Milk yield, oxytocin and β-endorphin gradually normalize during repeated milking in unfamiliar surroundings

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SUMMARY. For six successive milkings, six dairy cows were relocated immediately before milking to an unfamiliar operating theatre, a procedure previously shown to inhibit oxytocin release and milk ejection. Two control milkings were performed in familiar surroundings. After milk flow had ceased, two i.v. injections of 1 i.u. oxytocin were given to remove the remaining milk. Milk flow was recorded continuously and blood samples were taken every minute during milking and 10 min after milking. During the first milking in unfamiliar surroundings, no oxytocin was released. Thereby, only 13% of the total milk yield, the cisternal milk, was available and the alveolar milk fraction could only be removed after injection of oxytocin. During subsequent relocations oxytocin release steadily increased toward the control level, although the timing of oxytocin release remained delayed as compared with controls. However, the milk fraction available before oxytocin injection increased with increasing number of removals, following an asymptotic approach to control levels. The concentrations of β-endorphin, cortisol (and perhaps also of prolactin) gradually declined with the number of times the animal was moved to unfamiliar surroundings, i.e. hormone concentrations gradually adjusted to control level. During milking, concentrations of prolactin and cortisol increased, while β-endorphin concentrations decreased (except for the first relocation). We conclude that milking-related oxytocin release and therefore milk ejection adapted gradually to repeated relocations to unfamiliar surroundings. This adaptation was inversely related to β-endorphin concentrations, so it is possible that oxytocin release was suppressed by high circulating β-endorphin concentrations.

Milk removal can be disturbed under various conditions and can principally be divided into peripheral and/or central inhibition of milk ejection (Bruckmaier & Blum, 1994).

Peripheral inhibition is characterized by catecholamine action at the level of the mammary gland, even if oxytocin (OT) is released normally during milking (Lefcourt & Akers, 1984; Gorewit & Aromando, 1985; Roets & Peeters, 1985; Blum *et al.* 1989; Bruckmaier *et al.* 1991). Peripheral inhibition of milk ejection is due to α-adrenergic receptor stimulation (Blum *et al.* 1989; Bruckmaier *et al.* 1991, 1992, 1993; Hammon *et al.* 1994).

Central inhibition of milk ejection due to no or reduced release of OT can be totally abolished by administration of exogenous OT in physiological amounts and is found in primiparous parturient cows (Bruckmaier *et al.* 1992), in cows during peak

oestrus (Bruckmaier & Blum, 1994) and in cows milked in unfamiliar surroundings (Bruckmaier *et al.* 1993, 1994*a*, *b*).

In a previous investigation, milk yield was dramatically diminished and OT concentration remained basal when cows were displaced immediately before milking, while plasma concentrations of prolactin, cortisol and β-endorphin were elevated (Bruckmaier *et al.* 1993). A transient reduction of milk yield of dairy cows was demonstrated after relocation to new housing systems (Varner *et al.* 1983).

The goal of this work was to examine if and how cows adapt to unfamiliar surroundings if they are relocated repeatedly before milking.

MATERIALS AND METHODS

Animals

The six experimental cows (three Simmental × Red Holstein, three Swiss Braunvieh × Brown Swiss) in weeks 10–33 of their second to third lactation belonged to the herd of the Swiss Federal Research Station for Animal Production, Posieux. In their familiar barn the cows were housed in tie stalls in groups of 24 animals and fed on maize silage, hay and concentrates according to their individual production levels.

Materials and experimental procedure

Experimental milkings were performed during routine milking times from 06.00 to 08.00 and from 16.00 to 18.00 at a vacuum level of 45 kPa, a 65:35 pulsator ratio and a pulsation rate of 60 cycles/min, using 'Harmony' clusters (Alfa Laval, S-147 21 Tumba, Sweden). Milk flow was continuously recorded with a strain gauge system and conveyed to a strip chart recorder as described by Schams *et al.* (1984). At 6 h before the first milking, indwelling catheters were inserted into the left jugular vein of all cows for blood sample collection and OT injection.

The cows were milked at six successive milkings in the operating theatre of the research station. The first relocation was either at morning (three cows) or at evening milking (three cows). Each time the cows were relocated immediately before milking. In addition, two control milkings were performed in the familiar barn. Milking was started after a 1 min manual teat stimulation (Fig. 1). After milk flow had ceased 1 i.u. OT (a physiological dose) was injected intravenously (Fig. 1). The udder was completely emptied by a second OT injection (1 i.u., i.v.) and machine stripping (Fig. 1).

