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 empirical 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 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 (CL) were analysed by ELISA from one centre and subsequently concentrations of CL, cortisone (CN), prednisolone (PL) and prednisone (PN) were analysed by mass spectrometry. Urinary concentrations of PN and PL were found to be of similar magnitude to CL. CL, CN and PL concentrations were reciprocally correlated, but PN was not correlated with CL and only weakly correlated with the other three GCs. The strongest associations between GC concentrations and pup conditions and procedures were negative correlations of PN with growth rate of individual pups and body weight. Differences in GC concentrations, particularly CL, between pups in different social groupings (pups alone, paired or in groups of more than two) were also detected. On the basis of our results, we suggest that harbour seal pups should be fed 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, nutrition, welfare, captive environment

INTRODUCTION

New-born harbour seal pups found stranded on the shoreline without the mother (termed '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, usually after a period varying between about six weeks to 4 months.

Harbour seal pups are highly precocial, and free-living pups spend around 60% of their time in the water from birth [1, 2]. Average birth weight is about 11 kg and pups nurse from their mother usually for 3–4 weeks, or up to 6 weeks in some populations [2–4]. The fat content of the milk increases from about 41% at birth to 50% by day 7 until weaning [5], with pups typically achieving an average net weight gain of 0.4 to 0.6 kg/day [2–4]. During the nursing period, free-living pups are constantly in close proximity to their mother [6–8] until about 10 days of age, when mothers sometimes leave their pups onshore in the haul-out group for a few hours while they make foraging trips offshore [9, 10]. Reproducing – for orphan pups in rehabilitation ('rehab') – this concentrated nutrition and key features of the amphibious and social environment of free-living pups is obviously challenging.

Harbour seal pups in rehab will usually not suckle from a bottle. Some centres feed pups by force-feeding with whole fish or tube-feeding fish 'soup', with little or no growth or weight gain by pups until they are past natural weaning age and feeding freely on whole fish [11, 12]. Other centres enhance pup weight gain by tube-feeding pups enriched formulae using dedicated high fat, multi-milk based formulations; however, average weight gain nevertheless rarely achieves 0.3 kg/day [12–14].

Rehab facilities in the British Isles and N. America are usually designed to maintain orphan harbour seal pups in isolation and in dry pens for some weeks after admission [12, 13, 15], a principal reason for this being to prevent potential transmission of infectious agents [16]. Less often, centres house these pups with access to a pool, either in pairs [14] or in small groups [17], where they are able to experience species-typical sensory stimulation and muscle movement during swimming and social interaction.

The glucocorticoid (GC) hormone cortisol is widely considered to be a physiological marker of an animal's 'stress' status [18, 19], with high GC levels usually considered to be an indicator of poor

animal welfare in captivity [20], although with the caveat that studies have shown inconsistency between high cortisol levels and other welfare measures [21, 22]. A study of harbour seal pups in rehab found higher plasma cortisol levels in pups being tube-fed than in pups (at a later rehab stage) feeding on fish, suggesting that the tube-feeding procedures might be stressful [23]. The same study found that plasma cortisol levels in the rehab pups were higher than in free-ranging pups and suggested that pups from rehab are not physiologically equivalent to free-ranging weaned pups [23].

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 [19, 20] and has been trialled successfully in free-living Weddell seals [24]. GC measurement in urine has the advantage that protein-bound GCs are excluded from the kidney filtrate and so urinary GCs are a reflection of the free hormone in blood over the time that the urine collects between voiding [20, 25].

The present study is a preliminary report on urinary GC concentrations in harbour seal pups in five rehab centres with different feeding and care systems. In addition to cortisol, we analysed urine concentrations of the three other closely related GCs, cortisone (CN), prednisolone (PL) and prednisone (PN). To achieve this, we used mass spectrometry for multiple steroids, as recommended by Koren and colleagues [26].

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 [2–4], with only two pups (in centre IV) being slightly above this weight, at 12–13kg (Table 1). The pups in this study 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 [10]. The pups in this study were all therefore believed to be genuine ‘orphans’.

Table 1. 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	“	“	“	“	“	“	“	5	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

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, including the rehab captive environment (S1 Photos), food constituents and feeding methods during the sampling period (Table 1). 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 (S1 Photos), see also Table 1 for individual pup details).

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* (omitted from Table 1; only pups at centres II and IV had free access to water during the sampling period). Pups were weighed by centre personnel on alternate days at centre II and approximately once a week at the other centres. Estimated pup weight on sampling day was calculated from the nearest pup weighing and average growth rate during the sampling period.

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; 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 visibly uncontaminated with faeces and with at least 10 ml were used in the analyses.

Since a study of plasma cortisol levels in captive harbour seals found a diurnal pattern of plasma cortisol levels, peaking at 01:00 and waning around 13:00 [27], the time of day of each sample collection was recorded and samples 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).

Glucocorticoid (GC) analysis

The urinary glucocorticoid (GC) analyses were carried out at three laboratories. Lab 1was at Riseholme Park, University of Lincoln; Lab 2 was at the Institute of Global Food Research, Queens University, Belfast, and Lab 3 was the Chemical and Immunodiagnostic Sciences Branch, Veterinary Sciences Division, Agri-Food & Biosciences Institute (AFBI), Belfast. Seventeen samples from Centre I (2012) were analysed by ELISA for cortisol (CL) at Lab 1. Eleven duplicate samples from Centre 1 and a further 8 from centre II (2013) were analysed at Lab 2 for CL by ultra-performance liquid chromatography – tandem mass spectrometry (UPLC–MS/MS) [28, 29]. Ninety-six further samples collected from centres II, III, IV and V in 2014–15 were analysed at Lab 3 for CL, cortisone (CN), prednisolone (PL) and prednisone (PN) by UPLC–MS/MS. These 96 samples included seven in which the sample donor pup (one of a pair) was not identified; these seven 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 excluded from subsequent analyses. The full list of samples for each of the three analytical laboratories and methods are given in 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 concentration (ng GC mg/Cr) in each sample [24]. Creatinine (Cr) concentrations at all three laboratories were analysed using a colorimetric method modified from the Jaffe reaction, in

147 which creatinine in an alkaline solution reacts with picric acid to form a coloured complex [30]. For the
148 basic method, a standard Cr solution is prepared for use as a reference. A picric acid solution is added
149 to the urine sample and the Cr standard. The colour reaction is started by adding sodium hydroxide.
150 The resulting absorbance of each sample is then measured using a spectrophotometer in order to finally
151 obtain the Cr concentration of the urine sample. The detailed methods of Cr determination followed by
152 each laboratory are given in S3 Protocol and S4 Protocol.

153 *Analysis of urinary cortisol concentrations by ELISA*

154 The principle of the ELISA assay is the binding of a specific antibody to an enzyme-labelled target
155 antigen (CL in this case). The enzyme activity is measured using a solid substrate that changes colour
156 when modified by the enzyme. The intensity of the colour depends on the CL concentration and is read
157 on a microplate.

158 Urinary CL concentrations in 17 samples from centre I in 2012 were determined at Lab 1 using a
159 cortisol ELISA kit (Cat No ADI-900-071) for colorimetric detection from Enzo Life Sciences (UK)
160 Ltd. The details of the procedures undertaken are given in S3 Protocol. This method is stated to be for
161 plasma, saliva, serum or urine from any species. The cross-reactivity with other steroid hormones is
162 stated by the manufacturer to be Cortisol (100%), Prednisolone (122.35%), Corticosterone (27.68%),
163 11-deoxycortisol (4%), Progesterone (3.64%), Prednisone (0.85%), Testosterone (0.12%) and ≤ 0.10%:
164 Androstenedione, Cortisone and Estradiol.

165 The method (S3 Protocol) followed the kit instructions. Because the concentration range of CL in
166 harbour seal urine was not known, a test ELISA run was initially carried out to estimate the urine
167 sample dilutions needed for the middle calibrator of the ELISA kit. After converting the ELISA colour
168 changes on a microplate, the CL concentrations in the original sample were obtained by multiplying by
169 the dilution factors, and converted to mM/L using the molecular mass of cortisol. These values were
170 then divided by mM creatinine to obtain the cortisol/creatinine (CL/Cr) molar ratio.

