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Sheep wool cortisol as a retrospective measure of long-term HPA-axis activity and links to body mass

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Abstract

In recent years, hair cortisol analysis has been suggested as a powerful retrospective measure of long-term hypothalamic-pituitary-adrenal (HPA) axis activity in numerous mammal species. In contrast, research evaluating the use of wool as a marker of long-term HPA-axis activity is still scarce, and wool differs from hair in a number of ways. Here, we assess repeatability and differences in wool cortisol concentrations (WCC) across (i) the wool shaft, (ii) two body locations, and (iii) time in n=33 Welsh mountain barren ewes (*Ovis aries*). We also (iv) investigate effects of grazing-related changes in body mass on WCC, and (v) assess effects of the washing procedure during sample preparation on WCC. Cortisol concentrations were repeatable but differed significantly across the wool shaft indicating that, provided wool growth rate is known, a single sample per individual could be used as a retrospective cortisol 'timeline'. WCC were significantly higher in shoulder than in back samples and no correlation between these two body locations was found, highlighting the importance of sampling from the same body location for repeated measures. The increase in body mass during grazing corresponded with a decrease in WCC which was significantly negatively correlated with body mass (and positively with age), suggesting that WCC can be used as a marker of

study revealed significant within- and between-individual differences in WCC and highlights a number of advantages but also methodological considerations of using WCC as a retrospective measure of long-term HPA-axis activity in sheep.

Keywords

Chronic HPA-axis activity, Cortisol, Wool, Sheep,

1. Introduction

Measuring physiological stress, i.e. glucocorticoids (GCs) released from the adrenal cortex (cortisol or corticosterone depending on the species, [1]) in response to activation of the hypothalamic-pituitary-adrenal (HPA) axis, is central to understanding how animals respond to and cope with changes in their social and physical environment ("stressors", e.g. social status, resource availability; for reviews see [2-4]). The methodology/sample medium used to quantify GCs depends largely on the timescale in question, i.e. whether short-term or long-term HPA-axis activity is to be assessed, as well as the feasibility of sample collection [4]. Non-invasive techniques for monitoring long-term HPA axis activity are particularly important in animal conservation and welfare (reviewed by [1, 3, 5, 6]).

In recent years, hair cortisol analysis has become a commonly used tool for the retrospective assessment of long-term HPA axis activity in numerous mammal species, including, for example, non-human primates [7, 8-13], humans [14, 15, 16], carnivores [17-20], ungulates [21, 22], lagomorphs [23], rodents [24] (for reviews see e.g. [25, 26]). Hair sample collection is relatively easy and non-invasive, and although in most cases the animal will have to be held or restrained (but see [10, 17]), there is no impact of handling on hair cortisol concentrations. Relatively small quantities

Despite the growing body of literature evaluating the use of hair cortisol as a marker of long-term HPA-axis activity in a wide range of species (see above), wool (i.e. the fleeces produced by sheep and other animals such as goats and yak [27]), has received relatively little attention (but see [21, 28, 29, 30]). This is surprising given that the welfare of farmed and experimental sheep is of central interest [31] and effects of chronic stressors and external factors on wool cortisol concentrations (WCC) have been described [21, 28-30]. In June 2017, nearly 35,000 sheep were recorded on agricultural holdings in the UK (https://www.gov.uk; Livestock numbers in England in the UK; accessed 22/05/2018). Furthermore, sheep are social and relatively long-lived animals with complex, large brains, and thus, are a popular model in behavioural (e.g. [32, 33]), cognition (e.g. [34, 35]), and neurological research (e.g. [36, 37]).

Using wool cortisol to monitor HPA axis activity as an indicator of sheep welfare would be of great benefit because wool presents a potential advantage over hair given that wool (as opposed to hair) fibres grow almost continuously and may be used as an indicator of a cortisol 'timeline' or 'retrospective calendar of HPA activity' [6, 25]. However, segmental wool cortisol analysis, i.e. the cutting of samples in segments that differ with respect to their proximity to the animal's skin), has, to our knowledge, not yet been conducted. In addition, wool differs markedly from hair with regards to follicle and fibre characteristics [25] which, in turn, differ also between breeds and also within a sheep's body (e.g. [38, 39]). Together, these features of wool offer potential for new insight into the mechanisms of cortisol incorporation into the fibre as well as sources of hair cortisol variation [25].

