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Poor repeatability of cortisol responses to ACTH in beef heifers: is the ACTH challenge a suitable measure for stress research in cattle?

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ABSTRACT

This study investigated the repeatability and influences of the time of the day (**TOD**) and horn status on cortisol responses to ACTH administration in heifers. Sixty-four heifers were subjected to three ACTH challenges. The first challenge (**C1**) took place at the age of 2 mo. Balanced for peak cortisol responses at C1, the heifers were assigned to one of two rearing conditions: horned (**H+**) or disbudded (**H−**). At the age of 15 months, the second (**C2**) and third (**C3**) challenges took place, 7 d apart from each other at the same TOD. For cortisol analysis, saliva was sampled in 30-min intervals from 30 min before to 150 min after each ACTH injection. The area under the curve (**AUC**) of cortisol was calculated with respect to the ground (**AUC_G**) and to the increase (**AUC_I**). Between C2 and C3, AUC values did not differ ($P > 0.10$), intra-class correlation coefficients (**ICCs**) indicated poor repeatability (AUC_G: ICC = 0.24 and AUC_I: ICC = 0.26) and no correlations were found. The TOD had no effect on AUC values in C2 ($P > 0.1$), while in C3, they were greater in the morning than in the afternoon (for both AUC_G and AUC_I, $P < 0.05$). The H+ and H− heifers showed similar cortisol responses in C3, but in C2, horned heifers had greater AUC levels ($P < 0.05$). From C2 to C3, AUC values increased and decreased for heifers tested in the morning and afternoon, respectively. This was more pronounced in H+ than in H− heifers (interaction effect P <0.05). The results indicate poor to lacking repeatability for ACTH challenges performed within the same physiological state. While TOD and horn status partly contributed to the cortisol responses' variance, the poor repeatability critically questions the use of repeated ACTH challenges for stress research in cattle.

Implications

The ACTH challenge is used in repetition as a measure for stress and welfare. Hence, information about the repeatability of ACTH challenges are sparse, albeit fundamental. This study investigated the repeatability and influences of the time of the day and horn status on cortisol responses to ACTH administration in beef heifers. The results indicate poor to lacking repeatability for ACTH challenges and therefore critically question the use of repeated ACTH challenges for stress research in cattle.

Specification table

Subject	***Physiology and Functional Biology***
Type of data	Table, Figure
How data were acquired	ELISA R
Data format	Raw, calculated (AUC_I, AUC_G)
Parameters for data collection	Salivary samples were collected from 32 horned and 32 disbudded grazing heifers during two ACTH challenges seven days apart from each other. Half of the animals of each horn status were challenged in the morning, the other half in the afternoon.
Description of data collection	Salivary samples were collected using a cotton swab.

(***continued on next page***)

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Data source location	Institution: Agroscope City/Town/Region: Posieux Country: Switzerland Latitude and longitude (and GPS coordinates, if possible) for collected samples/data: 46°50'23.0"N 6°34'31.9"E 46.839708, 6.575537
Data accessibility	Repository name: zenodo Data identification number: 5495580 https://zenodo.org/record/5495580

Introduction

The use of ACTH challenges for evaluating stress or welfare in animals is important, as modified cortisol responses to ACTH may reflect the experience of prolonged stressful stimuli (Mormede et al., 2007). There is general agreement about individual variation of cortisol responses to ACTH (Ladewig and Smidt, 1989; Verkerk et al., 1994; Verkerk and Macmillan, 1997; Bertoni et al., 2005), but information about the repeatability of ACTH challenges are sparse and incomplete, albeit fundamental. The few existing cattle studies suggest weak to moderate repeatability. So far, this has been associated with physiological state changes between challenges in the experimental animals used. For example, repeatability was lacking or weak in young calves aged 3–26 wk (Van Reenen et al., 2005) and lacking in dairy cows at different lactation stages (Gross et al., 2018); weak repeatability was associated with great physiological state change in bulls (Reiche et al., 2020b).

Despite their frequent use, ACTH challenges in cattle do not follow a standardised protocol. Between-experiment variations are large and concern for example doses of injected ACTH. Low-dose tests have been proposed in different species, including cattle, with the advantages of provoking maximal cortisol responses with minimal ACTH use (Lay et al., 1996), with the assumption that they would be more sensitive than higher-dose tests and reflect "the true physiological capacity of the adrenal cortex" (Verkerk et al., 1994). However, low-dose ACTH challenges (0.002 IU ACTH/kg $BW^{0.75}$) failed to reveal significant differences in cortisol responses between tethered and free-moving bulls, which were observed in high-dose tests (1.98 IU ACTH/kg $BW^{0.75}$, Ladewig and Smidt (1989)), challenging the utility of low-dose tests for stress research and questioning even more the underlying causes of dose–effect differences.