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

173 Immunoassays – as the ELISA described above – may be used to determine the concentration of a single
174 steroid hormone (assuming cross-reactivity is not considered to be high), but if the concentrations of
175 several steroids are required, either separate kits for each steroid must be used (requiring splitting of the
176 sample), or the steroids need to be separated by chromatography. Liquid chromatography coupled with
177 tandem mass spectrometry (LC–MS/MS) can quantify either one or multiple steroids at wide-ranging
178 concentrations, while avoiding the issue of antibody cross-reactivity [26].

179 For this study of seal pup urine glucocorticoid steroids, ultra-performance liquid chromatography
180 coupled with tandem mass spectrometry (UPLC–MS/MS) was employed by Lab 2 to determine urinary
181 cortisol (CL) concentrations of 19 samples collected in 2012–13 and by Lab 3 to determine
182 concentrations of the four GCs, CL, cortisone (CN), prednisolone (PL) and prednisone (PN) in 96
183 samples collected in 2014–2016. The CL analyses of the 2012-2013 samples by Lab 2 were carried out
184 alongside a separate study of CL and CN concentrations in cattle [31], while the analyses of CL, CN, PL
185 and PN in the 2014–2016 samples by Lab 3 were carried out according to the procedures for analysis of
186 bovine liver as part of the routine statutory Veterinary Drug Residue testing programme in N. Ireland.
187 The UPLC–MS/MS methods employed by both Labs 2 and 3 are described in full in S4 Protocol.

188 *Method validation*. The seal pup urine samples were analysed by UPLC-MS/MS at laboratories where
189 the analytical method validation was previously undertaken for analysis accuracy/recovery, intra- and
190 inter-day repeatability and linearity for bovine urine in Lab 2 [31] and bovine, porcine and ovine liver in
191 Lab 3, in accordance with EU guidelines (2002/657/EC). The method validation procedures are described
192 in S4 Protocol.

193 **Statistical analyses of GC results**

194 Statistical analyses were carried out in the XLSTAT-pro statistical package, using ANOVA, correlation
195 and regression, and non-parametric Kruskal-Wallis or Mann-Whitney tests. Statistics were carried out
196 both (*1*) on median GC concentrations for individual pups and (2) on all GC concentrations from all
197 samples (*2*). To take into account the rehab environment variables, analysis of covariance (ANCOVA)
198 was carried out using the R statistical package [32].

199 **RESULTS**

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

202 The range of creatinine (Cr) concentrations in the 115 samples analysed by MS was 115−15576 μM/L,
203 (mean 3219, median 2273 μM/L). The range of glucocorticoid (GC) concentrations (ng/mg Cr) was
204 greatest for cortisol (CL) (0–8062 ng/mg; next for cortisone (CN) (0–4853 ng/mg), with a lower range
205 for prednisolone (PL) (0–236.6 ng/mg) and prednisone (PN) (0–142.5ng/mg). The complete set of raw
206 data is given in S2 Dataset, a summary of the dataset is given in Table 1 and the range of MS results on
207 a $\log_{10}$ scale is presented in Fig 2.

208 Fig 2. Scattergram of urinary concentrations of four GCs from all samples analysed by UPLC MS/MS.
209 (n=CL−115; CN, PL, PN – 96). Concentrations ng/mg creatinine on $\log_{10}$ scale. Horizontal lines indicate
210 medians and + markers indicate means.
211

212 The median values for CL, PN and PL were similar to each other (36.9, 31.1 and 31.57 ng/mg Cr
213 respectively), while median CN concentrations were an order of magnitude higher (390.4 ng/mg Cr).
214 There were seven samples with CL and PL concentrations below the detection limit (Fig 2). Six of
215 these samples had low Cr concentrations (range 115−683 μM/L). One sample which gave a very high
216 CL concentration (2030 ng/mg Cr) also had a low Cr concentration (444 μM/L). Because some very
217 high or very low results for concentrations from dilute samples, the median concentrations rather than
218 the means are used in continuing analysis of the data.

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

222 Table 2. Correlation (Pearson coefficient) between concentrations of four GCs (ng/mg creatinine) by
223 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

224

225 The ratios of mean and median CL concentrations to the other three GCs from all samples from which

226 all four GCs were analysed are given in Table 3.

227

228 Table 3. Ratios of mean and median concentrations (ng/mg Cr) of Cortisol to each of the other GCs in
229 all samples for which all four GCs were analysed (n=96 samples).
230

	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

231

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

233 11 of the 17 samples from centre I were analysed for cortisol by both UPLC-MS/MS and by ELISA,
234 The correlation (Pearson coefficient) between the MS and ELISA results was 0.795 (2-tailed P <0.01),
235 although the mean concentrations for the ELISA results were about 13% higher on average than for
236 MS (Table 4).

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

238

	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	

239

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

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

Supplementary file S2) were separated from the continuing analyses, which considered pups from rehab days 2 onwards.

Effect of time of day of sampling on GC concentrations

Distribution of sampling from rehab day 2– indicated that the majority of 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 5).

Table 5. 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 pups 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.

Analysis of variance (ANOVA) and covariance (ANCOVA) of GC concentrations (ng/mg creatinine) according to variables considered in study

The results for goodness of fit, R^2, by the ANOVA were similar for median GC concentrations for individual pups and for all samples from all pups (Table 6): 47% and 49% of the variability of PN concentrations was explained by the four and seven variables respectively, while less (25–36%) of the variability of CL, CN and PL was explained by the same variables. The P value of the F test indicates significant influence of the four variables only for PN for (*1*) the median individual pup concentrations.

The F test for GC concentrations for all samples indicates significant influence of the seven variables for (*2*) all GC concentrations, but the highest level of significance for PN (Table 6).

Table 6. ANOVA for GC concentrations (ng/mg creatinine) results

		CORTISOL (CL) N = 34(*1*); 96(*2*)				**CORTISONE (CN)** N=30(*1*); 78(*2*)			**PREDNISOLONE (PL)** N=30(*1*); 78(*2*)			**PREDNISONE (PN)** N=30 (*1*); 78(*2*)		
	df				**df**									
Goodness of fit R^2														
1	25	**0.289**			22	**0.340**			**0.286**			**0.466**		
2	84	**0.246**			67	**0.356**			**0.296**			**0.487**		
		Mean sq	**F value**	**Pr(>F)**		**Mean sq**	**F value**	**Pr(>F)**	**Mean sq**	**F value**	**Pr(>F)**	**Mean sq**	**F value**	**Pr(>F)**
ANOVA-Fisher's F test														
1	11	45857	1.27	0.302	10	247642	1.618	0.183	51440	1.26	0.315	781	2.74	**0.033**
2	11	104240	2.49	**0.009**	10	600346	3.71	**0.001**	1369	2.81	**0.006**	1996	6.35	**<0.0001**

1: Results from median concentrations for individual pups; 4 variables considered (*growth rate*, *centre*, *social group*, *sex*)

2: Results from concentrations in all samples from all pups; 7 variables considered (*growth rate*, *weight on sample day, rehab day, Centre*, *social group*, *free water access*, *pup sex*)

GC concentrations according to social group and rehab centre

Because five pups from centre IV changed *social group* during the sampling period (Table 1), the GC concentrations from all samples (rather than medians for each pup) from pups in each of the social groups are visualised in Fig 3. The CL, PN and PL concentrations differed according to social group (P=0.006, 0.008 and 0.004 respectively, Kruskal-Wallis test), with social group 2 (paired pups) having relatively lower concentrations (Fig 3). The CN concentrations did not differ between the social groups (P=0.402).

