COPING STRATEGIES IN DAIRY COWS

Coping strategieën bij melkkoeien

H. Hopster

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Herman Hopster

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Promotor: **Dr. P.R. Wiepkema,**
                 Emeritus hoogleraar in de Ethologie

Co-promotor: **Dr. Ir. H.J. Blokhuis**
                 Hoofd van de afdeling Gedrag, Stressfysiologie en Management,
                 DLO-Instituut voor Dierhouderij en Diergezondheid (ID-DLO),
                 Lelystad

COPING STRATEGIES IN DAIRY COWS

De mens hoort te weten
dat vanuit de hersenen,
en vanuit de hersenen alleen,
geluk, verrukking, vreugde,
evenals smart, droefheid, vertwijfeling
en leed ontstaan

Hippocrates, 400 voor Christus

CONTENTS

General introduction

1.1. Evolutionary adaptation

Modern dairy cows originate from the aurochs (*Bos primigenius*) which is thought to have been distributed during the Pleistocene from Asia over all Europe and Africa (Epstein and Mason, 1984). Examination of mitochondrial DNA, used for determination of phylogenetic relationships and sequence variation among European, African and Indian cattle populations, strongly support the idea that genetically discrete aurochs strains were domesticated on different continents. Phylogenetic relations suggest that both European and African ancestors were of a *Bos taurus* nature, whereas zebu cattle apparently developed from a separate origin (Loftus *et al.*, 1994; Bradley *et al.*, 1996). Moreover, by using rates of mitochondrial evolution estimated from other species, these studies further substantiated that the two lineages diverged during the Pleistocene; *ie* long before cattle were domesticated for the first time.

Based on the social organization in bovines (American bison, barren-ground caribou; Wynne-Edwards, 1962) and African ungulates (Leuthold, 1977), it may be assumed that under primeval conditions, *Bos taurus* adopted different degrees of gregariousness, depending on environmental conditions, *ie* available food-resources. It is likely that they tended to form larger groups in open, prairie-like country than in bushed or wooded habitats. Also sedentary and migratory *Bos taurus* populations may have differed markedly in several aspects of their social organization.

Local strains of *Bos taurus* were domesticated in a fertile region near the rivers Tigris and Euphrates that extended from the present Turkey to Iran, probably around 6300 BC (Epstein and Mason, 1984). Earliest domesticates spread from these regions to Africa and all over Europe. Recent studies, however, provide support for the existence of a centre of domestication in Africa as well. Archeological remains of the European ancestor indicate an impressive figure with a withers-height of approximately 180 cm and horns of 50 cm in circumference at the horn-base. From the Neolithic to the Copper and Bronze Ages, the size of cattle in Central and East Europe gradually decreased to a dwarf size only about 100 cm high. During the Roman domination, this trend was interrupted by the great ability of the Romans in breeding livestock which effected a distinct increase in size. The loss of this expertise towards the Middle Ages may have led to some renewed decrease in mean cattle size. This was finally reversed again by planned breeding at the start of the modern age.

Domestication also affected the behaviour of cattle, and not just their morphology. In many species, the behaviour of domesticated animals, in contrast to their wild ancestors, seems to be weaker and less affected by environmental factors. They express a lower intensity of maternal and locomotor behaviour, are less ready to flee or fight and have weaker alarm reactions. Moreover, their behaviour is less controlled by chronobiological stimuli and they have looser social bonds (Hemmer,

1990).

Present day breeds were developed because of the industrial revolution, when the human population markedly increased and thus the demands for dairy produce and beef. Selection for coat colour and production traits, combined with Darwinian selection under the influence of environmental conditions, resulted in a further differentiation in breed, variety and type. Presently, the world's estimated 1.2 billion cattle produce 46 millions tons of beef and 480 millions tons of milk annually (Albright and Arave, 1997).

In summary, cattle, described in the fifteenth century as 'wild animals, so strong that they can lift trees as well as armed knights with their horns', were transformed over thousands of years into generally placid 'foster mothers of the human race', ie modern dairy cows (Albright and Arave, 1997).

1.2. Adaptation to acute stress

Evolutionary changes over many generations appeared appropriate for adapting species to never ending environmental changes. New variants and well-adapted individuals were advantageous and supplanted less well-adapted ones. Differences between individual animals in ability to adapt to changes in the physical and social environment thus underlie the evolutionary adaptation of a species.

The individual's adaptability is determined by its potential to maintain and/or re-establish a dynamic balance, or *homeostasis*, in various biological processes despite changes in its living environment which constitute continuous threats to homeostasis. In this thesis, *stress* is defined as a condition where expectations and/or objectives (genetically programmed, established by prior learning or deduced from circumstances) do not match the current or anticipated perceptions (observed or sensed) of the internal or external environment. Physical and/or psychological factors that cause, support or magnify such a mismatch are called *stressors*. The behavioural and physiological responses that compensate for this discrepancy and restore homeostasis, are termed *stress responses*.

The study of how individuals respond to stressors has a long history. A brief outline of the current concept of the stress response will be given below. For full reference on this topic see Henry and Stephens, 1977; Levine and Ursin, 1991; Johnson et al., 1992; Wiepkema and Koolhaas, 1993; Dantzer, 1994; Goldstein, 1995; Stratakis et al., 1995.

1.3. Outline of the current stress concept

An individual reacts to a stressor by activating a complex repertoire of behavioural and physiological stress responses. By this, it seems to balance the costly operation of removing the stress through fighting or fleeing against minimizing the energy costs by displaying behavioural inhibition. Both types of reactions serve the animals' ability to survive or cope successfully with the stressor by either reducing the effects of adverse environmental stimuli or by minimizing the costs of the response. These different patterns of **coping** behaviour are termed 'active' (fight/flight) or 'passive' (conservation/withdrawal) responses and correspond with the activation of distinct central nervous and neuroendocrine pathways (Fig. 1.1).

PERCEIVED
STRESSOR

early experience
genetics
environmental requirements

ACTIVE COPING   PASSIVE COPING

behavioral activation	behavioral inhibition
sympathetic system	hypothalamus-pituitary-adrenocortical system
adrenalin / noradrenalin	CRH > ACTH > Cortisol

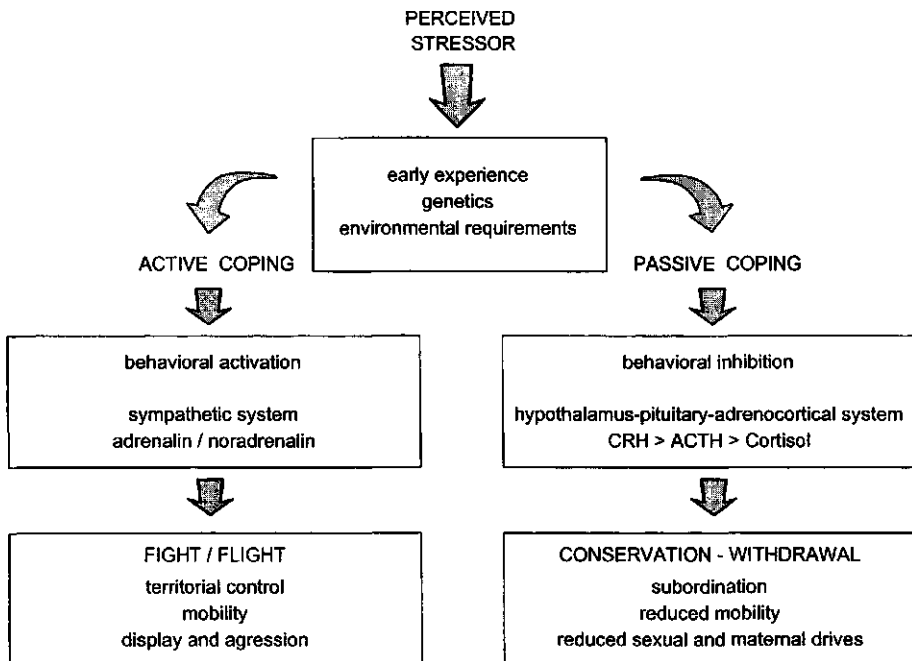| FIGHT / FLIGHT	CONSERVATION - WITHDRAWAL
territorial control	subordination
mobility	reduced mobility
display and agression	reduced sexual and maternal drives

Figure 1.1. Illustration of the different coping strategies and their typical behavioural and physiological stress response patterns (adapted from Henry and Stephens, 1977).

Thus active coping is associated with overt behavioural activity and activation of the sympathetic system, as manifested by noradrenaline release at nerve endings and secretion of adrenalin from the adrenal medulla into the bloodstream. Passive coping, in contrast, is characterized by behavioural inhibition and activation of the hypothalamus-pituitary-adrenocortical system, as demonstrated by increased concentrations of corticotrophin releasing hormone, adrenocorticotrophic hormone and glucocorticoids.

It is generally agreed that both ways of coping may be equally successful in maintaining and/or re-establishing homeostasis, but may have different consequences for health and welfare of the individual, particularly when coping is difficult and/or takes much time. Henry and Stephens (1977) integrated both the active and passive behavioural and physiological response patterns into one comprehensive biological model of coping. Note that this representation of mechanisms involved in the adaptive response to environmental change is obviously an over-simplification of the complex regulatory processes which govern the animal's homeostasis. What is important here, is that different ways of coping with stress involve different behavioural and physiological responses which can be quantified as changes in behaviour (activity, emotional response), in stress hormones (catecholamine and glucocorticosteroid concentration) and in autonomic regulation reflected in, for example, altered cardiac output (heart rate, blood pressure).

Successful adaptive responses can be either specific to a stressor or apparently nonspecific. Stress responses that seem to be nonspecific are observed when stressors are contaminated with psychological stimuli that arise in various stress situations (Mason, 1971). The significance of psychologic factors for the response to stress has also been proved in experiments where exactly the same stressor induced different reactions in subjects that were in different psychologic conditions (Weiss, 1970, 1972). Weiss' experiments made clear that not just the adverse event as such, but the ability to predict or to control its occurrence, determine to what extent stress pathologies will develop. Since then, these psychological characteristics of stressors, *ie* their predictability/controllability, are known as their most potent elements.

Besides the quality of the stressor (physical or psychological), its strength and its duration (acute, chronic), also characteristics of the individual animal (inherited genetic make up, previous experience, social position) determine whether the animal will respond predominantly actively or passively. Extensive research in man (Ursin, 1980; Henry and Stephens, 1977) and animals (Bohus et al., 1987; Benus, 1988; Hessing et al., 1994; Koolhaas et al., 1997) indicates that both animals and man respond in a rather individual way when exposed to a stressor. Individuals perform idiosyncratic coping behaviour as indicated by different physiological and behavioural reactions during different social- and non social experiments. Based on this literature, it was hypothe-

sized that individual dairy cows may also differ consistently in the way they respond to environmental stressors.

1.4. Aim and scope of the study

The physical and social environment of modern dairy cows is crucial for their welfare, health and production. The tendency to generalize about which housing and management is adequate to meet the needs of all animals may however not always be appropriate. Different individuals may make different demands upon the environment and may appraise similar housing and management conditions differently. At present, however, we do not know whether individual dairy cows do differ consistently over time in their behavioural and physiological stress responses, nor do we know whether these responses are consistent across situations. The main question in the present study therefore, is whether individual dairy cows display different but individual-specific patterns of physiological and behavioural responses that enable them to successful maintain homeostasis in a frequently changing environment. If so, similar environmental conditions (housing, management) may have different consequences for the welfare and health of different individual animals. Moreover, the objective assessment of stress responses in individual cows will be useful in predicting their response to various stress-inducing factors in their environment as well as the risk of developing stress and pathologies that may result from modern husbandry systems.

1.5. The experiments

First, a heart rate monitor developed for use in human sports and exercise, will be validated for measuring a stress response in dairy cows (Chapter 2). Because of the great sensitivity of catecholamines to environmental stress as well as their very short half-life, it would be virtually impossible to reliably measure concentrations of adrenalin and noradrenaline in free-moving dairy cows. Therefore, heart rate change will be examined instead as an indirect measure of sympathetic/parasympathetic nervous system activity. Additionally, a standard manipulation procedure will be introduced for triggering a stress response.

Next, the effects of venepuncture on plasma cortisol concentrations will be studied (Chapter 3). In these experiments, the first aim is to develop a method of blood sampling that can be used for the collection of only a few samples from many cows. Because these samples have to be used for studying the effects of experimental stressors on plasma cortisol concentrations, it is important that the collection of blood samples itself does not elicit confounding effects.

After validation of methods for heart rate measurements and blood collection, the stress-inducing qualities of different experimental manipulations will be evaluated. In this context the acute stress response of dairy cows to the removal of a calf after bonding will be studied (Chapter 4). Further, it will be investigated whether the interruption of a behavioural routine can serve to trigger a stress response in dairy cows (Chapter 5). In addition, the stress-inducing properties will be studied of a mixture of handling, novelty and isolation, called a 'novel environment' test (Chapter 6). Also the short- and long term consistency of individual differences in response to this test will be evaluated.

The novel environment test enabled selection of primiparous cows that showed either low or high concentrations of plasma cortisol in response to this test. Cows from these two groups will be temporarily isolated in their second lactation and used for investigating differences in stress response and in their host defence against intra-mammary administration of *Escherichia coli* endotoxin (Chapter 7). In Chapter 8, the major findings of the Chapters 2-7 will be discussed and evaluated in the light of current theories about the control of animal behaviour, the generation of emotional expressions and the existence of coping styles.

Validation of a heart rate monitor
for measuring a stress response
in dairy cows

Hans Hopster and Harry J. Blokhuis

Institute for Animal Science and Health (ID-DLO)
Research Branch Zeist
P.O. Box 501, 3700 AM Zeist, The Netherlands

ABSTRACT

In three experiments, a commercial non-invasive heart-rate monitor, (PST Polar® Sport Tester) was validated for use with dairy cows. First, 10 monitors were tested using a pulse generator, which produced pulses at levels between 30 and 240 bpm. PST values and generated beats were highly correlated (r = 0.97) but with varying delay (5-55 sec) depending on pulse-rate level.

In a second experiment, 10 dairy cows were quietly standing or exercising on a treadmill while their heart rates were recorded by direct-wire electrocardiograph (ECG) as well as the PST. Correlations between ECG and PST values during standing (0.88) and exercising (0.72) were significant and differed between cows. During standing, inter-beat interval and PST standard deviations were significantly correlated ($r_s = 0.76$), as were their coefficients of variation ($r_s = 0.89$).

The third experiment showed that the heart rate of 10 dairy cows increased rapidly immediately after an individual was isolated from the herd. Cows showed no obvious signs of being hampered by the equipment during 1-hr trials.

Change in heart rate may be a relevant parameter for studying an animal's response to a stressor. As heart-rate variability is thought to serve as an important mechanism for adaptability in humans, this aspect needs further attention in farm animals. The PST is suitable for measuring both heart rate and heart-rate variability in dairy cows.

INTRODUCTION

Change in heart rate may be a suitable parameter for studying both the quality of a stressor as well as an animal's response to environmental challenge. In numerous studies with different animal species, including farm animals, cardiac responses to various manipulative procedures have been investigated. In cattle, heart rate has been recorded during different branding procedures (Lay et al., 1992a) and in various rearing conditions (Le Neindre, 1989). Effects of handling procedures like restraint, transport, venepuncture (Stephens and Toner, 1975), sorting and loading onto a vehicle (Stermer et al., 1981) and handling by humans at calving (Hemsworth et al., 1989) and during milking (Hemsworth et al., 1989; Royle et al., 1992) have been studied. Besides husbandry procedures, the effect of more experimental manipulations on heart rate has been explored. For example, Kondo and Hurnik (1988) studied the reaction of cows to spatial novelty, while others applied electrical shocks (Lefcourt et al., 1986) or audible signals (Arave et al., 1991).

It is very important that subjects are not invasively instrumented for measurements of heart rate (Spodick, 1980), since this may modify the individual's physiologic milieu

and responsiveness. Therefore, telemetric recording of surface ECG is often used (Kovalčik et al., 1988; Le Neindre, 1989; Hargreaves and Hutson, 1990; Lay et al., 1992b; Royle et al., 1992). Even then, additional stress from handling or restraint may bias the measurements. Lay et al. (1992b) reported that despite the experimenters' serious attempts to avoid extra handling stress, the animals still flinched and tried to move away when the electrodes were glued to the skin. This may have caused high pre-treatment heart rates. Therefore, simple and non-invasive procedures are preferred.

To calculate beats per minute (bpm), both the time elapsed during a small number of beats (Lefcourt et al., 1986; Hargreaves and Hutson, 1990) and the average heart rate during a longer period (Hemsworth et al., 1989) can be used. If longer periods are used, it is important to know how these averaged heart rates relate to the momentary heart rate, since heart rate varies with minimum delay (< 5 sec; Lefcourt et al., 1986).

Recently Porges (1992) proposed that, compared with heart-rate level as such, the physiologic variability in heart rate may be a better indicator of both the status of the nervous system of the individual and its capacity to respond to environmental demands. Goldberger (1991) suggested that competing influences of both the sympathetic and the parasympathetic branches of the nervous system on the sinus node may be recorded as a type of chaotic heart-rate variability. In humans, this variability has often been determined by differentiating high- and low-frequency components of heart-rate fluctuations (Murata et al., 1992; Kunitake and Ishiko, 1992; Kamada et al., 1992). Goldberger (1991) also suggested that the heart-rate variability may indicate the individual's state of health. Heart-rate variability therefore needs further attention in farm animals.

Telemetric heart-rate meters, which have been developed to record the heart rate in humans doing sport or exercise (Karvonen et al., 1984), may also be suitable for transferring signals from free-ranging animals without disturbing or restraining them. Recent studies of both the precision and accuracy of one of these systems, the Polar® Sport Tester (PST) portable heart rate monitor (Polar Electro Oy, Finland), indicate that it provides valid readings of the heart rates of young children and adults across a wide range of exercises (Treiber et al., 1989; Seaward et al., 1990). Even during maximal-intensity endurance activities, electrical noise artefacts, created by motion and muscle contraction, did not significantly affect the system's values. Evans and Rose (1986) and more recently Sloet van Oldruitenborgh-Oosterbaan et al. (1988), tested the suitability of a modified version of the PST for use with the horse. These authors concluded that the PST provided valid and reliable recordings of the heart rate of the horse.

We anticipated that the PST might also be suitable for studying heart rate responses to environmental stressors in dairy cows. Because these changes can take place suddenly, it was also important to identify the delay between computed PST values and the momentary heart rate. Our experiments were designed to determine the accuracy and

reliability of a modified version of the PST system for use in dairy cows. We tested the heart-rate monitor in three studies under both laboratory and field conditions: 1) a precision test, using a pulse generator, 2) an accuracy test, with cows exercising on a treadmill, and 3) a reliability test of the system while cows were moving freely in a cubicle house. Moreover, we looked at whether the PST is also suitable for studying heart-rate variability in dairy cows.

ANIMALS, MATERIALS AND METHODS

The Heart-Rate Monitoring Device

In our experiments, the transmitter and the wrist monitor of the PST were used. The transmitter was attached to a girth belt, supplied by the manufacturer for use with horses (Polar® Horse Tester). This belt had two 17.5 cm² circular, stainless-steel plate electrodes, which were connected to the transmitter. One electrode was placed behind the scapula on the right withers (Fig. 2.1, No. 1); the other electrode was situated on the left ventral abdomen (Fig. 2.1, No. 2). Wash-leather pouches, covering the electrodes, could be wetted with water to improve the conductivity between the cow's skin and the electrodes.
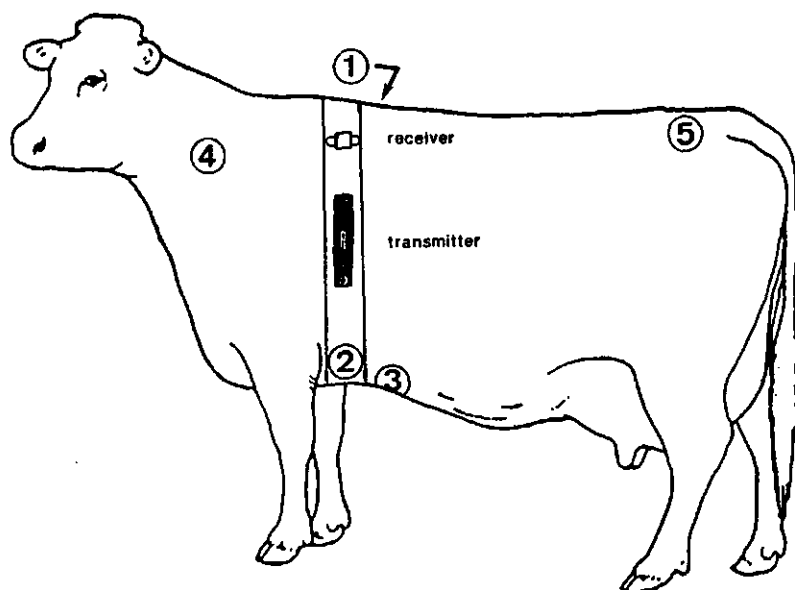

Figure 2.1. Sketch of a cow with girth belt, PST equipment, three direct-wire (3, 4, 5) and two PST electrodes (1, 2). For further explanation see text.

The heart-rate signal was telemetrically transmitted within a range of 1 m to the wrist monitor, which was attached to the girth belt. The monitor calculated heart rates, based on a pulse-to-pulse time-averaging algorithm, at 5, 15 or 60 sec intervals (Seaward et al., 1990). A sampling period of 160, 500 and 2020 min, respectively, could be saved in the memory. The first reading was calculated from the first four pulse values. Thereafter, heart rates were calculated with a moving average process, which was described by Karvonen et al. (1984). Using the manufacturer's software program, one can later transfer the data for further analysis. Physical dimensions of the PST have been reported by Treiber et al. (1989).

Precision Test

The technical performance of the PST monitors was compared by situating 10 monitors horizontally around one central transmitter in a circle with a radius of 50 cm, one on each 30° radial. The two monitors situated at 0° and 180° did not receive the signal properly, so these sites were excluded. The monitors were placed in such a way that the buckleless end of each watchstrap was directed towards the centre of the circle. The transmitter in the centre was connected with a heart-rate simulator, which generated an electrical pulse with an amplitude of 2 mV and a duration of 70 msec. The frequency of the pulse could be varied from 30 to 300 bpm. Each monitor computed average heart-rate values at 5-sec intervals. Before the recording of pulse rate began, the pulse generator was tuned to 30 bpm. All the monitors were started and began to record the signal at 5-sec intervals, saving the data in their memories. Next, the frequency of the simulated heart rate was raised by steps of 30 bpm each minute, from 30 bpm up to 240 bpm. Subsequently, the frequency of the signal was turned down stepwise to 30 bpm.

Finally, the generated heart rate was suddenly increased from 30 to 180 bpm. After 1 minute, it was turned down abruptly to 30 bpm. According to this procedure, the same 10 monitors were tested five times. In all five trials, the same transmitter was used. Heart-rate values, stored in the monitors' memories during the trials, were then transferred to a microcomputer.

Pearson correlations between PST values and simulated heart-rate values were calculated for each monitor for each trial. To determine intra- and inter-monitor effects, an analysis of variance was carried out using Fisher's Z-transformation:

$$Z = \ln \left[(1 + r) / (1 - r) \right] / 2 \qquad (1)$$

where r is the correlation coefficient (Zar, 1984, p. 310). In contrast to r, Z values estimate a population parameter, which is normally distributed. After analysis, Z values were transformed retrogressively into correlations:

$$r = (e^{2Z} - 1) / (e^{2Z} + 1) \qquad (2)$$

Accuracy Test

On two successive days, the heart-rate monitor was tested on a total of 10 lactating Holstein-Friesian dairy cows, aged between 2 and 9 yr. A direct-wire ECG-recording system (Mingograf-34®, Siemens-Elema) was used to record the ECG of the cows while standing and exercising on a treadmill. On each experimental day, five cows were restrained in the feedgate at 07.30 hr Five different sites on the cows were clipped and shaved (10 cm x 10 cm). Spots 3, 4 and 5 (Fig. 2.1) were cleaned with methylated spirit. On these three sites, disposable heart-rate electrodes (type HP® 14445A, Hewlett-Packard, Waltham, USA), provided with supplementary electrically conductive gel (Spectra® 360, Parker Laboratories, Orange, USA) were glued to the skin with an additional adhesive (Bisontex®, Perfecta Chemie, Goes, The Netherlands). After these preparations, the cows were loaded onto a truck and transported (20 min) to a treadmill at the Veterinary Faculty, University of Utrecht. One after the other, while the four other cows stayed on the truck, each animal completed the following test.

