84
Ratio of CL: other GC (median)		0.09	1.18	1.13

Comparison of cortisol/creatinine concentrations analysed by ELISA and UPLC MS/MS

11 of the 17 samples from centre I were analysed for CL by both UPLC-MS/MS and ELISA. The correlation (Pearson coefficient) between the MS/MS and ELISA results was 0.795 (2-tailed P <0.01), although the mean concentrations for the ELISA results were about 13% higher on average than for MS/MS (Table 4).

Table 4. Cortisol concentrations (ng/mg creatinine) comparing UPLC-MS/MS with ELISA

	n	Minimum	Maximum	Median	Mean	SD	Correlation (Pearson coefft)
MS/MS	11	21.8	221.2	73.5	86.9	54.5	0.795
ELISA		46.0	211.0	98.0	98.1	47.0	

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

Since pups' GC concentrations when they first arrive at the centre are likely to reflect their stranding conditions, handling and transport to the centre, concentrations in samples taken on rehab days 0–1 were tabulated separately from samples from rehab days 2 onwards (Table 5). The single or median concentrations for each pup in days 0–1 (n= 8 pups sampled for CL, 6 pups for PN, CN and PL) were compared with single or median concentrations in day 2 onwards (n= 34 pups sampled for CL, 34 for PN, CN and PL). CL, PN and PL concentrations were generally higher on days 0-1 (Mann-Whitney U test; 1-tailed $P < 0.05$ for each GC), although CN concentrations did not differ ($P = 0.328$). Since the aim of the study was to investigate the influences of the rehabilitation environment on pup GC levels, and not the pups' pre-admission history, the 12 samples taken from 8 pups on days 0–1 (Table 5 and Supporting file S2) were separated from the continuing analyses, which considered pups from rehab days 2 onwards.

Table 5. Summary of individual pup data and results of urinary glucocorticoid analysis (GC results in ng/mg creatinine)

									CORTISOL		CORTISONE		PREDNISOLONE		PREDNISONE	
Centre	**Pup name**	**Sex**	**Date of entry to rehab**	**Entry weight (kg)**	**Feeding method**	**Growth rate (kg/d)**	**social group**	**n (samples)**	**range**	**median or single**	**range**	**median or single**	**range**	**median or single**	**range**	**median or single**
Rehab day 0–1																
I	Orlando	M	21/07/12	10.1	Fish FF-HF	NA	1	1	221.23		NA	NA	NA	NA	NA	NA
I	“	“	“	“	“	“	“	“	211†							
I	Phocina	F	25/07/12	8.2	Fish FF-HF	NA	1	1	287†		NA	NA	NA	NA	NA	NA
II	Ula	F	05/07/14	7.1	Tube	NA	1	1		8062		4853.00		46.00		249.00
III	Rowan	M	24/06/14	7.8	Tube	NA	1	2	5-16	10.50	405-418	411.80	38-57	47.49	14-26	19.56
III	Beech	M	06/07/14	10.3	Tube	NA	1	1		14.28		407.00		30.00		13.62
III	Polly*	F	18/07/15	6.5	Tube	NA	1	1		486.6		455.03		114.93		236.59
IV	Urmel	F	05/07/15	10.6	Tube	NA	1	2	68-124	96.43	61-240	150.64	88-143	115.56	55-132	98.95
IV	Julio	M	08/07/15	11.5	Tube	NA	1	3	110-447	145.14	279-788	483.99	30-79	57.42	49-124	52.65
IV	Schultze	F	08/07/15	6.9	Tube	NA	1	1		4028.9		459.75		206.4		25.42
Rehab day 2 onwards																
I	Barry	M	20/06/12	8.94	Fish FF	0.08	1	7	33.6-106.8	62.83	NA	NA	NA	NA	NA	NA
I	“	“	“	“	“	“	“	9	61–134†	98†	NA	NA	NA	NA	NA	NA
I	Pascal	M	23/06/12	9.07	Fish FF	0.03	1	3	21.8-123.3	110.35	NA	NA	NA	NA	NA	NA
I	“	“	“	“	“	“	“	6	64-1164†	84†	NA	NA	NA	NA	NA	NA
II	Maxi	M	19/07/13	11.1	Tube	0.28	2	7	9.4-36.4	15.78	NA	NA	NA	NA	NA	NA
II	Mini	F	19/07/13	9	Tube	0.29	2	1		14.8	NA	NA	NA	NA	NA	NA
II	Ula	F	05/07/14	7.1	Tube	0.28	2	9	0.0-49.0	20.78	123.7-871.7	293.00	6.3-47.8	21.74	0.0-40.7	15.45
II	Earendil	M	07/07/14	7.4	Tube	0.27	2	3	6.6-21.3	20.16	231.2-693.4	444.82	9.2-14.8	11.76	8.8-11.2	9.09
II	Coral	F	03/07/16	8.5	Tube	0.29	2	6	9.2-206.7	39.14	69.5-571.2	153.34	5.4-27.0	20.35	10.4-45.2	32.46
II	Pearl	F	05/07/16	8.3	Tube	0.3	2	6	2.7-365.3	14.83	20.9-403.0	59.05	6.7-34.4	10.43	8.2-53.3	11.76
III	Elm	M	20/06/14	8	Tube	0.22	2	2	20.7-51.6	23.86	320.4-850.1	585.20	20.7-51.6	36.18	0.0-16.7	8.35
III	Birch	M	13/06/14	7.5	Tube	0.26	1	1		0.00		291.03		63.84		0.00
III	Fern	F	02/07/14	7.75	Tube	0.18	1	1		20.51		445.16		23.09		19.31
III	Finn	M	03/07/15	8.5	Tube	0.25	1	2	1.0-1.2	1.09	101.9-187.1	144.50	5.3-9.7	7.51	24.0-51.5	37.73
III	Isla	F	16/07/15	8	Tube	0.1	1	1		13.85		67.54		10.61		90.21
III	Klamath	M	30/06/15	7	Tube	0.23	1	1		11.73		327.63		39.22		91.30
III	Rowan	M	24/06/14	7.75	Tube	0.25	1	1		1.83		384.96		35.92		7.32
IV	Bärchen	M	29/06/15	12.2	Fish HF	0.16	3	1	10.67	10.67		259.85		22.97		27.31
IV	Beate	F	03/07/15	10.6	Tube	0.26	2	2	0.0-175.1	87.54	782.2-984.2	883.22	21.9-23.8	22.85	0.0-21.5	10.75