Fig 3. Scattergrams of distribution of urinary concentrations of four GCs according to social group

Social group categories: 1 (alone), 2(paired) and 3 (≥3 pups). Samples from rehab d. 2 onwards; n=96 samples for CL, n=78 samples for PN, CN and PL. Horizontal lines indicate medians and + markers indicate means

The distribution of each GC (considering all samples from all pups) differed between *centres* (Fig 4; P<0.0001 for each GC; Kruskal-Wallis test). Centre IV samples had overall the highest CL, CN and PL concentrations, whereas Centre II had the lowest CL, PN and PL concentrations. Scatterplots for median concentrations for individual pups from each centre (not shown) showed the same pattern, still with significance (CL – P = 0.003; PN – P = 0.011; CN – P =0.021, PL – P = 0.029; Kruskal-Wallis test).

Fig 4. Scattergrams of distribution of urinary concentrations of four GCs.
(all samples from rehab day 2 onwards) in each rehabilitation centre I–V. Horizontal lines indicate medians and + markers indicate means; n= 96 samples CL, 78 for PN, CN and PL.

The ANCOVA indicated which variables significantly influenced the variability in the GC concentrations. Considering (*1*) – median GC concentrations for individual pups – PN was significantly influenced by average *growth rate* during the sampling period, but not significantly by the other three variables; the other GCs were not significantly influenced by any of the four variables (Table 7). Considering (*2*) – GC concentrations of all samples – PN was most strongly influenced by *growth rate*, but also by *pup weight on sample day* (Table 7). However, the concentrations of the other GCs were

308 also apparently influenced by *growth rate* and *Centre* (PL), *growth rate*, *weight on sample day* and

309 *Centre* (CN) and *weight on sample day*, *Centre* and *social group* (CL) (Table 7).

310

311

Table 7. ANCOVA for GC concentration (ng/mg creatinine) results

		CORTISOL (CL) N=34(*1*); 96(*2*)				CORTISONE (CN) N=30(*1*); 78(*2*)			PREDNISOLONE (PL) N=30(*1*); 78(*2*)			PREDNISONE (PN) N=30(*1*); 78(*2*)		
Variables	**Df**	**Mean sq**	**F value**	**Pr(>F)**	**df**	**Mean sq**	**F value**	**Pr(>F)**	**Mean sq**	**F value**	**Pr(>F)**	**Mean sq**	**F value**	**Pr(>F)**
(*1*) MEDIAN VALUES FOR INDIVIDUAL PUPS (rehab day 2 onwards)														
Growth rate	1	45292	1.30	0.265	1	260203	1.74	0.200	766	1.96	0.175	3242	11.76	**0.002**
Centre	4	148981	1.07	0.392	3	252221	1.69	0.198	739	1.89	0.160	291	1.05	0.388
Social group	1	141227	4.05	0.055	1	583406	3.90	0.060	1558	3.98	0.058	1045	3.79	0.064
Sex	1	26977	0.77	0.387	1	62396	0.42	0.525	248	0.63	0.434	237	0.86	0.364
(*2*) VALUES FOR ALL SAMPLES (rehab day 2 onwards)														
Growth rate	1	97249	2.32	0.132	1	1586864	9.94	**0.002**	3927	7.51	**0.008**	13842	44.30	**5.9E-09**
Weight on sample day	1	265843	6.34	**0.014**	1	1287950	8.07	**0.006**	0	0	0.983	2274	7.28	**0.009**
Rehab day	1	19576	0.47	0.496	1	444196	2.78	0.100	1358	2.60	0.112	973	3.11	0.082
Centre	4	106516	2.54	**0.046**	3	705575	4.42	**0.007**	1747	3.34	**0.024**	742	2.38	0.078
Social group	1	182494	4.35	**0.040**	1	345182	2.16	0.146	11	0.02	0.884	143	0.46	0.501
Water access	1	7155	0.17	0.681	1	3246	0.02	0.887	86	0.16	0.687	319	1.02	0.316
Sex	1	105517	2.52	0.117	1	217899	1.37	0.247	120	0.23	0.634	6	0.02	0.893

1: Results from median concentrations for individual pups; 4 variables considered (*growth rate*, *centre*, *social group*, *sex*)

2: Results from concentrations in all samples from all pups; 7 variables considered (*growth rate*, *weight on sample day, rehab day, Centre*, *social group*, *free water access*, *pup sex*)

Pup growth rates

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). Regression of individual pup growth rate during the sampling period by entry weight (Fig 5) 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 5. Regression of pup growth rate (kg/day) by entry weight (kg)

The entry weights of the six pups from centre II ranged from 7.1 to 11.1 kg (Table 1). Frequent weight records for these pups over a four-week period indicated similar linear growth rates for all the pups (S5 Supporting Information), irrespective of their entry weight (range 0.27–0.30; Table 1), $R^2 =$ 0.97–0.99, $P < 0.0001$).

Regressions of GC concentrations by pup growth rates, pup weight on sample day and days since entry into rehab

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 lower concentrations of PN in pups with higher growth rates (Table 8a; Fig 6).

Fig 6. Regression of median GC concentrations (ng/mg creatinine) for each pup by growth rate (kg/day)

Linear regressions of GC concentrations in each sample (n=78) by pup estimated *weight* showed a significant negative correlation for all four GCs, with the strongest effect of pup weight (explaining 24% of the variability) on PN concentrations (Table 8a). However, because of the opportunistic nature of the urine sampling, pups at centres II–V were sampled at different weight ranges (Fig 7), with the

338 centre explaining a further 19% of the PN variability (combined R^2=0.436). There was no association
339 found between the weight of pups in centre I and CL concentrations (Table 8c), but the range of pup
340 weights in centre I over the sampling period was very small, owing to low growth rates (Table 1).

341 **Table 8.** Values of R^2 for regressions of concentrations (ng/mg creatinine) of each GC (Cortisol – CL,
342 Cortisone – CN, Prednisolone – PL, Prednisone – PN) by pup growth rate, estimated pup weight on
343 sample day and rehab day (day 2 onwards)

344

345

346

	(a) All 34 pups in the study n=34 (CL), 30 (CN, PL, PN) (pup medians) n= 96 (CL), 78 (CN, PL, PN) (all samples)				**(b) Six pups in centre II** n= 32 samples (CL), 24 (CN, PL, PN)				**(c) Four pups in centre I** n= 17 samples (ELISA)	
	CL	**CN**	**PL**	**PN**	**CL**	**CN**	**PL**	**PN**	**CL**	**CN/PL/PN**
Growth rate	0.036	0.051	0.056	**0.276**	NA	NA	NA	NA	NA	NA
Pr > F	*0.285*	*0.230*	*0.210*	*0.003*						
Weight	**0.051**	**0.145**	**0.073**	**0.242**	0.079	**0.193**	**0.190**	**0.348**	0.001	NA
Pr > F	*0.027*	*0.001*	*0.017*	*<0.0001*	*0.119*	*0.032*	*0.033*	*0.002*	*0.928*	
Rehab day	**0.05**	**0.145**	**0.126**	**0.16**	0.089	0.139	**0.278**	**0.369**	0.025	NA
Pr > F	*0.034*	*0.001*	*0.001*	*0.0002*	*0.098*	*0.072*	*0.008*	*0.002*	*0.544*	

347 Fig 7. Regression of Prednisone concentrations (ng/mg creatinine) by estimated pup weight (kg) on
348 day of sampling for pups from each centre II–V

349

350 Pups generally gain weight during rehab, as exemplified by the six pups in Centre II, which were
351 weighed regularly over a six-week period (S5 Supporting Information) Therefore, over all the pups in
352 the five centres of this study, pup weight is conflated to a considerable extent with rehab day. The
353 effect of rehab day (from day 2 onwards) was considered separately for the Centres I and II pups, for
354 which there were samples taken over a relatively long period. For the centre II pups, the regressions
355 showed a significant negative correlation with rehab day for PN and PL – most strongly for PN – but
356 the correlation was non-significant for CL or CN (Table 8b). For the centre I pups, there was no
357 association between rehab day and CL (Table 8c).

DISCUSSION

Choice of urine sampling and UPLC-MS/MS for sample analysis

Our choice or urine sampling was advantageous in that it caused no additional stress to the pups beyond their routine care. Its disadvantage was that samples could only be collected opportunistically when the pup happened to urinate during routine feeding or other care. The wide variability of GC concentrations found, even between samples from the same pup, is consistent with the conclusion of Cook [20] that the use of singly voided samples is not useful in assessing an animal's stress response. Although the variability might be in part due to the different times of day of sample collection [27], we found no significant difference between the samples collected at different times. The reason for this might be partly that the concentrations in a urine sample likely represent average circulating blood plasma concentrations over the preceding few hours [33].