The cow was unloaded, placed on the treadmill, and supplied with the girth belt and the PST wrist monitor attached to it. To improve the conductivity between the cow's skin and the electrodes, the wash-leather pouches covering the electrodes were wetted with water and greased with gel. Next, the ECG recorder wires were connected to the supplied electrodes. The amplified signal from the ECG recorder was sampled (100 Hz) using specific microcomputer-based hardware and software applications. Binary data were stored on disk for further analysis. During 10 min of the cow quietly standing, ECG values and PST values were recorded simultaneously. Then the cow was trained to walk on the treadmill. After 5 min of training with vocal and tactile encouragement, all the cows walked on the treadmill at a constant speed of 4-5 km.hr^{-1}. During another 10 min of exercise, the heart rate of the cow was recorded as described earlier. Finally, all heart-rate equipment was removed and the cow was reloaded onto the truck.

Data from ECG recordings were processed using a program that detects the maximum amplitude (R) of each action potential caused by ventricular contraction, known as the QRS-complex of the ECG, and calculates momentary heart rates (bpm) using intervals between these maxima. From each of the 20 files, recorded during resting and exercising as well, 85 successive 5-sec periods, without any failures in the ECG recordings, were used for analysis. The following methods were used to calculate ECG heart rates. Momentary heart rate, average heart rate, and average heart rate based on the last four R-R cycles, were computed for each of the periods and correlated with the PST values. To examine the delay, which may be caused by the averaging algorithm of the PST, ECG values and PST values were shifted 0-, 5- and 10 sec with respect to one another. Finally, the correlations were transformed into Z values. To estimate the effects of exercise (motion), method of computing meaningful heart-rate values

(method), delay in PST processing (shift), and random cow effects, an analysis of variance was carried out including the main effects and interactions (Genstat 5 Committee, 1987).

To investigate whether variations in PST values reflect the variability in inter-beat intervals (IBI), a period of 100 successive 5-sec periods was extracted from the ECG data that were recorded when the cows were quietly standing. Variation in IBI was correlated with variation in PST values. Because it has been suggested (Murata et al., 1992) that the coefficient of variation in the IBI is useful for objective assessment of the autonomic nervous system function, this parameter was computed for both the electro cardiographic IBI's (CV_{IBI}) and the PST values (CV_{PST}). For practical reasons, ratios have been multiplied by 100.

Reliability Test

Ten representative cows were selected from a group of 33 Holstein-Friesian dairy cows. The cows differed in age (2-9 yr) and number of days in lactation (22-243). The animals were used to going out to pasture after milking in the morning and to staying inside after milking in the evening. Each cow was tested as follows. Before going out to pasture, the cows were fed a restricted amount of maize silage. During feeding, all the cows were restrained at the feedgate, and one cow was selected and fitted with the Polar® girth belt as described in the previous section. As a reference, the cow's heart rate was recorded for 10 min while standing at the feeding rack with the herd. Then the exit door was opened, allowing all the cows, except the experimental animal, to go out to pasture. After the herd had left the cubicle house, the door was blocked with a wooden barrier and the isolated cow was released from the feedgate. During a 1-hr period, the cow's heart rate was recorded by the PST system at 5-sec intervals. Time spent walking and standing was recorded simultaneously using an Epson PX4 hand-held computer, supplied with the Observer® event-recording software program for observational research (Noldus et al., 1989).

RESULTS

Precision Test

A representation of the pulse-generator values and the PST values during the precision test is shown in Fig. 2.2. The graph shows a clear time lag between the generated pulse and the values produced by the PST monitor. PST values only adapt gradually to the generated values. The lag varies with the pulse rate. Decreasing the pulse rate within 1 sec from 60 bpm to 30 bpm takes the PST values about 55 sec to adapt, whereas less than 10 sec is needed to reach the same level of bpm when switching from 240 bpm to 210 bpm. From the difference in delay between PST values and generated pulses after

changing from 30 bpm to 60 bpm (25 sec) compared with the lag after switching from 60 bpm to 30 bpm (55 sec), it appears that the level that has been reached after switching primarily determines the magnitude of the delay.

The way PST values adapt to the sudden rise and fall in pulse rate support this. As shown at the curve end, increasing the pulse frequency from 30 bpm to 180 bpm induced very little delay. However, when the pulse rate was switched back to 30 bpm, the PST values took about 15 sec to adapt. Moreover, after this large step, the PST values remained unchanged for a while before adapting suddenly and completely, which is different from to the gradual adaptation found after changes of only 30 bpm.
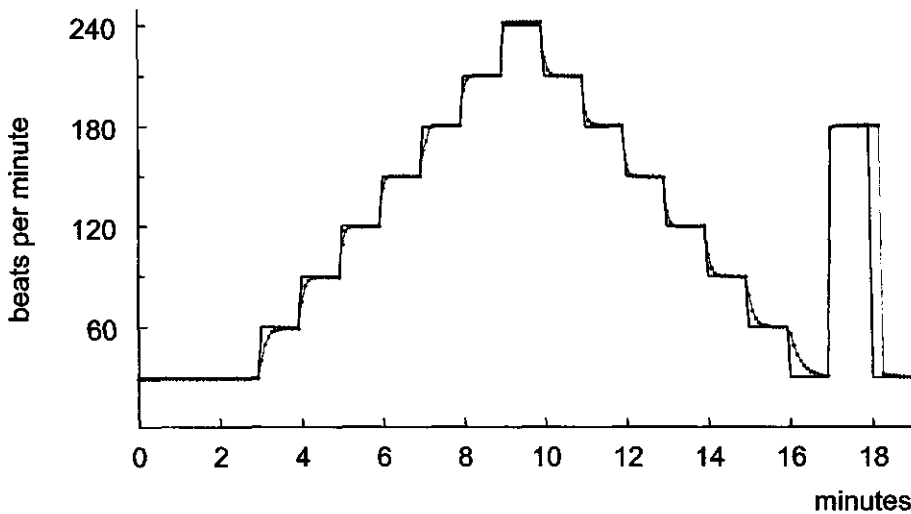
Figure 2.2. Generated pulse-rate (solid line) and PST values (dotted line) in beats per minute during stepwise changes in pulse rate of 1 min duration.

Apparently different algorithms are used to comply with various changes in heart rate.

Notwithstanding the variable time lag between the generated pulse rate and PST values, correlations between these two parameters (10 monitors; five trials) were high and ranged from 0.92 to 0.98. The analysis of variance revealed that monitor effects were highly significant and accounted for 74.6% of the total variation. On average, however, differences in correlations between monitors were small and did not exceed 5%. Trial effects were absent. Three PST monitors computed heart-rate values whose correlations were significantly lower than the grand mean (Table 2.1).

Table 2.1. Mean correlations (five trials per monitor) between the generated and the PST computed pulse rate recorded during 1-min stepwise changes in pulse rate (30-240 bpm) for each of the 10 monitors tested, derived by transforming Z back to original values.

Monitor										
1	2	3	4	5	6	7	8	9	10	Mean
0.957*	0.982	0.950*	0.940*	0.958	0.982	0.982	0.978	0.981	0.968	0.971

* Correlations which are significantly lower (P ≤ 0.05) than the mean.
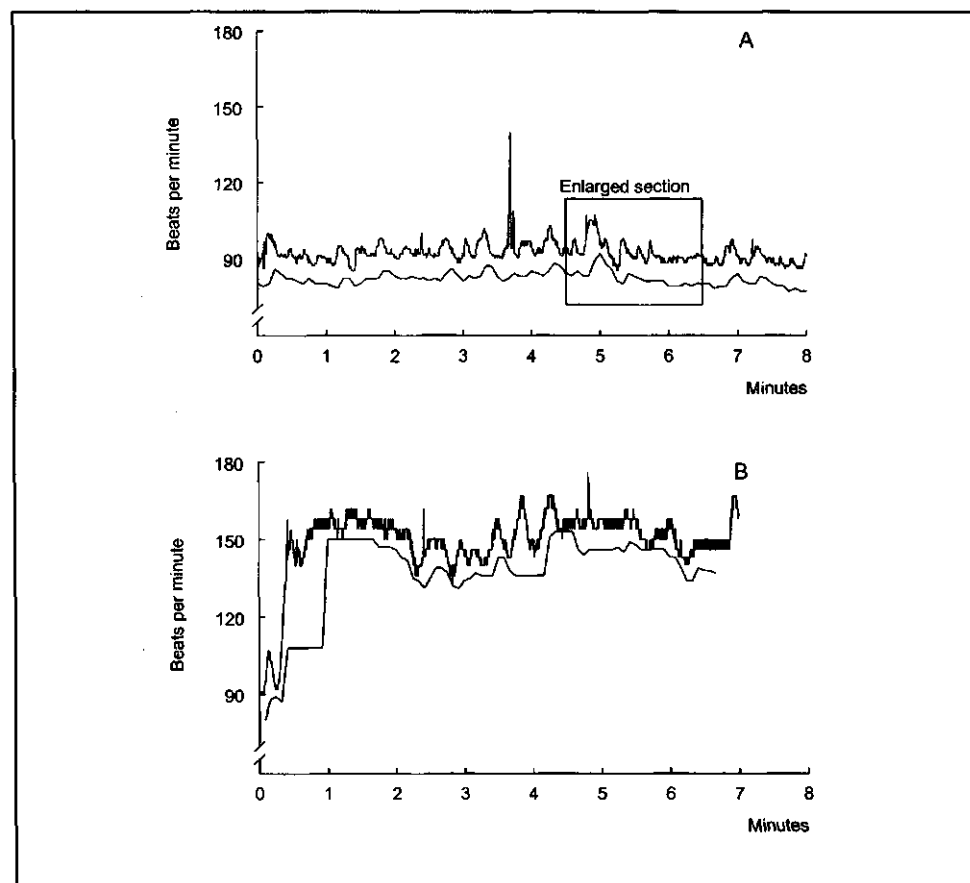
Figure 2.3. Example of the momentary heart rate (ECG values: upper line) and PST computed heart-rate (PST values: lower line), recorded during quietly standing (A) or exercising (B) on a treadmill. For clarity, PST values have been reduced by 10 bpm.

Accuracy Test

Before the onset of the exercise training, the cows were put in a quiet position on the stationary treadmill. During a 10-min period, heart rate (PST values) stabilized and

varied between cows from 65 to 97 bpm on average. Fig. 2.3A and 2.3B both show
typical examples of the simultaneously recorded momentary heart rate, derived from the
direct-wire ECG, and the PST values during standing and exercising, respectively. From
both graphs it is clear that there is, in general, some delay between the actual heart rate
and the PST computed heart rate. An enlarged section from Fig. 2.3A is shown in Fig.
2.4 and demonstrates the lag, which, at this level of heart rate, is between 5 and 10 sec.
At the start of the exercise, heart rate increased rapidly. During this rapid incline in heart
rate, PST values were computed with about 30 sec delay (Fig. 2.3B). It sometimes
happened that the system's figures remained at the same level for a while and then rose
abruptly by a large step. Sometimes sudden changes in heart rate, which lasted for some
seconds, generated flat lines. As PST values were averaged over a longer period of time,
momentary heart rate-curves were smoothed.
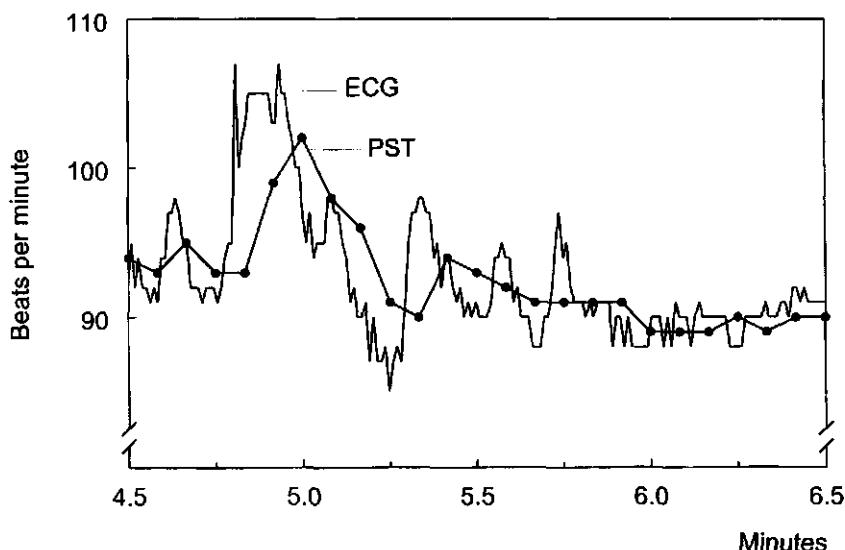
Figure 2.4. Enlarged section of Fig. 2.3A without the 10-bpm reduction of PST values. ECG values:
solid line; PST values: dotted line.

Correlations were calculated between PST and ECG heart rates, and the effects of
different methods for calculating heart rate from the ECG and the influence of different
shifts between ECG and PST values on these correlations are shown in Table 2.2. From
the analysis of variance it is obvious that correlations depend significantly on the
animal's individual characteristics. Cow effects accounted for one third of the total
variation in correlations. Additionally, each of the three main effects (Motion, Method
and Shift) contributed significantly to the correlations between ECG and PST values,
although these effects are much smaller than the effect caused by the individual animal.

The two factor interactions *Shift x Motion* and *Shift x Method* had a significant effect on the correlations. Method of computation was not related to standing or exercising.

Table 2.2. Source of variation, degrees of freedom (df), sum of squares (SS) and F probability (F prob.), resulting from the analysis of variance on Z-transformed correlations between ECG and PST values.

Source of variation	df	SS	F prob.
Cow (random effect)	9	2.515	≤ 0.001
Motion	1	0.422	≤ 0.001
Method	2	0.153	0.021
Shift	2	0.666	≤ 0.001
Motion x method	2	0.000	0.999
Motion x shift	2	0.426	≤ 0.001
Method x shift	4	0.201	0.039

Table 2.3 shows that, 0-, 5- or 10-sec shifts in computation, while the cows were stationary, the 5-sec shift improved the correlation significantly compared to the 0-sec shift. When the delay was increased to 10 sec, the correlation declined again. When the cows exercised on the treadmill no influence was found from the applied 5- and 10-sec shifts, probably because of a smaller delay during exercise when heart rate is higher.

Table 2.3. Correlations between ECG-derived heart rates and 0-, 5- and 10-sec shifted PST values averaged for 10 dairy cows; heart rate is analysed as a momentary heart-rate and as heart-rate means over, respectively, 5-sec intervals and the last four R-R cycles of each interval when cows were stationary or exercising on a treadmill.

	Shift (sec)		
	0	5	10
Method			
Momentary	0.61[ad]	0.79[bd]	0.67[ad]
5-s average	0.81[ae]	0.82[ad]	0.66[bd]
Four R-R average	0.72[ad]	0.83[bd]	0.67[ad]
Motion			
Stationary	0.78[ed]	0.88[bd]	0.65[cd]
Exercising	0.66[ae]	0.72[ae]	0.68[ad]

[a-c] Different superscripts within rows indicate significant contrasts (P ≤ 0.05; t-test).
[d, e] Different letters within columns indicate significant contrasts (P ≤ 0.05; t-test).

Average heart-beat values, without accounting for any delay and based on 5 sec of ECG recordings, correlated significantly better (r=0.81) with the PST computed heart rates than both the momentary heart-rate and the heart-rate mean over the last four R-R cycles (r = 0.61 and 0.72). When PST values were shifted by 5 or 10 sec, differences between methods disappeared. Compared with when the cows were stationary, exercise on the treadmill reduced the correlation significantly, except when the 10-sec shift was applied. When the results of individual cows were studied in more detail it appeared that cows with low correlations between ECG values and PST values frequently showed straight lines in their PST heart-rate curves during exercise.

Inter-beat interval variability. For 10 stationary cows, the mean IBI varied from 615 to 917 msec and PST values ranged consequently from 65.3 to 97.4 bpm (Table 2.4). IBI and PST standard deviations ranged from 15.0 to 70.0 msec and from 1.44 to 6.39 bpm, respectively, and were significantly correlated (r_s=0.76; P≤ 0.05). Neither parameter depended on the level of their related averages (P = 0.21 and 0.96 respectively). Three cows showed a relatively high variability in both IBI and PST values, which was even more clearly reflected in the coefficients of variation (r_s=0.89; P≤ 0.01). One of them (No. 3) combined high variability with a low heart rate. This particular cow had been raised at a children's zoo.

Table 2.4. PST values and inter-beat intervals [Mean ± SD (CV)] recorded from 10 quietly standing cows during 100 successive 5-sec intervals.

Cow No.	PST values (bpm)		Inter-beat intervals (msec)	
1	89.0 ± 6.39	(7.18)	677 ± 53.7	(7.93)
2	83.0 ± 5.52	(6.65)	728 ± 54.8	(7.53)
3	65.3 ± 4.32	(6.62)	917 ± 70.0	(7.63)
4	69.3 ± 2.25	(3.25)	864 ± 28.7	(3.32)
5	91.8 ± 2.80	(3.05)	653 ± 29.2	(4.47)
6	71.6 ± 1.95	(2.72)	836 ± 27.6	(3.30)
7	90.6 ± 2.42	(2.67)	659 ± 21.2	(3.22)
8	83.4 ± 2.19	(2.63)	719 ± 31.5	(4.38)
9	72.9 ± 1.44	(1.98)	821 ± 22.3	(2.72)
10	97.4 ± 1.59	(1.63)	615 ± 15.0	(2.44)

Reliability Test

During ten 1-hr isolation tests, heart rate only once showed a sudden fall, followed by some missing data. When the cows were standing at the feedgate among the other cows, their heart rates varied from 76 to 105 bpm on average. After isolation, heart rates rose immediately with an increase between 30 and 116 % of the average value before isolation. A typical example of a cows heart rate during the test is given in Fig. 2.5.

As expected, heart rate was affected by the activity of the cow. Average and maximum heart-beat values correlated significantly with the time spent walking (r_s = 0.72 and 0.73, respectively). Average heart rate during the period preceding isolation correlated with the average heart rate ($r_s = 0.66$), but not with the maximum heart rate during isolation ($r_s = 0.05$). In contrast to heart-rate curves monitored during exercising on the treadmill, flat lines appeared only for very short periods.

After release from the feedgate, only one cow started gambolling for a few seconds, apparently to take off the heart-rate belt. In general, the cows did not show obvious signs of being hampered by the equipment.
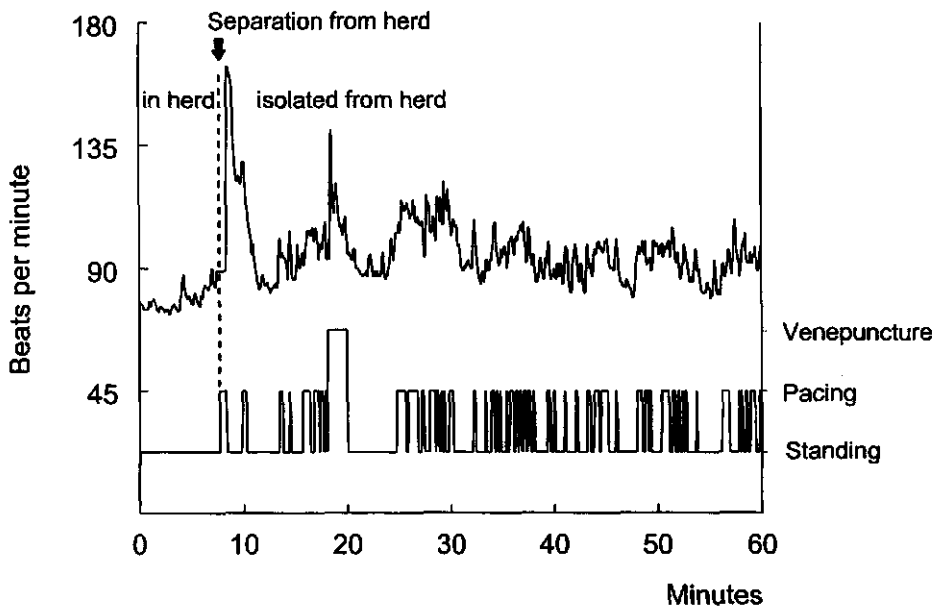

Figure 2.5. Typical example of heart-rate (upper line) and activity pattern (lower line) monitored during the isolation test.

DISCUSSION AND CONCLUSIONS

System Performance

The first part of the experiment showed that the values from the 10 tested monitors correlate significantly with the generated signals. Correlations ranged from 0.92 to 0.98. Sloet van Oldruitenborgh-Oosterbaan et al. (1988) found slightly higher correlations when they tested the equine equivalent of the PST using a pulse generator. Except for the heart-rate range that was tested (35-240 bpm), they did not report any details of the test.

When the generated values were changed stepwise, the PST values adapted gradually, which is presumably due to the PST algorithm for processing heart-beat signals. Unfortunately, but for obvious reasons, the manufacturer does not supply detailed information about the PST pulse-measuring algorithm. The 3-4 sec averaging time, indicated by Karvonen et al. (1984) at heart rates of 150 bpm, is slightly shorter than the 10 sec we found after switching to a pulse rate of 150 bpm.

Differences between various monitors were significant but small. Because monitors were not moved between trials, monitor effects could not be separated from site effects. As there is indeed evidence that the location of the monitor may influence its recording ability (two particular sites could not be used because the monitors did not function on these spots), placing the monitor at a proper angle to the transmitter is important. During our field experiments, we attached the wrist monitor at right angles to the transmitter and buckled it onto the girth belt; it appeared to function properly. The same holds for transferring the monitors' data to the computer. Using the interface supplied by the manufacturer did not cause any problems in all three studies.

Cow heart rates during rest ranged from 65 to 97 bpm and were similar to the results found by others (Lefcourt et al., 1986; Kondo and Hurnik, 1988; Kovalčik et al., 1988; Lay et al., 1992b; Royle et al., 1992; Rulquin and Caudal, 1992). With a 5-sec shift and regardless of the method of computation of ECG values, correlations between ECG values and PST values were on average 0.88 when the cows were standing still on the treadmill. This correlation is not as high as that found by Treiber et al. (1989) in children during rest. The difference may originate from the method used to compute heart-rate values. Treiber et al. (1989) calculated total ECG heart-rates for 1-min periods and correlated these values with the mean of 12 successive PST readings, recorded at 5-sec intervals. In the present study, 85 PST values and momentary ECG values based on much smaller (beat to beat) intervals were correlated. Correlations may be lower because of a higher variation in short-term heart-rate values when not smoothed by averaging over longer periods. From our study, it is clear that even averaging heart rate over short periods (5 sec) results in higher correlations between PST and ECG heart rates compared with direct correlations between PST and momentary heart rates.

Motion, *ie* exercising on a treadmill, affects the correlation between ECG and PST values in a negative way. Correlation decreased from 0.88 to 0.72 on average but showed profound individual differences, determined as highly significant cow effects. Surprisingly, correlations found by Sloet van Oldruitenborgh-Oosterbaan et al. (1988) and Evans and Rose (1986) with similar equipment in exercising horses were close to 1.0. These authors, however, recorded PST values at 15-sec intervals during the last 45 sec of each exercise stage. After averaging, these values were correlated with ECG values recorded in the same period. Clearly, in the studies with horses, the precision of the system in recording a steady-state heart-rate was tested rather than the accuracy to record a variable momentary heart-rate.

Cows with low correlations between ECG and PST values during exercise frequently showed straight lines in their PST heart-rate curves. This may have been caused by movements of the electrodes on the skin, by artefacts caused by muscle contraction, or by a highly variable heart-rate of the animal, factors that can all be related to the individual cow. However, the ECG heart-rate variability in phases where the PST generated straight lines compared with the heart-rate variability in periods in which the PST produced reliable curves did not differ significantly, and thus there is no evidence that these straight lines are caused by the variability of the heart-rate itself. Therefore, artefacts are likely to be the cause. Because of its error-detection algorithm (Seaward et al., 1990), the PST rejects artefact signals which induce changes of more then 40 bpm (P. van der Weijden, Support®, Almere, The Netherlands, pers. commun., 1992). In the meantime the last correct value seems to be maintained until the system again receives reasonable pulses. In the present study, this has been clearly demonstrated by switching the pulse generator suddenly from 180 bpm to 30 bpm (Fig. 2.2). From the intensive body movements of the exercising cow and based upon the results of extensive heart-rate measurements with cows and calves, we postulate that irregularities are caused by movements of the electrodes on the skin rather than by muscular activity. Our recent efforts to improve the recordings with cows and calves by using flexible and rubber-coated PST electrodes, designed for human application, have been very successful.

