Milk cortisol response to group relocation in lactating cows

Nataliya Poscic, Gianfranco Gabai, Bruno Stefanon, Laura Da Dalt, Sandy Sgorlon

Supplementary material

Detailed materials and methods

Animals and management

Collection of milk samples for cortisol analysis was performed on commercial dairy farm in Northern Italy. The farm involved adheres to a high standard of animal care that includes providing cows with nutritious diets, healthy living conditions and good veterinary care on best practice manual under the supervision of the licensed veterinarian. All animals were clinically healthy, were in their first, second or third lactation and were *kept under the same feeding and management conditions*.

The cows were housed in one free stall barn with cubicle design and automated milking *parlour*. Cows were milked twice a day, had free access to water and were fed *ad libitum* once a day at 7:30 am a *total mixed ration* (TMR) based on corn silage and formulated to cover nutrient requirements (INRA, 1989). To ensure that no dietary variations occurred during the time window of the study, the ration formulation and the offered amount were recorded using registrations of the TMR mixed feeder. Automatic individual animal identification and milk yield recording system allowed to record individual variations in daily milk yield during all the sampling period.

Experimental design and sample collection

The experiment was performed on Holstein Friesian (HF) and Norwegian Red (NR) cows and consisted of two studies (Supplementary Table S1), which were aimed at measuring the concentration of milk cortisol without perturbations and following the relocation of animals between production groups.

In Study 1 cows were not relocated to any other production group and were considered as the control group. These animals were used for evaluation of the inter-day variations of milk cortisol level. For that, milk samples were collected from individual animals twice a day during morning (6:00 AM) and afternoon (6:00 PM) milking for three consecutive days.

In Study 2 cows were relocated from the Post-partum group to the group of Fresh cows (PF), from the group of Fresh cows to the High production group (FH) and from the High production group to the Low production group (HL) (Supplementary Table S1). Milk samples from these animals were collected individually once a day during the evening milking (6:00 PM) for five consecutive days, starting from two days before the relocation date. Relocation date occurred on the morning of day 3.

Milk was collected into two 50ml sealed *tubes* without *preservative,* frozen within 2 hours after the collection and stored at -20°C until cortisol analysis. On the first day of each study, an additional 50ml aliquot of milk was collected from each animal into a tube containing preservative and analysed for the milk fat and protein percentage, lactose content and somatic cell count (SCC) using FT-MIR technique (FOSS, Denmark).

Experimental procedures used in the research comply with the Italian legislation on animal care (DL n.116, 27/1/1992) and were approved by the bioethics committee of the University of Udine. Before the beginning of the experiment, an oral *informed consent* was obtained from the farm owner and from the farm veterinary practitioner.

Cortisol assay

The whole *milk* samples were *first defatted* by centrifugation (1,500 x g, 4°C, 15 min). Skimmed milk (0.2 ml) was transferred in a glass tube and cortisol was extracted twice with 4.0 ml dichloromethane. The mixture was shaken for 15 min at 250 x g using a mechanical shaker and after the decantation supernatant was transferred into a fresh glass tube. The extracted solution was

evaporated by heating in a hot water bath (50°C) for 2 h. After the complete drying, 0.1 ml assay buffer (PBS, 0.1% BSA, pH 7.4) supplemented with 0.01 g thimerosol (sodium ethylmercurithiosalicylate) was added into the tube and mixed for 10 min (Waki et al., 1987).

Skim milk (0.05 ml) extracts were assayed by a solid-phase microtitre RIA (Gabai et al., 2006). Briefly, a 96-well microtitre plate (Optiplate, Perkin-Elmer Life Science, Boston, MA, USA) was coated with a goat anti-rabbit γ–globulin serum, by incubating overnight the antiserum diluted 1:1000 in 0.15 mM sodium acetate buffer, pH 9, at 4°C. Further the plate was washed twice with PBS 0.1% BSA, pH 7.4 (RIA buffer) and incubated overnight at 4°C with 0.2 ml of the anti-cortisol serum diluted 1:8000. The antiserum (Centro Medico Diagnostico Emilia, Bologna, Italy) was raised in the rabbit against cortisol-3 carboxymethyloxime–BSA and showed the following cross reactions: cortisol 100%, prednisolone 44.3%, 11-deoxycortisol 13.9%, cortisone 4.9%, corticosterone 3.5%, progesterone <0.01%.

Prior to the assay the plate was carefully washed with RIA buffer, and then standards (1.56–400 pg/well), quality control, unknown extracts and tracer (1,2,6,7–3H-cortisol, Perkin-Elmer Life Sciences, 30 pg/well, specific activity: 3700 GBq/mmol) were added to a final volume of 0.2 ml. The plate was incubated overnight at 4°C, thereupon the incubation mixture was discarded and the wells were washed with RIA buffer. Immediately after washing 200 μl of scintillation cocktail (Microscint 20, Perkin-Elmer Life Sciences) was added to each well and the plate was counted on the beta-counter (Top-Count, Perkin-Elmer Life Sciences). All samples were assayed in duplicate. The sensitivity of the assay was defined as the dose of hormone at 90% binding (B/B_0) and was 3.125 pg/well. The intra-assay and inter-assay coefficients of variation in high and low cortisol quality control plasma samples were between 5.9% and 9.1 % and between 13.5% and 15.1% respectively.