In this study, we assess repeatability and differences in WCC across (i) the wool shaft, (ii) two body locations, and (iii) time. We also (iv) investigate effects of grazing-related changes in body mass and age on WCC. In addition, we (v) evaluate effects of the sample washing procedure prior to hormone extraction and analysis on WCC. Sample washing is commonly used to remove sweat, sebum and other contaminants but assessments of its impact on cortisol concentrations are rare for

2. Methods

2.1 Study animals and data collection

Data were collected from n=33 Welsh mountain barren ewes (*Ovis aries*), born between 2010 and 2013 (randomly selected by the farmer), and located at Bangor University's Henfaes Research Centre, Abergwyngregyn, North Wales (53°13'13.75" N, 4°0'34.88" W). The sheep were housed outdoors during the entire study period. Between 12th May 2016 and 31st October 2016, the sheep were subjected to two 1-month long grazing periods (12th May - 16th June and 29th September - 31st October) in a semi-improved enclosed 11.5 ha upland pasture, as part of the Uplands-N_2O project (http://uplands-n2o.bangor.ac.uk/). We used this opportunity to investigate WCC as a retrospective measure of HPA-axis activity and links to body mass. The sheep were weighed before and after grazing (mean±sd: 38.8±6.5 kg). Wool samples were collected during attachment/removal of movement sensors used for other studies (e.g. see [40]). The work and methods used were approved by Swansea University's Animal Welfare and Ethical Review Group (Reference IP-1516-5) and by Bangor University's College of Natural Sciences Ethics Committee (Ethics approval code CNS2016DC01).

2.2 Wool sample locations and collection

A total of n = 150 wool samples were collected (see below; Figure 1), using commercially available pet clippers (BaoRun P6). The wool samples were stored in aluminium foil and labelled paper envelopes at room temperature until extraction [7, 21, 30].

To asses repeatability in WCC across time and investigate effects of body mass and age on WCC, n=120 samples were collected from the lower back, before and after two 1-month grazing periods (n=4 for n=27 individuals; n=2 for 6 individuals which were only sampled during one of the two grazing periods; Figure 1). Following grazing, regrown wool was collected from the same area as before grazing to ensure the WCC measured reflected HPA axis activity during the grazing period ('shave-reshave'; see e.g. [41]). Note that back samples collected during wool sampling 1 (Figure 1) were also used to evaluate effects of the washing procedure on WCC (see below).

Shoulder wool samples

N=30 samples were collected from the top of shoulder between the scapula bones during wool sampling 1 (12th May 2016) and were cut into three segments of 2.5cm length (A-C, with A being proximal and C distal to the skin; n=90 sub-samples; Figure 1) to assess repeatability and differences in WCC across the wool shaft. Segment A was also used to assess repeatability of WCC across the two body locations.

2.3 Hormone extraction and analysis

Wool samples were processed following published procedures (e.g. [7, 21]). In brief, approximately 250mg of each wool sample were washed twice with 5ml isopropanol. Isopropanol was removed and samples were then placed in individual aluminium weighing boats and allowed to dry at room temperature for seven days under a protected hood [42]. Following drying, approximately 50 mg of wool were placed in 2 mL microcentrifuge tubes, finely cut using scissors, and weighed (dry weight mean ± SD=0.052±0.003 g, n=190). Subsequently, 1 mL of methanol was added to each

Following extraction, samples were vortexed for ten minutes and centrifuged for 5 minutes (VWR micro star 17/17R centrifuge; 13,300 rpm). Then 600µl were transferred to a glass tube and were dried under a stream of nitrogen gas at 38°C. Once dried, samples were resuspended with 400µl of the assay diluent supplied with the enzyme immunoassay kit [21]. Samples were analysed for cortisol concentrations using a commercially available salivary cortisol enzyme immunoassay kit (Salimetrics LLC, State College, PA). The kit has been used to analyse hair cortisol concentrations in sheep [28] and numerous other species including humans [43-46], non-human primates such as chimpanzees [47], rhesus macaques [48-51], pig-tailed macaques [52], ring-tailed lemurs [53] and other mammals such as bears [54] brown hares [23], dogs [55], cows [56], angus bulls [57] and pigs [58, 59]. Later matched samples from the same individual were run on the same plate in duplicate. N=8 samples were diluted 1:2 and re-analysed since they fell outside the standard curve range. The sensitivity of the assay was 0.007µg/dL. Intra-assay variability ranged from 2.1% to 5.4%. Inter-assay coefficients of variation were 5.7% for high and 8.6% for low quality controls, respectively, which were run in quadruplicate on each plate (n=9 plates). Cortisol concentrations were converted from µg/dL and are expressed as pg/mg of hair [60].