IV	Chigoma	M	03/07/15	10.3	Tube	0.01	2/3	2	66.7-178.6	122.67	371.8-827.1	599.47	40.3-48.0	44.18	36.7-57.3	47.00
IV	Daggi	F	25/06/15	10.7	Fish HF	0.17	3	1		68.43		357.34		45.78		26.83
IV	Eddi	M	01/07/15	13.1	Tube	0.17	2/3	4	44.4-79.9	62.26	281.6-741.0	592.26	25.2-60.9	28.21	31.6-66.7	40.23
IV	Henry	M	03/07/15	10.1	Tube	0.18	3	3	89.2-243.6	103.84	588.9-896.6	668.15	34.8-61.7	42.89	37.6-51.2	39.38
IV	Jens	M	26/06/15	8.8	Fish HF	0.23	3	3	41.5-60.3	44.10	383.3-515.5	464.13	30.8-36.3	34.35	36.8-42.2	39.40
IV	Julio	M	08/07/15	11.5	Tube	0.03	1	1		82.6		442.35		66.01		76.38
IV	Kawamba	F	06/07/15	10.4	Tube	0.13	1	1		84.19		417.66		37.67		55.18
IV	Lisi	F	01/07/15	11	Tube	0.14	2/3	4	31.0-704.8	61.11	320.1-2233.2	633.94	37.94-57.97	42.92	35.6-58.2	48.52
IV	Schultze	F	08/07/15	6.9	Tube	0.1	1/3	2	298.4-2030.9	1164.60	2109.4-2787.4	2448.40	72.6-101.3	86.96	23.1-74.0	48.57
IV	Urmel	F	05/07/15	10.6	Tube	0.11	1/2	4	90.3-236.9	178.24	440.5-1295.4	862.47	38.4-95.8	62.50	46.7-70.6	56.62
IV	Yukari	F	01/07/15	10	Tube	0.22	3	2	79.6-104.7	92.13	304.0-395.8	349.91	23.0-31.4	27.19	47.5-53.4	50.43
V	Aquaman	M	07/07/15	10.2	Tube	0.1	1	3	5.56-90.48	36.87	291.1-904.8	407.08	34.9-43.3	38.09	20.3-40.8	20.65
V	Blackbird	M	04/07/15	7.4	Tube	0.05	2	3	0.0-37.2	8.38	223.7-958.6	363.30	23.1-80.5	74.87	0.0-45.5	12.52
V	Hawkeye	M	09/07/15	8.35	Tube	0.11	1	4	0.0-138.3	47.00	162.1-941.4	586.78	0.0-92.7	70.00	31.9-139.7	59.64
V	Medusa	F	27/06/15	7	Tube	-0.1	2	2	0.0-11.5	5.76	236.7-521.1	378.87	42.0-55.8	48.90	0.0-25.3	12.65
V	Mystique	F	21/06/15	9.6	Fish HF	0.06	2	1		61.25		599.28		33.871		32.70
V	Wolverine	M	06/06/15	7.8	Fish HF	0.19	2	2	27.7-28.1	27.89	337.6-374.3	355.91	32.3-49.7	41.02	28.8-45.5	37.12