Analysis of the methods for calculating ECG reference values showed that the PST values most accurately reflect the average value of the previous 5-sec interval (r = 0.81). Shifting the ECG and PST values 5 sec with respect to one another, compared with the 0-sec shift, did not improve the 5-sec average significantly. In contrast, the 5-sec shift did give a better representation of the actual (momentary and four R-R average) heart rate. Compared with the 5-sec shift, the 10-sec shift reduced correlations significantly, no matter which calculation method was used. To summarize, the PST values in general adjust to the ECG values within 5 sec. These results agree with the

outcome of the first part of our experiment and are supported by those of Karvonen et al. (1984).

The variability in heart-rate during standing, represented by the CV in IBI, is properly reflected by the CV of the PST values. Evidently, the averaging and error-detection algorithm of the PST equipment does not restrict its potential for recording heart-rate variability during standing. However, caution should be exercised when changes in heart-rate cross the error detection threshold from one recording interval to another. These cases can be detected from the curves by sudden alterations after a period of ostensibly steady heart rate.

Two of the advantages of non-invasive heart rate monitoring systems adapted for external mounting on cattle are that extended preparations can be avoided and the system can easily be moved from one cow to another. Using a girth belt to hold the electrodes in place may involve problems with the electrodes slipping out from under the girth or other causes of failure to obtain adequate records. In the third part of our experiment, heart rate was recorded continuously for periods of more than 1 hr. The problems described above, which were reported by Hemsworth et al. (1989) and Sloet van Oldruitenborgh-Oosterbaan et al. (1988), were absent in our experiments. No obvious malfunctioning was seen, nor were there any serious signs of the system hampering or exciting the cows. Only once did a cow start gambolling to get rid of the girth belt. We assume that cows need only a limited time to adapt to the system, which can easily be achieved by applying a sham girth belt beforehand.

Measuring a Stress Response

The cardiac response is considered to be an objective way of displaying the autonomic nervous system's response to psychological stressors. Increased and decreased heart rates are associated with a dominating influence of the sympathetic and the parasympathetic nervous system, respectively. As environmental challenge activates both branches of the autonomous nervous system, change in heart rate has been viewed as an index of the psychological impact of an external event (Herd 1991). This dual innervation also causes heart-rate in mammals to vary continuously, as suggested by Goldberger (1991) and Porges (1992). Both authors suggested that this variability serves as an important mechanism for adaptability. Thus, variability in heart rate may indicate both the individual's stress response and the vulnerability to stress. From the present study it appeared that both the heart-rate and its variability were significantly reflected by the PST values, and we therefore conclude that the PST is suitable for measuring heart rate and heart-rate variability in dairy cows.

Effects of repeated jugular puncture
on plasma cortisol concentrations
in loose-housed dairy cows

Hans Hopster, Joop T.N. van der Werf, Jo H.F. Erkens and Harry J. Blokhuis

Institute for Animal Science and Health (ID-DLO)
P.O. Box 65, 8200 AB Lelystad, The Netherlands

ABSTRACT

The following effects of venepuncture on plasma cortisol concentrations were investigated. In three experiments with in total 30 dairy cows: 1) effects of taking a single blood sample; 2) effects of repeated sampling once daily; 3) effects of frequent sampling within an hour, carried out during repeated sessions on alternating days.

In cows accustomed to handling, jugular puncture once daily during 6-12 successive days did not affect cortisol concentrations in single samples. Likewise, when 15 cows were sampled in a row once a day, in groups of five, starting 15 min apart, the order of sampling within and between groups did not influence plasma cortisol concentrations. Repeated sampling within a day yielded contradictory results. In cows accustomed to handling, taking a single blood sample by venepuncture did not have any effect on plasma cortisol 18 min later. In contrast, heifers which were less used to handling showed an average increase in cortisol concentrations when 5 samples were collected by venepuncture 15 min apart. During serial sampling on three alternate days, no signs of habituation could be detected at the group level. Striking differences appeared between individuals in the maximum effect of repeated venepuncture on cortisol concentrations, in the time at which the effect reached its maximum and in the consistency of the response pattern over successive series.

The results of this study show that in cows that were accustomed to handling and to being restrained in a self-tethering feed gate, baseline cortisol concentrations can be measured in single blood samples that are collected by jugular puncture within one minute after first approaching the cow. When successive blood samples need to be collected within 15-20 min, however, jugular puncture may induce an increase in cortisol concentration which seems to depend on the handling experience of the animals and on individual differences. Evaluation of the adrenocortical activity of loose-housed dairy cows using low dosages of adrenocorticotrophic hormone in cows that are not accustomed to handling therefore seems less suitable for studying structural changes in the steroidogenic capacity of bovine adrenocortical cells.

INTRODUCTION

The hypothalamus-pituitary-adrenocortical (HPA) axis is a vital neuroendocrine regulatory system for adaptation of animals to environmental changes. Disturbing factors in the environment affect the activity of the HPA axis, either temporally or permanently. Although temporal activation of the HPA axis may reflect a sound adaptive response with minor consequences for the animal's HPA axis capacity, effects of chronic environmental stress may involve a structural change in the steroidogenic capacity of the adrenocortical cells, indicating that the welfare of the animal is at risk (Broom, 1988).

Stimulation of the adrenal cortex by peripheral administration of ACTH and measuring cortisol concentrations is commonly used to study the reactivity of the adrenal cortex in companion (Feldman et al., 1982; Peterson and Kemppainen, 1993) and farm animals (Beuving and Vonder, 1986; Borell and Ladewig, 1989; Linden et al., 1990; Munksgaard and Løwendahl, 1993; Bowers et al., 1993).

In dairy cows, this method has been used to study chronic effects of housing (Redbo, 1993; Munksgaard and Løwendahl, 1993), temperature (El Nouty et al., 1978) and social stressors (Munksgaard and Løwendahl, 1993). Furthermore, acute effects of management practices such as social isolation (Hopster and Blokhuis, 1993; Munksgaard and Simonsen, 1996), transport (Warriss et al., 1995) or veterinary procedures (Alam and Dobson, 1986) have been investigated using plasma cortisol concentration as a response variable.

One of the difficulties in determining baseline cortisol concentrations and in studying adrenocortical response to physiological or psychological stimuli is that the HPA axis is extremely sensitive to disturbing factors in the environment. When these are not controlled for in the experiment, environmental disturbance may confound accurate measurement of adrenocortical effects. Since low dosages of ACTH are preferred for testing adrenocortical sensitivity (Dickstein et al., 1991; Crowley et al., 1991), environmental factors will easily disturb the test because the response to ACTH will be within the same range as the animal's adrenocortical response to everyday environmental stressors. This means, for example, that stress caused by catching and restraining before sampling, together with venepuncturing itself might induce endogenous release of ACTH and thus confound the results of a stimulation test.

In experimental circumstances, researchers try to avoid this difficulty by taking blood samples through a permanent cannula, generally fitted into the jugular vein about 24 hr prior to sampling. A problem, however, arises when the use of a cannula is not possible or allowed, for instance on commercial dairy farms. Another problem with cannulation is that, while in commercial loose housing systems, the constant activity and close cow to cow contact makes it unlikely that cows retain indwelling jugular cannula for a long time. Finally, the use of a cannula is less convenient when only a limited number of samples is needed from many cows. In all of these cases, venepuncture is an alternative, provided that the stress of handling and venepuncturing can be quantified or avoided.

Effects of single and repeated venepuncture on peripheral cortisol concentrations in dairy cows were reported by Alam and Dobson (1986). Their cows were housed in tied standings. However, no information is available on the effects of venepuncture on plasma cortisol concentrations in loose-housed dairy cows. In the present study the following effects of venepuncture were distinguished: 1) effects of the taking of a single sample; 2) effects of repeated sampling once daily; 3) effects of frequent sampling within

an hour during repeated sampling sessions of 1 hr duration, carried out on alternating days. Here, we also address the question whether the procedure of repeated jugular puncture is a suitable alternative for cannulation in the study of adrenocortical function in dairy cows.

ANIMALS, MATERIALS AND METHODS

Animal management
Holstein Friesian and Holstein x Dutch Friesian crossbred dairy cows were used. In three experiments cows were housed at the Institute's experimental farm in cubicle stalls. They were milked twice daily and fed roughage and concentrates according to normal farming practice. A restricted amount of maize silage was given once daily to entice the cows to come to the feed gate. In experiments 1 and 2, maize silage was given after cows were milked in the evening. In experiment 3, cows received maize silage at 10.30 hr. A self-tethering feed gate caused the cows to tether themselves during maize feeding. The cows were restrained in the feed gate for approximately 1 hr every day over at least two weeks in order to get them accustomed to this routine. All experiments described in this study were approved by the ID-DLO Institutional Animal Care and Use Committee.

Blood sampling
At least 30 min elapsed between milking and any sampling. Cows were equipped with a halter at least one day before any sampling, in order to habituate them to wearing one and to facilitate handling during the test. Blood sampling took place when cows were restrained in the feeding rack and all cows stayed there until blood was drawn from the last experimental cow. There were always two persons involved in taking jugular blood samples. One person attached a rope to the halter and securely pulled the cow's head to the side to stretch her neck. The second person obstructed jugular blood flow by applying some pressure with the thumb in order to engorge the vein before puncturing it with a sterile needle (21G x 1.5 inch, Venoject®, Terumo Europe N.V., Belgium). Blood was collected in 10 ml vacuum tubes (Vacuette®, Greiner B.V., The Netherlands) and stored on ice. Heparin (Exp. I and II) or ethylenediaminetetraacetic acid (EDTA, Exp. III) was used as an anticoagulant. After collection, samples were centrifuged (10 min, 3000 rpm) and aliquots of plasma stored at -20°C awaiting cortisol assay.

In experiments I and II, effects of repeated sampling once daily were studied. In experiments I and III, effects of frequent jugular puncture on the concentration of cortisol in successive samples was studied with intervals of 15-18 min.

Experiment I

While all the cows were at the feedgate, single blood samples were collected from two cows (A and B) on six successive days for baseline cortisol concentrations (Table 3.1). From each cow, a second blood sample was collected 18 min after the first on every second day. According to this schedule, 20 cows (parity: 1-5; days in lactation: 43-303) were sampled in total during 10 successive weeks. Sampling time was recorded for each blood sample as the time between attaching the rope to the halter and the completion of filling the vacuum tube. Sampling took place between 16.00 and 17.00 hr.

Table 3.1. Time at which blood samples were collected from cows (A,B) on successive days. Experiment I.

Sampling time	Days					
	1	2	3	4	5	6
t0	A, B	A, B	A, B	A, B	A, B	A, B
t18	A	B	A	B	A	B

Experiment II

Fifteen out of the 20 cows from experiment I were used in experiment II, about eight months later (parity: 2-6; days in lactation: 6-257). Cows were randomly distributed over three groups (A,B,C) of five animals (Table 3.2). Single blood samples were collected from all individual cows within groups, starting at 17.00, 17.15 or 17.30 hr respectively. Groups were randomly assigned to a 3 x 3 Latin square design with 4 replications. Thus blood sampling was performed during 12 successive days (Table 3.2). Within groups, individuals were randomly allocated to the order of sampling (1-5).

Table 3.2. Period in which blood samples were collected from cows in different groups (A,B,C; n=5) on successive days. Experiment II.

Sampling time	Days					
	1,7	2,8	3,9	4,10	5,11	6,12
17.00 - 17.15	A	B	C	B	C	A
17.15 - 17.30	B	C	A	A	B	C
17.30 - 17.45	C	A	B	C	A	B

Experiment III

Ten primiparous dairy cows (days in lactation: 128-154) were sampled after one another five times between 11.00 and 12.00 hr with 15 min intervals on three alternate days (Table 3.3). Sampling time was recorded as described in experiment I.

Table 3.3. Time at which bloodsamples were collected from cows on different days. Experiment III.

	Days
Sampling time	1, 3, 5
t0	All cows
t15	All cows
t30	All cows
t45	All cows
t60	All cows

Cortisol determination

Cortisol was measured using a time resolved fluoro immunoassay which has been developed for measurement of cortisol concentrations in unextracted bovine heparin or EDTA plasma (Erkens et al., 1998). Samples from individual cows were run in duplicate. Recoveries of cortisol amounts, ranging from 0.1-1.6 ng, added to plasma were $102.9 \pm 11.4\%$. The detection limit for a 20 μl sample was 0.5 ng.ml^{-1}. The intra-assay coefficients of variation for control samples with concentrations of 71.1, 39.2 and 10.3 ng.ml^{-1} were 8.2, 7.9 and 11.3% (n = 16) respectively. The corresponding inter-assay coefficients of variation were 5.5, 9.1 and 11.5% (n = 19).

Statistics

In accordance with the lower detection limit of the assay, cortisol concentrations below this limit were fixed at 0.5 ng.ml^{-1}. Pairwise comparisons were based on the Student's t test. A one-sample t-test was used for analysing differences in cortisol concentrations between successive blood samples. Fixed effects for day of sampling (Exp. I-III), sampling time and sampling order (Exp. II), and components of variance for random cow effects were estimated by REML (eg Engel, 1990; Searle et al., 1992, Ch. 6). Overall tests for main effects and interactions were derived from the Wald test (Buist and Engel, 1992).

Repeatabilities were estimated from the components of variance ($\rho = \sigma^2_{cow} / (\sigma^2_{cow} + \sigma^2_{rest})$). Standard errors are given between brackets; $\pm$ precedes standard deviances. All calculations were performed with the statistical programming language Genstat 5 (Genstat 5, 1993).

RESULTS

Experiment I

Sampling time was 65 ± 7.4 sec for the first and 61 ± 6.0 sec for the second sample. The interval between the two samples was 18.2 ± 0.07 min. In first samples (n = 20), average cortisol concentration was 2.6 (0.24) ng.ml^{-1}. On days 1 to 6, these concentrations were 2.8 (0.45), 2.2 (0.44), 2.41 (0.44), 2.58 (0.44), 2.73 (0.46) and 2.76 (0.49) ng.ml^{-1} respectively. No significant differences between sampling days were detected. The repeatability for cortisol concentrations was 0.09 ± 0.08.

Table 3.4. Cortisol concentrations (ng.ml^{-1}) in dairy cows (mean and standard error) in the first and second (n=10) blood samples, collected 18 min apart by jugular puncture on six successive days.

Blood sample	t0			t18		
Day	Mean	±	SEM	Mean	±	SEM
1	3.81	±	0.56	1.91	±	0.20**
2	2.07	±	0.38	1.84	±	0.53
3	3.25	±	0.94	2.19	±	0.42
4	3.16	±	0.71	2.61	±	0.72
5	2.54	±	0.31	1.43	±	0.15*
6	3.46	±	1.20	2.48	±	0.55

Asterisks indicate significant (* $P \le 0.05$; ** $P \le 0.01$) differences in average cortisol concentrations between first and second samples.

Table 3.4 shows the effect of taking the first jugular blood sample on cortisol concentrations 18 min later. No increase in plasma cortisol concentration was detected on any day. On the contrary, cortisol concentrations in the second samples tended to be lower than in the first samples. On days 1 (P $\le$ 0.01) and 5 (P $\le$ 0.05) this decrease was significant.

Experiment II

The average cortisol concentration was 3.2 ± 0.38 ng.ml^{-1} and cortisol concentrations varied little between days (Table 3.5). The order in which cows were sampled both within and between groups did not affect plasma cortisol concentrations. Plasma cortisol concentrations in cows that were sampled as number 1 within a group (3.2 ± 0.62 ng.ml^{-1}) did not differ significantly from those that were sampled as number 5 (2.3 ± 0.60 ng.ml^{-1}). In addition, plasma cortisol concentrations in the group that was sampled first (3.0 ± 0.52 ng.ml^{-1}) did not differ from those in the group that was sampled second (3.0 ± 0.50 ng.ml^{-1}) or third (3.6 ± 0.52 ng.ml^{-1}). Similar to what was found in experiment I, the repeatability (0.13 ± 0.07) of cortisol concentrations was low.

Table 3.5. Cortisol concentrations (ng.ml^{-1}) in dairy cows (mean, standard error; n = 15) in blood collected by jugular puncture on twelve successive days.

Day	1	2	3	4	5	6	7	8	9	10	11	12
Mean	3.1	4.4	2.3	3.6	3.7	3.2	3.9	3.2	4.1	1.3	2.1	3.4
SEM	0.86	0.84	0.90	0.86	0.90	0.84	0.93	0.93	0.86	0.90	0.90	0.93

Experiment III

On average it took 35 sec $\pm$ 6.4 to securely tie the head of the cow and fill the vacuum tube with blood. In Fig. 3.2 (Mean), average cortisol concentrations are presented for the first, the second and the third serial blood sampling. No significant differences in average cortisol concentrations were found between series on any of the sampling times. In 93.3% of all t0 samples, cortisol concentrations were below 4.0 ng.ml^{-1}. Average cortisol concentration at t0 was 2.2 (0.21) ng.ml^{-1}. At t15, t30, t45 and t60 average cortisol concentrations were 3.6 (0.51), 4.7 (0.96), 5.9 (0.89) and 6.1 (0.66) ng.ml^{-1} respectively. As compared to the average cortisol concentration at t0 the increase was significant (P $\leq$ 0.002) at all times. Significant increases, found at t30, t45 and t60 as compared to t15 (P $\leq$ 0.05), indicate that after an initial increase in cortisol at t15, concentrations of cortisol further increased during the next 15 minutes. After 30 minutes, no further increase in cortisol concentrations was detected. Cortisol concentrations in blood sampled at t45 and t60 were not different from those at t30. Likewise, concentrations at t45 and t60 did not differ significantly.

Repeatabilities of the cortisol concentrations were 0.07 ($\pm$ 0.20), 0.03 ($\pm$ 0.21), 0.07 ($\pm$ 0.21), 0.15 ($\pm$ 0.21) and 0.29 ($\pm$ 0.23) at t0, t15, t30, t45 and t60 respectively.

Striking differences appeared between individual animals in the maximum effect of repeated venepuncture on cortisol concentrations, with regard to the moment at which the effect reached its maximum and to the consistency of the response pattern over successive series. In Fig. 3.1 individual response patterns are ordered from animal A to J by the increasing area under the curve, averaged over successive serial samplings. Cortisol concentrations in cows A to D were stable during the first 30 min with only little variation between successive sessions, but increased for the first time at 45 min after the first venepuncture. Although repeated venepuncture induced an increase in cortisol, concentrations in these cows remained below 10 ng.ml^{-1} In many cases in cows E to H, plasma cortisol had already increased in the second blood sample and showed more variation within and between successive sessions. In these cows, maximum concentrations were only incidentally beyond 10 ng.ml^{-1}.
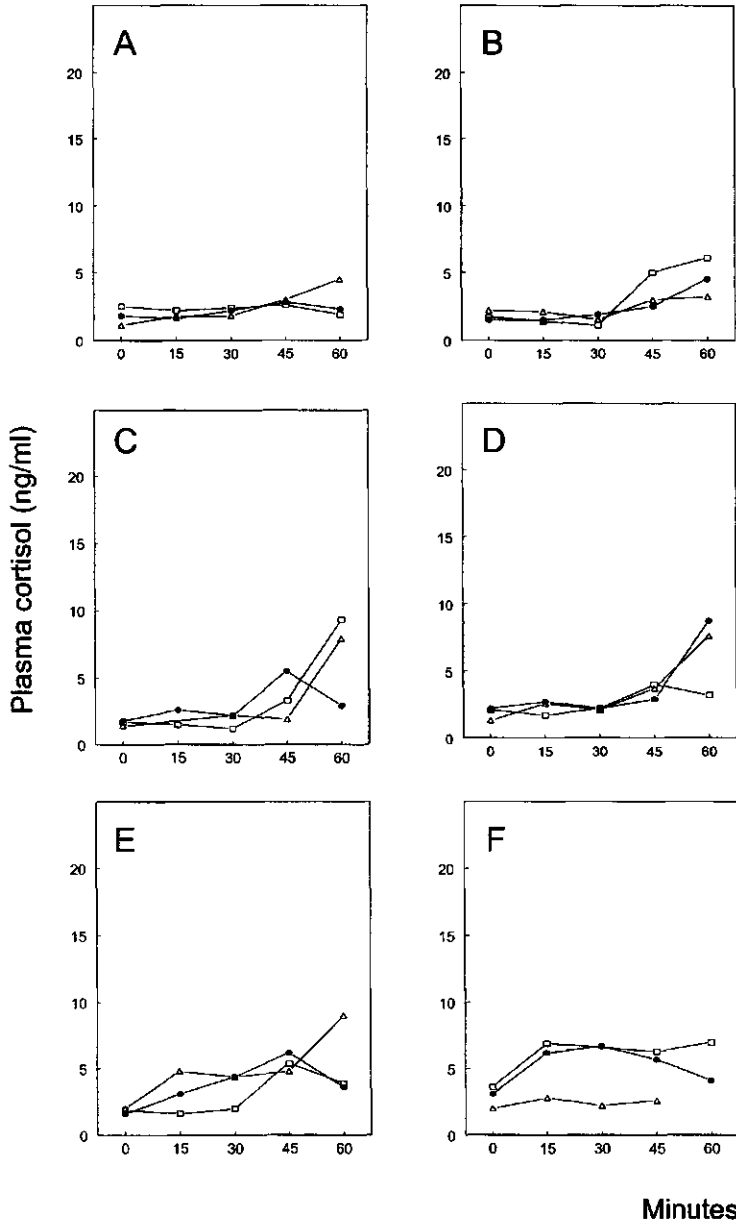
Figure 3.1, Part I. Concentrations of cortisol (ng.ml^{-1}) in serial blood samples, collected from dairy cows (A-F) at three alternate days by repeated jugular venepuncture with intervals of 15 min. In all graphs, different markers indicate the first (triangles), second (squares) and third (closed circles) serial sampling session.