2.4 Statistical analysis

All statistical analyses were conducted in R [61]. Data, if necessary, were log-transformed for subsequent analysis. Linear mixed models (LMMs) were performed using *lmer* and *lmertest* [62, 63]. Model diagnostics for all LMMs were performed using graphical procedures (Q-Q plot and standardized residuals vs. fitted values). Co-linearity of multiple fixed effects was controlled for by calculating Variance Inflation Factors for standard linear models excluding the random effects [64].

To test for differences in cortisol concentrations across the hair shaft a LMM (LMM1) was used, including WCC as response variable and 'segment' as categorical fixed effect, and controlling for age of subjects by including year of birth (categorical fixed effect). To test for repeatability in WCC across the three segments as well as time, the intraclass correlation coefficient (ICC) was calculated using the *ICC* package [65]. To assess repeatability in WCC across shoulder (segment A) and lower back samples a Pearson's correlation was used. A paired sample t-test was used to test for differences between shoulder and back WCC. A Pearson's correlation and paired sample t-test were also used to test for a correlation and difference between washed and unwashed samples. To test for repeatability in WCC of samples collected before and after grazing, a LMM (LMM2) was used, with WCC_{after} fitted as response and WCC_{before} fitted as fixed effect. 'Grazing period' was included as categorical fixed effect (one versus two). To assess changes in WCC and body mass during grazing, two LMMs were used, including body mass (LMM3) and cortisol (LMM4), respectively, as response variable and 'grazing period' and 'context' (before versus after) as categorical fixed effects. To investigate the link between body mass and WCC a LMM was used (LMM5). WCC was included as response variable and body mass was fitted as categorical fixed effect. To control for age-related differences in body mass, 'cohort' (three or four *versus* five or six years of age) was included as categorical fixed effect.

3. Results

3.1 Repeatability of WCC across the wool shaft and across body locations

Cortisol concentrations varied significantly across the wool shaft (range: 15.0-226.4 pg/mg), and were significantly higher in segments B (LMM1: estimate±SE=0.49±0.07, t=7.35, p<0.001; Figure 2)

182 significant effect of Sheep ID on wool cortisol concentrations (LMM1: random effect, p < 0.001;. ICC
183 = 0.240, 95 % CI = 0.022 to 0.484; see also Figure 2). Wool cortisol concentrations of shoulder
184 (segment A) and back samples were unrelated (Pearson's r = 0.094, p = 0.620, n = 30), and were
185 significantly higher in shoulder than back samples (t = -6.346, p<0.001, n=30).
186
187 *3.2 Wool cortisol in washed versus unwashed samples*
188
189 Wool cortisol concentrations of washed and unwashed samples were significantly positively
190 correlated (Pearson's r = 0.739, p < 0.001, n = 30; Figure 3A). Although the differences were small
191 (washed mean±sd: 77.6±28.3 pg/mg; unwashed mean±sd: 69.0±25.2 pg/mg), washed samples had
192 significantly higher wool cortisol concentrations (t = 2.408, df = 29, p = 0.023, Figure 3B).
193
194 *3.3 Repeatability of WCC across time and effects of body mass and age*
195
196 WCC before and after grazing (range: 16.8-171.3 pg/mg) were significantly positively correlated
197 (LMM2: estimate±SE=0.42±0.13, t=3.12, p=0.003; Figure 4A) and were significantly higher before
198 than after grazing (LMM3: estimate±SE=0.36±0.07, t=5.31, p<0.001; Figure 4B), and significantly
199 lower in the second than in the first grazing period (LMM3: estimate±SE=-0.24±0.06, t=-4.09,
200 p<0.001; Figure 4B). Sheep body mass was significantly lower before than after grazing (LMM4:
201 estimate±SE=-4.29±0.61, t=-7.07, p<0.001; Figure 4C), and significantly higher in the second than in
202 the first grazing period (LMM4: estimate±SE=3.68±0.63, t=5.83, p<0.001; Figure 4C). Cortisol
203 significantly negatively correlated with body mass (LMM5: estimate±SE=-0.02±0.01, t=-2.65,
204 p=0.009; Figure 4D). WCC were significantly higher in five and six year old sheep compared to three
205 and four year olds (LMM5: estimate±SE=0.27±0.10, t=2.62, p=0.013; Figure 5).

Cortisol analysis from hair represents a relatively recent development in stress physiology research and has the potential to be a powerful tool in understanding welfare in livestock because it allows for the assessment of chronic or long-term HPA-axis activity [1, 4, 25]. Given the structural differences between hair and wool and the lack of methodological studies on wool cortisol, the present study set out evaluate the suitability of wool cortisol as a measure of long-term hypothalamic-pituitary-adrenal (HPA) axis activity in sheep.