In a previous work, we reported the repeatability and, furthermore, influences of physiological state change on the repeatability of ACTH challenges and the presence and absence of effects of horn status and time of the day (**TOD**), respectively, on cortisol responses in young bulls (Reiche et al., 2020b). In the mentioned study, horned bulls showed greater cortisol responses than disbudded. The effect of horn status on cortisol responses of female cattle has not yet been studied. To complete and extend our knowledge about the repeatability and influencing factors of ACTH challenges, the objective of the present work was to investigate the influences of physiological state change, time of the day, and horn status on cortisol responses to low-dose ACTH challenges and their repeatability in cycle synchronised, non-pregnant beef heifers.

Material and methods

Animals and housing

The experiment was embedded in a larger study with 71 crossbred heifers (Limousin × dairy breed) in which further traits related to animal behaviour were investigated (Reiche et al., 2020a). The heifers were purchased in June 2016 as calves at a mean age of 6.5 wk in two cohorts (first cohort: n = 36, second cohort: n = 35), which started the experiment as two replicates 2 wk apart. After arrival on the experimental farm of Agroscope, Posieux, Switzerland, the animals went through a medical checkup. Only healthy animals were selected for the study. Seventeen to 18 heifers per pen were housed on deep litter in four pens that were situated in the same building and were identical in construction and equipment. At the age of 9 wk, half of the heifers were disbudded under administration of sedation (xylazine, 0.1 mg/kg BW), local anaesthesia of the horn bud (2% lidocaine, 10 ml/calf) and analgesia (meloxicam, 0.5 mg/kg BW). Afterwards, the animals were divided into two groups of exclusively horned and groups of exclusively disbudded animals (see section 'Grouping'). The heifers of different pens did not have visual or physical contact. The space allowance was 3.7 m^2/calf from arrival to weaning and 6 m^2/heifer afterwards. Until weaning, the animals were fed milk replacer, ad libitum hay and a compound feed (UFA 117 Prevacox, UFA, Herzogenbuchsee, Switzerland) and after weaning a roughage-based fattening diet of aftergrowth (hay) and corn silage. At the age of 13 mo, in May 2017, 16 heifers of each pen, i. e. 64 heifers in total, were selected, balanced for BW and behavioural reactions in a novel object test at 11 mo of age (Reiche et al., 2020a), and were brought up to a farm (1 200 m above sea level) in the Swiss canton of Jura, about 70 km away from the experimental farm, for the summer grazing period. Only these 64 animals were considered in the present experiment. At the arrival on the farm, the heifers of each rearing pen were subdivided into two groups, again balanced for BW and reactions in a behaviour test at the age of 11 mo (Reiche et al., 2020a), resulting in eight grazing groups (four horned and four disbudded groups) of eight animals each. The eight groups were rotated separately on 64 paddocks with a mean surface of 34.7 a, on a total surface of 22 ha. The pastures were permanent pastures composed predominantly of grasses (50–70%). Rotation on pastures was managed using plate meters (Farmworks Plate Meter F200, Jenquip, Feilding, NZ) to measure sward heights. The animals changed paddocks at a sward height of 4–5 cm. Due to regular weighing in intervals of 5 wk and rotation on pastures, the heifers were accustomed to handling by stockpersons.

ACTH challenges, grouping, and disbudding

First ACTH challenge

The first ACTH challenge (**C1**) was performed in calves (mean age ± SD: 54 ± 17 d; mean BW ± SD: 74.3 kg ± 6.9 kg). Maximally eight calves of one pen were challenged either in the morning (0830–1200 a.m.) or afternoon (1330–1700 h p.m.). Challenges and sampling were performed as described by Reiche et al. (2020b) by administering intravenous ACTH (Synacthen, 25 IU/mL) injections of 0.5 IU/kg $BW^{0.75}$, corresponding to 0.17 IU/kg BW (Gottardo et al., 2002; Szűcs et al., 2003), and sampling saliva for cortisol determination at −30, 0, +30, +60, +90, +120, and +150 min before and after the injection of ACTH. The saliva samples were taken by sampling devices (Salivette®, Sarstedt, Nümbrecht, Germany). Handling was gently carried out, and environmental noise was avoided. The saliva samples were immediately centrifuged and stored at −20 °C until further analysis.