Data calculation and statistical analysis

All the data were stored in a spreadsheet using Microsoft Office Excel (2010, Microsoft Corp., Redmond, WA) and statistical analyses were performed with the SPSS package (SPSS,1997). Before analysis of variance, normality of independent variables was tested by the Kolmogorov-Smirnov non-parametric test. Cortisol concentrations in milk were not normal distributed and a natural logarithm transformation was applied before statistical analysis.

The productive data of milk in Study 1 were analysed with the following General Linear Model:

$Y_{iz} = \mu + Breed_i + a*DIM + \varepsilon_{iz}$

Where:

Y_{iz} = dependent variable;

μ = general mean;

$Breed_i$ = Fixed effect for Breed, with i from 1 to 2;

a*DIM = linear effect for days in milk;

ε_{iz} = residual error

The productive data of milk in Study 2 were analyzed with the following General Linear Model:

$Y_{ijkz} = \mu + Breed_i + Group_j + Parity_k + a*DIM + \varepsilon_{ijkz}$

Y_{ijkz} = dependent variable;

μ = general mean;

$Breed_i$ = Fixed effect for Breed, with i from 1 to 2;

$Group_j$ = Fixed effect for Group of lactation, with j from 1 to 3;

$Parity_k$ = Fixed effect for Parity, with k from 1 to 2 (level 2 includes parity 2 and 3);

a*DIM = linear effect for days in milk;

ε_{ijkz} = residual error

To analyze milk cortisol concentrations in Study 1, the following mixed model with repeated measures was used:

$Y_{ijkwz} = \mu + Breed_i + Day_j + Cow(Day)_{jk} + Time_w + Breed*Day_{ij} + \varepsilon_{ijkwz}$

Where:

Y_{ijkwz} = dependent variable;

μ = general mean;

$Breed_i$ = Fixed effect for Breed, with i from 1 to 2;

Day_j = Fixed effect for the Day of sampling, with j from 1 to 3;

$Cow(Day)_{jk}$ = Random effect of the k^{th} Cow within Day of sampling, with j from 1 to 3;

$Time_w$ = Fixed effect for Time of sampling, with w from 1 (AM) to 2 (PM);

ε_{ijkwz} = residual error

In Study 2, the following mixed model with repeated measures was used to analyse milk yield and milk cortisol concentrations:

$Y_{ijklwz} = \mu + Breed_i + Group_j + Day_l + Cow(Day)_{kl} + Parity_w + Breed*Day_{il} + Breed*Parity_{iw} + Group*Day_{jl} + Breed*Group*Day_{ijl} + \varepsilon_{ijklwz}$

Where:

Y_{ijklwz} = dependent variable;

μ = general mean;

$Breed_i$ = Fixed effect for Breed, with i from 1 to 2;

$Group_j$ = Random effect for Group of lactation, with j from 1 to 3;

Day_l = Fixed effect for the Day of sampling, with l from 1 to 5;

$Cow(Day)_{kl}$ = Random effect of the k^{th} Cow within Day of sampling, with l from 1 to 5;

$Parity_w$ = Fixed effect for Parity, with w from 1 to 2;

ε_{ijklwz} = residual error

Least square difference test was applied to assess significant differences between means.

Supplementary Table S1. Number of cows sampled in PF, FH and HL groups in Studies 1 and 2.

Breed	Group			Total
	PF	FH	HL	
Study 1				
NR		7		7
HF		13		13
Total		**20**		**20**
Study 2				
NR	6	6	14	26
HF	24	3	23	50
Total	**30**	**9**	**37**	**76**

HF: Holstein Frisian; NR: Norwegian Red; PF: from postpartum to fresh cows; FH: from fresh to high production cows; HL: from high production to low production cows.

Supplementary Table S2. Effect of breed, day of sampling and time of sampling on cortisol concentration in milk (ln of pg/ml) in Study 1. Milk samples were collected in three consecutive days (D1, D2, D3) at 6:00 AM and 6:00 PM.

	ln (pg/ml)	SE	
Breed			
HF	6.13	0.05	ns
NR	6.24	0.07	ns
Day of sampling			
D1	6.09	0.08	ns
D2	6.26	0.07	ns
D3	6.20	0.06	ns
Time of sampling			
AM	6.24	0.05	ns
PM	6.13	0.06	ns
Breed x Day of sampling			ns
General Mean	6.18	0.04	

HF: Holstein Frisian; NR: Norwegian Red; AM - before noon milk sampling; PM - after noon milk sampling; ns: not significant