4.1 Repeatability of WCC across the wool shaft, body locations, and time

The majority of studies conducting segmental hair analysis found no difference between segments [7, 9] or measured lower cortisol concentrations in segments more distal from the skin [8, 18, 22, 66], potentially due to the 'wash-out effect' (see [15]) or diffusion from areas of high to low cortisol concentration [7]. Neither mechanism can explain the opposite effect, i.e. higher concentrations in distal compared to proximal segments, as found in the present study (see Figure 2) and two other recent studies [47, 67]. Although we did not quantify wool growth rate of shoulder samples, we can speculate that higher WCC in more distal segments may reflect seasonal patterns in HPA axis activity (see e.g. [67, 68, 69]). Our result provides support for using WCC as a retrospective timeline of long-term HPA-axis activity in sheep ('cortisol calendar'). It is possible that the long, continuous anagen phase in wool growth make wool fibres more suitable for use as retrospective cortisol calendars than hair fibres [25].

Despite consistent individual differences in WCC across the wool shaft (for similar results see [8]), WCC of shoulder and lower back samples were unrelated and WCC of back samples were higher. The observed pattern likely is a result of the differences in wool characteristics within a sheep's body and/or cortisol incorporation into fibres (e.g. [38, 39]). Previous work suggests local

incorporation into hair [6]. An alternative explanation relates to the repeatedly reported anterior-posterior trend in staple length, fibre diameter, and medullation, in which the wool fibres become shorter, coarser, and more medullated from the front to the rear of the sheep (reviewed by [38]). Given that both body regions sampled in the current study run along this anterior-posterior axis, it is possible that the differences in wool cortisol concentrations between shoulder and back samples is due to longitudinal variation in the structure of wool fibre characteristics. The more medullated fibres at the posterior end of the sheep may, for instance, provide more area for cortisol to diffuse into from the blood.

4.2 Effects of sample washing on WCC

Washing of hair samples was introduced to remove any glucocorticoids that may have been deposited externally on the hair through contact with sebum and sweat [7, 25]. A number of studies have validated the washing process with regards to the optimum washing solvent (methanol, isopropanol) and the number of wash cycles [7, 18, 54]; however only one study on cattle, to date, has directly compared cortisol concentrations of washed and unwashed hair samples and found higher concentrations in unwashed samples, suggesting that washing does indeed remove external glucocorticoids [22]. Surprisingly, we found the opposite effect in the present study – cortisol concentrations were slightly but significantly higher in washed compared to unwashed wool samples (see Figure 3B). This could be due to a combination of three potential reasons: First, wool, compared to hair samples, is springy and absorbent, making it more difficult to remove the entire volume of washing solvent (personal observation). Any remaining solvent evaporates during sample drying, leaving behind (potentially unevenly distributed) steroid hormones as residue on the hair surface. Consequently, total WCC measured include cortisol extracted from hair and cortisol residue,

much larger sample used for washing (here: 250mg) than what is used for extraction and analysis (here: 50mg). Third, wool fibres have a smaller diameter than hair fibres (reviewed by [25]), and this is partly due to the arrangement of the cortical cells in the cuticle layer, i.e. minimal overlapping of the cortical cells in the cuticle, resulting in the cuticle being single layered [70, 71]. Hair fibres, on the other hand, have considerable overlapping between the cortical cells, leading to the cuticle being up to 10 cells thick [70, 71]. The relative thinness of the wool fibre cuticle could mean that the wash-solvent starts to breakdown the wool fibre resulting in cortisol to be more readily extracted during extraction with methanol. Future studies are needed to further explore the effects of washing on WCC.

4.3 Effects of body mass and age on WCC

Corresponding with an increase in body mass during grazing, WCC decreased during both grazing periods (see Figure 4AB). Furthermore, WCC and body mass were significantly negatively correlated, indicating that sheep of lower body mass had higher WCCs (see Figure 4C). A similar relationship between hair cortisol concentrations and body condition (fatness) has been recently shown in polar bears (*Ursus maritimus*, [20]), suggesting that hair/wool cortisol can be used as a marker of body condition and nutritional status. Note, however, that in humans positive relationships between hair cortisol and various measures of body condition, including weight, body mass index, waist circumference and waist:hip ratio have been found (reviewed by [25]).

The effect of body mass on WCC occurred independent of sheep age and we found higher WCC in older sheep (see Figure 5). In primates, older individuals have been shown to have lower hair cortisol concentrations [11-13]. It would be interesting to compare WCC across a wider age range, e.g. including lambs and very old individuals, than in the present study (3-6 years).