Grouping

After C1, the animals were allocated into two rearing groups of either exclusively horned (**H+**) or exclusively disbudded (**H−**) animals. The groups were balanced for peak cortisol values, BW, and behavioural reactivity during behaviour tests (Reiche et al., 2020a). This grouping procedure was carried out on each of the two replicates. This resulted in four groups (two rearing conditions × two replicates) of maximally 18 animals. Each of the four rearing groups was housed from grouping in one of the four pens until the start of the summer grazing period.

Disbudding

The disbudding of the H− calves used in this study (n = 32) was performed at a mean age of 63 d by using a hot iron (Buddex TM, Kerbel Corp., Buchbach, Germany) after the administration of sedation, local anaesthesia, and analgesia according to the common Swiss standard procedure.

Second and third ACTH challenge

At the age of 15 mo, the heifers were challenged again two times at an interval of 1 wk (mean age ± SD: 457 and 464 (±17) d in the second (**C2**) and third (**C3**) challenge, respectively; mean BW ± SD (in C2): 419.3 ± 27.8). Seventeen days before C2, the heifers received an intra-vaginal progesterone-releasing device (Eazi-breedTM CIDR® B, Zoetis, Switzerland). Six days after the application of the latter, the heifers received a 2-ml intramuscular injection of prostaglandin-2-alpha (Estrumate®, MSD Animal Health, Switzerland), and on the following day, the devices were removed. Consequently, ovulation should have occurred 2 d after the removal of the devices (i.e., 7 d before C2). At C2 and C3, a low dose of 0.03 IU ACTH/kg $BW^{0.75}$, corresponding to 0.007 IU/kg BW, was used [dose between Bertoni et al. (2005) and Van Reenen et al. (2005)]. The intravenously injected doses were calculated on the BW recorded 7 to 10 d before C2 with a livestock scale (Grüter, Eschenbach, Switzerland). Half the animals, balanced within the rearing groups, were ACTH challenged at the same TOD as in C1 (n = 32), the other half on a different TOD than in C1 (n = 32). Saliva sample times were the same as in C1 (see section 'First ACTH challenge'). The heifers were ACTH challenged either in the morning (0800–1130 h) or in the afternoon (1330–1700 h) in a familiar outdoor circuit. Half of the grazing groups of each rearing condition were challenged in the morning, the other half in the afternoon. For saliva sampling, the heifers were quietly driven into a familiar livestock scale with a head gate. Environmental noise was avoided. Seven days after C2, all the heifers were challenged again by the same dose of ACTH at exactly the same time as 7 d before.

Sample analysis

Saliva samples were analysed by a commercially available ELISA kit (Demeditec Diagnostics, Kiel, Germany). The sensitivity of the assay was 0.019 ng/mL, the intra-assay CV was ≤15%, and the inter-assay CV was ≤15%.

Statistical analysis

Statistical analysis was carried out on the data set (see specification table) using the R environment (version 3.6.3) (Core, 2018). Following Pruessner et al. (2003), the area under the curve (**AUC**) was calculated with respect to the ground (**AUC_G**) and the increase (**AUC_I**). To analyse the repeatability between challenges, data of C2 and C3 were used. The analysis was carried out on square root transformed data to meet the model assumptions. Pearson correlations were computed for AUC values among C2 and C3 by rearing conditions. The correlations were checked for robustness to exclude the possibility that rearing group and replicate effects were being confounded with correlation. Robustness was achieved when i) no outliers caused or negated the correlation (checked within each of the two replicates) and ii) when the inclusion of the factor replicate did not remove statistical significance in an analysis of covariance. In a first step, following Reiche et al. (2020b), two separate correlation analyses for AUC values in C2 and C3 were carried out for the subgroup of animals tested in C2 in the morning and in the afternoon, respectively. As the correlation coefficients of both AUC_G and AUC_I did not show robust or significant correlations in any of the subgroups, further analyses were carried out using the complete dataset. Linear mixed-effect models were computed using the lme4 (Bates et al., 2014), multcomp (Hothorn et al., 2008), and sjstats (Lüdecke, 2018) packages for calculating intra-class correlation coefficients (**ICCs**). The latter had been described previously as "best practice" for evaluating repeatability (Taff et al., 2018). One linear model aimed to investigate the effects of the challenge number, TOD, and the rearing condition on AUC values. Fixed predictors were introduced through the creation of a new factor variable by combining the challenge number, the rearing group, and TOD. Contrasts were designed between challenges (C2 vs C3), TODs (morning vs afternoon in C2, C3), and rearing conditions based on the challenge number (H+ vs H− in C2 and C3). Random effects were animal and replicate. In addition, a similar model was run for the subset of data of C2 and C3 to calculate ICC values between C2 and C3. A third model aimed to evaluate the AUC change from C2 to C3 (obtained by subtraction of the C2 from the C3 value), which included TOD, the rearing condition, and their interaction as fixed, with the replicate as the random factor. All the models met the assumption that inclusion of the fixed predictors improved the model over the null model (which included no predictors). The means are presented with SE, unless otherwise specified. The significance was set at P <0.05 and tendency for 0.05 < P <0.10.