Laboratory evaluations

Blood samples (10 ml) were collected via a catheter at 1 min intervals from 2 min before the start of milking until the end of milking. An additional sample was taken at 10 min after milking. The blood samples were treated with heparin anticoagulant (50 USP units/ml blood), cooled on ice and centrifuged immediately after the experiment at 1500 ***g*** for 20 min. The plasma was stored at −20 °C until determination of hormone concentrations. Plasma OT concentrations were determined by radioimmunoassay after extraction with C_{18} cartridges (Waters SEP-PAK C_{18}, Millipore Corporation, Milford, MA 01757, USA) as described in detail by Schams (1983). Recovery (50–70 %) was dependent on the amount of extracted plasma (0·5–1·0 ml). OT for iodination and standard was purchased from Bachem Feinchemikalien AG (CH-4416 Bubendorf). ^{125}I was used to label OT using chloramine T. The reaction was stopped with sodium pyrosulphite and the ^{125}I-labelled products were purified on a Sephadex C50 column (Pharmacia LKB

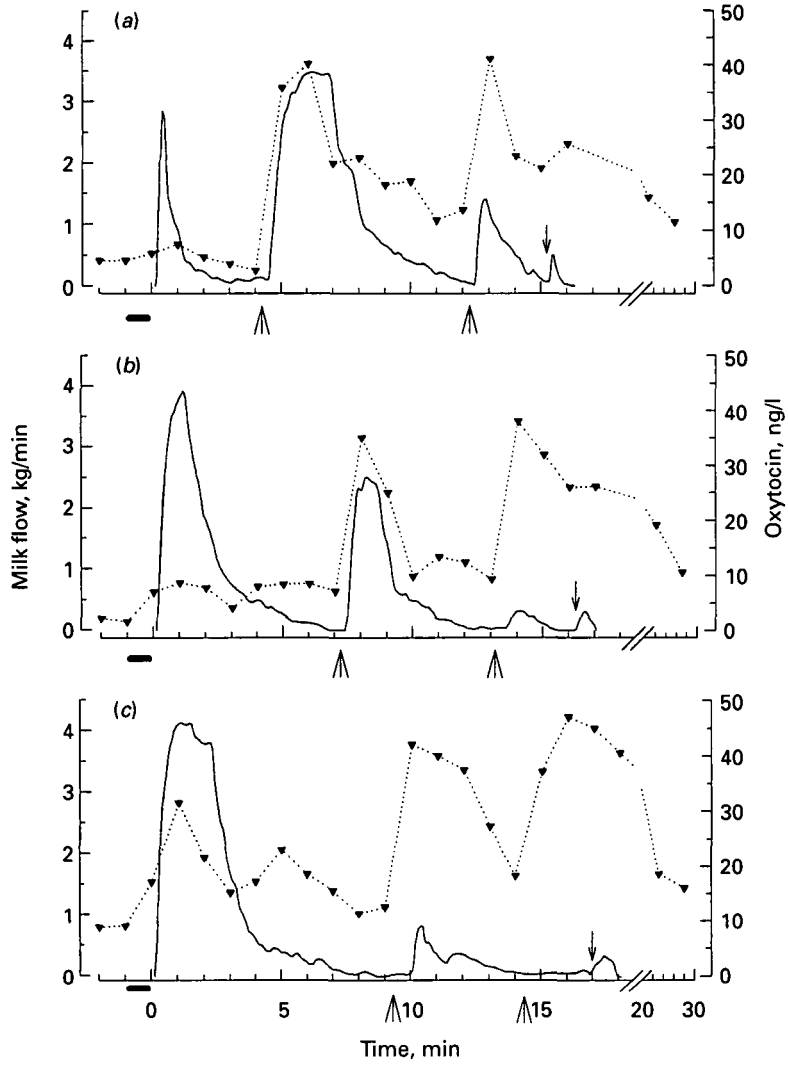

Fig. 1. ▼, Concentration of oxytocin and ——, milk flow rate in one cow before, during and after milking in (*a*) unfamiliar surroundings (first relocation), (*b*) unfamiliar surroundings (third relocation) and (*c*) its familiar barn. ▬, Teat stimulation; 0, start of milking; ⋔, i.v. injection of 1 i.u. oxytocin; ↓, stripping.