Figure 3.1, Part II. Concentrations of cortisol (ng.ml⁻¹) in serial blood samples, collected from dairy cows (G-J) at three alternate days by repeated jugular venepuncture with intervals of 15 min. Average concentrations (ng.ml⁻¹) and standard errors are given in the graph 'MEAN'. In all graphs, different markers indicate the first (triangles), second (squares) and third (closed circles) serial sampling session.
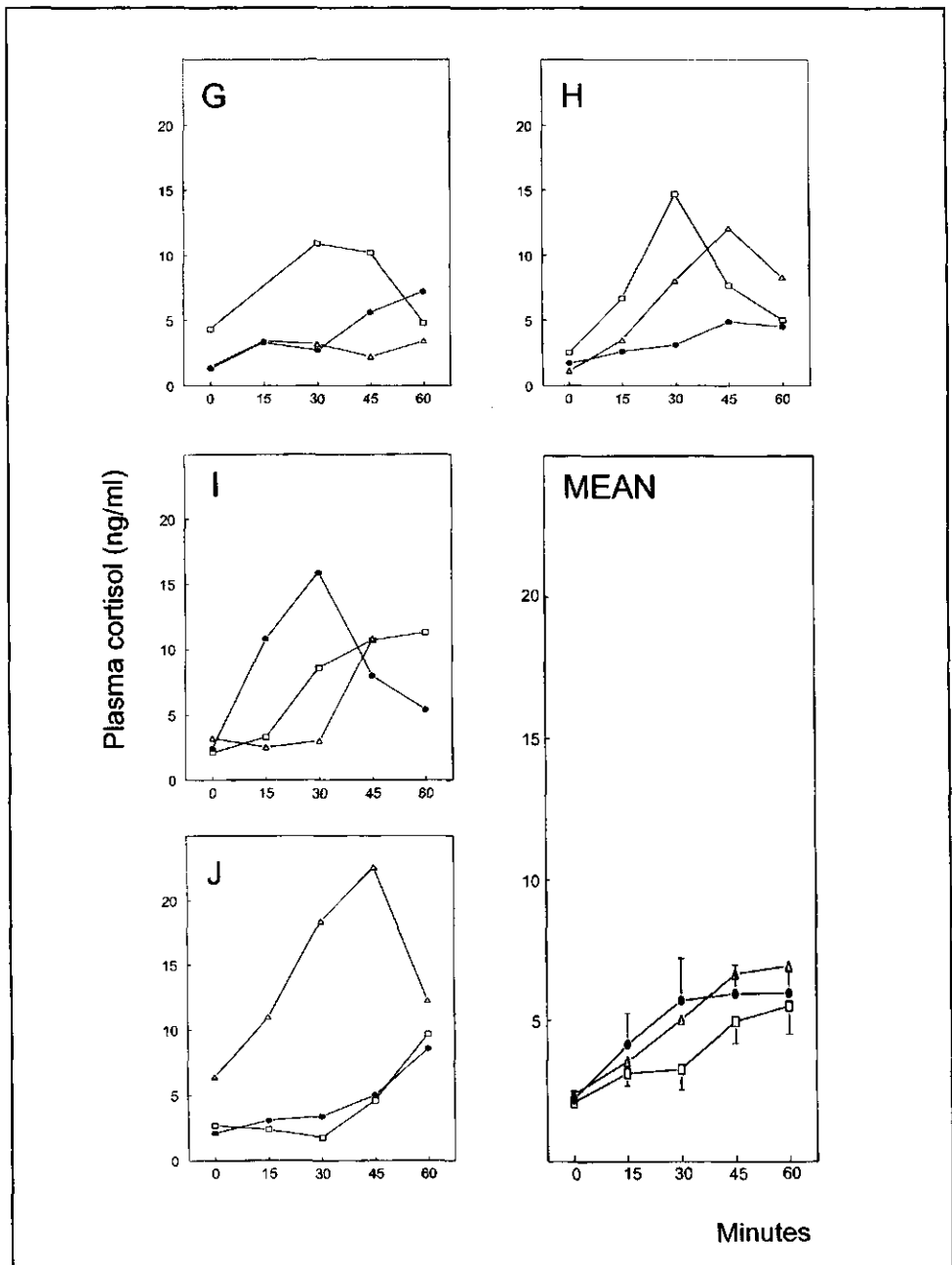
In cows I and J cortisol patterns show that concentrations incidentally increase beyond 20 ng.ml^{-1}.

Behavioural observations during repeated jugular puncture in experiment III were limited to the subjective scaling of the animals' fearfulness by the experimenters. Cow A was the most docile one and cow E showed the most marked fear response during venepuncture.
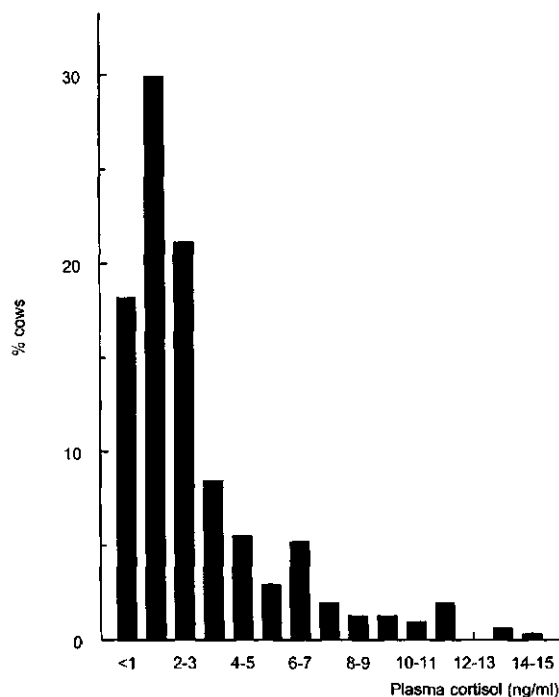
Figure 3.2. Distribution of cortisol concentrations (ng.ml^{-1}) in first blood samples of any dairy cow on any experimental day, collected by single jugular puncture, as a percentage of the total number of samples (n=307)

Baseline cortisol

Data for concentration of cortisol, determined in all first blood samples of any cow on any day of the three experiments were pooled in order to establish a frequency distribution of baseline plasma cortisol concentrations in dairy cows sampled by jugular venepuncture. In total, the results of 307 samples were used from 30 different animals. The average cortisol concentration was 2.89 ± 2.67 ng.ml^{-1} (median 2.0). Fig. 3.2 illustrates that 69.4% of the samples had cortisol concentrations below 3 ng.ml^{-1} and 13.7% of the samples contained 6 ng.ml^{-1} cortisol or more.

DISCUSSION

Effects of the taking of a single sample

The average concentrations of cortisol in the first samples (2.89 ng.ml^{-1}) are in line with values found by others in undisturbed dairy cows. For example, frequent blood sampling through a cannula (15 min interval during 48 hr) in cows that were housed in a rigidly controlled environment revealed that, on average, these samples contained between 2 and 6 ng.ml^{-1} cortisol (Lefcourt et al., 1993). In addition, serial blood sampling in cannulated dairy cows (30 min intervals, from 08.00 to 15.30 hr) resulted in cortisol concentrations between 0.5 and 4.0 ng.ml^{-1} (Munksgaard and Simonsen, 1996). In addition to the average concentration of cortisol, which is in agreement with the results of these previous studies, the variation in individual values is also within the same range. In the present study, cortisol concentrations ranged between 0.5-15.0 ng.ml^{-1} whereas Lefcourt et al. (1993) found concentrations between 1 and 17 ng.ml^{-1} . As both Lefcourt et al. (1993) and Munksgaard and Simonsen (1996) made it plausible that cortisol concentrations in their studies reflected baseline concentrations and because we found similar concentrations in the first samples, we safely consider these concentrations of cortisol as baseline concentrations. The relative high values which we incidentally found, may have been due to 1) circadian and ultradian rhythms that are incorporated in the study; 2) the pulsatile release of cortisol that may have resulted in temporal peaks with amplitudes up to 17 ng.ml^{-1} (Lefcourt et al., 1993) and; 3) psychological factors that may have disturbed a cow in the period prior to the taking of the first blood sample. Low repeatabilities in cortisol concentrations in first samples show that in our study individual dairy cows did not differ consistently in baseline plasma cortisol concentrations.

Effects of repeated sampling once daily

Our results show that in dairy cows, the daily taking of a single blood sample by jugular venepuncture is a suitable method for collecting blood if baseline cortisol concentrations need to be determined. No effects on cortisol concentrations were found when cows were punctured once daily on a number of successive days. On the one hand, this could be expected because it has been demonstrated that about 16 min elapses after a single venepuncture before peripheral cortisol starts to increase (Alam and Dobson, 1986). We collected blood within 60 sec of first approaching the cow, thus being ahead of a possible rise in cortisol concentration. On the other hand, repeated sampling once daily did not evoke anticipatory stress when being blood sampled in a row. This indicates that cows perceive jugular puncture as not very stressful. As 69.4% of the samples had cortisol concentrations of less than 3 ng.ml^{-1}, it is evident that we successfully

avoided pre-test arousal by letting the cows tether themselves in the feed gate for feeding.

In summary, the procedure of sampling cows in a row at the feed gate did not result in differences between baseline plasma cortisol concentrations, caused by the order of sampling.

Effects of frequent sampling

With regard to the effects of taking blood samples by jugular puncture and the effect on the concentration of cortisol in successive samples, we found contradictory results. Although taking two blood samples 18 min apart (Exp. I) did not yield any evidence that the procedure itself affected cortisol concentrations, more frequent sampling 15 min apart (Exp. III) caused average cortisol concentrations to increase significantly. Furthermore, a 3 times repetition of the serial sampling did not reveal any indications that cows on average (Fig. 3.2 Mean) habituated to the procedure of repeated venepuncture.

Contradictory results are also found in the literature. Alam and Dobson (1986) reported a maximum increase in cortisol concentrations in dairy cows of 8.6 ± 4.6 ng.ml^{-1}, 16.6 ± 7.6 min after a single venepuncture. Likewise, venepuncture increased cortisol concentrations in men (Ginsberg et al., 1988; Meeran et al., 1993). In dogs, however, repeated venepuncture did not result in increased concentrations of cortisol (Knol et al., 1992). Nor did caudal venepuncture affect cortisol concentrations in heifers within 30 min after venepuncture (Veissier and Le Neindre, 1988).

Confounding effects of handling

We suggest that contradictions in the literature as well as in the current study could be associated with differences in the extent that animals are accustomed to handling. 'Handling effects' are reported in different species. Heerden en Bertschinger (1982), for example, reported that taming jackals clearly reduced their adrenocortical response to venepuncture. Furthermore, it has been proven in sheep (Bassett and Hinks, 1969) and in silver fox vixens (Moe and Bakken, 1996) that accustomization to repeated venepuncture significantly reduces the effect of blood sampling on cortisol concentrations

In the present study, the cows in Exp. I were previously involved in another test and had been intensively handled accordingly (Hopster et al., 1998a). Heifers in Exp. III, in contrast, had no such handling experiences. We speculate therefore, that the differences in effects of jugular venepuncture on plasma cortisol concentrations found between Exp. I and III, are associated with differences in the extent that the animals were accustomed to handling.

Individual differences

As individual cows in Exp. III differed in genetic background and in previous experience and were not accustomed to intensive handling, differences in personality traits (perception, character, HPA-axis reactivity) may have affected their adrenocortical response to repeated venepuncture (Fig. 3.1 A-J). An intriguing question is whether intensive handling could transform H- or I-patterns into A-patterns and which handling-program is required for that. These are relevant questions for further studies into the existence of interindividual differences in stress responsiveness in dairy cows. This is particularly relevant because this type of research implicitly requires that individual animals are (repeatedly) exposed to various experimental situations which also means that the animals will be handled many times to various degrees.

Whether animals should be trained or not to repeated jugular venepuncture depends in the first place on the experimental question. Especially when effects of human interaction are studied, for example, it is inappropriate to train the animal beforehand. When handling effects must be prevented from confounding treatment effects and handling can not be controlled for in the experiment however, it is necessary to accustom the animals to handling. Apart from the experimental question itself, it is important to consider the relative contribution of the venepuncture-induced increase in cortisol as compared to the increase resulting from the exposure to the experimental stressor. Independent of the question involved, it is important to stress Reinhardt's (1991) assertion that 'the description of the experimental animal's handling prior to and during venepuncture is a methodological issue which needs to be specified in order to account for a possibly data-biasing variable'.

In conclusion, the results of this study show that in loose-housed dairy cows that were accustomed to handling and to being restrained in a self-tethering feed gate, baseline cortisol concentrations can be measured in single blood samples that are collected by jugular puncture within one minute of first approaching the cow. When successive blood samples need to be collected within 15-20 min however, jugular puncture may induce an increase in cortisol concentration which seems to depend on the handling experience of the animals and on individual differences. This handling induced rise in cortisol concentration, relative to the treatment effect, will determine whether repeated jugular puncture is a suitable method for collecting serial blood samples in loose-housed dairy cows. Evaluation of the adrenocortical activity of loose-housed dairy cows using low dosages of ACTH in cows that are not accustomed to handling seems therefore a less suitable method for studying structural changes in the steroidogenic capacity of the bovine adrenocortical cells.

Acute effects of cow-calf separation on heart rate, plasma cortisol and behaviour in multiparous dairy cows

Hans Hopster[a], Janet O'Connell[b] and Harry J. Blokhuis[a]

[a] Institute for Animal Science and Health (ID-DLO),
Research Branch Zeist,
P.O. Box 501, 3700 AM Zeist, The Netherlands

[b] Teagasc, Moorepark Research Centre, Fermoy,
Co. Cork, Ireland

ABSTRACT

Removing the calf after bonding may induce acute stress in the dairy cow. We examined the responses of dairy cows immediately after the removal of their calves. Eight dairy cows were successively separated from their calves on the third day post-partum and heart rate and behaviour of the cows were recorded. In addition, the cows were blood sampled before and after the separation for cortisol analysis. After separation, cows vocalised initially and moved to the feeding rack but started feeding shortly after that. Heart rate effects were restricted to the first minutes after separation and cortisol effects were not found. We conclude that multiparous dairy cows respond only mildly immediately after their calves have been removed. Feasible explanations for the observed mild response are discussed.

INTRODUCTION

Unconfined semi-wild cows (Camargue and Bos indicus) near calving look for a hidden place to give birth and stay apart from the herd for several days, according to Schloeth (1961) and Reinhardt (1980). During this time, mother-infant ties are established, ensuring the calf of food, care and protection.

Under farm conditions, remaining with the dam for at least 24 hr post-partum facilitates the calf to stand up, suck and eliminate (Edwards and Broom, 1982; Le Neindre, 1984; Metz and Metz, 1984, 1986; Metz, 1987) and facilitates earlier and larger intakes of colostrum which in turn leads to higher immunoglobulin concentrations in the calf (Selman et al., 1971; Petrie, 1984). Stott et al. (1979) suggested that the latter may be explained not only because mothered calves drink more, but also because they absorb the immunoglobulins in the colostrum better. Thus, keeping cow and calf together for some time may benefit the calves' health.

In most studies of maternal behaviour, cows were separated from their calves at different times post-partum and for various periods (Kurosaki et al., 1983; Kent, 1984; Kent and Kelly, 1987; Hudson and Mullord, 1977). Few refer, however, to how dairy cows respond immediately after the removal of their calves and thus we do not know whether this induces stress in the dairy cow.

The present experiment is part of a larger project which aims at elucidating the existence of stable individual characteristics mediating general stress responsiveness in dairy cows. The cow-calf separation might be used as one of the paradigms for studying individual differences in response of dairy cows to acute stress. To investigate its suitability for inducing acute stress, we studied behavioural and physiological responses of dairy cows after they were separated from their calves 48-72 hrs after birth.

ANIMALS, MATERIALS AND METHODS

Animals and housing

At least 1 week before calving, each of eight Holstein-Friesian cows (parity 2-5) was moved to an individual, straw bedded calving pen adjacent to the group of dry cows they originated from. Individual calving pens had solid walls and, except when standing at the feeding rack in the front of the pen, cows were visually isolated from other cows. After parturition (videorecorded) all cows behaved maternally while staying with their calves continuously for 2-3 days.

Cows were fed hay twice a day (07.30 and 16.00 hr) and received concentrates according to their requirements. During the nursing period, calves could suckle their mother except when cows, while remaining in the pen, were machine milked twice daily.

At 09.00 hr on the third day post-partum, calves were removed from their mothers and transported to a calfshed at a distance of about 100 m. Thus, the attachment period varied between 2 and 3 days. After the experiment, cows returned to the dairy herd before the next milking.

Behavioural observations

From 15 min before to 15 min after the separation, the behaviour (standing, feeding, vocalising) and location of the cow (feeding rack, remaining space) were recorded continuously with a hand-held computer, supplied with the Observer® event-recording software program for observational research (Albonetti et al., 1992). During the observations, the experimenter stood in front of the pen across the feeding alley.

Heart rate recordings

Heart rate was recorded at 5 sec intervals in beats per minute (bpm) using a non-invasive heart rate monitor, validated for use in dairy cows (Hopster and Blokhuis, 1994b). Occasionally and for short periods, this heart rate monitor generated flat lines (Fig. 4.1, Cow 493) or zero values (Fig. 4.1, Cow 493 and 522) owing to problems with the system described elsewhere (Hopster and Blokhuis, 1994b). Heart rate values in these periods were considered as missing values. To avoid confounding effects of venepuncture on behaviour or heart rate, the analyses were restricted to 10 min before and after calf removal.

Blood collection

Blood was collected by jugular puncture at 15 min before and after the separation (Veissier and Le Neindre, 1988; Hopster and Blokhuis, 1994a). Blood was saved in heparinised tubes (Venoject®, Terumo Europe N.V., Belgium) at room temperature

according to Reimers *et al.* (1983). Samples were then centrifuged and aliquots of plasma were stored at -20°C awaiting cortisol assay. For each blood sample, bleeding time was recorded as the time between entering the pen and the removal of the needle after blood collection.

In our first test, we collected an extra blood sample 2 min before the separation (Fig. 4.1, Cow 521). Because of its interference with heart rate around the removal of the calf, however, sampling at -2 min was omitted with the other cows. For Cow 521, heart rate values during sampling at -2 min are considered as missing values.

Statistics
We divided the total observation time into two pre-separation (P1, P2) and two post-separation (P3, P4) periods of 5 min each (Fig. 4.1). Differences between periods in behaviour and average heart rate were tested with the Wilcoxon's matched-pairs signed-rank test and the paired *t*-test, respectively.

RESULTS

Individual heart rates, behaviour recordings during the test as well as plasma cortisol values 15 min before and after the removal of the calf are illustrated in Fig. 4.1. On average, it took 78 ($\pm$ 49.2) sec and 62 ($\pm$ 33.7) sec to catch and bleed the animals before and after the separation respectively. During venepuncture, indicated by the vertical lines in the graphs, heart rate in all cows increased rapidly for a short period. Average heart rate during blood collection before and after the separation was 93.3 ($\pm$ 5.61) bpm and 96.1 ($\pm$ 6.98) bpm respectively which was significantly (P $\leq$ 0.05) higher than in periods P1 and P4. When the calf was removed, heart rate increased rapidly in all cows and then quickly returned to previous levels. Feeding (solid horizontal bars) was connected with higher heart rates in some cows and drinking was associated with increased heart rate in Cow 468, between 6 and 3 min before removing the calf.

Average heart rates within 5-min and 1-min periods before and after the separation differed significantly between various periods (Fig. 4.2). When calf and cow were still together (open bars), heart rate was 81.1 bpm, 81.2 bpm and 83.2 bpm during the first 5 min, the second 4 min and the 1 min period before the removal of the calf respectively. Although mean heart rate during the minute before separation tended to increase, this increase was only 2 bpm and probably reflected the cows' reaction to the experimenter entering the pen. Immediately after the removal of the calf (shaded bars), average heart rate accelerated significantly (P $\leq$ 0.05) compared with the mean heart rate during the previous 10 min.
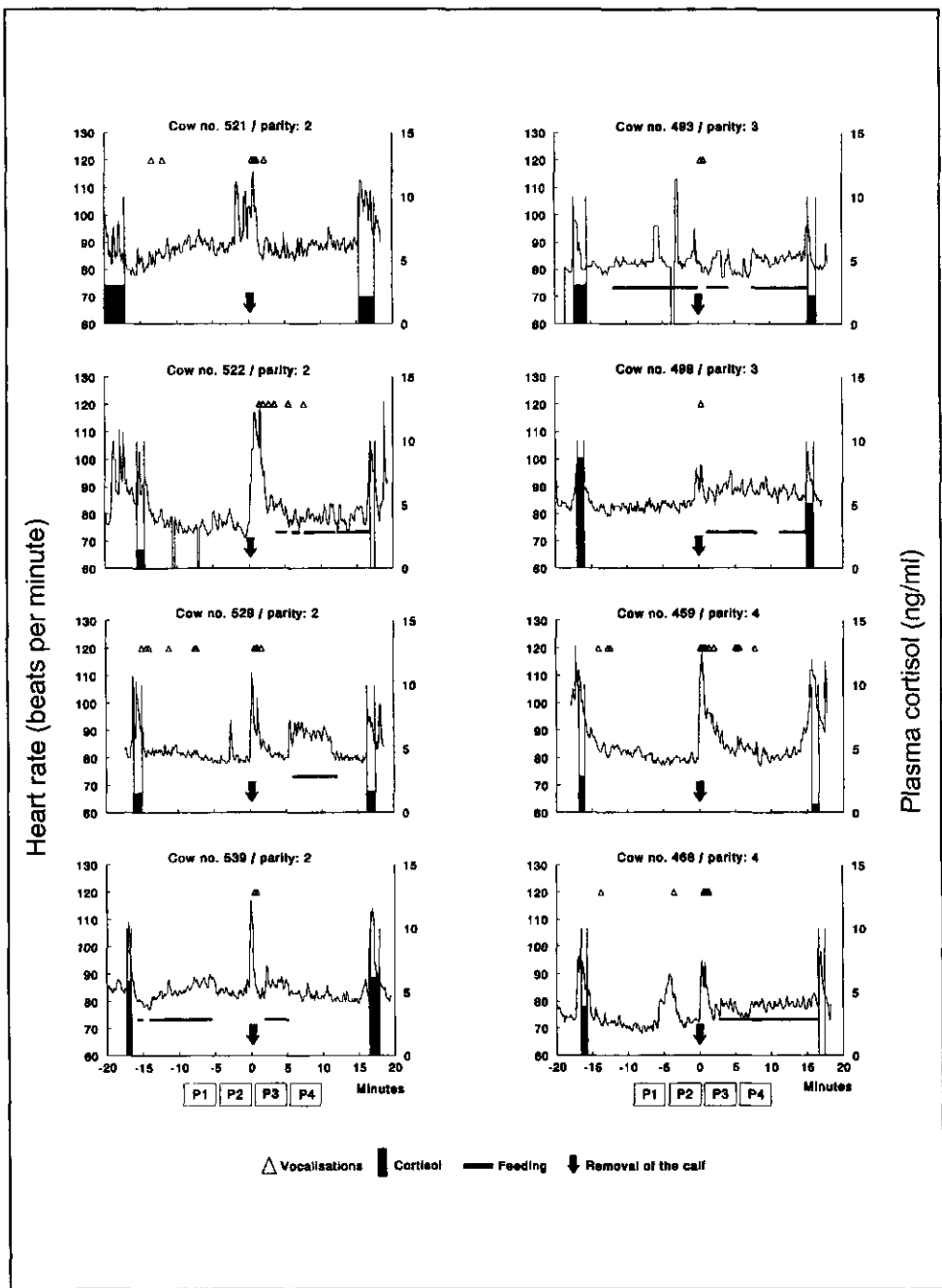
Figure 4.1. Heart rate (left Y-axis), plasma cortisol (right Y-axis), vocalizations and feeding time for each of the eight multiparous cows during observation. Vertical lines indicate the start and end of the blood sampling. The total test is divided into two pre-separation (P1, P2) and two post-separation (P3, P4) periods.
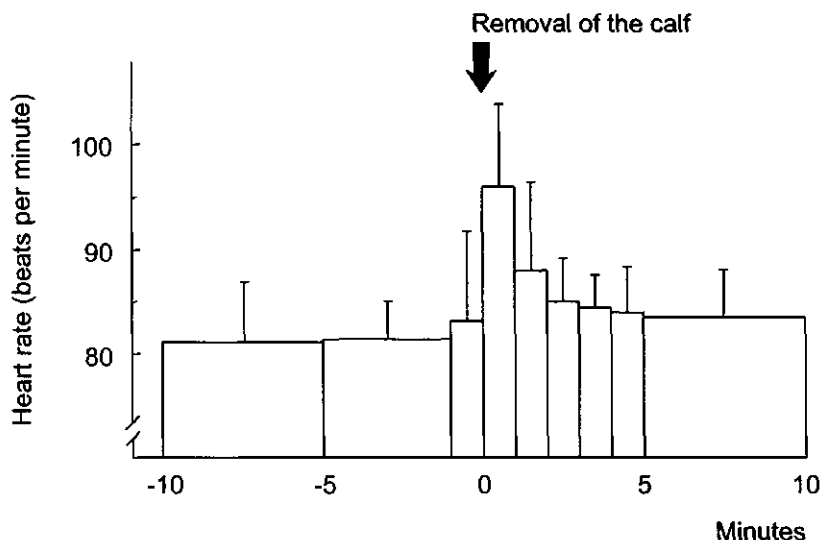
Removal of the calf

Figure 4.2. Average heart rate and standard deviation in beats per minute during different periods before (open bars) and after (shaded bars) the removal of the calf.

Heart rate averaged 96.0 bpm in the first minute but slowed down to 88.0 bpm in the second minute after separation. During the next three 1-min and last 5-min periods, no significant ($P > 0.05$) differences were found between periods and heart rate was equal to the mean heart rate before separation. During observations, only two cows spent time at the feedgate when the calf was present. When the calf was taken away, both cows stayed at the feeding rack while four others moved there. Cows tended to spend more time at the feedgate in the periods after removal (P3, 190 ± 95.2 sec; P4, 190 ± 124.9 sec) than in the two periods before the removal of the calf (P1, 75 ± 138.9 sec; P2, 97 ± 139.0 sec). When we restricted the analysis to cows which were not at the feeding rack before separation, these six cows spent on average significantly more time ($P \leq 0.05$) there in periods P3 (116 ± 119.8 sec) and P4 (154 ± 138.7 sec). During the first period after separation, these cows were feeding 47.8% of their time at the feedgate feeding, which increased up to 68.5% in the second 5-min period after separation.