Results

Descriptive statistics of salivary cortisol concentrations and influence of time of the day and rearing condition

The heifers' cortisol responses in the three challenges are presented in Fig. 1. In C1, the mean baseline (**BL**) (1.6 and 1.7 ng/mL for 1st and 2nd BL value, respectively), peak (12.5 ng/mL), and AUC values (AUC_G: 1 332.5 ng/mL × min; AUC_I: 1 048.6 ng/mL × min) were higher than in C2 (BL value both 0.4 ng/mL; peak: 5.4 ng/mL; AUC_G: 458.8 ng/mL × min; AUC_I: 382.4 ng/mL × min) and C3 (1st and 2nd BL value 0.4 and 0.3 ng/mL, respectively; peak: 5.2 ng/mL; AUC_G: 415.6 ng/mL × min; AUC_I: 349.6 ng/mL × min). While the mean cortisol concentrations 150 min after ACTH administration remained markedly higher than the mean BL values in C1 (mean value 150 min postinjection: 4.4 ng/mL), they almost returned to BL levels in C2 and C3 (C2: 1.1 ng/mL; C3: 0.9 ng/mL). Time of day had no effect on the AUC_G and the AUC_I in C1 and C2 (P > 0.1), but in C3, the cortisol concentrations were greater in the morning than in the afternoon (for both AUC_G and AUC_I P <0.05; Table 1). While in C1 and C3, the horn status did not influence the AUC values; in C2, the H+ heifers showed greater AUC_G and AUC_I than the H− heifers (Table 1).

Differences and repeatability between challenges

The values of AUC did not differ between C2 and C3 (Table 1, both P > 0.10), but an analysis of the AUC change from C2 to C3 showed an increase and decrease of AUC values for heifers challenged in the morning and afternoon, respectively, which were more pronounced in H+ than in H− heifers (interaction effects for AUC_G and AUC_I both P <0.05; Fig. 2).

The correlations between the AUC values measured in C2 and that measured in C3 were lacking in H+ heifers (AUC_G: r = −0.15; AUC_I: r = −0.08; both P > 0.10; Table 2), while in the H− heifers,

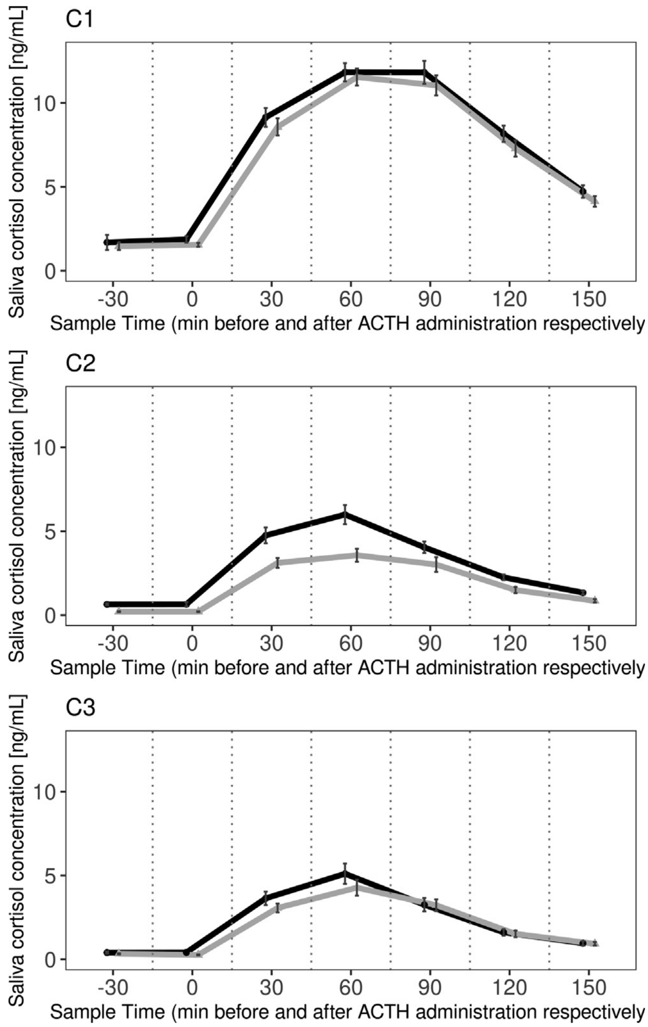

Fig. 1. Temporal progression of cortisol concentrations (ng/mL) of horned and disbudded beef heifers in response to ACTHadministration by rearing group and ACTH challenge number. Black and light grey lines represent the means of heifers of horned and disbudded rearing conditions, respectively. ACTH was administered at time 0. Error bars show SE. Abbreviations: C1, C2 and C3 = first, second and third ACTH challenge, n = 64 for each challenge.