Biotechnology AB, S-751 82 Uppsala, Sweden). The antiserum against OT, which was raised in a rabbit, was highly specific for OT and did not cross react with vasopressin and other pituitary hormones (Robinson, 1980). The standard curve ranged from 1·5 to 200 ng/l; the intercept at 50% relative binding was at 25 ng/l. The assay allowed us to measure ⩾ 1·0 ng/l if 1 ml plasma was extracted. Intra-assay and interassay CV were 10 and 12% respectively. The assay procedure was carried out according to Schams (1983). Prolactin, cortisol and β-endorphin were determined by radioimmunoassay as described previously by Bruckmaier *et al.* (1992).

Evaluation of results and statistical analyses

For statistical evaluation, seven phases were defined during milking time as shown in Table 1. OT and prolactin were analysed in all blood samples. The OT and prolactin concentrations are presented as mean values for phases 1, 2, 3 and 5 and

Table 1. *Phases during the course of the experiment*

Phase	Definition
1	Time before stimulation was started (after relocation)
2	First 2 min of milking
3	Between 2 min of milking and the first oxytocin injection
4	Last 2 min before the first oxytocin injection
5	First 2 min after the first oxytocin injection
6	Last 2 min before the end of milking
7	At 10 min after the end of milking

phases 1, 4, 6 and 7 respectively. For cortisol and β-endorphin, only one blood sample each was analysed for phases 1, 4, 6 and 7 and phases 1, 4 and 7 respectively.

Changes during milking compared with premilking baseline values were tested for significance by paired t test($P < 0{\cdot}05$). Within-phase differences between relocations and control were tested for significance by ANOVA ($P < 0{\cdot}05$) using the SAS program package, release 6.08 (SAS, 1990).

RESULTS

Milk yields and milking characteristics

Total milk yields were similar in unfamiliar ($12{\cdot}8 \pm 0{\cdot}6$ kg) and familiar ($12{\cdot}7 \pm 0{\cdot}8$ kg) surroundings. Unless indicated otherwise, all values are given as means ± SEM.

The milk fraction spontaneously received after prestimulation and machine onset rapidly increased from the first to the second and third relocations (Table 2, Fig. 1). Thereafter it increased more slowly until the sixth removal, when it reached approximately control levels (Table 2). Consequently, the milk fraction ejected in response to the first OT injection was continuously decreasing from the first to the sixth relocation and control. The small last milk fraction ($4{\cdot}5 \pm 0{\cdot}4$ %), collected after the second OT injection, was not significantly different between relocations and control milkings.

Peak flow rate of the milk fraction received spontaneously after machine onset greatly increased from the first to the second and then less markedly up to the sixth relocation to reach control values ($1{\cdot}42 \pm 0{\cdot}54$, $3{\cdot}21 \pm 0{\cdot}50$, $3{\cdot}82 \pm 0{\cdot}31$ and $3{\cdot}93 \pm 0{\cdot}24$ kg/min respectively), i.e. in an asymptotic manner. This peak flow rate was negatively correlated with the peak flow rate in response to the first OT injection ($3{\cdot}58 \pm 0{\cdot}14$, $3{\cdot}46 \pm 0{\cdot}29$, $1{\cdot}56 \pm 0{\cdot}33$ and $1{\cdot}11 \pm 0{\cdot}25$ kg/min respectively; $r = 0{\cdot}39$, $P < 0{\cdot}05$).

The lag time from the first OT injection until the start of milk flow increased with decreasing amounts of milk remaining in the udder after removal of the spontaneous milk fraction. Thus, lag time had already returned to control levels by the third relocation (27 ± 2, 29 ± 1, 35 ± 4 and 34 ± 2 s at the first, second and third relocation and at control milkings respectively).