During the first 5 min after separation all cows vocalised on average 4.8 ($\pm$ 3.3) times, which is significantly more ($P \leq 0.05$) than the infrequent vocalisations in all three other periods. Cows vocalised at a low amplitude and with their mouths closed.

On average, no significant effect of the separation on cortisol in plasma could be demonstrated. Plasma cortisol averaged 3.8 ± 2.4 ng.ml^{-1} before and 2.2 ± 2.3 ng.ml^{-1} after separation. Individual differences were small and values did not exceed basal cortisol levels.

DISCUSSION AND CONCLUSIONS

The acute response of multiparous dairy cows to the removal of their calves is moderate and short-lived. After separation all cows vocalised and half of them moved to the feeding rack. This may be a sign that cows responded maternally and looked for their calf since the feedgate was the only place where cows, from the inner side of the calving pen, could observe the outer world. However, this interest was quickly replaced by feeding and at the second minute after separation, the heart rate had already decreased.

Previous studies of maternal behaviour yield contradictory results. For instance, Houwing et al. (1990) found no signs of restlessness but an increase in maintenance behaviour when dairy cows were separated from their calves at about 10 hr post-partum. Furthermore, Kurosaki and co-workers (1983) separated cows and calves within 2 hr post-partum, observed their behaviour during the next 60 hr and compared this with the behaviour of non-isolated cows in that period. Isolated cows showed longer lying times and showed no young-seeking behaviour, suggesting that isolated cows were not severely stressed.

However, the findings of Hudson and Mullord (1977) indicate distinct maternal responses after cows were separated from their calves, after 5 min of contact by shifting the cows to an adjoining paddock. The separation lasted 1-24 hr and cows were not milked. These cows remained at the shed-end of the paddock in the neighbourhood of their calf and vocalised continually and were restless.

An explanation for these contradictory results may be found in the studies of Poindron and Le Neindre (1980) with sheep, who postulate that initially, maternal interest in the neonate is hormone facilitated. A few hours post-partum a neurosensory control develops as a result of sensory information provided by the young, which gradually replaces the hormonal regulation of maternal behaviour.

From that perspective, cows separated from their calves shortly after calving may become restless and start vocalising (Hudson and Mullord, 1977) because their hormones urge them to do so. In addition, when cows and calves are separated, for example at 3-4 weeks post-partum, as was reported by Hill and Godke (1987), the sensory information provided by the calf (bellowing and fence walking) may have caused the cows to become excited and nervous. Thus, we hypothesize that with the removal of the calf, the accompanying sensory information also faded and stress was reduced.

This may explain why cows, after separation, initially went to the feedgate and vocalised silently. According to Kiley (1972) these "mm" calls can be interpreted as a short-distance (less than 10 m) recognition signal, particularly between the cow and her calf. Therefore, the increased "mooing" observed during the first 5 min after removal of the calf should help the calf find its mother, rather then being a sign of distress. Finally,

when the calf disappeared and was out of sight, the recognition call lost its value and cows started feeding.

The small behavioural changes found indicate that the removal itself is not perceived as a potent stressor for the cow. This is supported by the physiological data. Effects on heart rate were small and were restricted to the first minute after the removal of the calf. Although the heart rate effect in this period is obvious, it is unclear whether the removal of the calf is the major factor being responsible for this. After all, the experimenter entering the pen and approaching cow and calf interferes with the separation. Because the heart rate had already increased during the minute before the separation when the experimenter walked towards the pen, there is evidence that this factor is important. The average heart rate of the cows after this initial increase was still a little higher than before the separation. This may result from the increased feeding activity following separation, as shown by Price et al. (1993) in farmed red deer.

Separation had no effect on concentration of plasma cortisol and supports the findings of Hudson et al. (1975) and Cockram et al. (1993) who studied calf and lamb removal respectively. Cortisol values were within the normal basal spectrum, recorded by Lefcourt et al. (1993) in cannulated lactating Holstein cows.

We conclude that the cow-calf separation after 2-3 days of maternal attachment of itself does not evoke strong behavioural and physiological responses in the multiparous dairy cow. However, these findings must be used with caution since the number of animals used in the study as well as the observation time were restricted. Nevertheless, the cow-calf separation paradigm seems to be less suitable for studying individual differences in response to environmental stressors in dairy cows.

Side preference of dairy cows in the milking parlour and its effects on behaviour and heart rate during milking

Hans Hopster, Joop T.N. van der Werf and Harry J. Blokhuis

Institute for Animal Science and Health (ID-DLO)
P.O. Box 65, 8200 AB Lelystad, The Netherlands

ABSTRACT

Side preference of dairy cows in a two-sided milking parlour was studied under practical
and experimental conditions. Historical data, originating from the computerized identifi-
cation of cows over a period of 28 months, revealed that 23 out of 89 cows showed
significant (P ≤ 0.05) left-side preference in more than 75% of their lactation months.
Twelve of these cows showed this side preference in all months. For the right side of the
parlour, these numbers were 20 and 11 respectively. Two groups of eight cows, show-
ing either significant or no side preference, were selected. These cows were individually
tested for parlour side preference during 18 successive milkings. Test outcomes con-
firmed historical results for all but one cow. By milking the eight individual cows show-
ing side preference alternately on both their habitual side (H-cows) and their nonhabit-
ual side (N-cows), effects of side preference on behaviour, heart rate and milk produc-
tion were examined in six successive afternoon milking sessions. The order of testing as
well as the side of the parlour on which each animal was milked was alternated on a
daily basis. Eight cows not showing side preference acted as controls (C-cows). N-cows
used significantly more (P ≤ 0.05) time to enter the milking parlour (86 sec) than H-cows
(20 sec) and C-cows (31 sec). When feeding during milking, N-cows paused signifi-
cantly more (P ≤ 0.05) often (0.89 times per min) in contrast to H-cows (0.49) and C-
cows (0.51). During the first minute of milking, the increase in heart rate as well as heart
rate variability was significantly higher (P ≤ 0.05) in N-cows (21.8% and 3.2 respec-
tively) than in H-cows (16.7% and 2.0). No differences in milk production between
treatment groups could be detected. From this study, it can be concluded that:
1)individual cows differ consistently in side preference in the milking parlour; 2) a
substantial proportion of the cows showed consistent side preference in spite of changes
in their social environment or in other environmental factors; 3) side preference of dairy
cows in the milking parlour seems to be a consistent behavioural routine with only
unimportant implications for the welfare of cows if it were to be interrupted.

INTRODUCTION

In two-sided herringbone and tandem milking parlours, dairy cows are exposed to dual-
choice situations twice daily. In general during milking, two doors or two gates allow
entry to either the right or the left side of the parlour. Field observations, in which the
parlour side preference of dairy cows in herringbone (Gadbury, 1975) and parallel
(Tanner et al., 1994) parlours was recorded, provide strong evidence that differences
exist between individual dairy cows in side preference and in their consistency of
choice. A consistent side preference for one side of the milking parlour could suggest
that parlour side is a highly significant factor for a dairy cow. If so, environmental factors

which prevent the cow from choosing her preferred side may bring about stress responses during milking.

Studies in heifers (Grandin et al., 1994) and calves (Arave et al., 1992) indicated that cattle are reluctant to change their initial choice. Therefore, consistent side preference in the milking parlour could indicate a habit formed after an initial, possibly random, choice. In that case, the consequences of not being able to be milked on the preferred side of the milking parlour may have less significance for the cow and the word 'preference' has possibly only little significance.

Another intriguing aspect of side preference in the milking parlour is that only a certain proportion of the cows in a herd seem to routinely visit one side of the milking parlour, whereas other animals visit both sides of the milking parlour at random (Tanner et al., 1994). From studies in rats and mice, it is known that differences in routine formation between individual animals originate from fundamental differences in coping behaviour (Benus et al., 1987, 1988, 1990). If differences between dairy cows in side preference reflect similar differences in coping behaviour, side preference may be a significant individual characteristic of dairy cows, implying physiological (Korte et al., 1996b) and neuroendocrinological interindividual differences (Koolhaas et al., 1983).

The limited number of observations in the study of Gadbury (1975) and the results in the abstract of Tanner et al. (1994), however, do not provide sufficient insight into the changes in individual parlour side preferences over time, nor do these studies yield experimental data, suitable for examining the relevance of individuality in preference for milking parlour side. Therefore, we conducted a study with two major aims. First, to investigate the differences in side preference between cows and their consistency of choice, and second, to determine whether to be milked on a particular parlour side has any significance for the behaviour and physiology of the cow during milking and for milk production.

ANIMALS, MATERIALS AND METHODS

Analysis of historical data

Firstly, cows showing either high or no consistency in side preference in the milking parlour were selected in the following manner. Historical data were used, originating from the computerized identification of cows in the milking parlour during a period of 28 successive months. Data comprised milking stall and cow number combinations from 70,081 individual milkings, collected at the experimental farm of ID-DLO. In total, 89 Holstein Friesian and Holstein x Dutch Friesian crossbred dairy cows were involved. Cows were milked twice daily in a double-three open-tandem parlour. Both sides of the parlour were identical. In the parlour, cows stood nose to tail in individual stalls with

entry and exit through side passages. In each stall, the identity of the cow was automatically recorded and concentrates were administered during milking according to milk production level. Individual milk production was recorded automatically with a milk meter (Fullflow, Fullwood, Ellesmere, U.K.). In general, cows went to pasture between the morning and evening milkings during the summer period (May-October) but were kept inside after milking in the evening. During the winter season, cows were permanently kept indoors in a cubicle house with two rows of cubicles, separated by a slatted floor. Cows had free access to the feed gate.

The composition of the herd varied over time due to normal culling and replacement activities. Consequently, individual cows contributed different numbers of milkings to the overall data set. The lactation period for each cow consisted of periods of 30 successive days (months) starting from calving, and thus each of these months comprised a possible total of 60 milkings. No distinction was made between morning and evening milkings. The number of individual milkings per month might be less than 60, for instance if cows were dried off, if data were lost due to technical failure or if months were cut short by the end of the data collection. Months in which less than an arbitrarily chosen number of 20 milkings remained, (757 milkings in 72 months), were excluded from further analysis. This resulted in an average number of 55.2 recorded milkings per month.

Side preference (SP) and consistency score (CS, measuring consistency of SP) were calculated for each month. For explanation about the methods and statistics used in calculating SP and CS, please refer to Appendix A. Individual cows were classified according to their average CS. Finally, eight cows that showed significant and consistent side preference, and eight cows that did not were selected (see Appendix A) for use in both the free and the enforced parlour side choice tests. Parities of these cows ranged from one to five. The experiments described in these sections were approved by the ID-DLO Institutional Animal Care and Use Committee.

Free parlour side choice

The collection of the historical data as described above had taken place under farm conditions in which social and other factors connected to the cows being milked in a group, might have influenced the cows' selection of parlour side. Following this period of historical data collection, a test was carried out to examine whether social factors had been affecting cows' choices.

In the test, a waiting room was separated from the collecting yard by a fence and a selection gate (see Fig. 5.1). The waiting room was in front of the milking parlour. The left and the right side of the parlour were identical and cows had access to each side of the milking parlour through remotely controlled pneumatically operated swing doors. During milking, when there was an empty stall on both sides of the milking parlour, the
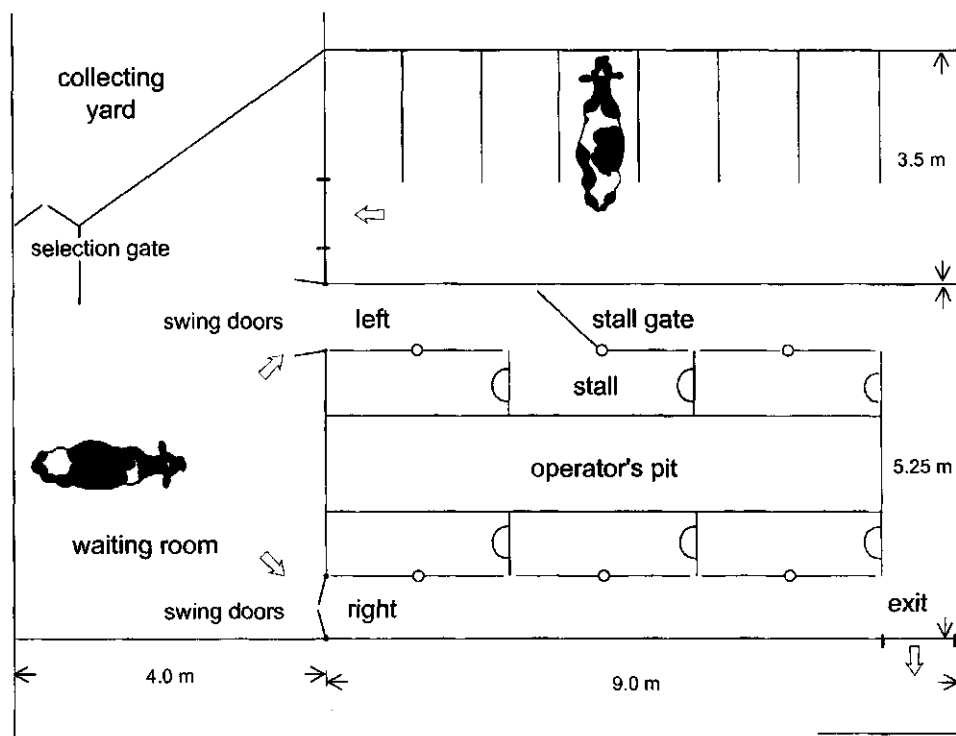
Figure 5.1. Lay out of the experimental facilities.

left and the right swing doors at the entrance of the milking parlour were opened simultaneously. Then, also the first selection gate was opened, allowing one cow to enter either side of the milking parlour voluntarily and without disturbance. This procedure was repeated for each of the 16 individual cows during 18 successive milkings, except for two cows which were dry at that time.

Enforced parlour side choice
The significance of side preference in the milking parlour was studied by milking the 16 selected cows alternately on the left and right side of the milking parlour, according to the following procedure. Cows from both groups (ie showing side preference and not showing side preference) were paired according to stage of lactation. Each pair was tested during six afternoon milkings within one week. Consequently, the entire experiment took eight weeks. On the first day of every week, a cow showing side preference

and a cow not showing side preference was milked on the left and right side of the milking parlour respectively. During the following five successive testing days, the order of testing as well as the side of the parlour on which each animal was milked was alternated on a daily basis. As a result, cows showing side preference were milked three times on both their habitual and nonhabitual side and cows not showing side preference were milked three times on both the left and the right side of the milking parlour.

The testing procedure consisted of the following steps and observations. Two days before the first day of testing, the two experimental cows were equipped with a halter in order to habituate them to wearing a halter and to facilitate handling during the test. On testing days, when the herd was collected from the pasture for milking, the two experimental cows were separated from the herd at the gateway of the pasture and taken to a cubicle barn next to the milking parlour. Each cow was loosely tied up in a cubicle by a rope connected to her halter. The animals were then equipped with a heart rate monitor which recorded the average heart beat at 5-sec intervals (Hopster and Blokhuis, 1994b). For five minutes, starting seven minutes before the first experimental cow entered the waiting room, the heart rate of the two cows was recorded as a reference. In the meantime, milking of the nonexperimental cows had started. After at least two cows had been milked at the middle position on the left side of the milking parlour, this stall was reserved for milking the first experimental cow. This cow was then released and led into the waiting room. After one minute, the left swing doors were opened, giving the cow access to the left side of the milking parlour. If she did not enter the parlour within the next minute, the experimenter gently encouraged her to go in. As soon as the cow had passed the swing doors, these doors were closed automatically and the stall entrance-gate was closed after she had entered the milking stall. After being automatically identified, she received 1 kg of concentrates, her udder was cleaned, the milking cluster was attached and milking began. Milk production was automatically recorded by a milk meter (Fullflow, Fullwood, Ellesmere, U.K.). As soon as the milk flow decreased below 0.2 $kg.min^{-1}$ the cluster was automatically removed, the stall exit-gate was opened and the cow left the stall and returned to the cubicle house. Here, the heart rate belt was removed. This procedure was then repeated for the second experimental cow on the other side of the milking parlour.

During the test, behavioural events as well as times that marked specific phases in the milking procedure (Table 5.1) were recorded with a hand-held computer, installed with The Observer® event-recording software program for observational research (Albonetti et al., 1992). During milking, the observer stood in the rear of the operator's pit.

Table 5.1. Definition of relevant events and periods during which behaviour and heart rate were recorded.

Events / Periods	Definition
Assistance	Gentle encouragement by the experimenter for the cow to enter the parlour.
Step	Lifting one of the hind legs.
Feeding break	Moving the head out of the concentrates trough..
Reference	Five-minute period, starting 7 min before the first experimental cow was moved to the waiting room.
Entry	Time between entry into the waiting room and closure of the swing doors of the parlour.
Latency to enter the milking parlour	Interval between opening and closing the swing doors at one of the parlour sides.
Latency to enter the milking stall	Interval between opening the swing doors and closing the entrance-gate.
Milking	Period between distribution of concentrates and removal of the milking cluster.
First minute of milking	First minute after distribution of concentrates.
Last minute of milking	Last minute before removal of the milking cluster.

Statistical analysis

Treatments were defined as cows showing side preference, milked at their habitual side (H), cows showing side preference, milked at their nonhabitual side (N) and cows not showing side preference milked at both the right and the left side of the parlour, acting as a control group (C).

A mixed analysis of variance (Searle et al., 1992), using repeated measures was employed. Fixed effects for treatments (H, N, C) and parlour sides (L, R) and components of variance for random cow effects were estimated by REML (eg Engel, 1990; Searle et al., 1992, Ch. 6). Overall tests for main effects and interactions were derived from the Wald test (Buist and Engel, 1992). Pairwise comparisons were based on Student's t test. As a measure of adaptation, heart rate variability was calculated as the ratio between heart rate standard deviation and average heart rate (Hopster and Blokhuis, 1994b). For practical reasons, this ratio was multiplied by 100. Calculations were performed with the statistical programming language Genstat 5 (Genstat 5 Committee, 1993).

RESULTS

Analysis of historical data

A total of 1328 months were extracted from the data set and of these, 1256 months met the criterion of more than 20 milkings. The average number of lactation months per cow was 14.1 (standard deviation of 6.87; range: 3-24). Of the 1256 lactation months analysed, cows were identified significantly more often on the right-hand side of the milking parlour in 36.3% of months and on the left-hand side in 38.2% of months when compared to the opposite side. In 25.5% of the lactation months, visits to the left- and right-side of the milking parlour were evenly distributed. Out of the 89 cows, 23 animals showed significant left-side preference and 20 cows showed significant right-side preference in more than 75% of their lactation months.
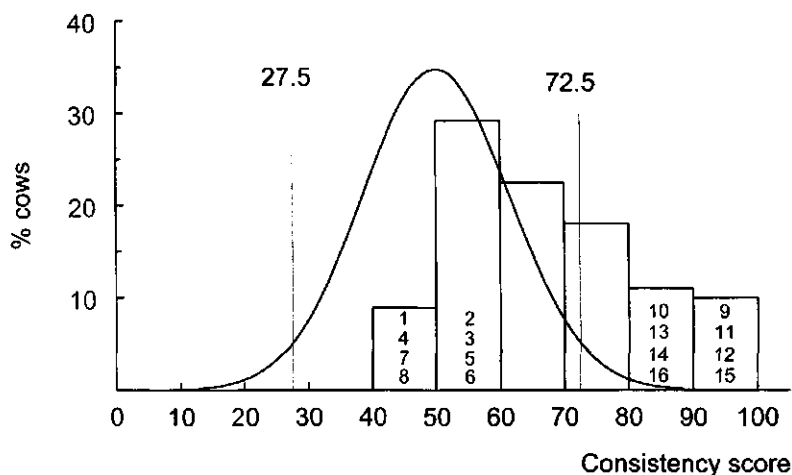
Figure 5.2. Distribution of consistency scores (solid line), calculated from 10^5 series of randomly simulated left- and right-side choices with a length of 20 successive choices. Vertical lines indicate the lower and upper limit of the 95% confidence interval. Open bars reflect the percentage of cows, classified by average consistency score. Consistency scores are derived from historical data, collected under farm conditions from 89 dairy cows. Cow numbers within bars refer to the position of selected cows within the distribution.

Table 5.2. Individual consistency score for successive lactation months and during consecutive lactations in cows not showing side preference (n=8) and cows showing side preference (n=8) in the milking parlour.

No.	Successive lactation months, numbered as months before the testing of side preference under individual conditions																											
	28	27	26	25	24	23	22	21	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
Cows not showing side preference																												
1	58	54^L	40	43	52	54	45	46^R	44			47	47	56	54	53	38	53	48	46	50			51^L	66^L	54^L	54^L	34
2		59	62^R	67^R	56^R	50^R	80^R	55^R	59	43			49	53	61	42	47	45	37	46	69^L	67^L	89^L			59^L	47	59
3		59^L	65^L	71^L	60^L	85^L	58^L	47^L			52	49	46	53^R	57	49^L	55	41	67^L	49	38			51	56	51^R	43	46^R
4				47	40	48	45^R	53	52^R	41	51	57	52^L			37	66	47	44	43	57	53	44	58^L	39	52^R	55	61^R
5											65	51^R			47	53^R	58	57^L	52^L	69^L			71^L	53	58	43	53^L	74
6												48	56	51^L	47	67	61^R	48^L	65^L	61^L			63^L	57	52	53^L	59	64^R
7																									40	48	57	33
8																										48	52	32
Cows showing side preference																												
9	96^R	80^R	88^R	93^R	89^R	85^R	95^R	93^R			89^R	98^R	92^R	93^R	98^R	98^R	97^R	100^R	97^R	100^R			97^R	93^R	97^R	100^R	100^R	100^R
10		39	41	54^L	59^L	83^L	83^L	93^L	97^L	97^L		98^L	98^L	92^L	93^L	95^L	95^L	95^L	90^L	98^L	93^L	98^L			89^L	95^L	97^L	95^L
11				98^R	85^R	88^R			84^R	95^R	93^R	88^R	81^R	83^R	95^R	97^R	98^R			91^R	100^R	97^R	95^R	98^R	100^R	97^R	97^R	100^R
12													56^L	56^L	85^L	93^L	97^L	81^L	97^L	100^L	93^L	91^L			93^L	100^L	98^L	100^L
13																				64^R	69^R	88^R	88^R	97^R	98^R	100^R	97^R	95^R
14																							53^L	75^L	92^L	91^L	91^L	77^L
15																							84^L	100^L	95^L	100^L	100^L	100^L
16																									75^R	87^R	97^R	90^R

Superscripts indicate significant side preference (P ≤ 0.05) for the left (^{L}) or right (^{R}) side of the milking parlour.

Twelve and eleven of these cows showed preference for the left and right side respectively in all months.

In all, there were 138 months (11%) in which cows exclusively visited one particular side of the parlour. In these cases individual CS's reached the maximum of 100 which indeed means that the animal only entered one side of the parlour during the entire lactation month. This is in contrast to the minimum CS of 30, incidentally found, which means that the cow showed a great deal of alternation between the left and the right side of the parlour.

Average CS's of individual cows ranged from 44 to 97. It is evident that CS's and the extent to which left- or right-side preference reached statistical significance were positively correlated. Cows with the lowest average CS's only showed a significant ($P \leq$ 0.05) side preference in 23% of the number of months. Cows with average CS's between 70-100 showed a significant ($P \leq 0.05$) side preference in over 90% of their months. As explained in Appendix A, two groups of eight cows were selected from both ends of the consistency score classification (Fig. 5.2). In order to illustrate changes in side preference of the selected cows over time, individual consistency scores are presented for all their successive lactation months, with superscripts indicating significant preference ($P \leq 0.05$) for the left or right side of the milking parlour (Table 5.2).

In only 1.9% of all months in the 'cows showing side preference' group, no significant side preference could be detected. This is in contrast with the 'cows not showing side preference' group, in which in 62.6% of the months cows did not show any side preference. The average CS's for the group showing and not showing side preference was 89.6 ($\pm$ 5.50) and 51.8 ($\pm$ 5.34) respectively.