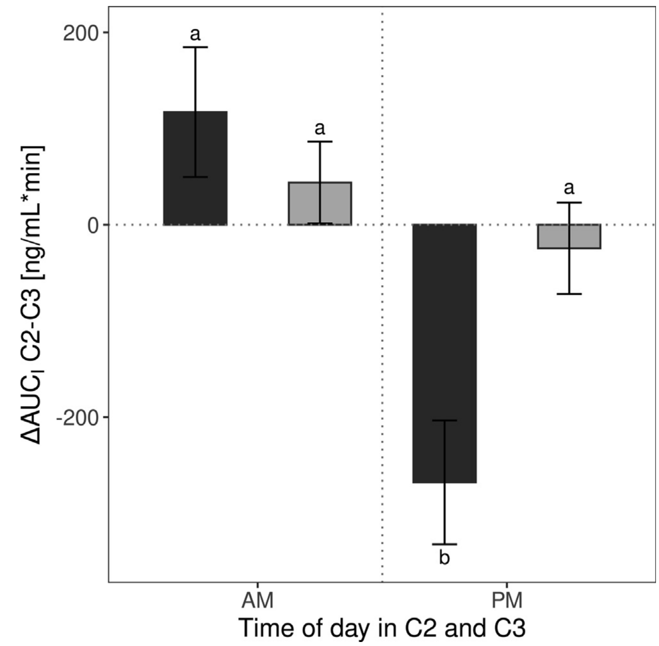

Fig. 2. Change of AUC_I of horned and disbudded beef heifers between the second and third ACTH challenge by time of the day and rearing condition. Dark and light grey bars represent means of heifers of horned (n = 32) and disbudded (n = 32) rearing conditions, respectively. Different letters indicate statistical significance of Tukey's posthoc pair-wise comparisons (P <0.05). Abbreviations: AM = ACTH challenge took place in the morning (0800–1130 h); PM = ACTH challenge took place in the afternoon (1300–1730 h), n = 32 each; C2 and C3 = second and third ACTH challenge.

Table 2
Pearson correlation coefficients of area under the curve values between the second and third ACTH challenge in horned and disbudded beef heifers, presented by rearing condition.

	Horned (n = 32)	Disbudded (n = 32)
AUC_G	−0.15	0.49[a]
AUC_I	−0.08	0.46[a]

Abbreviations: AUCG and AUCI = Area under the curves of cortisol with respect to ground and increase, respectively.
[a] correlation only robust in one of the two replicates.

they were moderately positive (AUC_G: r = 0.49; AUC_I: r = 0.46; both P <0.05; Table 2), but not robust, as a positive correlation was only present in one of the two replicates (replicate 1: AUC_G: r = 0.63, AUC_I: r = 0.62, both P <0.05; replicate 2: AUC_G: r = 0.08, AUC_I: r = −0.01, both P > 0.10). The intra-class correlation coefficients (ICCs) of AUC_G and AUC_I for the repeatability between C2 and C3 were weak (0.24 and 0.26, respectively; Table 1).

Author's point of view

The present study confirmed the technical feasibility of measuring cortisol concentrations in saliva during low-dose ACTH challenges in heifers, which had previously been reported for ACTH challenges with higher doses in young bulls (Reiche et al., 2020b), calves (Negrao et al., 2004), and dairy cows (Schwinn

Table 1
Effects of challenge number, time of the day and rearing condition on the area under the curve (AUC) of horned and disbudded beef heifers.