Overall, our study highlights a number of advantages but also methodological considerations of using WCC as a retrospective measure of long-term HPA-axis activity in sheep. Given the pronounced between- and within-individual variation in WCC, any study aiming to use wool samples to assess sheep welfare and responses to environmental challenges should obtain repeated samples from the same body location of a representative portion of the population in question. To reliably obtain a cortisol timeline, wool growth rate should be quantified. Future research will benefit from further evaluating the effects of sample washing prior to analysis and exploring the links between wool characteristics and WCC.

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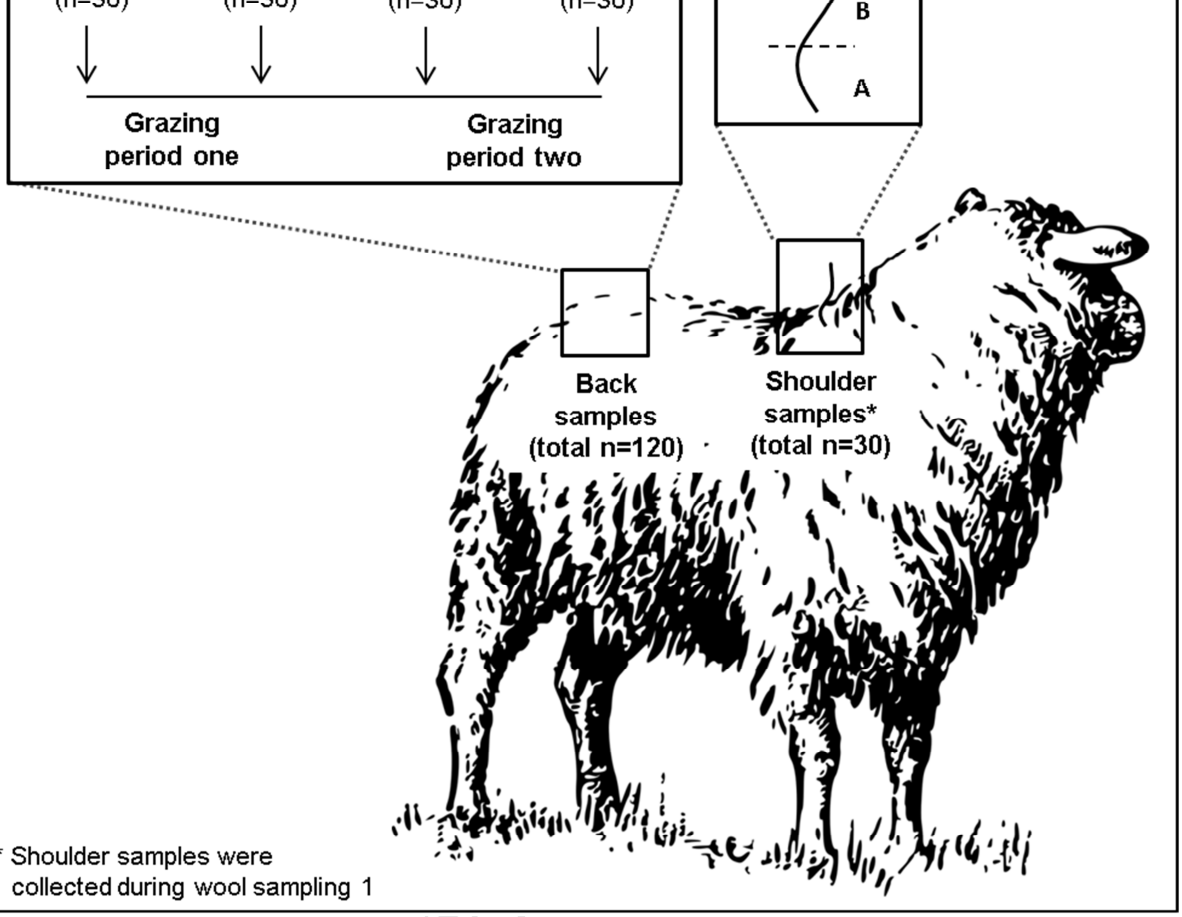
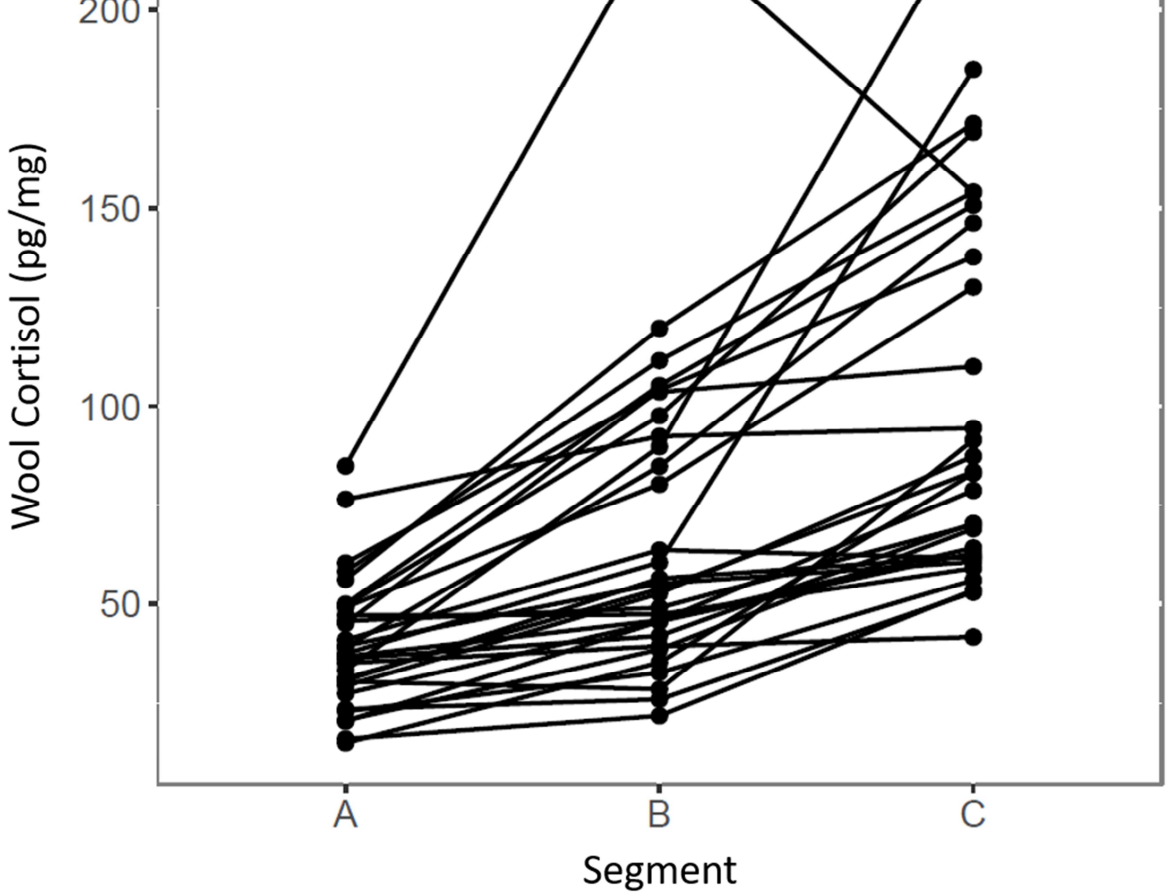
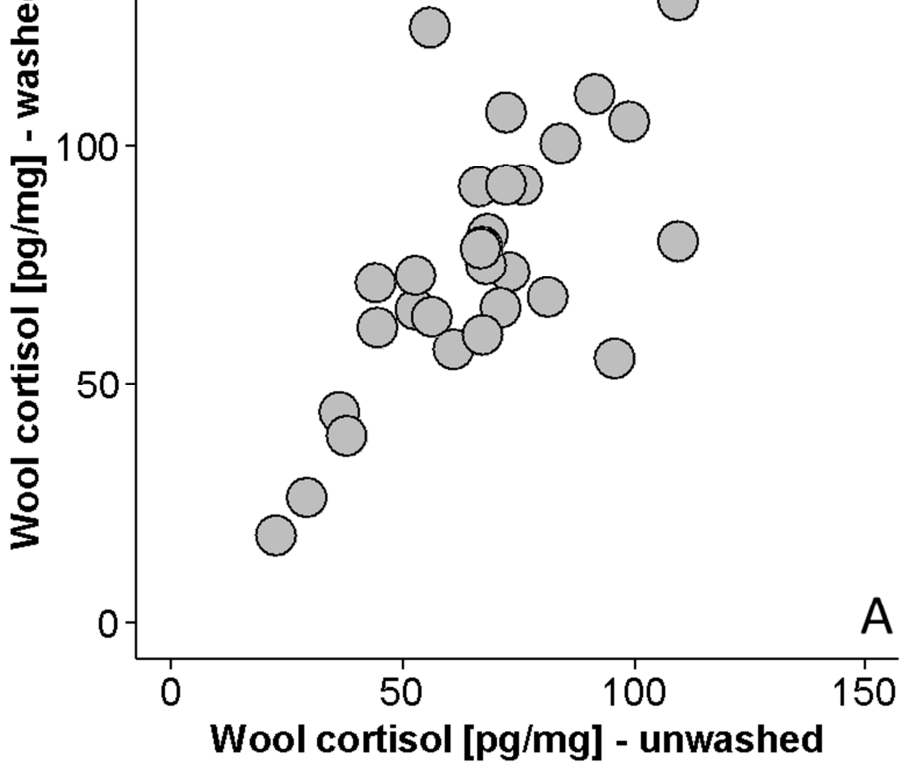
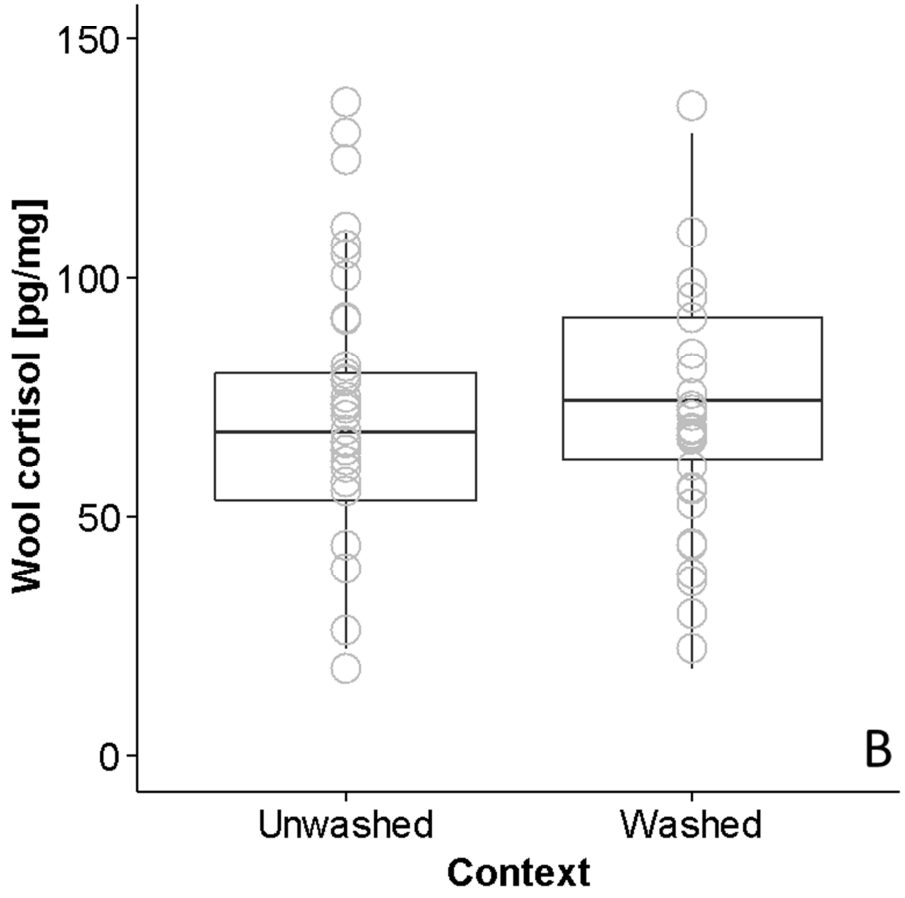