† results from ELISA, FF – force-fed, HF – hand-fed, Tube – stomach gavage; *'Polly' died on rehab day 1

Effect of time of day of sampling on GC concentrations

Distribution of sampling from rehab day 2 onwards indicated that most of the samples were taken in the morning 6-hour period (0:700–12:59) and afternoon (13:00–18:59), with fewer in the evening (19:00–00:59) (Fig 1). Comparison of concentrations of CL and PN in each of the three 6-hour periods between 07:00 and 00:59 indicated no significant differences in concentrations of either of these GCs according to time of day of sampling (Table 6).

Table 6. Median concentrations of urinary cortisol and prednisone (ng/mg creatinine) and significance of differences between each of three 6-hour time periods between 07:00 and 00:59. (samples from rehab day 2 onwards).

Time period	**07:00–12:59**	**13:00–18:59**	**19:00–00:59**	
CENTRE	Median concentration ng/mg creatinine			P value
Cortisol				
I	84.0†	97.0†	no samples	0.846
II	22.27	11.50	15.78	0.258
IV	82.6	298.38	87.17	0.994
V	27.67	45.01	no samples	0.859
Prednisone				
II	26.88	11.55	8.72	0.091
IV	41.52	36.29	34.85	0.426
V	41.97	49.55	no samples	0.680

†– concentrations from ELISA; P values from Kruskal-Wallis 3-sample test or Mann-Whitney 2-sample test; number of samples as in Fig 1.

Differences between the five centres in pup GC concentrations recorded

The distribution of GC concentrations (median or single value for each pup) analysed by UPLC-MS/MS is shown in Fig. 3. Centre IV samples had overall the highest CL, CN and PL concentrations, whereas Centre II had the lowest CL, PN and PL concentrations. Comparison between centres by analysis of variance (ANOVA) indicated that the centre explained 92% of the variability in PL concentrations (DF=29, Pr>F= <0.0001), 31% of PN variability (DF=29, Pr>F= 0.019), but only 13% of CL (DF=33, Pr>F= 0.379) and 19% of CN (DF=29, Pr>F= 0.127).