	Morning (n = 32) vs afternoon (n = 32)		Horned (n = 32) vs disbudded (n = 32)		Second (n = 64) vs third (n = 64) ACTH challenge
	Estimates	P value	Estimates	P value	P value
AUC_G					
C2	−2.83	0.99	19.42	<0.001	0.45
C3	21.89	<0.001	4.23	0.96	
AUC_I					
C2	−3.61	0.99	14.87	0.017	0.48
C3	20.16	<0.001	3.06	0.99	

Abbreviations: AUCG and AUCI = Area under the curves of cortisol with respect to ground and increase, respectively; C2, C3 = second and third ACTH challenge, respectively.

et al., 2016). The results obtained during the ACTH challenges of similar doses in bulls are in line with the salivary cortisol concentrations in C1 and the greater BL values in the first compared to the later challenges, which possibly reflect the animals' habituation to handling through regular weighing and pasture rotation (Reiche et al., 2020b). In comparison to C1, the lower dose used in C2 and C3 decreased the AUC levels, which is in line with previously published works (Lay et al., 1996; Reiche et al., 2020b) and which was associated with lower peak concentrations and shortened cortisol responses. The two latter observations are in contrast and accordance with, respectively, dose–response studies reporting an association between greater ACTH doses and prolonged cortisol responses in bulls and beef heifers (Lay et al., 1996; Verkerk and Macmillan, 1997). The dose dependence of the cortisol peaks in the present study may be related to the greater quotient between the lowest and highest dose used and the lower dosage levels (0.007 IU/kg BW and 0.17 IU/kg BW) compared to other studies [0.125 IU/kg BW and 0.5 IU/kg BW (Lay et al., 1996); 0.08 IU/kg and 0.3 IU/kg BW (Verkerk and Macmillan, 1997)].

The present study reports poor (in terms of ICC) and lacking (in terms of correlation coefficients) repeatability between the ACTH challenges. The observed, lacking repeatability between low-dose challenges 1 week apart from each other is lower than the moderate repeatability found in bulls within the same between-challenge interval and at a similar age (ICC = 0.52; Reiche et al. (2020b)). Several differences between the two studies might have contributed to this finding, including sex, housing, and ACTH doses. Female calves had greater and tendentiously shorter cortisol responses than male calves of a similar age (mean: 49 d) upon receiving similar ACTH doses (Reiche et al., 2017); heifers receiving testosterone showed reduced cortisol responses to ACTH than non-treated heifers (Boissy and Bouissou, 1994). Cortisol responses to ACTH are suspected to change with the lactation stage in dairy cows (Gross et al., 2018) and are influenced by the menstrual cycle in women (Kirschbaum et al., 1999). Although the heifers in the present study were non-lactating, non-pregnant, and cycle synchronised, the possibility that the sex impacts on the repeatability of ACTH challenges cannot be excluded. This should be clarified in future—not least because mostly dairy cows are subjected to stress research.

Another difference between the present and earlier similar experiments with bulls (Reiche et al., 2020b) concerns the ACTH dose used in C2 and C3, which was lower in the present study than in the former. While salivary cortisol concentrations in response to higher ACTH doses can reach supraphysiological levels, those in C2 and C3 of the present study are comparable to concentrations that can be reached under severe stress conditions, for example, at slaughter (Reiche et al., 2019). It is possible that the measured cortisol concentrations under such lower-dose conditions not only represent the adrenals' response to the injected ACTH, but may also represent other ongoing endogenous stimuli. The latter may include ACTH secretions from the pituitary gland and potentially from lymphocytes or vasopressin and non-ACTH-mediated adrenocortical activities, which are situation dependent, as they are sensitive to environmental and physiological changes (Senn et al., 1995; Bornstein and Chrousos, 1999; Dixit et al., 2001; Mormede et al., 2007). Inevitably, both meteorological and nutritional conditions, as well as food and water intake, change from 1 week to the next, and such changes might have been more pronounced in the heifers grazing on pasture than in the stall-fed bulls. On the one hand, such non-ACTH regulated mechanisms may not have been overwritten, as it might be the case in higher-dose ACTH challenges with maximum cortisol responses. Further research is needed to understand the influence of injected ACTH doses on the repeatability of adrenal responses, including the possible interactions between exogenous and endogenous cortisol responses and pathways and the possible nutritional or environmental factors that might contribute to the variance of responses. On the other hand, the repeatability of cortisol responses to ACTH was only moderate, even when using greater ACTH doses in bulls (Ladewig and Smidt, 1989; Reiche et al., 2020b). Therefore, the previous hypotheses might not be limited to low-dose ACTH challenges. As weak to moderate repeatability of ACTH challenges seems to be the rule rather than an exception in cattle (Table 3), it is evident that cortisol responses to ACTH are to a certain, even important, amount explained by other factors than the administered ACTH. In other species, namely healthy humans and horses, in which, albeit rarely, the repeatability of cortisol responses to ACTH has been investigated and expressed by means of correlation coefficients or ICC values, the repeatability, while ranging from poor to high, was also mostly only moderate (Table 3). The apparent, generally limited repeatability of the ACTH challenge may be related to several factors. Besides the above-mentioned interferences, such as cortisol being a labile trait (as discussed in Reiche et al. (2020b)), another factor might be adrenal adaptations. The administration of ACTH not only stimulates the release of cortisol but also causes physiologic, molecular, and morphological responses, for example, by upregulating certain mRNA and causing structural changes on the adrenal cell level (Bornstein and Chrousos, 1999). In dairy cows, repeated ACTH administration (every 8 h over 3 d) increased and decreased cortisol responses to ACTH on the second and third day, respectively, in comparison to the first day (Gwazdauskas et al., 1980). It is therefore possible that administered ACTH and the associated relatively high cortisol concentrations activate acute short-term adaptation mechanisms of the HPA axis. Such mechanisms need further investigation, especially with regard to optimal between-challenge intervals. Although it is difficult to draw conclusions from the few studies listed in Table 3, the greatest repeatability seems to be associated with shorter intervals (cattle: 1–7 d; horses: 2 wk; human: 24–48 h), suggesting that rather short between-challenge intervals could be promising for possible future research on the repeatability of cortisol responses (Ladewig and Smidt, 1989; Vestergaard et al., 1997; Park et al., 1999; Van Reenen et al., 2005; Widmer et al., 2005; Ghadir and Azziz, 2006; Gross et al., 2018; Reiche et al., 2020b).