Our GC analysis of the first set of samples intended for cortisol (CL) was carried out using an ELISA kit with known high cross-reactivity to prednisolone (PL), which undoubtedly explains at least in part why the ELISA CL concentration results for the centre I samples were on average 13% higher than the duplicate samples analysed by UPLC-MS/MS. This cross-reactivity with similarly-structured steroids is a recognised problem with any form of immune-assay (IA) for CL levels in humans, where other synthetic GCs may be present [34, 35], but at the time of our ELISA analysis in 2012, the MS analysis of the 2014–16 samples had not been carried out and therefore the existence of significant amounts of naturally-occurring PL in our seal pups was not suspected. As Koren and colleagues [26] have pointed out, distinguishing between similar steroids in a single sample requires a mass-spectrometric technique which can unequivocally discriminate between these similar GCs.

Comparison of seal pup urinary GC concentrations with other species

Cortisol. This is the first investigation into urinary GCs concentrations in harbour seals, and baseline levels of urinary CL for healthy pups are not known. Urinary CL has only been studied in one other

seal species, the Weddell seal (*Leptonychotes weddelli*) in the wild. Concentrations in juveniles <1 year were much higher than in the harbour seal pups of our study, with means of ~930–2370 ng/mg Cr by radio-immuno-assay (RIA). However, it is thought that Weddell seal CL concentrations are unusually high amongst phocid species [24].

The overall median and mean of our seal pup CL concentrations (overall median 37 ng/mg Cr) were of the same order of magnitude as urinary concentrations found in cattle (~36–84 ng/mg Cr by UPLC-MS/MS [31]) and in free-range lambs (~20–46 ng/mg Cr by RIA [36]).

Cortisone. CN is a downstream oxidised metabolite of CL [37], with less than half of CL's biological activity [38, 39]. Except for a few samples with very high CL concentrations, CN concentrations in our pup urine samples were an order of magnitude higher than for CL, with the CL:CN ratio of median concentrations of 0.09: 1 (Table 3). This is much less than the average ratio for human athletes of 0.64:1 [40], and also much less than that reported from median concentrations in free-ranging Chianina cattle aged 1–2 years (1.43:1 in males, 1.02:1 in females [41]) and in young Friesian bulls in pre-slaughter enclosures (1.35–1.68:1) [31].

Prednisolone. The ratio of CL:PL median concentrations in our seal pups was 1.2:1 (Table 3), i.e. close to parity. In humans, urinary PL has been found to occur only in concentrations 100-times lower than CL [42]. Naturally-occurring PL has been found in low concentrations in Chianina cows (median CL:PL ratio of 25:1 (none present in bulls), and even lower concentrations in Friesian veal calves (mean CL:PL ratio range 29–64:1 [43]). It has been suggested [44] that neo-formation of PL 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). However, we believe the PL in our seal pup samples was endogenous since (i) the samples were not subjected to high temperatures, were free from faecal contamination, and were transferred to the freezer within minutes, (ii) concentrations were consistently at similar concentrations to cortisol and (iii) PL concentrations were significantly correlated with CL, which would not be the case with post-collection microbial action.

409 *Prednisone.* PN is an oxidised metabolite (or precursor) of PL. Synthetic PN is widely used as an
410 anti-inflammatory agent in the treatment of human conditions and, often illegally, in domestic cattle.
411 Endogenous urinary PN has so far been reported to be absent in Piedmontese and Friesian cattle [45]
412 and Chianini cattle [41], present in trace amounts in a small proportion of Friesian veal calves (CL:
413 PN ratio 43:1 [43]), but has not been reported in other mammal species. Its occurrence in our seal pup
414 urine, with the median CL:PN ratio of only 1.13:1 (Table 3), i.e. close to parity, was therefore
415 unexpected. The lack of inter-correlation between PN and the other three GCs might be due in part to
416 a feedback effect by circulating PN suppressing adrenal function, possibly affecting secretion of the
417 other GCs [46].

418 *Psychological stress and PL and PN concentrations in bovine urine.* Concentrations of PL and PN in
419 bovine urine above the detection limit have been found to be more frequent in conditions of extreme
420 psychological stress, such as slaughterhouse conditions [43, 45]. The median concentrations of PL
421 and PN of a similar order of magnitude to CL routinely found in our seal pup urine in all five centres
422 are unlikely to be related to extreme psychological stress, since relaxed social interaction and play
423 behaviours between pups – which would be unlikely in a highly stressful environment [47, 48] –
424 were typical of pups held with companion pups, as in centres II, IV and V (S.Wilson, pers. obs.). In
425 harbour seal pups, therefore, it seems that the trigger for PN and PL secretion is different from the
426 bovines studied hitherto.

427 **Possible association between levels of each GC and rehab procedures and conditions**

428 Interpretation of our data is challenging because of the large number of variables considered in the
429 analysis as well as potential variables not considered, in relation to the relatively small dataset. These
430 limitations are a result of our studying pups opportunistically in existing rehab centres, each with their
431 independent procedures, rather than according to any controlled or experimental design.

432 *Pup growth rate and weight on sample day*. The ANCOVA and regression analyses indicated that the
433 variables chiefly influencing PN concentrations were pup growth rate and pup weight on the sampling
434 day, but that actual weight also influenced CN, PL and CL concentrations. Elevated CL has

435 previously been associated with poor nutrition and growth in human children [49] and with the post-
436 weaning fasting period in harp seal pups [50]. High endogenous CL levels in fasting post-weaning
437 grey seal pups have been shown experimentally to increase protein catabolism, which may
438 compromise pup survival during the period between weaning and successful post-weaning foraging
439 [51].

440 Since free-living harbour seal pups suckled by their mothers on milk typically have an average net
441 mass gain of ~0.4 to ~0.6 kg/day from a birth weight of ~11 kg [2–4], even our rehab pups with the
442 highest weight gain (0.27–0.30 kg/day in centre II) have barely half the net mass gain of free-living
443 pups. Because the urinary concentrations of PN appeared to be the most closely linked negatively
444 correlated) of the four GCs to pup growth rate and weight, we suggest that its presence in our rehab
445 pups of nursing age may be an age-specific response to the failure of pups in rehab to achieve the high
446 normal growth rate of >0.5 kg/day in naturally suckling pups of this species and therefore it is likely
447 that the PN concentrations seen in our rehab pups may be much higher than in healthy free-living
448 suckling pups.

449 *Pup social group and Centre*. *Social group* was to a considerable extent conflated with *Centre*, since
450 all pups in centres I and III were social group 1 (isolated pups), all pups in centre II were paired, and
451 all pups in groups of more than two pups were in Centre IV. CL was the only GC that the ANCOVA
452 indicated had a significant association with *social group*, with social group 2 (paired pups) overall
453 apparently having lower CL than either isolated or group pups.

454 Rehab conditions in which pups were kept alone in dry pens (*social group* 1) evidently did not mimic
455 key aspects of the pup's natural social or physical environment, and might therefore be interpreted as
456 relatively poor welfare with possible chronic stress or distress due to lack of stimulation [52].
457 However, although the isolated *social group* 1 pups in centre I did have relatively elevated CL levels,
458 the similarly isolated pups in centre III had relatively low (and sometimes undetectable) CL. This
459 significant difference between these two pup groups might be due to negative feedback mechanisms

460 (18, 53) operating in the centre III pups, but other factors – possibly including the very low growth
461 rate and body mass – being over-riding for the centre I pups.

462 *Pup GC response to maternal separation and isolation.* On losing contact with their mother, harbour
463 seal pups vocalise [54], swimming around and attempting to follow other mothers or pups (S. Wilson,
464 pers. obs.), while pups captured in the field while separated from their mothers have higher CL levels
465 than pups captured with their mothers [55]. The elevated CL levels typical of this first 'acute' stage of
466 maternal separation trigger the energy release necessary to fuel the typical agitated activity and
467 vocalisation shown by a pup trying to reconnect with its mother (56). By the time orphan seal pups
468 enter rehab centres, they will already have suffered this initial first stage of maternal separation.