514 **Figure 1:** Wool sampling schedule and details of samples and subsamples for n=33 Welsh mountain
515 barren ewes. Wool samples were collected between 12th May 2016 and 31st October 2016.
516
517 **Figure 2:** Repeatability and differences in cortisol concentrations (pg/mg) along the wool shaft
518 (shoulder samples; segments A-C, with segment A being proximal and C distal to the skin; n=30
519 individuals). Note that non-transformed data are shown.
520
521 **Figure 3:** (A) Correlation in wool cortisol concentrations (pg/mg) between washed and unwashed
522 samples (n=30). (B) Difference in wool cortisol concentrations (pg/mg) between the washed and
523 unwashed samples (n=30). Boxes indicate medians (solid line), and lower and upper quartiles and
524 whiskers represent 1.5x the interquartile range. Open grey circles represent real data.
525
526 **Figure 4:** (A) Repeatability in wool cortisol concentrations of samples collected before and after two
527 1-month long grazing periods. Differences in (B) wool cortisol concentrations and (C) body mass
528 before and after grazing and between trials. Boxes indicate medians (solid line), and lower and upper
529 quartiles and whiskers represent 1.5x the interquartile range (B, C). (D) Correlation between body
530 mass and wool cortisol concentrations (n=120 samples, n=33 sheep). The line represents the
531 predicted effect of body mass on cortisol and shaded areas represent 95% confidence intervals.
532
533 **Figure 5:** Differences in wool cortisol concentrations between two age classes (3 or 4: n=19 sheep,
534 n=64 observations; 5 or 6: n=14 sheep, n=56 observations). Boxes indicate medians (solid line), and
535 lower and upper quartiles and whiskers represent 1.5x the interquartile range. Open grey circles
536 represent real data.