The excellent repeatability for ACTH challenges with an interval of 2 weeks found in adult horses (ICC = 0.90, Scheidegger et al. (2016) is contrasting and thus interesting. Although the reasons remain unclear, the study shows that certain experimental conditions may—at least in horses—allow for good repeatability.

In general, the ACTH stimulation test is used in repetition to investigate (chronic) stress and welfare states in animals. The results of the present and previous studies and the above argumentation suggest that its use in this context should be reconsidered. Chronic or prolonged experiences of stress implicate the exposure to a certain stressor over a certain time; if the ACTH challenge is not fairly repeatable over that time, cortisol responses to ACTH are not suitable indicators for stress-modulated modified HPA reactivity. Future research should either elucidate under which circumstances ACTH challenges may be repeatable, including ACTH doses, between-challenge intervals, and physiological and environmental factors. Additionally, alternatives to quantify stress need to be explored. Examples might be evaluating the salivary cortisol/-cortisone ratio (Bae et al., 2018; Binbin et al., 2020), HPA responsiveness not at the adrenal level, but on higher functional levels, as does the corticotropin releasing hormone challenge, for example, which is feasible in calves (Veissier et al., 1999) and used in human psychology combined with the prior administration of dexamethasone (Heim et al., 2008). Again, repeatability should be shown here likewise.

The present study revealed some additional results regarding horn status and TOD as they appear to be factors that may explain

Table 3
Repeatability of cortisol responses to ACTH in cattle, horses and humans.

Subject	Age range	Sample size	Sample medium	ACTH dose	Between-challenge interval	Sampling timepoint	Repeatability	Reference
Cattle								
Female calves	Similar age	20	Plasma	0.016 IU/animal	10 wk	3 (C1) and 13 (C2) wk of age	ρ = −0.05 (n.s.)	Van Reenen et al. (2005)
					13 wk	3 (C1) and 26 (C3) wk of age	ρ = −0.12 (n.s.)	
					23 wk	3 (C1) and 26 (C3) wk of age	ρ = −0.24 (n.s.)	
Dairy cows	Parity 2–7	23	Plasma	16 μg/100 kg BW	3 and 6 wk	prior to drying off (C1), week 3 (C2) and 9 (C3) of lactation	overall AUC_G: ICC = 0.08 overall AUC_I: ICC = 0.11	Gross et al. (2017)
Bulls	Similar age	16	Plasma	C1: 0.002 IU/kg $BW^{0.75}$ C2: 1.98 IU/kg $BW^{0.75}$	1 d	15 mo of age	r = 0.64	Ladewig and Smidt (1989)
Bulls	Similar age	81	Saliva	C1: 0.5 IU/kg $BW^{0.75}$	9 mo	43 (C1) and 331 (C2) d of age	AUC_G: r = 0.59, ICC = 0.21 AUC_I: r = 0.48, ICC = 0.41	Reiche et al. (2020b)
				C2 and C3: 1 IU/kg $BW^{0.75}$	7 d	331 (C2) and 338 (C3) d of age	AUC_G: r = 0.59, ICC = 0.53 AUC_I: r = 0.58, ICC = 0.52	
Horse								
Mares, geldings and stallions	4–20 y	22	Saliva	1 μg/kg BW	2 wk		r = 0.61; ICC = 0.90	Scheidegger et al. (2016)
					5 mo		r = 0.60; ICC = 0.33	
Human								
Women	20–35 y	11	Plasma	1 000 μg	1 mo	monthly; two to six (mean: four) challenges	ICC = 0.24	Ghadir and Azziz (2006)
Men and women	26–50 y	16	Plasma	250 μg	N/A	N/A (probably days to weeks)	r = 0.60	Vestergaard et al. (1997)
Men and women	35–57 y	20	Plasma	1 and 250 μg	24 h	4 challenges on four consecutive days (each between 0600 and 0900 AM)	1 μg: ICC = 0.78 250 μg: ICC = 0.64	Widmer et al. (2005)
Men and women	18–28 y	8	Plasma	1 μg	24–48 h	5 challenges on three consecutive days. Day 1 and day 2: 0800 AM and 1600 PM; day 3: 0800 AM.	r = 0.70–0.80	Park et al. (1999)