469 CL levels are expected to reduce during the next "depressive" stage of separation, when the pup is
470 likely to show reduced locomotion and other more passive behaviours [56, 57, 58]. Pups kept alone in
471 pens without water access (centres I and III) do usually appear inactive and passive (S. Wilson, pers.
472 obs.) and may possibly be in a continuing depressive phase, possibly involving a feedback mechanism
473 which suppresses the cortisol response [18, 58]. By contrast, pups maintained socially with water
474 access (centres II and IV) show alternating rest and activity periods, with much behaviour typical of
475 pups with their mothers, and do not show signs of a 'depressive' stage of separation (S. Wilson, pers.
476 obs.).

477 **Factors likely to affect urinary GC concentrations in orphan pups unrelated to rehab centres**
478 **conditions**

479 *Possible differences in pup GC concentrations due to regional population of origin*. The pups with
480 generally relatively lower CN and PL levels were mostly from Irish Sea centres (II and V) and the
481 lower CL-level pups were also mostly from Irish Sea centres (II, III and V). One possible suggestion
482 for future investigation is whether the relatively elevated levels of GCs in pups from the southern
483 North Sea centres (I and IV) compared with centres in the Irish sea (centres II, III and V) might be, at
484 least in part, due to elevated levels of persistent organic pollutants (POPs) in the southern North Sea
485 and Wadden Sea [59]. POP contaminants in maternal tissues may be transferred to the foetus, posing

health risks during development [60]. Studies *in vitro* [61] and in adult polar bears [62] have suggested that POPs can disrupt GC activity, potentially altering circulating CL levels. Future studies might investigate the POP concentrations in tissues of pups currently entering seal centres in the southern North Sea and Wadden Sea (Centres I and IV) compared with the Irish Sea and elsewhere.

Effects of interval between maternal separation and 'orphan' pup rescue. Several factors including maternal separation time, body condition and infection status may all influence pups' GC cortisol level on arrival at rehab centres. Pups separated for a few days may have opportunistic infections, often causing pneumonia with respiratory distress (as 'Ula' in centre II), which is known to cause an additional stress response in new-born babies [63]). We feel that our finding – that the GC concentrations in newly arrived pups was generally higher than from rehab day 2 onwards – justifies our omission of the results in days 0–1 from the main analyses. Our additional finding that body mass at the time of stranding and entry into the centre was unrelated to growth rate during the sampling period also suggests that poor body condition at stranding did not affect orphan pups' subsequent physical development during rehab.

Conclusions and recommendations

This study has been preliminary and empirical, recording associations between urinary GCs and variables in rehab conditions and procedures without a physiological explanation.

We found that the most significant association between GC concentrations and the variables considered was a negative relationship between prednisone (PN) and growth rate or body weight. If our preliminary findings should be confirmed by an extended, independent study with more controlled variables, this would provide a strong argument for providing pups with an appropriate high fat formula to maximise growth rate from an orphan pup's earliest days in rehab. Our results suggest that slow or negative growth rates [12] for some weeks would likely result in chronically elevated circulating PN levels, which could have a negative effect on a pup's future responses to stress as well as possible neural, cognitive and immune system impairment [18, 64]. Increasing average growth rate to an approximation of 0.3 kg/day, as in centre II, would have a positive and practical advantage of

enabling orphan pup release after 6–7 weeks [14]. Such a relatively early release potentially 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 [65].

Our study did not find a clear relationship between urinary GC levels and the pup's social or physical environment in rehab, although – since a significant effect of social group on CL levels was found – social and environmental variables as contributory factors could not be ruled out. We therefore consider that the psychological welfare of seal pups in rehab may be best assessed using multimodal indices of welfare, including behavioural as well as physiological components [25]. The latter could in future investigate the benefits of keeping pups socially using markers of positive emotion, such as oxytocin [66, 67].

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CONFLICTS OF INTEREST

The authors have no conflicts of interest

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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

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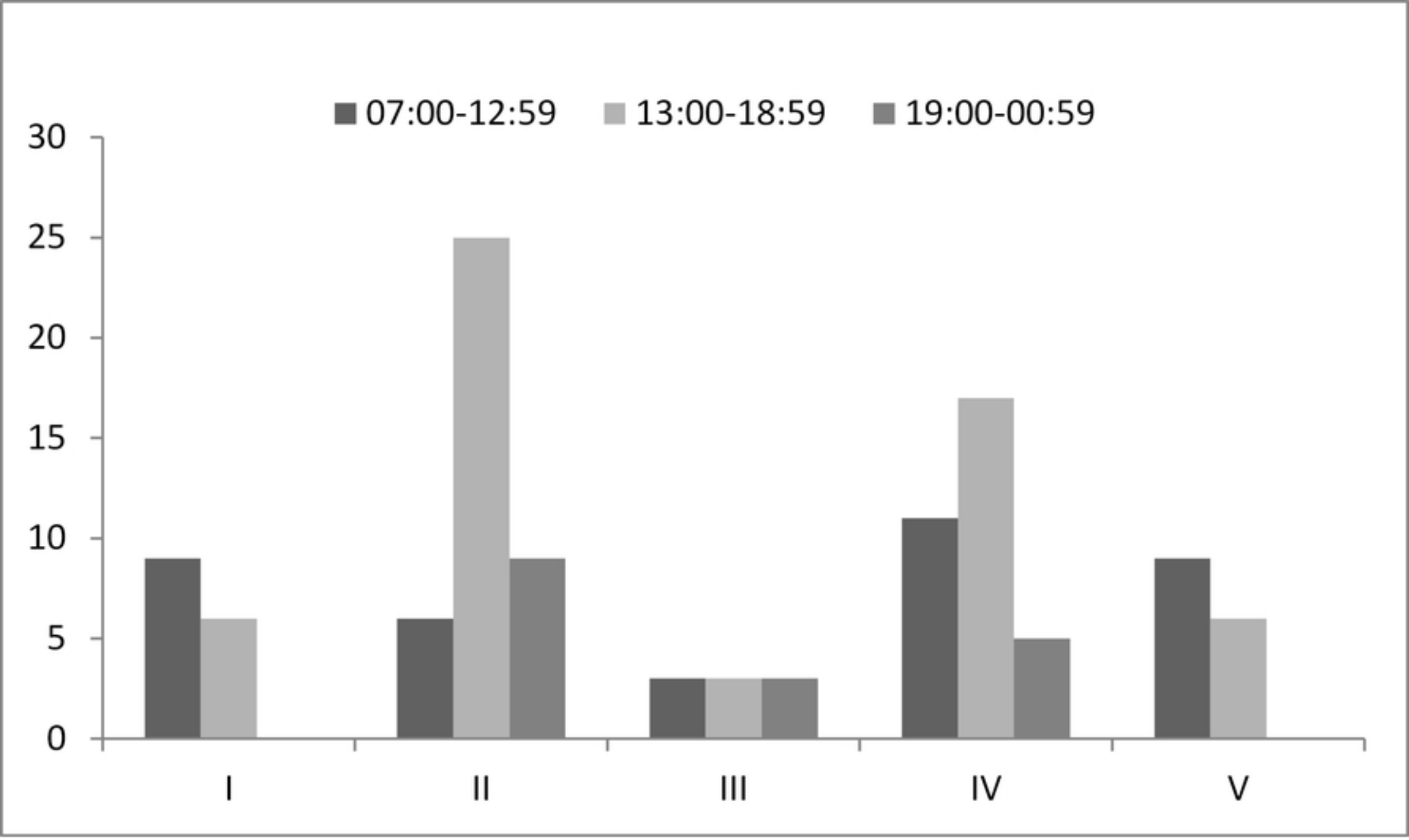
S2_dataset
S3_ELISA_protocol
S4_MS_protocol
S5_pup_growth_rates_centre_II