Free parlour side choice

To find out whether the change to the individual testing conditions influenced the cows' choices, we compared results that were based on all 18 successive side preference tests, with results from the last 15 and the last 12 tests. Inclusion of the first three tests resulted in different outcomes. One cow which showed a significant side preference when all 18 side preference tests were included, showed no side preference when the first three tests were skipped. Three cows initially showed no side preference but did display a significant side preference when the first three tests were excluded. A comparison between the last 15 and the last 12 milkings yielded no differences in side preference of individual cows. Therefore, we considered that the first three side preference tests were required for habituation to the individual testing conditions and therefore excluded them from the analysis. Accordingly, the results of this part of the experiment are based on the last 15 side preference tests.
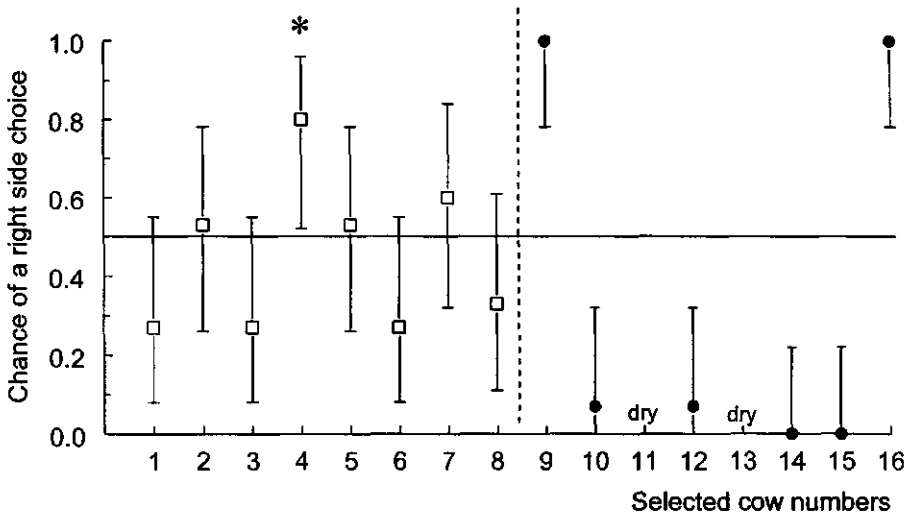
Figure 5.3. Binomial probability and confidence intervals for a right-side choice made by cows, classified by historical data as either not showing (numbers 1-8; open squares) and showing (numbers 9-16; closed circles) significant side preference in the milking parlour. Results are based on individual side preference tests during 15 successive milkings. The asterisk indicates that results of the side preference test do not match historical grouping.

For each cow, the probability of the right-side choice is illustrated by the markers in Fig. 5.3. The vertical lines on both sides of the markers reflect the lower and upper limits of the confidence interval, which obviously are reduced to zero as the probability approaches zero or one. The horizontal line at P = 0.5 indicates an equal chance that either the right or the left side of the parlour will be chosen. When this line intersects a confidence interval, the corresponding cow does not show any significant side prefer-ence for either of the parlour sides. Confidence intervals for cows showing significant side preference do not cross the equilibrium line. In Fig. 5.3, individuals are ordered along the x-axis in correspondence with the cow numbers in Table 5.2 to facilitate a comparison between the historical data and the results of the side preference tests. It appears that for 13 out of 14 cows the results of the side preference tests are comparable with historical data. Only cow number 4 exclusively chose the right side of the parlour when tested under individual conditions, whereas she had previously only shown significant right side preference in four out of 23 lactation months (Table 5.2).

Enforced parlour side choice
Out of the total number of 96 observations, three observations had to be excluded completely because of problems with the event recorder. From the remaining 93 recordings, on six occasions heart rate data were not suitable for further analysis in any of the given periods. For calculating average heart rate during entry, during milking and during the first and last minute of milking another eight, four, eight and one heart rate recordings, respectively, had to be excluded from the analysis for various reasons discussed by Hopster and Blokhuis (1994b).

Milking process
N-cows took significantly more time (P ≤ 0.05) to enter the milking parlour than H-cows and C-cows (Table 5.3). It should be remembered that cows which did not enter the parlour within one minute after the swing doors were opened, were gently encouraged to go in. N-cows received this assistance in 20/24 of the milking tests compared to 8/48 for the C-cows and 2/24 for the H-cows. Because N-cows were assisted relatively often, their latency was artificially shortened and differences between treatments in latency to enter the milking parlour were consequently underestimated. Besides a longer latency to enter the parlour, N-cows also used significantly more time (P ≤ 0.05) to enter their milking stall. On average, cows stayed 386 ± 18.4 sec in the milking stall and the milking cluster was attached for 356 ± 18.5 sec.

Duration of milking entailed over 90% of the total time spent in the milking stall. No significant differences in milking time were found between treatment groups. The maximum time it took to get the cow into the parlour and to milk her was measured as the time that elapsed between opening the swing doors of the parlour and opening the exit-gate of the stall. This time for the N-, C- and H-cows was 488 sec, 404 sec and 404 sec, respectively. The average milk yield per cow per milking was 10.2 ± 0.73 kg and did not differ between treatments.

Behaviour
The number of steps during milking did not differ between treatments and was 5.5 ± 2.28 on average. When corrected for milking time, differences between treatments were again not significant (Table 5.3). Significant differences (P ≤ 0.001) between treatments were found in the number of feeding breaks per minute during milking (Table 5.3). N-cows paused during feeding more often than H-cows and C-cows.

Table 5.3. Latency to enter the parlour and the milking stall and feeding breaks and steps during milking (mean ± SEM) for cows not showing side preference (C-Cows) and cows showing side preference, milked on either their habitual (H-Cows) or their nonhabitual (N-Cows) side of the milking parlour.

Periods / events	C-Cows	H-Cows	N-Cows
Latency to enter the milking parlour (sec)	31 ± 5.6[a]	20 ± 6.6[a]	86 ± 6.8[b]
Latency to enter the milking stall (sec)	9 ± 1.5[a]	8 ± 1.7[a]	16 ± 1.7[b]
Feeding breaks (n min^{-1}	0.51 ± 0.12[a]	0.49 ± 0.12[a]	0.89 ± 0.12[b]
Steps (n min^{-1})	1.55 ± 0.42	0.69 ± 0.44	0.57 ± 0.44

Different superscripts indicate significant differences (P ≤ 0.05) between treatments.

Heart rate

During the reference period, average heart rate as well as heart rate variability did not differ between treatments (Table 5.4). During entry, average heart rate increased 8.2 ± 1.01% compared to the average heart rate during the reference period, irrespective of the treatment. As during reference, no significant differences in heart rate variability were found during entry. During milking, as well as during its first and its last minute, the average heart rate of H-cows and N-cows was significantly higher than the heart rate of C-cows.

Table 5.4. Heart rate parameters (mean ± SEM) during milking for cows not showing side preference (C-Cows) and cows showing side preference that were milked on either their habitual (H-Cows) or their nonhabitual (N-Cows) side of the milking parlour.

Heart rate	Period	C-Cows		H-Cows		N-Cows	
Average (beats.min^{-1})	Reference	77	± 2.4	82	± 2.4	82	± 2.5
	Milking	84	± 2.3[a]	91	± 2.4[b]	93	± 2.4[b]
	First minute	90	± 2.6[a]	96	± 2.7[b]	99	± 2.7[b]
	Last minute	80	± 2.1[a]	86	± 2.2[b]	87	± 2.2[b]
Variability	Reference	2.8	± 0.18	3.0	± 0.24	2.9	± 0.25
	Milking	4.9	± 0.42[ab]	4.2	± 0.45[a]	5.3	± 0.46[b]
	First minute	2.6	± 0.25[ab]	2.0	± 0.32[a]	3.2	± 0.33[b]
	Last minute	1.7	± 0.22[ab]	1.5	± 0.23[a]	2.0	± 0.24[b]
Increase compared to	Milking	9.2	± 0.95[a]	11.0	± 1.18[ab]	13.7	± 1.23[b]
reference heart rate (%)	First Minute	16.9	± 1.63[ab]	16.7	± 1.89[a]	21.8	± 1.93[b]
	Last Minute	3.0	± 1.00[a]	5.3	± 1.18[ab]	6.6	± 1.21[b]

Different superscripts indicate significant differences (P ≤ 0.05) between treatments

No differences in average heart rate during these periods were found between H-cows and N-cows. In spite of the similarity in average heart rate between H-cows and N-cows, heart rate variability in N-cows was significantly (P ≤ 0.05) higher than in H-cows during

both the first and the last minute of milking as well as during the total milking time. Heart rate variability of C-cows had an intermediate position and did not differ from that in N-cows and H-cows.

Table 5.4 also illustrates that during the total milking time and during the last minute of milking, heart rate increased significantly more (P ≤ 0.05) in N-cows than in C-cows but was not significantly different from the increase that was found in H-cows. When, however, only the first minute of milking is considered, heart rate increased significantly (P ≤ 0.05) more in N-cows than in H-cows.

To examine the effects of milking on heart rate, we also analysed the maximum heart rate. The maximum heart rate during milking was significantly higher (P ≤ 0.05) for the N-cows (105±2.75 bpm) compared to the maxima found in H-cows (98±2.71 bpm) and C-cows (94±2.56 bpm). More specifically, during the first and last minute of milking, N-cows also had significantly higher (P ≤ 0.05) heart rate maxima when compared to the C-cows (first minute: 105±2.76 bpm vs 94±2.59 bpm, last minute: 90±2.25 bpm vs 82±2.15 bpm). Compared to the maximum heart rate of H-cows (98±2.73 bpm), the maximum heart rate of N-cows was significantly higher (P ≤ 0.05) but only during the first minute of milking.

Effects of parlour side

To avoid confounding the effects of differences between the left and the right side of the milking parlour, milkings were balanced over both sides of the parlour. Consequently, we were able to separate parlour side effects from treatment effects. The analysis did not reveal any significant treatment x parlour side interactions but showed that parlour side was indeed a significant factor in various respects. For example, latency to enter the parlour (left: 41±4.2 sec; right: 30±4.2 sec) and tachycardiac responses during entry (left: 10.1±1.16%; right: 6.2±1.19%) and during milking (left: 12.5±0.88%; right: 10.0±0.88%) were significantly higher in cows that were milked on the left than in cows that were milked on the right side of the milking parlour. Further differences were found in increase in heart rate during the first and the last minute of milking and in heart rate variability. No difference between parlour sides was found in maximum heart rate during entry and during milking, nor in the behaviour of cows during milking. In contrast to the greater effects on heart rate, milk yield was significantly (P ≤ 0.001) lower when cows were milked on the left side of the parlour (left: 9.8±0.74 kg; right: 10.6±0.74 kg).

DISCUSSION AND CONCLUSIONS

Side preference in dairy cows
From this experiment, it is obvious that many dairy cows have a strong tendency to enter the parlour on the same side during consecutive milkings. Under random selection of parlour side, it is expected that only 2.5% of the animals would have CS's of 72.5 or more (see Appendix A, Fig. 5.4). The analysis of historical data illustrated that over one third of the cows made nonrandom choices by showing a clear side preference in the milking parlour. With regard to the actual average number of milkings (55.2) per month, we used a rather conservative upper limit of the confidence interval. In other words, we even tended to underestimate the number of cows showing consistent side preference.

Our results are in accordance with previous studies. Gadbury (1975) found that 79 out of 200 cows (39.5%) significantly preferred one side of the parlour over the other and Tanner et al. (1994) reported that 47% of the cows in a large commercial dairy herd significantly preferred one particular parlour side during 90 successive milkings. In our study, some cows even showed an absolute left- or right-side preference. Furthermore, the individual tests revealed hardly any difference in side preference, compared to when side preference of the individual cow was recorded in the herd. Only one cow out of 14 consistently visited the right side of the parlour when tested individually, whereas she had shown no consistent side preference in the previous lactation months. Possibly, social influences prevented this cow from entering her preferred side of the parlour under normal farm conditions.

From these results, we conclude that in dairy herds a substantial proportion of the cows show a distinct individual side preference in the milking parlour, which persists over time despite changes in group size, group composition, stage of lactation and time of the year. Accordingly, side preference seems to be a stable characteristic of an individual dairy cow.

The significance of side preference for the cow
One reason for a consistent choice of a parlour side may be that animals experience important differences between the two sides and therefore tend to select the most attractive side or avoid the most unattractive.

In dual-choice tests, for example, it was demonstrated that cattle prefer to enter the arm of a Y-maze where they had previously received a food reward (Hosoi et al., 1995) and avoid the arm where they previously experienced restraint (Grandin et al., 1994). Moreover, we know that cows have a good spatial memory and can remember the association between the location and a food reward for periods of 8 hr (Bailey et al., 1989) and for 6 weeks (Kovalčik and Kovalčiková, 1986).

In our study, cows showing side preference in the milking parlour entered their nonhabitual side reluctantly. Differences in behaviour, average heart rate and heart rate variability during milking indicated that cows were more restless on the nonhabitual side. Heart rate variability of N-cows, however, was comparable to the variability in heart rate of quietly standing cows (Hopster and Blokhuis, 1994b). In addition, heart rate effects were within the normal range of tachycardiac responses during milking (Royle et al., 1992). In comparison, heart rate increased after exposure to novelty (Kondo and Hurnik, 1988), separation from the calf, venepuncture (Hopster et al., 1995) and stand-ing (Rulquin and Caudal, 1992) with 29%, 19%, 13% and 7%, respectively. The decline in tachycardiac effects, parallel to the decrease in the amount of physical activity in this series of studies suggests that heart rate effects are at least partly due to physical activity.

In our study, therefore, differences in heart rate effects between N- and H-cows may be partly caused by differences in physical activity during entry. In addition, also the experimenter's intervention, necessary for encouraging N-cows to go in, or differ-ences in feeding activity may have induced an increase in heart rate. Either factor could explain the heart rate effects that were found mainly in the first minute of milking. When averaged over the milking period, tachycardiac responses of N-cows and H-cows differed only little (2.7%) and not significantly. Moreover, milking the cows on their nonhabitual side of the parlour did not result in a stress-induced decline in milk produc-tion, comparable to the decline that was found when cows were milked in a novel environment (Bruckmaier et al., 1993).

In summary, dairy cows seemingly do not put a high value on being milked on their habitual side. We conclude therefore that the preference for parlour side was not particularly strong and that cows perceive at most small differences in aversion or attractiveness between parlour sides. Accordingly, dairy cows are not severely stressed when they are prevented from visiting their habitual side, but may feel at most uncom-fortable.

Differences between parlour sides

Regarding the negative effects of being milked on the left side of the parlour on heart rate and milk production, we can only speculate about their cause. At first sight, the two milking stalls did not differ in lay out. It is therefore improbable that the effects are caused by differences in lay out. Conversely, being milked on the left side of the parlour did not only reduce milk production, but also increased heart rate. Accordingly, it is also unlikely that the difference in milk production was due to differences in automatic milk recording between stalls. Most probably, differences in heart rate and in milk production resulted from different functioning of the milking clusters.

Side preference as an individual characteristic in dairy cows

Although small differences between parlour sides initially could have caused the development of side preference, it is hard to imagine that this determines consistency in side preference over long periods, as was found in this study. Therefore, also other factors likely contribute to the maintenance of side preference. Because side preference in dairy cows appeared to be a stable characteristic of an individual dairy cow and large interindividual differences were found, differences in side preference might originate from underlying factors, associated with the way individual animals differ in their ability to adapt their behaviour to changes in their environment.

In studies with male mice, for example, Benus et al. (1987, 1988, 1990) showed that some animals build up a routine very rapidly and do not react to minor environmental changes whereas others do not form routines and notice changes in their environment perfectly well. The authors concluded that the way in which the animals react to their environment reflects a differentiation in the extent of 'extrinsic', ie sensitive for environmental stimuli, versus 'intrinsic', ie insensitive for environmental stimuli, control of behaviour. Although individuals may be predisposed to one of these two modes of control, both means of control are not mutually exclusive but rather act in concert, with the behaviour as the overt expression of the actions of both control systems.

The question whether differences in the control of behaviour as reported in rodents, could also account for the differences in side preference in dairy cows is a fascinating one, but remains unanswered here. Further studies are required to draw conclusions on the association between side preference and behavioural and neuroendocrine responses to psychological stressors, ie coping styles. As suggested by Neveu (1996) in mice, selecting cows with different side preference scores and subsequently testing their response to various psychological stressors and physiological stimuli may be a sound experimental approach to further study the existence of coping styles in dairy cows.

APPENDIX A

Measuring side preference

An explanation is given for how side preference (SP) and consistency score's (CS's) are calculated and used to select cows showing and not showing significant and consistent side preference in the milking parlour. SP indicates which side of the parlour was predominantly chosen. CS's describe consistency in side preference.

Side preference

For a fixed period, it was calculated how many times the cow was milked on each of both sides of the milking parlour, left (L) or right (R), irrespective of the position of the milking stall within that parlour side. A confidence interval for the probability of a right side visit was constructed with the standard Clopper-Pearson approach, employing the relationship between cumulative binomial probabilities and the incomplete beta function (see Johnson et al., 1992, section 8.3). Significant deviation (two-sided, $P \leq 0.05$) from an even distribution over L and R, ie probability 0.5 not being in the interval was interpreted as an individual side preference of a cow in the milking parlour.

Consistency Score

For the same period, CS was calculated, being the ratio between the number of times that a given parlour side was visited twice in succession and the number of possible transitions between L and R in a series of successive milkings. For practical reasons, this ratio was multiplied by 100. For example, L-L-R-L-L-L-R-L-L produces eight possible transitions between L and R including four pairs of successive L's. Thus, $CS = (4/8)*100 = 50$. CS provides information about the dynamics of repeated visits over a long period, being another element of side preference.

Table 5.5. Example of consistency scores (CS) and side preference (SP) of various patterns of successive choices for either the left- (L) or the right (R) side of the milking parlour.

Choice		$c_i ... c_{i+10}$	CS	L	R	SP
Pattern	1	L R L R L R L R L R L	0	6	5	-
	2	L L R R L L R R L L R	50	6	5	-
	3	L L L R R R L L L R R	70	6	5	-
	4	L L L L L L R R R R R	90	6	5	-
	5	L L L L L L L L L L R	90	10	1	L ($P \leq 0.05$)

When, as shown in pattern 4 in Table 5.5, a cow repeatedly visits the left side of the milking parlour and after a while she shifts to the right side, the ratio between left and right choices is 6/5 and comparable to the ratios from patterns 1, 2 and 3. However, the fourth pattern is far more consistent than the first three. CS is used to distinguish type-1

patterns from type-4 patterns. At the same time, a comparison between the fourth and the fifth pattern shows that although the consistency score is the same, the underlying patterns may produce significant differences in side preference. Therefore, CS's were supplemented with superscripts that indicate significant side preference (see Table 5.2). In order to determine a null distribution for CS, under the hypothesis of no consistent side preference, two sets of 10^5 series of respectively 20 (set 1) and 60 (set 2) randomly selected L's and R's are generated by a computer. For all series, CS was computed. The average CS and the standard deviation were calculated for each set. Average CS for set 1 and set 2 were 49.99 and 50.02 and standard deviations were 11.467 and 7.999 respectively. As set 1 provided the most conservative estimate of the standard deviation, we used the critical values 49.99 ± (1.96 * 11.467) for selecting cows showing and not showing significant and consistent side preference (P ≤ 0.05) in the milking parlour.
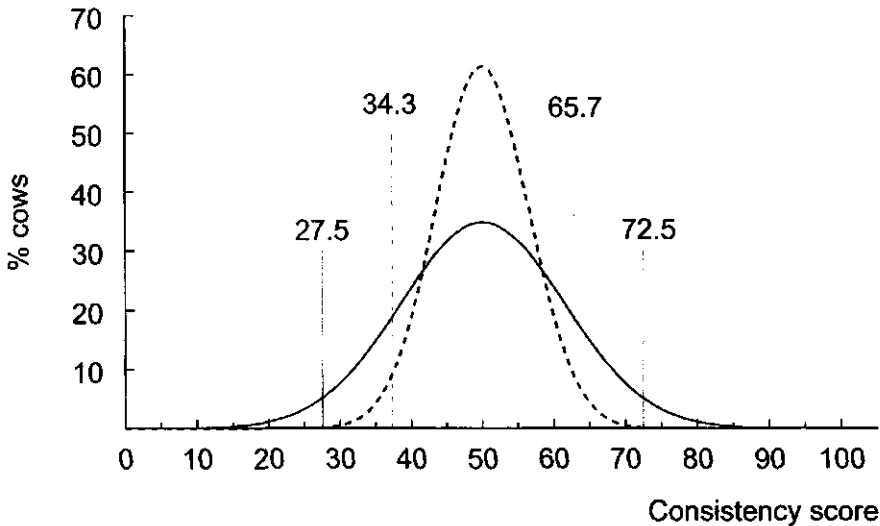

Figure 5.4. Distribution of consistency scores, calculated from 10^5 series of randomly simulated left- and right-side choices with a length of 20 (solid line) or 60 (dashed line) successive choices. Vertical lines indicate the lower and upper limits of 95% confidence intervals.

Short- and long-term consistency
of behavioural and physiological stress responses in dairy
cows during a novel environment test

Hans Hopster, Joop T.N. van der Werf, Bas Engel and Harry J. Blokhuis

Institute for Animal Science and Health (ID-DLO)
P.O. Box 65, 8200 AB Lelystad, The Netherlands

ABSTRACT

Short- and long-term consistency of individual differences in stress responses was
studied in dairy cows. Twenty (Exp. I) and 58 (Exp. II) cows were repeatedly tested in a
novel environment on three and two days within one week respectively. Eighteen cows
from Exp. II were re-tested one year later. Heart rate and behaviour (vocalisations,
eliminations, locomotion) were measured during the test; blood was collected prior to
and at the end of the test for cortisol analysis. Individual cows responded in a consistent
and idiosyncratic way to the novel environment test session. Consistency appeared in
behaviour, in heart rate and in plasma cortisol concentrations within one week. Consis-
tency of individual responses was also found for heart rate and plasma cortisol concen-
trations when tests were spaced 1 yr apart. Heart rate peaked while cows were moved
to the novel environment and decreased thereafter. The increase in heart rate was
significantly correlated with both locomotion time during the test and plasma cortisol
concentration at the end of the test. Handling prior to exposure to the novel arena,
besides the exposure itself, appeared to be an important stress-inducing element in the
novel environment test. The study provides evidence that individual dairy cows differ
fundamentally in the degree in which their actions are guided by environmental stimuli.
The treatment presented in this study is a valid method for the objective assessment of
this trait in dairy cows.

INTRODUCTION

In laboratory animals, a manipulation used to trigger the animal's stress responses is the
'open-field test'. In this test, the animal is moved from its home environment to a novel
enclosure which, in case of laboratory animals, is much larger and more brightly lit than
the home cage. In rats, exposure to the 'open-field' induces a variety of stress responses
eg defecation, tachycardia (Candland et al., 1967), immobility (Archer, 1973), and
increased plasma corticosterone concentrations (Weinstock et al., 1992).

        Since novelty per se is generally seen as a potent frightening element of many
stressors, exposure to a novel environment is also used for measuring emotional re-
sponses in farm animals (Beilhartz and Cox, 1967; Moberg and Wood, 1982; Romeyer
and Bouissou, 1992). In dairy cows, such procedures have been used to assess differ-
ences in temperament (Kilgour, 1975), to examine relationships between behavioural
reactivity and milk production (Kovalčiková and Kovalčik, 1982) and to study the effects
of different rearing (Arave et al., 1985) and housing conditions (Munksgaard and
Simonsen, 1996).

        Although the open-field test is generally accepted as suitable for measuring fearful-
ness in rats (Gray, 1991), one should be cautious when drawing conclusions about

which motivation underlies the response to this test in dairy cows. Besides similarities in general set-up of the open-field procedure, there are differences in dimensions of both animals and fields as well as in the species-specific behaviour of rats and cows (Munksgaard and Jensen, 1996). To avoid suggesting a misleading correspondence between the two species, we preferably term this test a 'novel environment test'.

The social isolation of a cow (Boissy and Le Neindre, 1997) and probably also the handling which is necessary for introducing her into the arena (Boissy and Bouissou, 1995) interfere with the animals response to the novel environment. In cattle, this response is therefore expected to reflect the combined action of different motivational systems of the individual, ie search for social contact, need for locomotor behaviour, exploration of escape possibilities and detection of possible threats. For studying specific motivations, the novel environment test is therefore presumably less appropriate (Munksgaard and Simonsen, 1996).