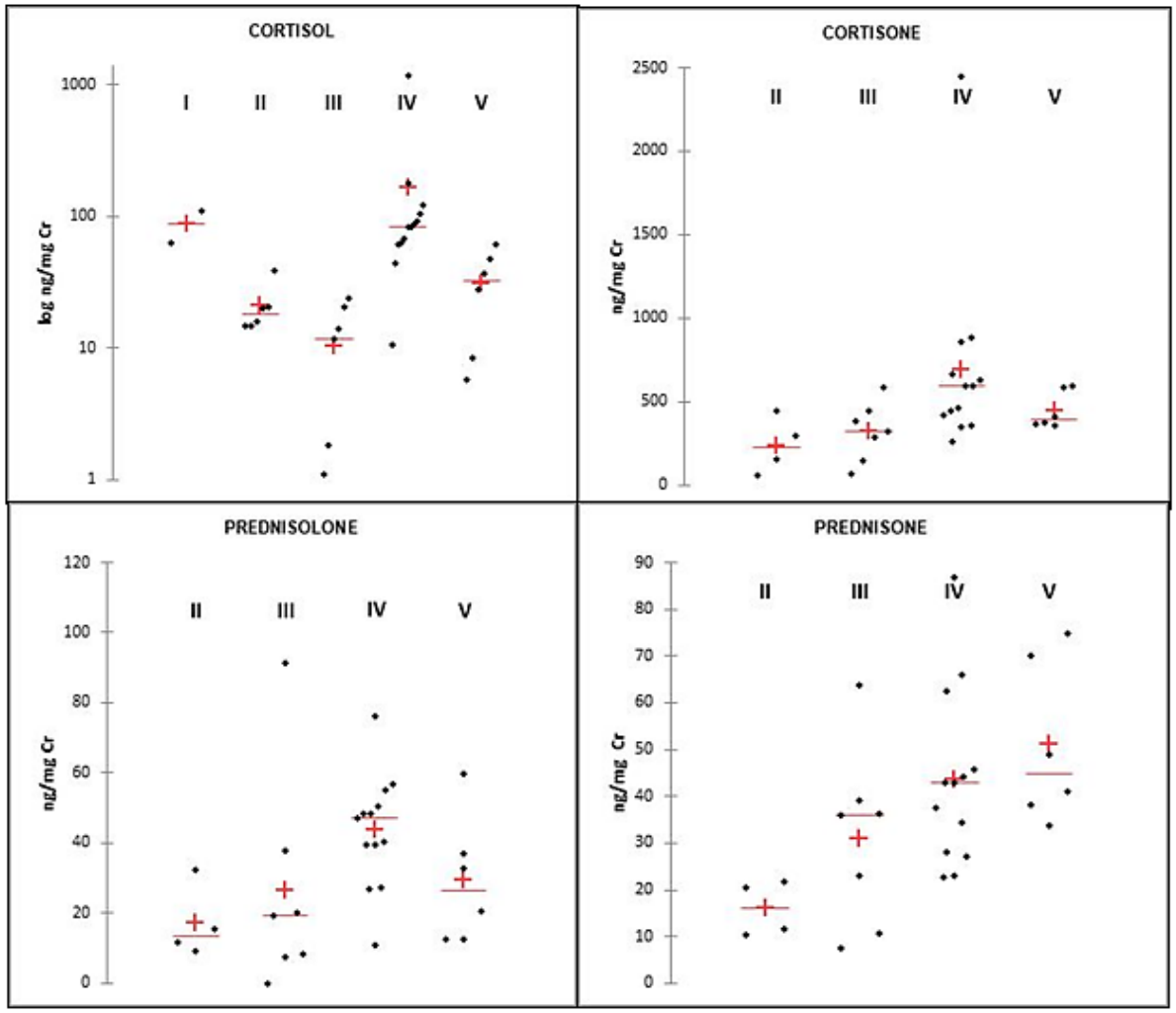

Fig 3. Scattergrams of distribution of urinary concentrations of four GCs. (median or single values for each pup sampled from rehab day 2 onwards) in each rehabilitation centre I–V. Horizontal lines indicate medians and + markers indicate means; n= 34 samples CL, 30 for PN, CN and PL.

Pup growth rates and correlations with GC concentrations

The average growth rate of pups at the five centres ranged from 0.06 kg/day (2 pups at centre I) to 0.29 kg/day (6 pups at centre II) (Table 1). Analysis of variance (ANOVA) of growth rates of each pup at the five centres indicated a significant difference between centres (n=34 pups, df=29, R^2=0.588, Pr>F <0.0001).

We considered the possibility that pup weight at entry to the rehab centre may have affected its subsequent growth rate at the centre. The entry weight of the pups ranged from 6.9 to 13.1 kg (n = 34 pups, Table 5). Regression of individual pup growth rate during the sampling period by entry weight

(Fig 4) indicated there was no relationship between entry weight and subsequent growth rate in our sample of pups ($R^2 = 0.010$, $P = 0.576$).

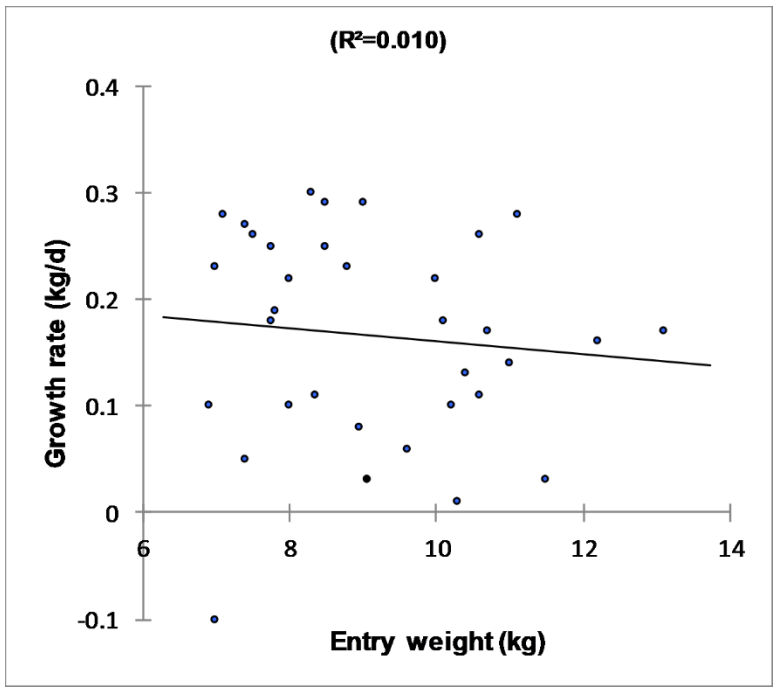

Fig 4. Regression of pup growth rate (kg/day) by entry weight (kg).