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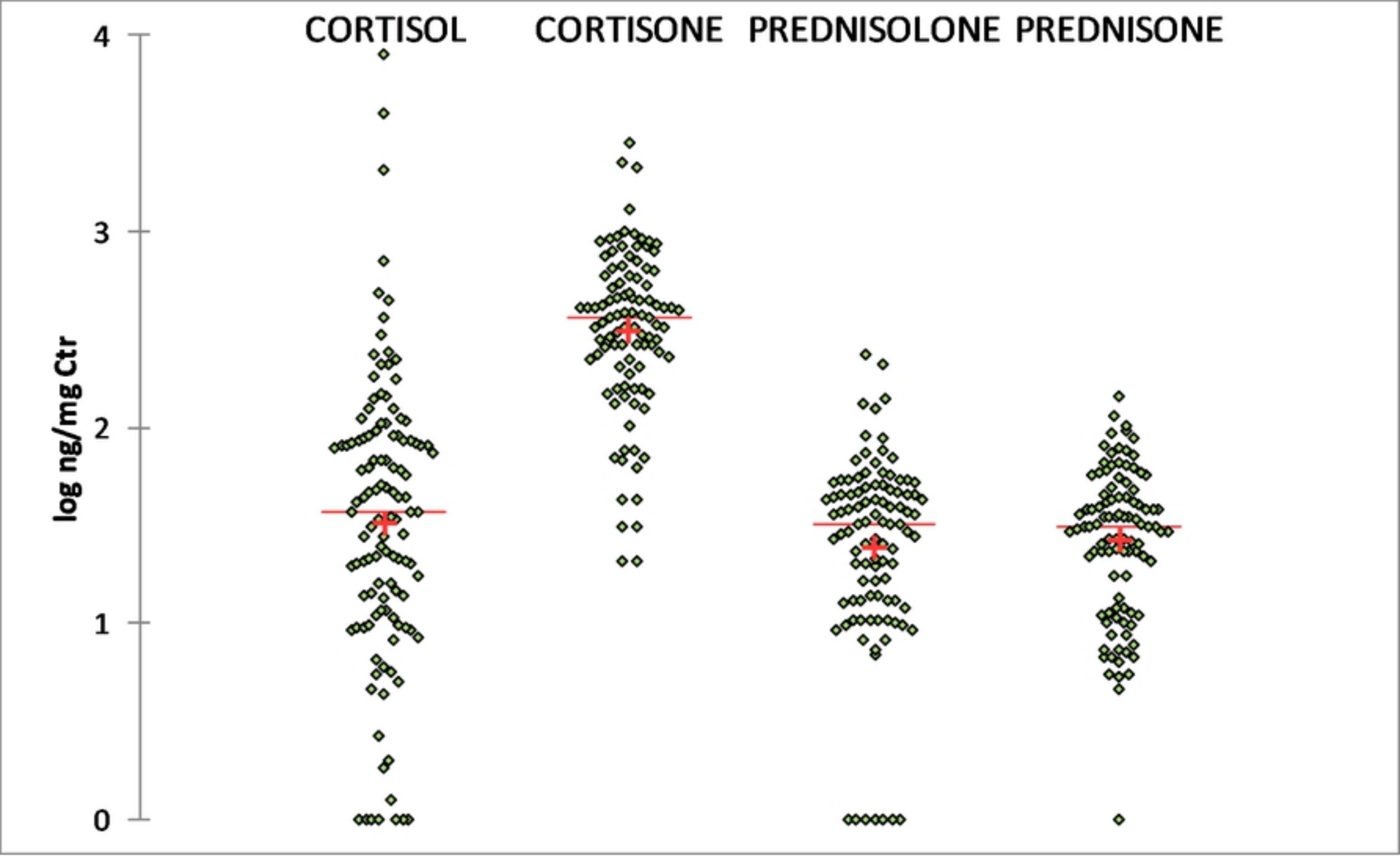
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CORTISONE
PREDNISOLONE
PREDNISONE
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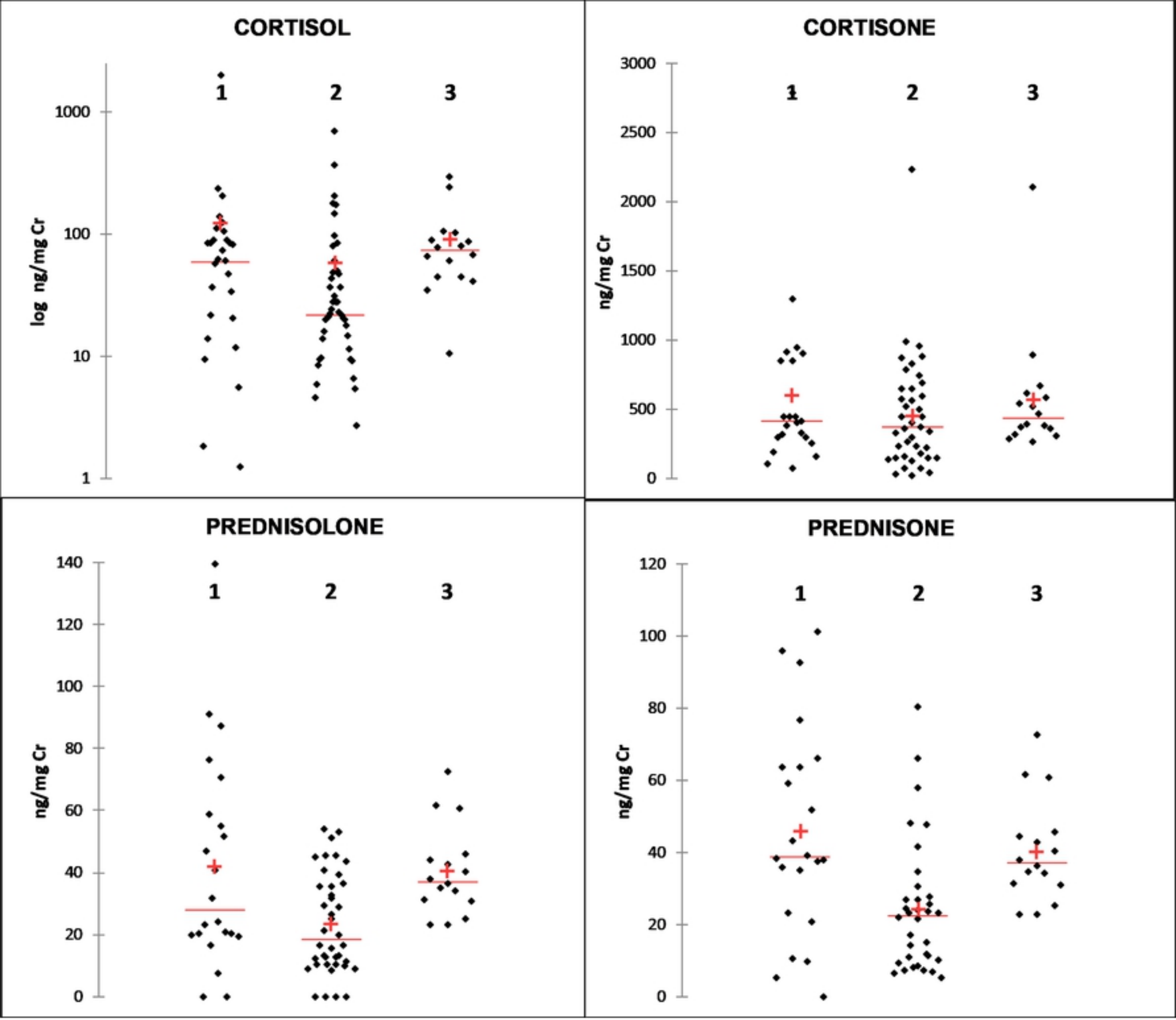
4
3
2
1
0