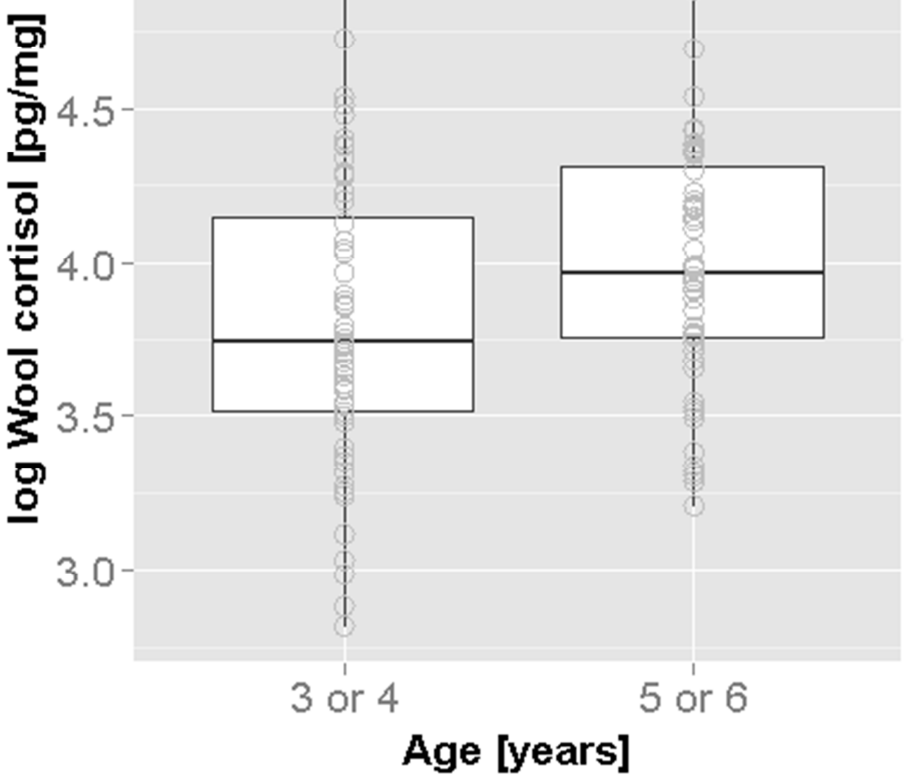


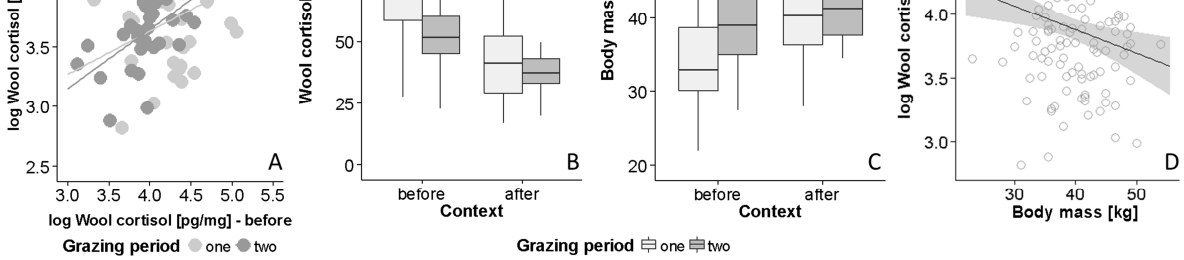
* Shoulder samples were collected during wool sampling 1




Wool cortisol [pg/mg] - washed
100
50
0
A
0
50
100
150
Wool cortisol [pg/mg] - unwashed


150
Wool cortisol [pg/mg]
100
50
0
B
Unwashed
Washed
Context





sheep
- WCC were not correlated across two body locations
- WCC were negatively correlated with body mass and positively with age