Linear regressions of median GC concentrations for each pup (n = 34 for CL, n = 30 for PN, PL and CN) by *growth rate* (kg/day) showed a significant negative correlation only for PN, indicating generally higher concentrations of PN in pups with lower growth rates (Fig 5).

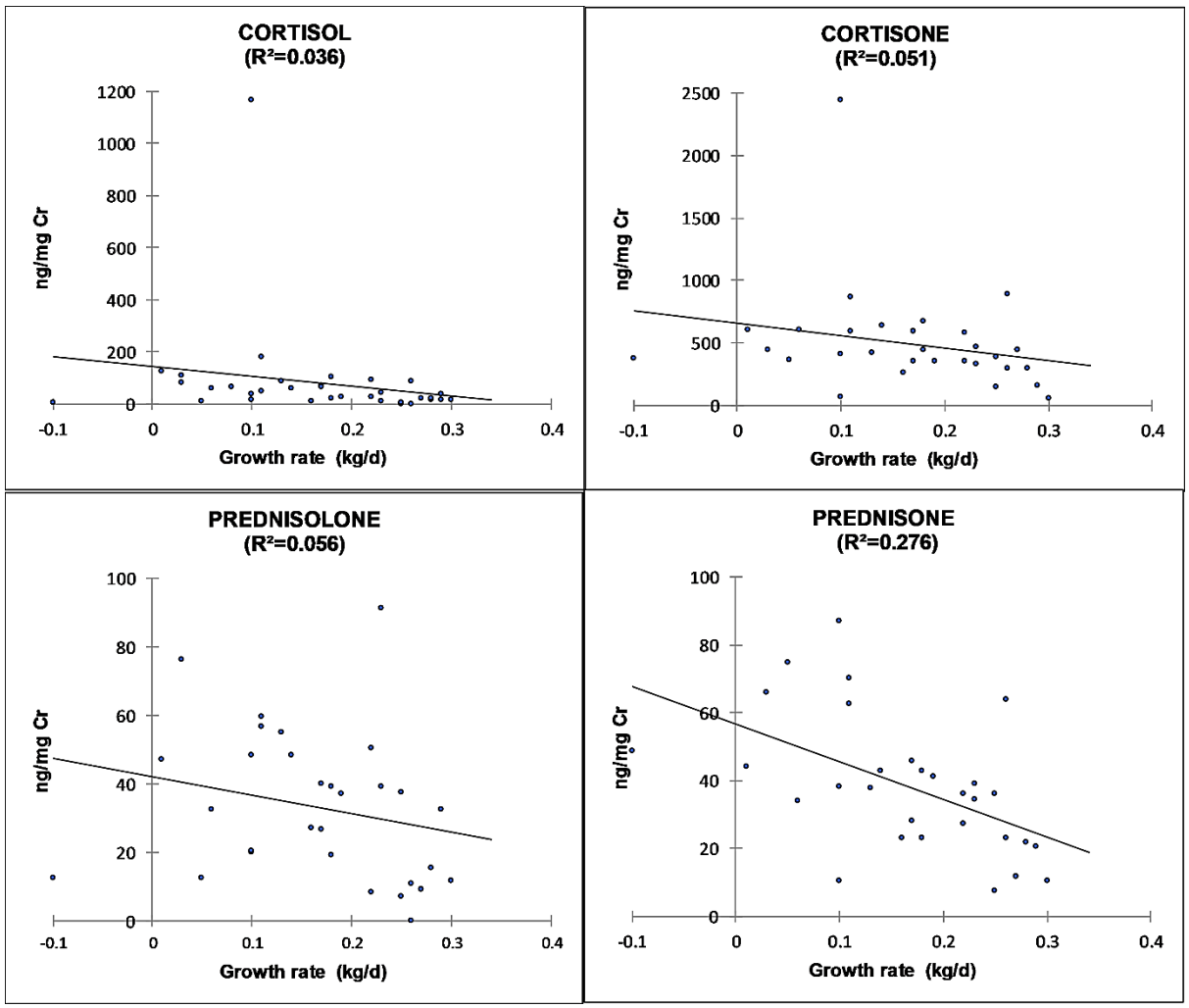

Fig 5. Regression of median GC concentrations (ng/mg creatinine) for each pup by growth rate (kg/day)

Analyses of covariance (ANCOVA)

In order to investigate how much of the variability in the concentration of a particular GC was explained by the variables considered (*growth rate*, *sex*, *weight on sample day*, free *water access social group*, *rehab day*), analyses of covariance (ANCOVA) were used (Table 7; Supporting file S5).