Oxytocin concentrations

Premilking basal plasma OT concentrations (phase 1) were similar during all relocations and in control milkings ($3{\cdot}4 \pm 0{\cdot}4$ and $5{\cdot}2 \pm 1{\cdot}2$ ng/l respectively). The increment of plasma OT concentration during the first 2 min of milking (phase 2) was significantly smaller in unfamiliar than in familiar surroundings and was not

Table 2. *Oxytocin concentration changes during the first 2 min of milking (phase 2) and from 2 min until oxytocin was injected the first time (phase 3) and spontaneously obtained milk fraction*

(Values are means ± SEM for $n = 6$)

Relocation no.	Changes in oxytocin, phase 2, ng/l	Changes in oxytocin, phase 3, ng/l	Spontaneous milk fraction, % of total
1	$0{\cdot}6 \pm 0{\cdot}6^{a}$	$-0{\cdot}4 \pm 0{\cdot}1^{a}$	13 ± 5^{a}
2	$0{\cdot}6 \pm 0{\cdot}6^{a}$	$1{\cdot}5 \pm 0{\cdot}8^{a}$	42 ± 9^{b}
3	$2{\cdot}0 \pm 1{\cdot}0^{a}$	$3{\cdot}4 \pm 2{\cdot}1^{ab}$	70 ± 6^{c}
4	$0{\cdot}5 \pm 0{\cdot}9^{a}$	$4{\cdot}6 \pm 2{\cdot}0^{ab}$	73 ± 7^{c}
5	$2{\cdot}9 \pm 1{\cdot}7^{a}$	$4{\cdot}0 \pm 2{\cdot}2^{ab}$	77 ± 7^{c}
6	$1{\cdot}6 \pm 1{\cdot}2^{a}$	$5{\cdot}1 \pm 2{\cdot}6^{ab}$	83 ± 4^{c}
Control†	$8{\cdot}0 \pm 3{\cdot}5^{b}$	$8{\cdot}3 \pm 2{\cdot}6^{b}$	86 ± 2^{c}

[a, b, c] Values in the same column without a common superscript letter were significantly different: $P < 0{\cdot}05$.
† Control, familiar surroundings.

significantly changed with repeated relocations (Table 2, Fig. 1). Even after 2 min milking (phase 3), the plasma OT concentration remained low during the first relocation. However, the OT rise during milking increased progressively from the first to the sixth relocation, although it never reached the values found in control milkings during the period of the experiment (Table 2). After the first OT injection (phase 5) the plasma OT concentration increased to a high level in all milkings but was lower in the first three than in the latter three relocations. During the last three relocations OT levels reached those in control milkings ($25{\cdot}6 \pm 2{\cdot}0$, $35{\cdot}4 \pm 4{\cdot}6$, $39{\cdot}3 \pm 5{\cdot}4$ ng/l respectively).

Prolactin concentrations

Plasma prolactin concentrations before milking (phase 1) were numerically but not significantly higher in relocations than in controls (Table 3). Values decreased from the first to the sixth relocation and finally reached values similar to those measured in control milkings. During milking (phases 4 and 6), plasma prolactin concentrations increased; however, this was significant only in familiar surroundings. At 10 min after the end of milking (phase 7) plasma prolactin concentrations remained the same in the first two relocations, but fell slightly in all other treatments.

Cortisol concentrations

Basal cortisol concentrations (phase 1) were elevated in the first two relocations (Table 3). During milking (phases 4 and 6), plasma cortisol concentrations were significantly higher in all milkings compared with controls, but the changes themselves were not significant. The increase was highest in the first relocation, and fell towards the sixth relocation to control values. Thus, cortisol concentrations at the end of milking (phase 6) decreased significantly from the first to the sixth relocation and control. At 10 min after milking (phase 7) plasma cortisol concentration was still numerically increased from the first to the fourth relocation but decreased slightly from the fifth to the sixth relocation and to values of control milkings; however, these changes were not statistically significant.

Table 3. *Concentrations of prolactin, cortisol and β-endorphin during milkings in unfamiliar and familiar (control) surroundings*

(Values are means ± SEM for $n = 6$)