An important question is whether the stress responses of a cow to a novel environment are reflecting relatively stable individual characteristics which show consistency over time. As far as we know, in dairy cows only Kilgour (1975) tried to answer this fundamental question by testing cows in a novel environment on three consecutive days. He found significant similarities between days for both ambulation and vocalisation. However, no information is available regarding the consistency of individual differences in physiological measures.

Apart from consistent individual differences in separate behavioural and physiological responses, interrelationships between these responses may further support the existence of a general characteristic, mediating stress responsiveness of the individual animal at the central level. Kilgour (1975), for example, found positive correlations between ambulation and vocalization scores in dairy cows. Furthermore, Boissy and Bouissou (1995) found that especially cows which showed a short latency to approach a novel object in a novel environment, also vocalised more frequently. These results argue for a common mechanism that controls the search for social contact (vocalisations), the exploration of escape possibilities (ambulation) as well as the investigation of possible threats (approach latency). With respect to physiological indices, the possibility that different time-lags may exist between the exposure to the stressor and its effect on different physiological parameters must be taken into account.

To substantiate the existence of consistent individual differences in reaction of dairy cows to environmental challenge, we examined their behavioural and physiological responses to a novel environment test. Individual cows were repeatedly tested with both one day and one year between successive tests. In addition, we studied relationships between behavioural and physiological measures. Finally, we addressed the question which motivational systems may be activated during the novel environment test session.

ANIMALS, MATERIALS AND METHODS

Housing and care

Two experiments were conducted, within the present study. In both, cows were milked and fed roughage twice daily and received concentrates during milking. In addition, cows were housed in the same cubicle barn and similar experimental procedures were used (Tables 6.1 and 6.2). Prior to the experimental period, animals were habituated for three weeks to being tethered in a self-tethering feed gate during feeding. To encourage the cows to tether themselves, maize silage (approx. 6 kg.cow[-1]) was supplied every day about 15 min before the testing was planned to start. After about one hour, all cows were released and went on pasture during the summer season (Exp. I) or stayed in the cubicle house during the winter period (Exp. II-A and -B). Two days prior to the first day of testing, experimental cows were equipped with a halter in order to habituate them to wearing one and to facilitate handling during the test. Tests were carried out between 16.00 and 17.00 hr (Exp. I), between 09.30 and 12.30 hr (Exp. II-A) or between 13.15 and 14.30 hr (Exp. II-B). All experiments were approved by the ID-DLO Institutional Animal Care and Use Committee and were carried out at the Institute's experimental farm in Lelystad.

Table 6.1. Observation schedule used in Exp. I, II-A and II-B (see Table 6.2 for key).

Experimental period	Starting time sequence (min)			Duration (min)
	Exp. I	Exp. II-A	Exp. II-B	
Ref	-7	-	-7	5
Sam1	-1	-	-11	approx. 1
ToBox	0	0	0	approx. 2
InBox	2	2	2	3
Arena1	5	5	5	5
Arena2	10	10	10	5
Arena3	15	-	-	5
ToPen	20	15	15	approx. 1
Sam2	21	21	21	approx. 1
Sam3	-	26	26	approx. 1

Novel environment

The arena was situated in a closed rectangular room (9 x 13 m^2). A corridor of approximately 35 m connected this room with the home environment of the animals. At the end of this corridor, a 'starting box' (2 x 1 m^2) outside one of the corners of a square test arena (6 x 6 m^2) permitted access to the arena through remotely controlled pneumatically operated swing doors. The arena had solid walls (2 m high) and a solid concrete floor. The room was equipped with a microphone and two video-camera's were mounted on the ceiling directly above both the arena and the starting box. These camera's were connected to a video recorder and monitor in a separate room. Both the starting box and the arena floor were pressure washed prior to each test. A small pen near the entry gate, equipped with a self-tethering feed gate, held the cow after she had left the arena.

Animals

Experiment I. Twenty Friesian dairy cows were randomly selected from the Institute's herd. On the first day of testing, cows varied in parity (2.4 ± 1.28), in days of lactation (226 ± 71), in daily milk production (23.3 ± 5.4 kg) and in body weight (578 ± 49 kg). Experimental cows made up one herd with 34 non-experimental cows.

Experiment II-A. To avoid effects of parity and stage of lactation that may confound the results, 58 Friesian dairy cows, all in first parity, were tested at the same stage of lactation, ie 66 ± 6.6 days post partum on the first day of testing. Cows varied in daily milk production (27.5 ± 3.4 kg) and in body weight (542 ± 39.8 kg).

Experiment II-B. Out of the 58 animals from Exp. II-A, 10 cows with low- (LC) and 8 cows with high (HC) cortisol concentrations were selected and re-tested one year later. Selection was based on plasma concentration collected 16 min after the cow had entered the novel arena. All these cows were in their second lactation at 81 ± 14.0 days after calving. Cows varied in daily milk production (34.5 ± 5.7 kg) and in body weight (601 ± 30.1 kg).

Experimental procedure

Experiment I. Sets of two cows were tested three times within a week (6 days) alternately with one day in between tests of the same individual. Successive tests for individual animals were labelled day 1, day 2 and day 3. The experimental period consisted of 10 weeks. On the day of testing, while the cow was standing in the feed gate, heart rate was measured according to methods reported previously (Hopster and Blokhuis, 1994b).

A blood sample was collected after at least 10 min of recording, using a procedure described elsewhere (Hopster et al., 1998c). A rope (approx. 1.5 m) was temporarily attached to the cow's halter whilst leading her through the corridor into the starting box. She was then left undisturbed for three min (Tables 6.1 and 6.2). Next, the swing doors were opened giving the cow access to the novel arena. Doors were closed after cows had entered the arena. Fifteen min later, the swing doors were opened again and the cow was led into the holding pen and tethered in the feed gate. Finally, a second blood sample was collected.

Table 6.2. Definition of different periods of the novel environment test and list of behavioural categories quantified in these periods.

Period / Behavioural category	Definition
Ref	Period (5 min) during which heart rate is measured in the experimental cow while standing at the feed gate
Sam1, Sam2, Sam3	Time between hitching a rope to the cow's halter before blood sampling and removing the needle after blood sampling
ToBox	Time between releasing the cow from the feed gate in the home environment and the closure of the starting box.
InBox	Time between the closure of the starting box (horizontal bar) and the opening of the front exit.
Standing free	Standing with the hind-part free from the horizontal bar which prevented the cow from escaping backwards
Head in normal position	Looking forward with the nose not lower than the front knees and not above back height
Arena1	Period (5 min) following InBox, during which the cow is in the novel arena.
Locomotion	Changing position of front legs, running or jumping
Elimination	Urination or defecation
Vocalisation	All kinds of vocalisation
ToPen	Time between re-opening the swing doors at the end of the novel environment test and the closure of the gate of the holding pen.

Experiment II-A. All cows were tested twice within a week, *ie* on Tuesday (day 1) and Thursday (day 2), according to the procedure that was used in Exp. I. In order to make the test more practicable the following modifications were introduced. To facilitate handling at the start of the test, 4-6 cows, amongst which the animals to be tested, were separated from the herd by a single bar approximately three hours before the time of testing and next to the entrance of the corridor. To minimize pre-test handling, no heart rate measurements were carried out during the test nor did we collect blood samples before testing. Furthermore, cows were individually driven through the corridor instead of being led and the stay in the novel arena was limited to 10 min (Tables 6.1 and 6.2). At the end of the test, cows were calmly driven from the novel arena into the holding pen where some feed was available to encourage them to tether themselves in the feed gate. A blood sample was collected at 16 and 21 min after cows had entered the novel arena (Tables 6.1 and 6.2).

Experiment II-B. Each cow was tested once according to the procedure in Exp. II-A, except for some minor adjustments (Tables 6.1 and 6.2). As in Exp. I, heart rate was measured during all different periods of the test and a blood sample was collected for measurement of baseline cortisol concentrations. The pre-test sample however, was collected prior to the measurement of the reference heart rate instead of immediately thereafter.

Behavioural observations and time-points
The behaviour of cows was analysed, either directly from a video screen or from video tape afterwards, using a portable computer installed with The Observer® event-recording software program for observational research (Albonetti *et al.*, 1992). This program was also used for recording time points, defining specific periods in the overall test procedure (Tables 6.1 and 6.2)

During Exp. I, video tapes of the animals' stay in the starting box were analysed for two measures of calmness. These measures were *Standing free* and *Head in normal position*. Different periods within the overall test session as well as behavioural categories are defined and abbreviated in Table 6.2.

Determination of plasma cortisol
Cortisol was measured using a time resolved fluoroimmunoassay which has been described elsewhere by Erkens *et al.* (1998). The intra-assay coefficients of variation for control samples with concentrations of 71.1, 39.2 and 10.3 ng.ml^{-1} were 8.2, 7.9 and 11.3% (n = 16). The corresponding inter-assay coefficients of variation were 7.4, 8.8 and 19.5% (n = 13). In accordance with the lower detection limit of the cortisol assay, concentrations below this limit were fixed at 0.5 ng.ml^{-1}.

Statistical analyses

A 5-min period that started 6 min before either *Sam1* (Exp. I) or *ToBox* (Exp. II-B) was used for calculating mean reference heart rate. All heart rate effects are expressed as percentages of this mean. For various reasons, discussed by Hopster and Blokhuis (1994b), heart rate could not always be properly measured. In Exp. I and II-B, in 7.3 and 5.6 % of all different periods respectively, data were unsuitable for further analysis and were considered missing values.

Spearman's rank correlation coefficients (r_s; Conover, 1980, p252) for individual physiological and behavioural parameters were calculated between days (Exp. I: day 1 vs day 2; day 2 vs day 3; day 1 vs day 3; Exp. II-A: day 1 vs day 2) as well as within days between different parameters. P-values of the test for associations between either days or parameters were based on a normal approximation for the distribution of Spearman's rank correlation (Gibbons, 1971, p232).

As an overall measure of association, *ie* a repeatability measure, Spearman's correlations for different parameters were averaged over pairs of days (Exp. I: n = 3) or for pairs of parameters over days (Exp. I: n = 3; Exp. II-A: n = 2). An overall P-value for association between days was derived with Friedman's test. The approximation for the Friedman test statistic based on an F-distribution, which is superior to the conventional chi-square approximation (Iman and Davenport, 1980), was employed. P-values for association between parameters were derived from the normal approximation for the distributions of the averaged Spearman correlations.

There is a simple one-one relationship between Spearman's correlations, averaged within parameters over days, Kendall's coefficient of concordance (Conover, 1980, p305) and the Friedman test statistic (Conover, 1980, p299). This implies that significance tests for association based on these three statistics are equivalent.

Statistical significance is considered at $P \leq 0.05$ for two-tailed analyses. Values are expressed as means $\pm$ SEM. All calculations were performed with the statistical programming language Genstat 5 (1993).

RESULTS

Selection of data

On average, cows adapted rapidly to the 15 min exposure to the novel arena. Differences between animals were greatest during the first 5 min. With regard to the stay in the novel arena, we therefore only present the results of the first 5 min (*Arena1*) for all three experiments.

In Exp. II-A and -B, we collected blood samples at both *Sam2* and *Sam3* for two reasons. Firstly to prevent accidental mistakes in measurements of cortisol concentrations and secondly, to check whether these concentrations changed in a similar direc-

tion in all animals. It appeared that in Exp. II-A and in Exp. II-B, cortisol concentrations at *Sam2* and *Sam3* correlated at the 0.1 % level ($r_s > 0.95$). We therefore decided to only present cortisol concentrations collected at *Sam2*. Moreover, concentrations at *Sam3* were consistently lower ($P \leq 0.01$) than at *Sam2*.

Short-term consistency

Mean rank correlations in Exp. I indicate significant agreement between days in rank orders for heart rate effects in all periods of the test (Table 6.3). These correlations show that the consistency in heart rate effect was strongest during *ToBox* and weakened during the periods thereafter. Fourteen out of 15 possible correlation coefficients which were calculated for the five different periods of the test between daily scores for heart rate effects (day 1 vs day 2, day 1 vs day 3, day 2 vs day 3) were significantly different from zero ($P \leq 0.05$; Mean $r_s = 0.67$; s.d. $= 0.15$).

Table 6.3. Mean Spearman rank correlations (average of bilateral correlations between days; n = 3) for heart rate effects in dairy cows, recorded during different periods of a novel environment test (see Table 6.2 for key).

Parameter	Period	Mean rank correlations between days
		Exp. I (20 cows, tested thrice)
Heart rate response, expressed as a percentage of baseline	Sam1	0.68***
	ToBox	0.86***
	InBox	0.64***
	Arena1	0.61***
	Sam2	0.56***

Statistically significant correlations are indicated: *** $P \leq 0.001$.

With regard to the time that was needed for bringing the cow from the home to the novel environment, mean rank correlations indicate significant but weak agreement between daily rank order for duration of *ToBox* in both Exp. I and II-A (Table 6.4).

For behavioural responses in Exp. I, the strongest agreement between successive tests was found in rank order for vocalisations during *InBox* and during *Arena1*, which was confirmed in Exp. II-A (Table 6.4). These findings were supported by analysis of 2 x 2 tables (Chi-square test) of the number of individuals that either vocalised or did not vocalise during successive days (data not shown). During all days in both Exp. I and II-A, over 60% of the cows never vocalised during *InBox* but less than 40% never vocalised during *Arena1*. On average, cows in Exp. I vocalised 3.3 ± 0.89, 3.9 ± 0.92 and 3.8 ± 0.88 times during *Arena1* on days 1, 2 and 3 respectively. Cows in Exp. II-A

vocalised in this period on average 1.7 ± 0.36 and 2.2 ± 0.43 times on day 1 and 2 respectively ($P = 0.095$).

Table 6.4. Mean Spearman rank correlations (average of bilateral correlations between days) for different parameters in dairy cows, recorded during different periods of a novel environment test (see Table 6.2 for key).

Period	Parameter	Mean rank correlations between days	
		Exp. I (20 cows, tested thrice)	Exp. II-A (58 cows, tested twice)
ToBox	Duration	0.26*	0.36*
InBox	Vocalisation	0.57***	0.55***
	Elimination	0.36**	NS
Arena1	Locomotion	0.50***	0.43***
	Vocalisation	0.66***	0.67***
	Elimination	NS	NS
Sam2	Cortisol concentration	0.56***	0.67***

Statistically significant correlations are indicated: * $P \le 0.05$; ** $P \le 0.01$; *** $P \le 0.001$.

Rank orders for locomotion time during *Arena1* appeared to correlate significantly over days in both Exp. I and II-A (Table 6.4). In Exp. I higher rank correlations for locomotion time between day 2 and day 3 ($r_s = 0.72$; $P \le 0.001$) compared to correlations between day 1 and day 2 ($r_s = 0.45$; $P > 0.05$), suggest that stability in rank orders for locomotion time increased after cows had been exposed to the test once.

Significant agreement between daily rank orders for elimination scores was only found in Exp. I during *InBox* (Table 6.4). Rank correlations between pairs of days showed that this agreement was exclusively attributed to the similarity between days 1 and 2 in cows' eliminative behaviour. This was confirmed by analysis of 2 x 2 tables (Chi-square test), ie 8 cows neither eliminated on day 1 nor on day 2 and 7 cows eliminated both on days 1 and 2 ($P \le 0.05$). According to Chi-square analysis, elimination during *InBox* tended ($P = 0.07$) to be consistent also in Exp. II-A where these numbers were 16 and 19 respectively.

Significant similarity over days was found in rankings for plasma cortisol concentrations in both Exp. I and II-A (Table 6.4). Plain concentrations of plasma cortisol during *Sam2* in Exp. I, showed stronger agreement between daily rank orders than the increase in plasma cortisol concentrations ($r_s = 0.46$; $P \le 0.05$). Together with the strong relationship between these two variables ($r_s = 0.94$; $P \le 0.001$) this justified the omission of a pre-test blood sample in Exp. II-A.

Long-term consistency
Both Fig. 6.1 and 6.2 illustrate that two groups of cows which showed distinct cortisol concentrations after having been moved to and having tested in a novel environment in their first lactation, still respond differently to this procedure after one year.

In HC-cows, increase in heart rate was significantly higher during *ToBox, InBox* and *Arena1* compared to LC-cows, (Fig. 6.1). From arrival in the starting box, heart rate gradually decreased in both LC- and HC-cows. In LC-cows heart rate was no longer significantly different from reference values during *Arena2* (P = 0.23) whereas the heart rate of HC-cows was still significantly increased in this period (P = 0.02).
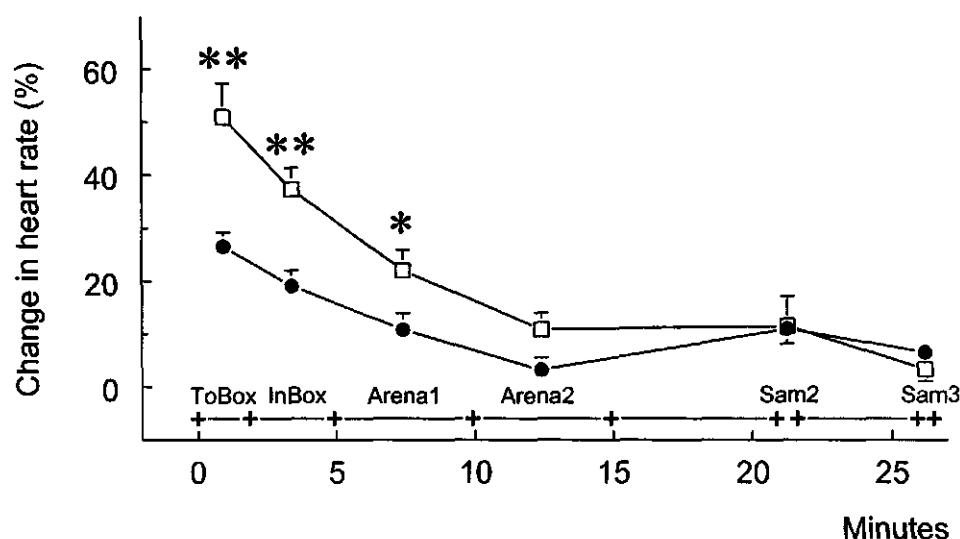
Figure 6.1. Heart rate changes (mean ± SEM) in dairy cows (2nd lactation) during different periods of a novel environment test (see Table 6.2 for key; plus signs under abbreviations mark transitions between periods). Cows showed either Low- (n = 10; closed circles) or High (n = 8; open squares) cortisol concentrations after having been exposed to the same test one year earlier. Heart rate changes are expressed as percentages of baseline heart rate. Statistically significant differences between LC- and HC-cows are indicated: * P ≤ 0.05; ** P ≤ 0.01.

Fig. 6.2 shows that, besides heart rate, cortisol concentrations in plasma, collected at *Sam2*, were also higher in HC-cows than in LC-cows. It is clear that, although cortisol concentrations were markedly higher in the first lactation, plasma cortisol responses of LC- and HC-cows were still significantly different in the second.

Contrary to these significant differences in physiological responses, behavioural responses were equal between groups or at most tended to be different (Exp. II-B). The duration of *Sam1* tended (P = 0.085) to be longer in HC-cows (102±6.84 sec) than in LC-cows (86±5.52 sec).

Likewise, we found a tendency (P = 0.064) that *ToBox* took more time in HC-cows (134 ± 17.6 sec) than in LC-cows (95 ± 6.03 sec). No further differences in behavioural responses were found between LC- and HC-cows in the second lactation.
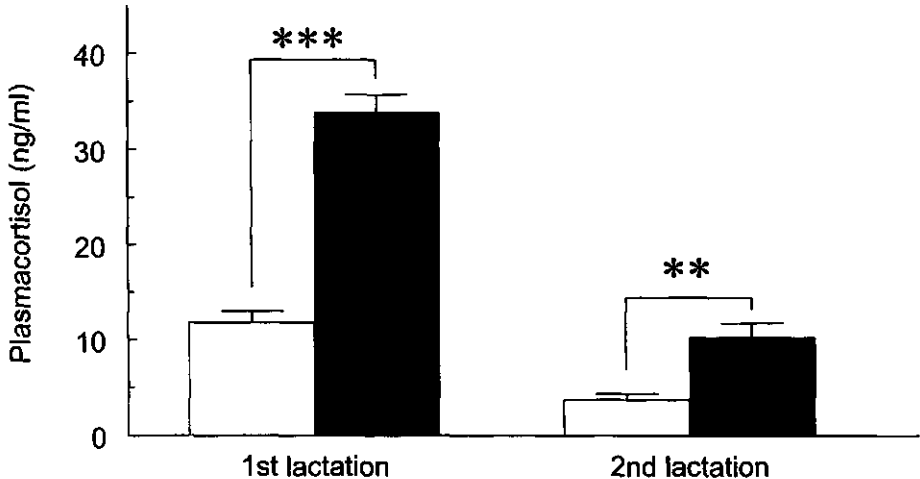

Figure 6.2. Cortisol concentrations (mean ± SEM) of dairy cows during *Sam2* (see Table 6.2 for key). Cortisol concentrations during *Sam2* of cows (n = 58), tested in first lactation were used for selecting cows with Low- (n = 10; open bars) and High (n = 8; closed bars) cortisol concentrations (1st lactation). These cows were re-tested one year later (2nd lactation). Statistically significant differences between LC- and HC-cows are indicated: ** P ≤ 0.01; *** P ≤ 0.001.

Relationships between behavioural and physiological responses

Results from Exp. 1 (Table 6.5) demonstrate that within the parameter 'Increase in heart rate', measurements during different periods of the test correlated with one another at or below the 5 percent level. Mean rank correlations between increase in heart rate and locomotion indicate that cows which showed a high increase in heart rate during *Sam1*, *ToBox* or *InBox* spent also much time on locomotion during *Arena1*.

Highest correlations between increase in heart rate and locomotion time were found when both parameters were recorded simultaneously during *Arena1*. This is most likely caused by locomotor activity which directly affects heart rate. Despite these relationships between heart rate response and locomotion time which were found in all periods of the test, significant correlations between heart rate response and plasma-cortisol concentrations (*Sam2*) were found only during *ToBox* and *Sam2* (Table 6.5). When calculated within days, correlation appeared to be completely absent on day 1, almost present on day 2 (r_s = 0.40, P = 0.15) and significant (r_s = 0.60, P = 0.029) only on day 3. Apparently, the association between heart rate increase during *ToBox* and cortisol concentrations during *Sam2* became stronger as the test was repeated.

Not a single significant correlation was found between plasma cortisol concentrations during *Sam2* and cows' responses (locomotion time, heart rate increase) during *Arena1*

Table 6.5. Mean Spearman rank correlations between parameters, recorded in dairy cows (n = 20) during different periods of a novel environment test (see Table 6.2 for key).

Parameter		Mean rank correlations between parameters						
		Increase in heart rate					Locomotion	Cortisol
	Period	Sam1	ToBox	InBox	Arena1	Sam2	Arena1	Sam2
Increase in	Sam1		0.34*	0.50***	0.53***	0.30*	0.33*	NS
heart rate	ToBox			0.54**	0.48**	0.58***	0.35*	0.37*
	InBox				0.78***	0.34*	0.45**	NS
	Arena1					0.40**	0.65***	NS
	Sam2						NS	0.44**
Locomotion	Arena1							NS

Statistically significant correlations are indicated: * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001.

Habituation to the test

Fig. 6.3 illustrates that in Exp. I, heart rate effects were lower as cows were repeatedly exposed to the test. The graph also shows that the highest increases in heart rate consistently occurred during *ToBox* (duration: 104±4.1 sec) and during *Sam2* (duration: 111± 4.0 sec). During *InBox* and during *Arena1*, heart rate gradually returned to reference values. As the test was repeated, heart rates returned to reference values at an earlier stage of the test. On days 2 and 3 heart rate returned to (P = 0.25) and tended (P = 0.075) to be lower than reference values respectively during *Arena1*, whereas on day 1 heart rate was still increased in this period (P = 0.002). On day 3, heart rate decreased to even below reference values during both *Arena2* and *Arena3* (P ≤ 0.001). Mean reference heart rate was stable over days, with 84±1.7, 86±2.1 and 87±2.0 beats per minute on days 1, 2 and 3 respectively; differences between days were not significant.