Median values of GC concentrations for each pup were used to consider the mixed effects of variables which did not change during the sampling period, i.e. pup *growth rate*, pup *sex* and free *water access* (Table 7a). This analysis indicated that less than 15% of the variability in CL, CN and PL concentrations was explained by these three variables. However, 32% of the variability of PN was explained by a combination of these variables, and pup *growth rate* alone brought significant

information to the model (P=0.002, Table 7a). The results indicated that neither pup *sex* nor free *water access* significantly influenced individual pup GC levels.

In order to consider the effect of *rehab day* and pup *weight* on sample day and *social group* as well as free *water access,* ANCOVA using the GC results for all samples was used. Since multiple samples were taken from some individuals over the sampling period, *pup ID* was also considered a variable, The results showed that 90% of the variability in CL concentrations and 71%, 71% and 75% of CN, PL and PN concentrations respectively were explained by a combination of these variables; *pup ID* contributed most to this variability for all GCs, most strongly for CL (Table 7b). The only other variable to influence GC levels significantly was pup *weight* in relation to PL (P=0.037, Table 7b), for which (considering all samples irrespective of pup ID) there was a negative correlation (R= -0.27, R^2=0.073, n=78, Pr>F = 0.02).

Table 7. Analysis of covariance (ANCOVA) of urinary GC concentrations of samples from pups (rehab day ≥2) showing the F probability values (type III sum of squares) for each variable considered.

a) Median values of GC concentrations for each pup ID in all five centres

	DF	CL (n=34 pups)	DF	CN (n=30 pups)	PL (n=30 pups)	PN (n=30 pups)
GOODNESS OF FIT						
R^2	30	0.147	23	0.130	0.138	**0.321**
ANOVA						
Pr>F	33	0.184	29	0.295	0.268	**0.016**
ANCOVA	Type III sum of squares analysis† Pr>F					
Growth rate	1	0.136	1	0.161	0.258	**0.002**
Sex	1	0.416	1	0.553	0.308	0.251
Water access	1	0.160	1	0.207	0.217	0.439

b) GC concentrations in all samples with pup ID from pups in all five centres

	DF	CL (n-96)	DF	CN(n=78)	PL (n=78)	PN (n=78)
No. pups		**34**		**30**	**30**	**30**
GOODNESS OF FIT						
R^2	56	0.903	42	0.712	0.710	0.745
ANOVA						
Pr>F	95	**<0.0001**	77	**0.0004**	**0.0001**	**<0.0001**
ANCOVA	Type III sum of squares analysis† Pr>F					
Pup ID	34	**<0.0001**	30	**0.005**	**0.0006**	**0.0002**
Weight	1	0.300	1	0.443	**0.044**	0.063
Rehab day	1	0.578	1	0.373	0.084	0.108
Social group	2	0.254	2	0.852	0.675	0.467
Water access	1	0.086	1	0.945	0.985	0.185

c) GC concentrations in all samples with pup ID from pups in centre II (all pups in social group 2 and with free water access)

	DF	CL (n=32)	DF	CN (n=24)	PL (n=24)	PN (n=24)
No. pups		**6**		**4**	**4**	**4**
GOODNESS OF FIT						
R^2	24	0.518	18	0.280	0.664	0.494
ANOVA						
Pr>F	31	**0.008**	23	0.270	**0.001**	**0.022**
ANCOVA	Type III sum of squares analysis† Pr>F					
Pup ID	5	**0.006**	3	0.948	**0.026**	0.264
Weight	1	**0.010**	1	0.942	**0.037**	0.271
Rehab day	1	**0.014**	1	0.984	0.056	0.347

† Sum of Squares, Mean Squares and F values are given in Supporting file S5

The potential influence of pup ID, weight and rehab day was also explored separately for the six pups in centre II, since these pups were sampled over a longer time period and had constant *growth rates*

(average 0.29 kg/day), *social group* (2 – paired) and free *water access* (yes) Tables 1, 5). The analyses indicated that 52%, 66% and 49% of the variability of CL, PL and PN respectively were explained by a combination of *pup ID*, *weight* and *rehab day*. *Pup ID* contributed significantly to the variability of CL and PL, but not to PN or CN (Table 7c). Pup weight contributed significantly to the variability of both CL and PL and rehab day also significantly influenced CL and, to a less significant extent, PL (Table 7c). Considering the centre II pups separately therefore indicates an influence of pup *weight* and *rehab day* on CL concentrations, which was not detected when all samples from all centres were considered together (Table 7b).