	Phase†	Phase 1, before stimulation	Phase 4, before oxytocin injection	Phase 6, end of milking	Phase 7, 10 min after end of milking
	Displacement				
Prolactin, μg/l	1	64·8 ± 30·6	115·9 ± 40·2*	109·3 ± 41·4	111·6 ± 45·6
	2	54·8 ± 16·6	81·7 ± 26·0	93·7 ± 35·9	95·1 ± 41·2
	3	52·7 ± 17·1	66·7 ± 26·3	73·1 ± 28·4	54·1 ± 21·9
	4	34·1 ± 9·9	62·9 ± 19·2	58·3 ± 18·0	45·1 ± 14·7
	5	41·8 ± 11·7	70·3 ± 20·0	67·5 ± 19·9	45·5 ± 12·7
	6	31·1 ± 7·9	62·1 ± 20·4	62·3 ± 22·2	47·9 ± 19·9
	Control	20·2 ± 3·6	95·4 ± 29·4*	82·3 ± 24·8*	54·3 ± 14·5*
Cortisol, μg/l	1	4·1 ± 1·6	10·6 ± 2·5[a]*	12·2 ± 2·5[a]*	15·9 ± 2·4[a]*
	2	3·8 ± 1·0	9·1 ± 1·7[ab]*	10·9 ± 2·0[ab]*	12·7 ± 3·9[ab]
	3	2·9 ± 0·5	7·2 ± 1·6[ac]*	7·5 ± 1·7[bc]*	8·0 ± 1·8[bc]
	4	1·8 ± 0·3	5·9 ± 0·7[bc]*	6·4 ± 0·6[bc]*	7·0 ± 1·3[bc]*
	5	3·5 ± 0·9	6·2 ± 0·8[bc]*	6·8 ± 0·9[bc]*	6·3 ± 1·2[c]
	6	2·2 ± 0·3	6·6 ± 1·0[ac]*	6·8 ± 1·2[bc]*	5·3 ± 0·9[c]*
	Control	2·4 ± 0·5	5·4 ± 0·8[c]*	5·9 ± 1·1[c]*	4·9 ± 0·6[c]*
β-Endorphin, ng/l	1	33·6 ± 9·5[ab]	39·4 ± 8·1[a]	ND	33·3 ± 9·2[a]
	2	39·0 ± 9·1[a]	29·6 ± 5·8[ab]	ND	28·0 ± 11·1[ab]
	3	46·5 ± 13·4[a]	23·0 ± 1·5[bc]	ND	14·9 ± 2·1[b]
	4	35·1 ± 8·8[ab]	21·2 ± 4·9[bc]	ND	13·1 ± 4·7[b]
	5	33·6 ± 7·4[ab]	21·9 ± 5·9[bc]	ND	19·2 ± 4·1[ab]
	6	24·1 ± 5·2[ab]	15·8 ± 3·4[bc]	ND	12·3 ± 2·5[b]
	Control	15·2 ± 3·4[b]	13·9 ± 3·6[c]	ND	13·8 ± 2·3[b]

ND, not determined.

[a, b, c] Values without a common superscript letter in the same column set were significantly different between displacements: $P < 0·05$.

* Value was significantly different from corresponding value in phase 1: $P < 0·05$.

† For full details, see Table 1.

β-Endorphin concentrations

Basal concentrations of β-endorphin (phase 1) were elevated in relocations as compared with controls (Table 3). During milking (phase 4), concentrations of β-endorphin decreased significantly from the first to the sixth relocation and to control values. During and after milking (phases 4 and 7), β-endorphin concentrations were starting to decrease again in all relocations except the first, where β-endorphin concentrations increased, although not significantly, further during milking. During control milkings β-endorphin did not change.

DISCUSSION

Basal concentrations (phase 1) of OT were similar at all milkings in unfamiliar and familiar surroundings and in the range found previously (Schams *et al.* 1984; Mayer *et al.* 1991; Bruckmaier *et al.* 1993).

In response to prestimulation and machine milking, OT was released as expected in the familiar barn, obviously causing complete alveolar milk ejection (phases 2 and 3; Schams *et al.* 1984; Mayer *et al.* 1991; Bruckmaier *et al.* 1993), and resulting in removal of the main milk fraction before OT was injected. In contrast, and in accordance with our previous investigations (Bruckmaier *et al.* 1993), after the first relocation OT concentrations remained at baseline levels during milking (phases 2 and 3), milk ejection did not occur and only a small amount of milk was removed which was comparable to the cisternal milk fractions found in other investigations (Bruckmaier *et al.* 1994*a*; Knight *et al.* 1994).

The OT release was delayed in all relocations; i.e. a more prolonged stimulus had to be applied before OT increased. However, after the second minute of milking until OT was injected, OT was gradually and slowly increasing from the first to the sixth relocation. OT concentrations remained below control level during the duration of the experiment, although the milk flow curves and milk yields were already normalized at the sixth relocation. This confirms the theory that after a certain threshold level any additional OT released has no further effect on milk removal (Schams *et al.* 1984).