Figure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1
2
3
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ng/mg Cr
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2500
2000
1500
1000
500
0
1
2
3
PREDNISOLONE
ng/mg Cr
140
120
100
80
60
40
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ng/mg Cr
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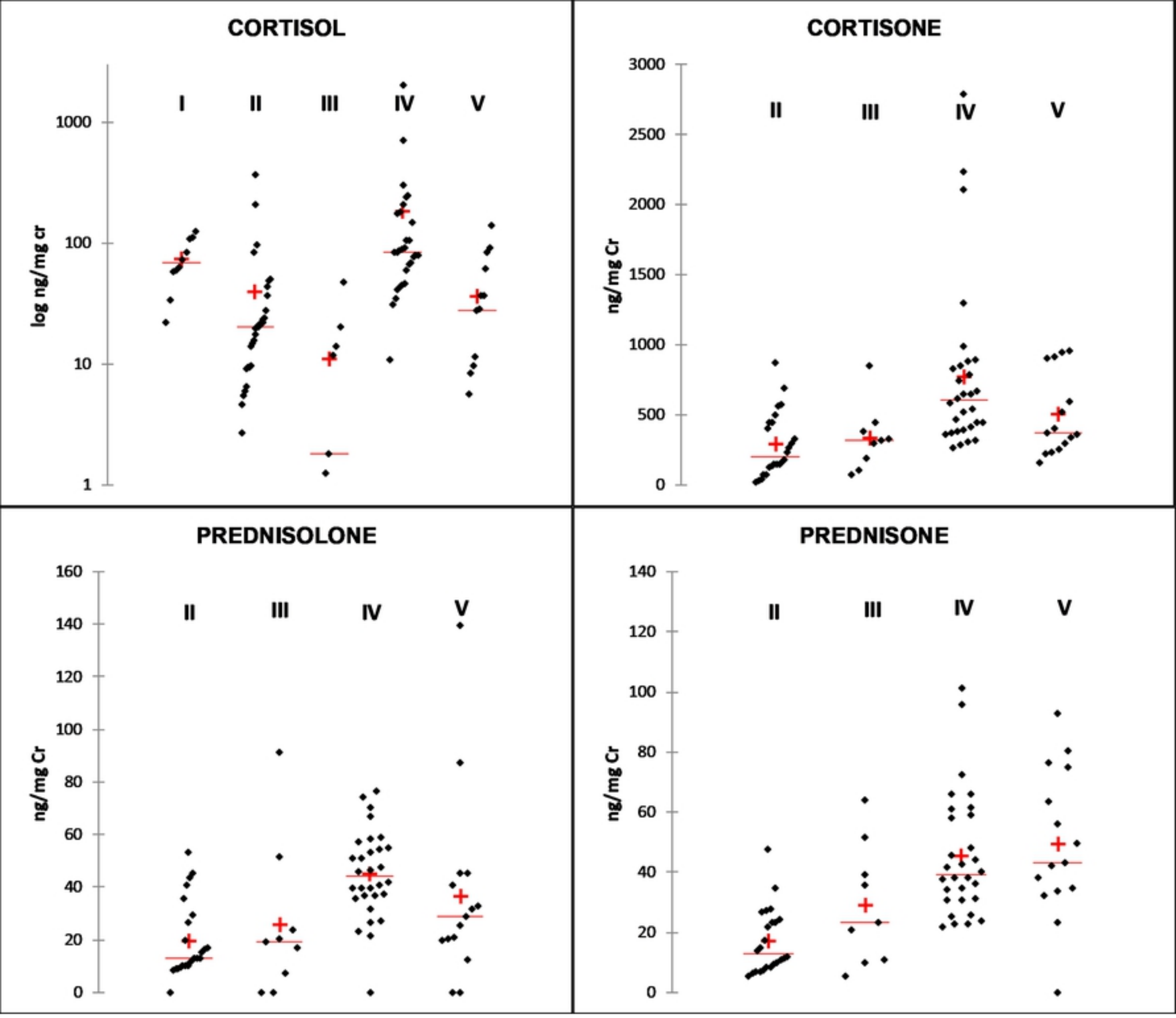
Figure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500
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II
III
IV
V
PREDNISOLONE
ng/mg Cr
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140
120
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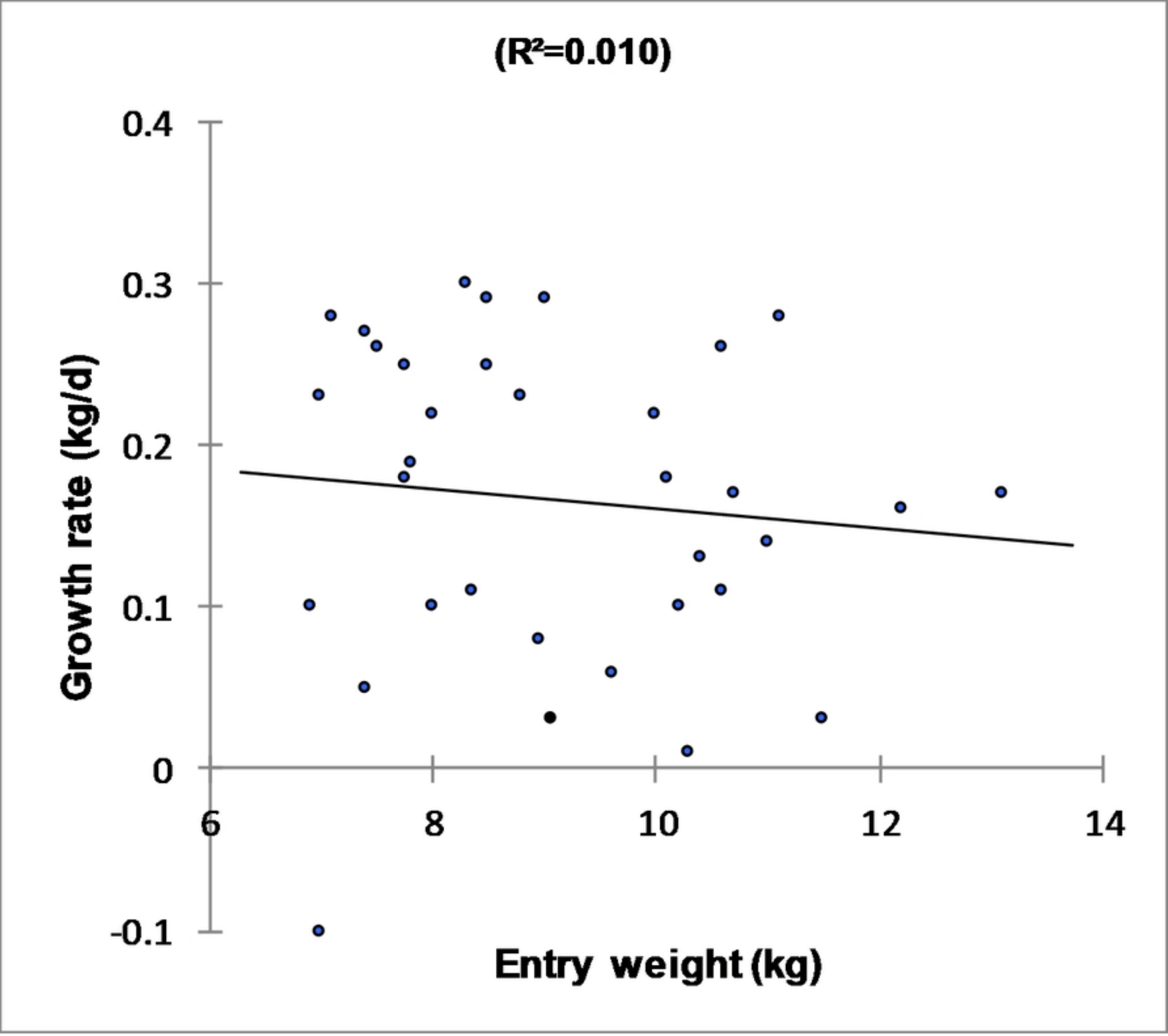
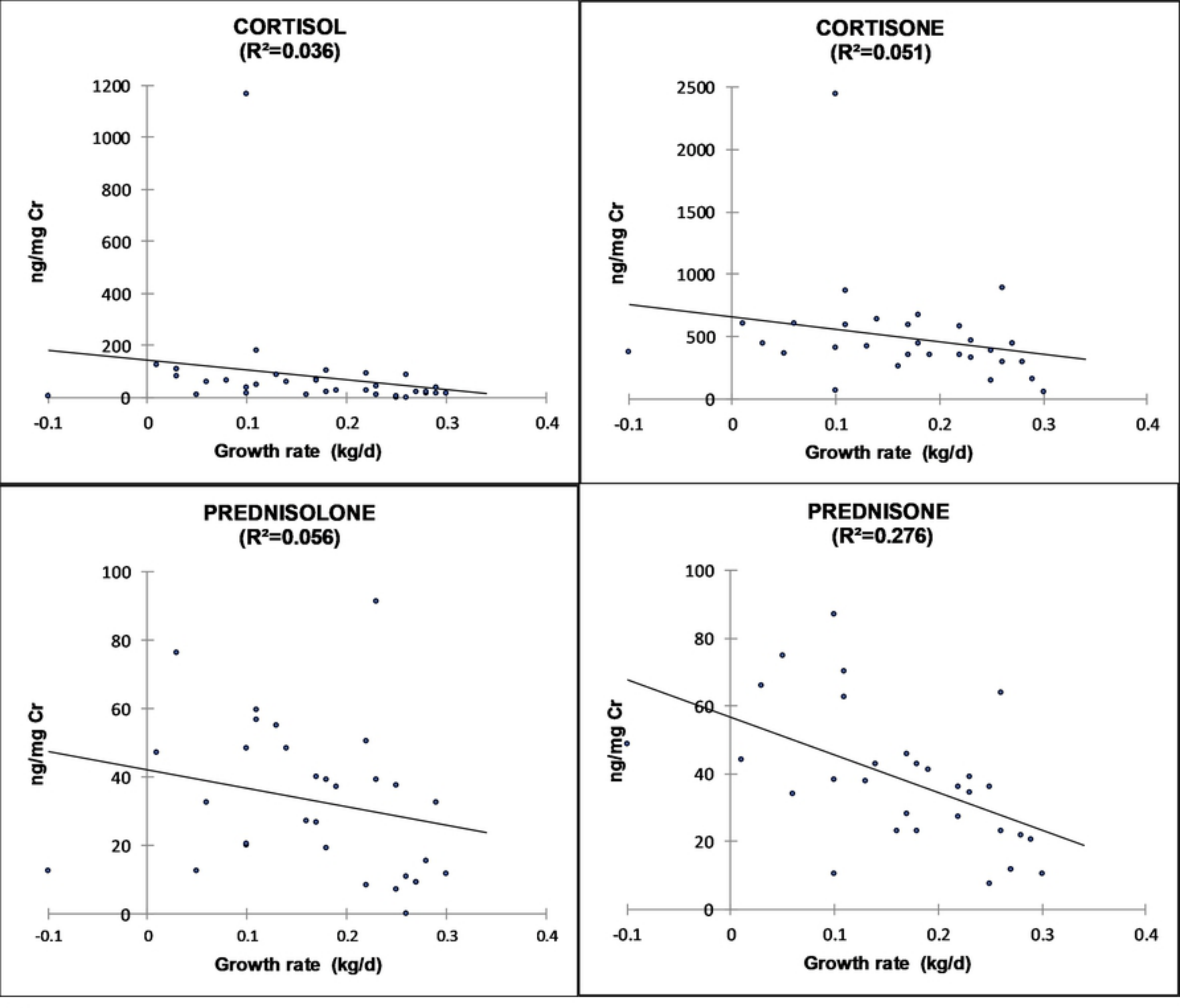
Figure