DISCUSSION

The occurrence of prednisolone and prednisone

The first and most striking finding from these analyses of harbour seal rehab pup urine was the high concentrations of naturally occurring prednisolone (PL) and prednisone (PN), occurring with the same order of magnitude as cortisol (CL). The ratio of CL:PL and CL:PN median concentrations in our seal pups was close to parity. By contrast, endogenous urinary PL and PN has been found to occur only in low concentrations in Chianina cows (CL:PL ratio of 25:1), even lower concentrations in Friesian veal calves (CL:PL ratio range 29–64:1, [48], and with a CL:PL ratio of ~100:1 in humans [59]. Similarly, endogenous PN has so far been reported to be absent in Piedmontese, Friesian and Chianini cattle [45, 47], although present in trace amounts (CL:PN ratio 43:1) in a small proportion of Friesian veal calves [48]. The present report of endogenous PL and PN in significant concentrations in rehab harbour seal pups appears to be the first such finding in a mammal species.

The influence of pup rehab variables investigated on GC concentrations

Pup growth rate

A prominent finding of this study was an apparently singular negative relationship between pup growth rate and concentrations of PN. This was clear from both the regression and analysis of covariance (ANCOVA), whereas the concentrations of the other three GCs appeared not to be

significantly influenced by growth rate. The unique sensitivity of PN concentrations in this regard is consistent with the lack of inter-correlation found between concentrations of PN and the other three GCs, indicating that it varies independently of them. Because harbour seal pups normally have a high growth rate during the suckling period, it is possible that elevated PN levels may be a response to the physiological processes accompanying slow growth rate of orphan pups in rehab relative to the species norm; even the pups in centre II, with the highest average growth rate of nearly 0.3 kg/day, could not achieve the harbour seal pup norm of 0.4–0.6 kg/day [2, 10, 12]. PN release may therefore increase glucose release to supply a slowly-growing pup with immediate energy. Free-living pups probably grow most slowly in the neonatal period while lactation is still becoming established, but even as neonates they spend much time actively swimming with their mothers in the sea; possibly in free-living pups PN plays a role in facilitating neonatal pup active swimming.

Pup weight

Pup weight appeared to influence PL concentrations more strongly than PN. The centre II data (although not the whole dataset) indicated also an effect on CL concentrations. The effect of low body weight on PL has not been previously reported. Elevated CL has previously been associated with poor nutrition and growth in human children [60] and with the neonatal period in harp seal pups (*P. groenlandica*), in which it is thought to be involved in the process of metabolising brown fat tissue to provide energy to the new-born pup [40]. Many of the pups during our sampling period were of low body weight, possibly therefore sending physiological signals similarly to that of the healthy neonate, and triggering PL and CL release.

Water access

There was no evidence from the ANCOVA that the variable *free water access* had a significant influence on concentrations of any of the four GCs, although it did make a greater contribution to CL concentrations than to the other GCs, and a greater contribution to CL than pup weight, social group or water access. Since pups typically spend 40–60% of their time swimming and diving [1, 2], and follow their mother into the water when disturbed [61, 62], precluding water entry by keeping pups in

a dry pen may be considered intuitively to be stressful. Possibly a stress response might be measurable following a single event of a pup being prevented from entering water – but possibly not when the pup's situation in a dry pen is constant, as in centres I, III and V.

Social group

The ANCOVA indicated that there was no significant influence of social group on GC levels. The context in which an effect might be expected to occur would be if isolated pups were to have elevated levels relative to pups held on pairs or groups, since the isolation could be akin to prolonged separation from the mother. However, as discussed above, orphan pups in rehab have most likely passed the stage of acute separation, when CL would be expected to be elevated, and be in the next 'depressive' stage, when cortisol levels would be expected to fall [37, 39]. Isolated pups in centres I and III sometimes gave distress calls or showed agitated behaviour when their keeper left the pen after feeding or cleaning, and our overall GC results cannot discount the possibility that CL levels would rise temporarily in such a context.

Rehab day

The ANCOVA indicated that rehab day contributed to CL concentrations, significantly in the case of centre II pups. PL concentrations were also influenced by rehab day, though not quite reaching significance. If a rehab environment is, indeed, causing a stress response to the pup, one might expect this to diminish with time in rehab.

Pup ID

Given the wide range of individual differences between pups sampled of growth rate, body weight on sample day, rehab day when sampled, social group and free access to water, it is not surprising that individual differences in levels of all four GCs were significant. However, in the situation of centre II, where growth rate, social group and water access were constant (Centre II pups), it appeared that pup ID had no effect on PN levels. This supports our overall finding, from all pups sampled, of a negative relationship between growth rate and PN levels.