Abbreviations: C1, C2 and C3 - first, second and third ACTH challenge; AUC_G and AUC_I - Area under the curve with respect to ground and increase, respectively. ICC – intra-class correlation coefficient.

a part of the cortisol response variability. However, due to the lack of repeatability and thus inconclusiveness of the true value, these results must be interpreted with caution. While the cortisol responses did not differ by horn status in C1, the horned heifers showed greater cortisol responses than the disbudded in C2 and, albeit only numerically, in C3. This is in line with previous results from bulls of different horn status and therefore supports the hypothesis of adrenal sensitisation or desensitisation in horned and disbudded animals, respectively (Reiche et al., 2020b). The varying adrenal reactivity values may be related to behavioural differences between horned and disbudded animals (Reiche et al., 2020a) or, alternatively, might be a consequence of the aversive experience of disbudding itself, for example, through possibly engendered chronic pain (Casoni et al., 2019). The result is another example of the relationship between horn status and physiology, as previously observed for muscle physiology, muscle proteome (Mato et al., 2018), milk metabolome, and lipidome (Wohlers, 2011; Baars et al., 2019). The underlying reasons for such physiological differences are not yet understood and need further investigation.

Although TOD did not influence cortisol responses in C2, the TOD at C2 influenced the AUC change from C2 to C3, by increasing and decreasing the AUC values in heifers tested in C2 in the morning and afternoon, respectively, causing, in C3, greater cortisol responses in the morning than in the afternoon. It is generally assumed that cortisol release in cattle underlies circadian and even ultradian rhythms (Mormede et al., 2007). In mice, the injection of dexamethasone followed by a decrease of cortisol levels at different TODs shifted, depending on injection time, the circadian rhythm of peripheral tissues, including liver and kidney (Balsalobre et al., 2000). Therefore, it might be that the ACTH administration interfered with or rather shifted the peripheral rhythms of cortisol release in the present study. Future studies should investigate the consequences of the injection time of ACTH (in relation to endogenous peak cortisol concentrations) on endogenous cortisol rhythms. At the same time, the changes in AUC between C2 and C3 were more pronounced in the horned heifers than in the disbudded ones. This finding is interesting and may reflect a faster or greater adaptation of the adrenal glands of horned heifers in response to low-dose ACTH stimulation. Thus, HPA axis-related differences between horned and disbudded cattle may not only concern the secreted amount of cortisol in response to ACTH but also other aspects of HPA axis reactivity.

ACTH challenges are frequently used for stress and welfare research in various species, since prolonged stress can modify cortisol responses to ACTH. To clearly attribute alterations in cortisol responses to ACTH to stress conditions, cortisol responses to ACTH need to be repeatable. The poor or even lacking repeatability between ACTH challenges of the present study is in line with the generally limited repeatability found in previous studies. The possible underlying reasons include the possible interactions of adrenal responses to injected ACTH with endogenous HPA activity and between-challenge adrenal adaptation. Questions arise about how such potential interactions may be related to ACTH doses and injection time, sex, environmental changes and horn status. So far, little is known about these issues. Further research on the influences on and consequences of ACTH challenges is needed to draw conclusions about whether—and if so, under which conditions—cortisol responses to ACTH may be sufficiently repeatable. Better knowledge in that respect might, finally, enable the establishment of an appropriate standardised protocol for ACTH challenges in cattle. If repeatability remains to be deemed insufficient, it should be concluded that the ACTH challenge is not suitable for quantifying stress in cattle and that other indicators need to be used instead.

Ethics approval

This study was conducted with respect to the Swiss laws of animal protection and were authorised by the cantonal veterinary office of Fribourg, Switzerland (No. 2015_21_FR).

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Declaration of interest

None.

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