It has been shown previously that OT is normally released during the entire milking procedure (Mayer *et al.* 1984; Schams *et al.* 1984), and continuously elevated concentrations of OT above a threshold level are necessary for complete milk removal (Bruckmaier *et al.* 1994*b*). Obviously, the OT release during early relocations was not sufficient to eject the entire alveolar milk fraction. Either the threshold level was exceeded only for a short period or the OT was not really above the threshold level, inducing only partial alveolar contraction and milk ejection. However, a high percentage of milk could be removed after the third relocation, although the OT increase was delayed and concentrations reached only ~ 50% of the control level. This finding provides additional support for the existence of a threshold level of OT. However, partial alveolar milk ejection seemed to be induced even if OT concentration was below this threshold level. Partial milk ejection was previously observed in response to the brief stimulus from teat cannulation, which caused a transient OT release (Mayer *et al.* 1991).

After the first OT injection (phase 5), the OT concentration was significantly elevated in all milkings and sufficiently high to eject almost all retained milk. The lag time from the first OT injection until the start of milk flow was therefore not influenced by an endogenous OT release. However, in accordance with previous

results (Bruckmaier *et al.* 1994*b*) the lag time depended directly on the amount of milk still remaining in the udder. Because this fraction was highest at the first relocation and consequently lag time was shortest, any effect of emotional stress on the responsiveness of the mammary gland to OT is very unlikely. Furthermore, the complete milk yield was not influenced by relocations and was similar in familiar and unfamiliar surroundings. Peripheral inhibition of milk removal, which was shown to occur after catecholamine administration with simultaneous normal OT release (Blum *et al.* 1989; Bruckmaier *et al.* 1991), could therefore be excluded. In addition, in the first relocation peak flow rate after the injection of OT was already comparable to the milk flow rate before OT injection in the familiar barn. Furthermore, we have shown that plasma concentrations of adrenaline and noradrenaline were always in the normal range under our experimental conditions (R. M. Bruckmaier & J. W. Blum, unpublished results), indicating that inhibition of milk ejection was solely due to disturbed OT release.

Elevated concentrations of cortisol and β-endorphin and perhaps also of prolactin indicate that relocations to unfamiliar surroundings provoked a considerable emotional stress. Elevated cortisol concentrations have previously been reported in cows after relocation (Varner *et al.* 1983), during transportation (Bremel & Gangwer, 1978) and during isolation from companion animals (Willett & Erb, 1972) and are considered to be a stress reaction. During the course of milking (phases 1 and 4), concentrations of cortisol were increasing in all milkings as in previous experiments (Bruckmaier *et al.* 1993), i.e. cortisol increased in a normal manner during milking even if the basal concentration was already elevated. Adrenocorticotropin (ACTH), the releasing factor for cortisol, and β-endorphin are derived from a common precursor in the pituitary (Eipper & Mains, 1980) and were shown to be released concomitantly in stressed rats (Guillemain *et al.* 1977). Furthermore, the administration of opioid peptides lowers the release of cortisol in cows through negative feedback (Nanda *et al.* 1992). The simultaneously elevated concentrations of β-endorphin and cortisol in unfamiliar surroundings during milking indicate a similar common regulation of ACTH and β-endorphin release in cows.

Until OT was injected the β-endorphin concentrations were falling except for the first relocation. Interestingly, the OT concentration during milking increased only if the β-endorphin concentration had decreased during milking. Consistent with this, OT was not released during the first 2 min of milking for all relocations. When β-endorphin increased during milking (first relocation), the OT concentration remained basal. While an inhibitory effect of cortisol on OT release in cows is unlikely (Mayer & Lefcourt, 1987), elevated circulating β-endorphin was shown to suppress OT release in rats (Bicknell *et al.* 1988) and mice (Haldar & Bade, 1981). Therefore, increased circulating β-endorphin after relocation to unfamiliar surroundings was possibly responsible for deficient OT release.

It has been shown that endogenous β-endorphin modulates prolactin release in several species (Barb *et al.* 1991) and that the administration of the opioid antagonist naloxone influences prolactin concentrations in ewes, calves and cows (Gregg *et al.* 1986; Johnson *et al.* 1989). It is likely that elevated concentrations of β-endorphin were in part responsible for the (not significantly) higher values of prolactin in unfamiliar surroundings.

In conclusion, while the concentrations of β-endorphin, cortisol and perhaps also prolactin were gradually declining with increasing relocation number, the milking-related OT release was enhanced. Thus, OT and prolactin release were probably modulated by high β-endorphin concentrations. In emotionally stressed animals

milk yields without OT injection were significantly smaller and time until OT was released was prolonged.

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