(R²=0.010)
0.4
0.3
0.2
0.1
0
-0.1
Growth rate (kg/d)
6
8
10
12
14
Entry weight (kg)

Figure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(R²=0.036)
ng/mg Cr
Growth rate (kg/d)
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(R²=0.051)
ng/mg Cr
Growth rate (kg/d)
PREDNISOLONE
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Growth rate (kg/d)
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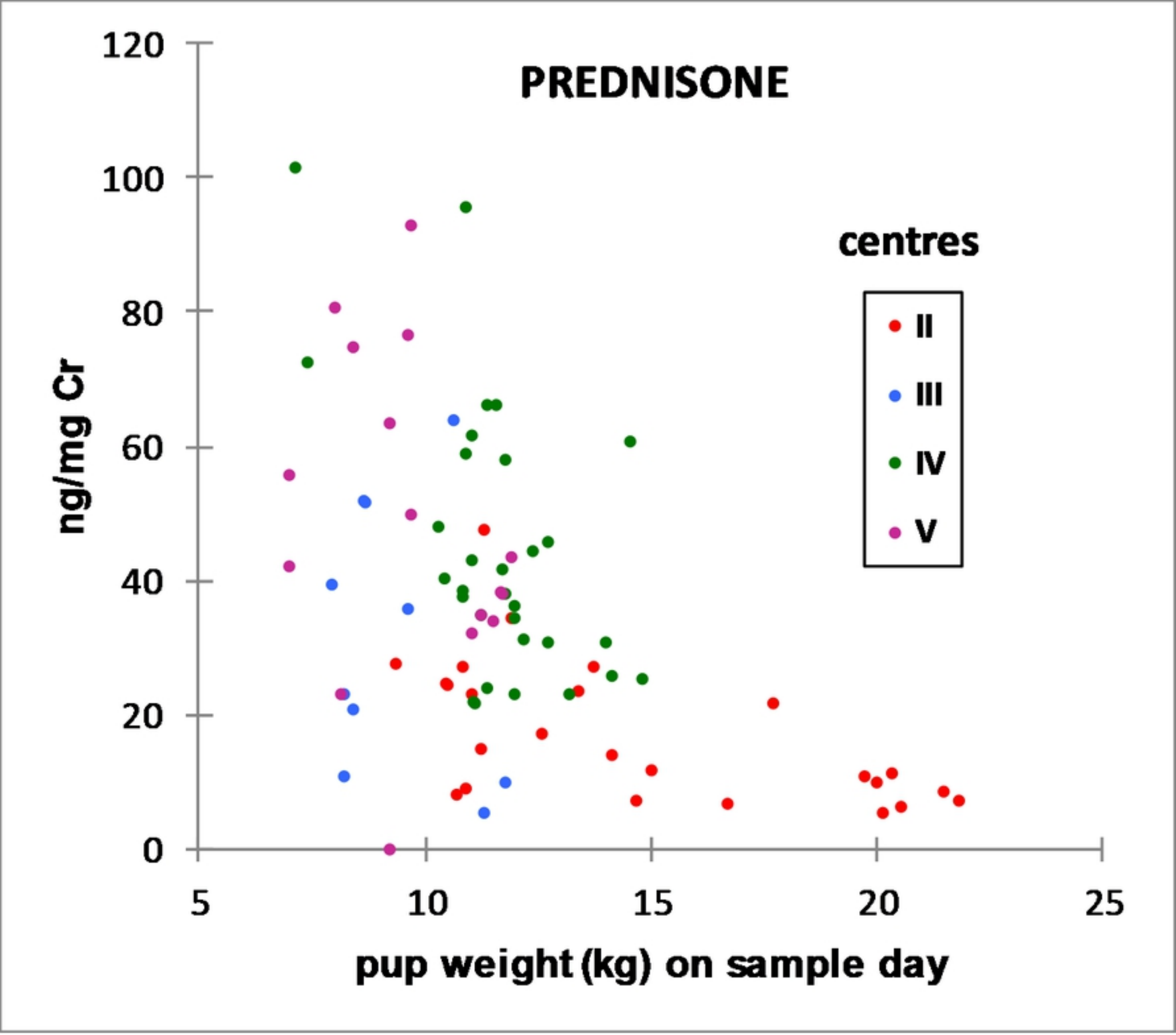
ng/mg Cr
Growth rate (kg/d)

Figure


PREDNISONE
ng/mg Cr
120
100
80
60
40
20
0
5
10
15
20
25
pup weight (kg) on sample day
centres
II
III
IV
V

Figure