Differences between the five centres in GC levels

Individual median or single GC levels in pups in the five different centres differed significantly only in PN and PL concentrations. For PN, this is consistent with our results indicating that relatively elevated PN concentrations were associated with relatively low growth rate (as in centre V) while relatively low PN concentrations were associated with relatively high growth rate (as in centre II; Table 1). For PL the reasons for relatively low concentrations in the centre II pups could be related to their heavier median weights (the sampling included later rehab days than at the other centres; Table 1), but the reason for the relatively high concentrations in the pups from centre IV does not seem to be related to especially low body weights, since these were not less than average pup weights in centres III and V (Table 1).

Possible differences in pup GC concentrations due to regional population of origin

One possible suggestion for future investigation is whether the relatively elevated levels of GCs in pups from the Wadden Sea centre IV compared with centres in the Irish sea (centres II, III and V) might be, at least in part, due to elevated levels of persistent organic pollutants (POPs) in the Wadden Sea [63, 64]. POP contaminants in maternal tissues may be transferred to the foetus, posing health risks during development [65]. Studies *in vitro* [66] and in adult polar bears [67] have suggested that POPs can disrupt GC activity, potentially altering circulating GC levels. It is likely that orphan pups stranding in the Wadden Sea and entering centre IV may have had higher POP contaminant levels transferred trans-placentally from the mother [64] than pups entering the Irish Sea centres II, III and V.

Appropriate study contexts for choosing mass spectrometry or immunoassay for monitoring GC levels in seals

Our own analysis of CL in centre I pups by ELISA and previous analyses of CL levels in seals by immunoassay [24, 35, 36, 40, 51, 53] were not able to take cross-reactivity with PL into consideration. Our continued study using mass spectrometry described above indicated that PL

concentrations may be significant in seals, at least in pups. However, since our study found that ELISA and mass spectrometry results for CL were closely correlated, as were also absolute concentrations of PL and CL determined by mass spectrometry, differentiation between CL and PL is probably not important if a particular study is concerned generally with a GC biomarker of stress in response to restraint, capture or mother-pup separation [35, 36]. However, if changes in seal pup body weight during a study period may be important [24, 40], mass spectrometry analysis to consider PL and PN levels could be more informative than immunoassay for CL alone.

Conclusions and recommendations

This preliminary and descriptive study began with the hypotheses that the rehab orphan harbour seal pups' GC levels, might be relatively high if held in isolation, without water and with slow growth rate, since these factors would be contrary to the pups' natural condition. Our results did not support the hypotheses concerning isolation or lack of water access but did support the hypothesis of elevated GC levels with slow growth.

Our results indicated that endogenous prednisolone (PL) and prednisone (PN) occurred in rehab harbour seal pup urinary concentrations in the same order of magnitude as cortisol (CL). This appears to be the first report of significant levels of endogenous PN and PL in a mammal species, and furthermore that PN and PL concentrations were negatively associated with growth rate and body weight respectively in the samples from pups in our study. These results would be consistent with the role of GCs in regulating energy storage and release, and therefore being elevated in orphan pups growing slowly in rehab compared with the normal very rapid growth in free-living nursing pups [2, 10, 12].

Since it is likely that chronically elevated circulating GCs during the early post-natal period could have a negative effect on a pup's future responses to stress as well as possible neural, cognitive and immune system impairment [33, 68], we would suggest that maximising pup growth during the first days and weeks in rehab, as in centre II in this study, could benefit pup health and welfare in the longer term. Rapid growth in the early weeks would also facilitate early release of pups [16], which

maximises the opportunity for pups to develop their survival skills alongside free-living pups from their original cohort at the time when the pups are initially dispersing from their natal sites and learning to forage during their first post-weaning weeks [16, 69, 70].

We were unable, from our sample set, to detect any significant association between any of the four GCs and variables that could be considered indicators of pup emotional welfare, such as whether pups had access to water and a companion pup. We consider, therefore, that potential improvements to the rehab environment in order to maximise positive emotional welfare may be best assessed using multimodal indices, including behavioural as well as physiological components [3, 29]. The latter could in future investigate the extent the benefits of keeping orphan pups socially, using markers of positive emotion, such as urinary oxytocin [71, 72].

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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 five seal centres. Four of the five centres (I, III, IV and V) were in the public domain and acting within relevant government guidelines. Centre II was a voluntary private practice 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 affect their welfare. No specific license for this study was therefore required.

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Supporting information

S1_pup_rehab_environment_photos
S2_dataset
S3_ELISA_protocol
S4_MS_protocol
S5_ANCOVA_SS