Signs of habituation were also found in the effect of the novel environment test on plasma cortisol concentrations. In Exp. I, mean concentrations in plasma, collected at *Sam2* tended to be lower (P = 0.08) on day 2 (12.3±2.20 ng.ml^{-1}) than on day 1 (18.7 ±2.74 ng.ml^{-1}) but were significantly higher on day 2 (P = 0.004) than on day 3 (8.6± 1.69 ng.ml^{-1}). Further evidence for habituation to the test was provided in Exp. II-A. It appeared again that mean cortisol concentrations in plasma, collected during *Sam2* were significantly higher on day 1 (20.8±1.39 ng.ml^{-1}) than on day 2 (15.3±0.97 ng.ml^{-1}).

Finally, adaptation was also found in behavioural responses over days. Cows in Exp. I generally showed more activity on day 1 than on days 2 and 3. During *InBox*, for example, cows spent significantly less time (P ≤ 0.05) on standing with their head in normal position on day 1 (107±4.6 sec) than on days 2 (133±4.8 sec) and 3 (146±4.3 sec). In addition, during *Arena1* they spent more time (P ≤ 0.05) on locomotion on day 1 (108 ±8.4 sec) compared to days 2 (70±10.5 sec) and 3 (55±10.0 sec). These findings were confirmed in Exp. II-A in which cows spent also more time on average (P ≤ 0.01) on locomotion on day 1 (91±5.2 sec) than on day 2 (78±5.1 sec) during *Arena1*.
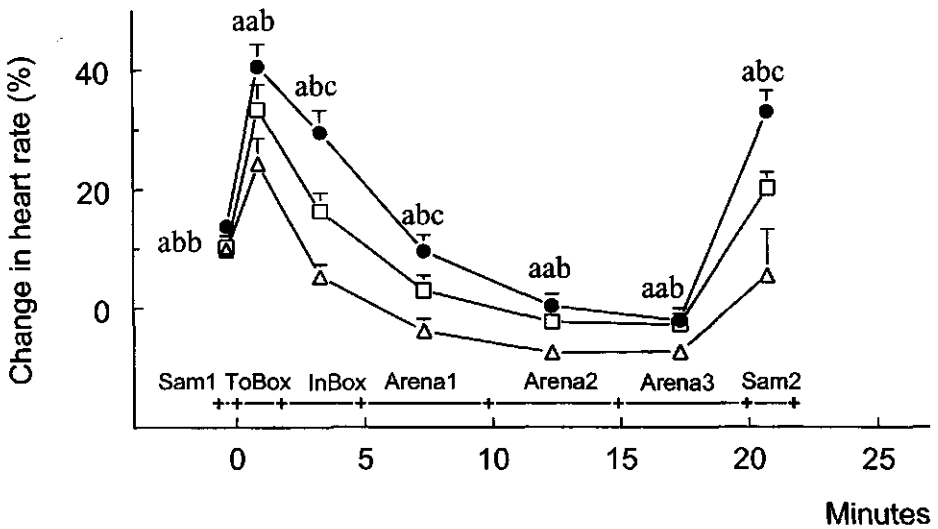
Figure 6.3. Heart rate changes (mean ± SEM) in dairy cows (n=20) during different periods of a novel environment test (see Table 6.2 for key; plus signs under abbreviations mark transitions between periods), conducted three times on alternating days (day 1: closed circles; day 2: open squares; day 3: open triangles). Different characters represent significant differences between days (P ≤ 0.05); character order corresponds with day number.

DISCUSSION

Consistency in heart rate response to handling

In both experiments, we found strong agreement in heart rate responses between days and between different periods of the test. The highest and most consistent increase in heart rate was observed while cows were moved from the cubicle house to the novel environment (*ToBox*; Fig. 6.1 and Fig. 6.3).

Changes in heart rate result from competing influences of the sympathetic and the parasympathetic nervous system. As psychological stressors as well as physical load activate these systems, the increase in heart rate during *ToBox* reflects a dominating

sympathetic stimulation, associated with both emotional and locomotor activity. From the difference in heart rate response between day 1 and day 3, however, we conclude that at least one-third of the total increase on day 1 was induced by the novelty of the treatment, since the distance to move and thus the physical activity required was similar. Kondo and Hurnik (1988) concluded that novelty rather than physical activity induced an increase in heart rate in dairy cows. They found a 30% increase in heart rate when cows were moved by a familiar handler from the stanchion barn to a novel environment. In contrast, heart rate increased only 5% when novelty was reduced by returning the cows from the novel to the home environment. In the present study, average increase in heart rate on day 3 was still 25% and thus much higher than in the study of Kondo and Hurnik (1988). This suggests that cows in our study were still distressed when they were moved to the novel environment for the third time. An explanation for this could be that cows in our study were used to moving freely in the cubicle house and that they were probably less familiar with a handler who stayed within their flight-zone while not being able to retire.

In both Exp. I and II-B, heart rate began to decrease as cows arrived in the starting box. This response pattern agrees with studies from Le Neindre (1989) and from Lay et al. (1992b) in cattle. In addition, a marked increase in heart rate was found at the end of Exp. I (Sam2; Fig. 6.3) at which time the cow was held by a rope and led to the holding pen. This contrasted to the absence of such an increase at the end of Exp. II-B (Sam2; Fig. 6.1), when the cow was not led but calmly driven to the holding pen. Probably, because the latter procedure corresponds more closely to the way these cows were usually handled. All these experimental findings strongly support our hypothesis that the close contact to the human handler was an important element in our study. The results further indicate that the heart rate response of dairy cows to a mixture of handling, social isolation and novelty is a stable individual characteristic that shows consistency over time.

Correlated adrenocortical response

Besides significant correlations between days in rank order for heart rate effects, also rank orders for plasma cortisol concentrations correlated significantly between days. Assuming that handling was indeed an important stress-inducing element in our study, it is likely that the cows' response is also reflected in the concentrations of plasma cortisol. Significant mean rank correlation between heart rate increase during both ToBox and Sam2 and cortisol concentrations during Sam2 (Table 6.5) provides evidence for parallel sympathetic and adrenocortical activation. Rank correlations between heart rate increase during ToBox and cortisol concentrations during Sam2, calculated for separate days, became stronger as cows were tested repeatedly. This suggests that the effects of handling gradually dominated effects of novelty and social isolation. Possibly because

cows habituated to novelty and isolation more rapidly. More evidence for such an important role of handling is provided by Exp. II-B. Cows, during first lactation classified as cows with either high- or low plasma cortisol concentrations during *Sam2*, showed different heart rate responses during *ToBox* when tested again one year later (Fig. 6.1).

The delay between the increase in both heart rate (*ToBox*) and cortisol concentrations (*Sam2*) is not surprising since catecholamines are rapidly discharged whereas corticosteroids are the end product of a neuroendocrine cascade of time consuming processes. In cows, Alam and Dobson (1986) found a lag-phase of approximately 16 min between the application of the stressor, *ie* venepuncture, and the maximum rise in plasma cortisol concentrations. Cortisol concentrations and heart rate increase, however, appeared to be associated also within *Sam2*, possibly because of significant correlations between *ToBox* and *Sam2* in heart rate effects. During both *Tobox* and *Sam2* however, cows had close contact with the handler. We conclude therefore that not only the increase in heart rate during *ToBox* but also the cortisol concentrations during *Sam2* about 20 min later, reflect the individual's response to being led to the novel environment. It is obvious that individual dairy cows differ considerably in their response to the test procedure. Some individuals showed strong cardiac and strong adrenocortical responses whereas others responded only moderately or weakly.

Interpreting the novel environment test in dairy cows

Mean rank correlations between days for behavioural responses were, although significant, generally lower than for physiological responses. Consistent individual differences in behavioural responses were found in both Exp. I and II-A in the duration of *ToBox*, in vocalisations during both *InBox* and *Arena1* and in locomotion time during *Arena1* (Table 6.4). Apparently, some individuals showed active behavioural responses in contrast to others, being less active. Kilgour (1975) considered these differences in temperament and reported similar results. Mean Spearman correlations for locomotion time (Exp. I: $r_s = 0.50$; Exp. II-A: $r_s = 0.43$) are in agreement with Kendall's Coefficient of Concordance for the number of squares entered during ambulation of $W = 0.57$ as found by Kilgour (1975).

With regard to the number of vocalisations however, cows in the present experiments seemed to vocalise less than cows in Kilgour's study. This could be related to differences in the degree of novelty, associated with the design and construction of the novel arena. In our study, the arena probably differed more from the cows' normal environment than in the study of Kilgour (1975) in which the arena was constructed outdoors, of posts and rails with a floor of pumice sand and wood shavings. Also, in a previous study (unpublished) we found that individual cows vocalised substantially less when isolated in a novel environment compared to isolation in their home environment. A novelty-dependent reduction in vocalisations was also found by Boivin *et al.* (1992)

who reported with cattle being isolated in a novel environment, that animals reared in a range system vocalised less than indoor reared animals. Since novelty per se is generally seen as a potent frightening element in many of the fear-inducing stimuli (Gray, 1991), vocalisations by cattle in a novel environment is considered to reflect the absence of fear (Boissy and Bouissou, 1995).

Significant correlations (Table 6.5) between the increase in heart rate during *Sam1*, *ToBox*, *InBox* and locomotion time during *Arena1* strongly suggest that stimuli which were encountered **prior to** the novel arena affected the behaviour of the cows **while in** the novel arena. As the present study also provides evidence that these stimuli are related to handling, we suggest that the response of loose-housed dairy cows to a novel environment is seriously confounded by their response to human contact before being introduced to the test arena. In addition, this study proves that the common use of a starting-box for standardization purposes is not effective in preventing pre-treatment handling from affecting the novel environment test responses, even when this period is as long as 3 min. Further research is required to study whether these findings also apply to tethered cows as these are probably more used to close human contact.

CONCLUSIONS

Loose-housed dairy cows respond consistently different to a novel environment test session. Consistency was measured in behaviour, in heart rate and in plasma cortisol concentrations within one week. Consistent responses were also found for heart rate and plasma cortisol concentrations over one year. Handling prior to the exposure to the novel arena, besides the exposure itself, appeared to be an important stressor in the novel environment test. The findings in this study strongly suggest that individual dairy cows differ fundamentally in degree in which their actions are guided by environmental stimuli. The treatment presented in this study offers possibilities for the objective assessment of this trait in dairy cows.

Stress enhanced reduction
in peripheral blood lymphocyte numbers
in dairy cows during
endotoxin-induced mastitis

Hans Hopster*, Joop T.N. van der Werf and Harry J. Blokhuis

Institute for Animal Science and Health (ID-DLO)
P.O. Box 65, 8200 AB Lelystad, The Netherlands

ABSTRACT

Two groups of Friesian dairy cows, which showed either high- (HC; n = 8) or low (LC; n = 10) plasma cortisol concentrations in response to a psychological stressor (novel-environment test) in their first lactation, were used one year later for studying the effects of psychological stress on various aspects of the host defence during endotoxin-induced mastitis. Social isolation was used as a stressor; endotoxin was used to activate the host defence.

HC-cows appeared to be more stressed by the experimental procedure than LC-cows. One hr after having been isolated, rectal temperature increased (+ 0.59°C) in HC-but not in LC-cows. Handling, related to the intra-mammary administration of endo-toxin, induced a higher increase in cortisol concentration in HC- than in LC-cows 1 hr after injection. In addition, HC-cows vocalised significantly more frequently (595 ± 222) than LC-cows (81 ± 24) between 1.5 and 9.5 hr after isolation.

Between 3 and 5.5 hr after endotoxin-treatment, cortisol concentrations increased profoundly in both LC- and HC-cows and peaked at 24.6 ± 3.8 and 22.8 ± 3.1 ng.ml^{-1} respectively. The rise in plasma cortisol concentration was associated with a decrease in the number of circulating lymphocytes between 0 and 8 hr post injection (PI). Between 8 and 10 hr PI, the number of circulating lymphocytes in HC- further decreased (40%) to 1.58 x 10^6 cells.ml^{-1} and remained so until 21 hr PI. This was not the case in LC-cows.

The results show that a differing sensitivity of dairy cows to environmental stress-ors is associated with significant difference in the number of circulating leukocytes following intra-mammary administration of endotoxin during a period of social isolation. The results further suggest that, apart from adrenocortical hormones, other factors related to general stress-responsiveness modulate the number of peripheral lymphocytes in dairy cows during endotoxin-induced mastitis. During environmental stress, these factors may enhance endotoxin-induced reduction in circulating lymphocyte numbers, particularly in cows that are sensitive to environmental stressors.

As to the clinical relevance of these differences in stress responsiveness, no differ-ences between LC- and HC-cows were found in disease incidence nor in duration of a disease episode during both 1st and 2nd lactation. However, HC-cows tended to pro-duce less milk than LC-cows.

INTRODUCTION

A complex network of communication exists between the central nervous system, the endocrine system and the immune system (for review see Ader et al., 1991; Rivier, 1995; Spangelo and Gorospe, 1995; Madden et al., 1995). The hypothalamus-pituitary-

adrenal (HPA) axis plays a significant role in the communication between the neuro-endocrine and the immune system. This control is exerted at various regulatory levels and is of vital importance for preventing the defence reactions from overshooting and thereby threatening homeostasis (Munck et al., 1984). Briefly, during inflammation, activated immune cells synthesize and secrete cytokines, several hormones and neuropeptides that stimulate both the hypothalamus and the pituitary to release corticotrophin-releasing factor and adrenocorticotropic hormone respectively. Conse-quently, the adrenal cortex is stimulated to synthesize and release glucocorticosteroids (GCs), the end-product of the HPA axis. GCs, on their turn, activate a long-loop inhibi-tory feedback response on both the immune cells and the HPA axis.

It is important to recognize that many immunosuppressive effects of GCs were observed after pharmacological (ie large) rather than physiological doses and using large doses of GCs do not necessarily reflect true immunomodulatory effects of dynamic physiological changes in the endogenous release of GCs. Furthermore, within physio-logical concentrations, GCs not only suppress, but may also enhance immune functions (for review see Wilckens and De Wijk, 1997). This argues for in vivo studies of immune function, where increased GCs concentrations in plasma result from stress-induced endogenous release of adrenocortical hormones.

The immune system is also regulated by signals from the central nervous system. Sympathetic nervous system innervation of primary and secondary lymphoid organs, for example, and the presence of adrenoceptors on cells of the immune system enables noradrenergic signalling to lymphocytes and macrophages from sympathetic nerves (Madden and Felten, 1995; Felten and Felten, 1991). The activation of the immune system, in its turn, is communicated to the central nervous system by various secretory products of the immune system (eg neuroendocrine hormones, neuropeptides). Given this genuine bidirectional communication between the neuroendocrine system and the immune system, differences in sympathetic activation and in activity of the HPA axis may have different immunomodulatory effects.

Within a normal population of conspecifics, individuals with extreme differences in stress-reaction pattern coexist (Henry and Stephens, 1977; Engel and Schmale, 1972). Distinct reaction patterns were found in rodents (Bohus et al., 1987; Sgoifo et al., 1996; Koolhaas et al., 1997) as reflected by relatively stable individual characteristics that show consistency over time and across situations. Evidence is accumulating that consis-tent individual differences in stress-reactions also exist in farm animals (eg in pigs: Hessing et al., 1994; in poultry: Jones et al., 1994; Korte et al., 1997; in cattle: Hopster and Blokhuis, 1994a; Van Reenen et al., 1995). These differences relate to differences in behaviour, in sympathetic activation and in activity of the hypothalamic-pituitary-adre-nal axis. Thus, under stress, immunoregulatory processes in individual animals will take place in different neuroendocrine environments and therefore individuals that respond

consistently different to psychological stressors, may also show different immune or inflammatory responses to infectious organisms or non-replicating antigens. In rats changes in various immune parameters, for example, depended on the social status of the individual animal (Bohus et al., 1991). Also in pigs, individual differences in immune reactivity appeared to be associated with their response to social and non-social challenges (Hessing et al., 1995). In spite of differences in immune response, it is not clear as to what extent these differences are clinically relevant and whether they associate with differences in disease incidence or in severity of a disease episode.

The present study was designed to investigate stress-induced changes in immune- and inflammatory responses in dairy cows which showed consistently different adreno-cortical response to psychological stress (Hopster et al., 1998b). Social isolation was used to trigger stress responses in individual cows and to identify individual differences in response. A low-dose endotoxin-induced mastitis model was used for studying possible effects of isolation-induced stress on various aspects of the host defence. Concentrations of cortisol in plasma were measured for studying adrenocortical effects of both isolation (Hopster and Blokhuis, 1994a) and endotoxin (Paape et al., 1974; Shuster et al., 1993). Plasma zinc concentrations were examined because these have extensive effects on immune function (Wellinghausen et al., 1997) and are appropriate indicators of the acute phase-response as well (Verheijden et al., 1982; Koj, 1985). Rectal temperature, heart rate, milk production and behaviour were recorded for investigating systemic effects. Disease incidence and production records were analysed to explore the clinical relevance of differences in stress-responsiveness in dairy cows.

ANIMALS, MATERIALS AND METHODS

The present study was conducted at the Institute's experimental farm at Lelystad. Animal experimentation had been approved by the Institute's Animal Care and Use Committee.

Animal care and animal housing
We used two groups of cows. Ten cows with low and eight cows with high plasma-cortisol concentrations in response to a short stay in novel environment were selected, according to methods reported elsewhere (Hopster et al., 1998b). This novel environment test was carried out one year before the present study. The average cortisol concentrations at the end of that test were 11.8 ± 3.92 for the low responders and 33.9 ± 5.15 ng.ml^{-1} for the high responders. Low- and high responders were labelled LC- and HC-cows respectively. All 18 cows involved were Holstein-Friesians at early lactation (88 ± 12.4 d post partum) in second parity and in clinically good health. Cows had milk somatic cell counts (SCC) below 500×10^3 cells.ml^{-1} and were free of specific mastitis pathogens in all quarters. They produced 34.5 ± 5.7 k of milk.day[-1]. At the start

of the experimental period the average body weight of LC- and HC-cows was 600 (±
27.1) and 602 (± 30.1) kg respectively. 7 LC-cows and 5 HC-cows were in calf at the
time of treatment; precaution was taken that the others were not in heat during 2 weeks
around treatment.

Before and after treatment all cows were housed in cubicles in a herd comprising
approximately 60 cows. Cows entered the herd after calving and received commercial
dairy concentrates to supplement roughage according to yield. Cows were milked at 14:
10 hr intervals in a double-three open-tandem parlour. Milk yield, milk temperature and
quarter milk conductivity were automatically recorded for each individual cow twice
daily. On the day of stress treatment (day = 0) and one hour before endotoxin was
administered (time = -1), experimental cows were moved to an isolated stanchion barn
and stayed there for 55 hr (Fig. 7.1).
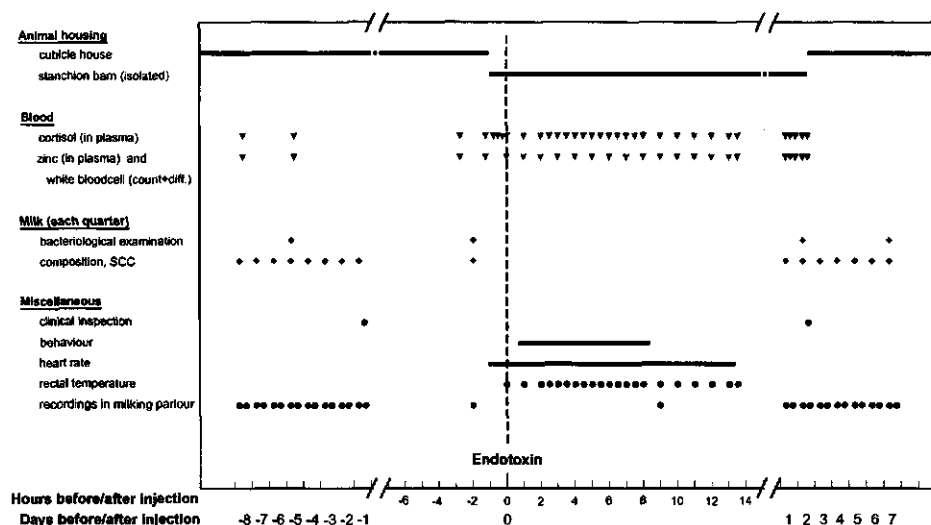
Figure 7.1. Experimental outline of animal housing conditions, sampling time-points (blood and milk)
and miscellaneous recordings during the experiment.

Endotoxin preparation

Endotoxin, a polyclonal stimulator of B-cells, was prepared according to the method
used by Shuster et al. (1993). Briefly, endotoxin from Escherichia coli O55:B5 (Sigma
Chemical Co., St. Louis, MO) was dissolved in pyrogen-free Earl's balanced salt solution
(EBSS, Life Technologies, Paisley, Scotland) in a final concentration of 2 μg.ml^{-1}. Sterile
syringes (10 ml) were filled with 5 ml filter sterilized (.2 μm) solution and stored (-20°C)
for future use. Solutions were thawed under refrigeration (4°C) overnight.

Experimental protocol

Cows participated in the experiment one by one for 16 days. On day -8, monitoring of milk SCC started as illustrated in Fig. 7.1. On day -1 at 14.00 hr, the animal was clinically inspected by a veterinarian and a cannula (Intraflon 2, Vygon, Ecouen, France) was fitted into the vena jugularis to facilitate frequent blood sampling.

After morning milking on day 0 at 06.45 hr the experimental cow was equipped with a Polar® Sport Tester (Hopster and Blokhuis, 1994b) and left loosely tied and undisturbed in a cubicle for 15 min to record baseline heart rate. At 07.00 hr, she was moved from the herd and relocated to a stanchion barn, tethered and isolated. Water was available through a water bowl.

Before endotoxin-treatment on day 0, average quarter milk SCC from day -8 to -6 was used to decide which hind quarter was to be infused with endotoxin. If both quarters had SCC below 200×10^3 cells.ml^{-1}, the left hind quarter was selected. If not, the quarter with the lowest SCC was selected. At 08.00 hr (day = 0, time = 0) endotoxin (10 μg) was aseptically injected through the teat canal. Thereafter, a mixture of the cow's usual amount of concentrates and roughage was provided ad libitum. The cow was milked twice daily in the milking parlour at 17.00 and 07.00 hr. Fresh feed was supplied in the stanchion barn after every milking. Frequent blood sampling ended at 13.5 hr (see Fig. 7.1) and the cannula was removed. The animal was returned to the cubicle house after clinical inspection at 14.00 hr on day 2 (= 54 hr PI). As illustrated in Fig. 7.1, monitoring of udder health continued until day 7.

Sampling procedures and measurements

During the experimental period, the following samples and measurements were taken (Fig. 7.1).

Blood

Sample collection: After application of the cannula, it was filled with sterilized citrate (32 g, dissolved in 1 litre of saline) to prevent blood clotting. Blood samples were collected through the cannula. Cannulae were flushed with citrate after each sampling. After the cannula had been removed, blood samples were again taken by venepuncture. Blood samples were collected into 10-ml evacuated tubes containing EDTA (Vacuette®, Greiner B.V., The Netherlands). Half of the sample volume was centrifuged (12 min, 3000 rpm), the other half was used for white blood cell count and differentiation. Aliquots of plasma were stored at -20°C. Samples were analysed in duplicate in all assays. Samples collected at -3 and -1.25 hr PI were used for reference.

Cortisol: Cortisol was measured using a time resolved fluoro immunoassay in unextracted bovine EDTA plasma (Erkens et al., 1998). The intra-assay coefficients of variation for control samples with concentrations of 71.1, 39.2 and 10.3 ng.ml^{-1} were 8.2,

7.9 and 11.3% (n = 16). The corresponding inter-assay coefficients of variation were 8.8, 10.1 and 12.9% (n = 17).

Zinc: Plasma zinc concentrations were determined by atomic absorption spectrophotometry.

White blood cell count: Total leukocyte counts (number of cells per microliter) in EDTA-anticoagulated blood samples were determined by electronic counting (Sysmex® F-800, TOA Medical Electronics, Kobe, Japan).

White blood cell differentiation: Blood smears were stained with a Hema-Tek slide-stainer. A total of 100 cells were counted, and the lymphocytes, monocytes and neutrophils (mature and immature) differentiated on the basis of morphology (Sanderson and Phillips, 1981).

Milk

Sample collection: milk samples from all quarters were collected aseptically during morning milking for bacteriological examination (International Dairy Federation, 1981).

Bacteriological examination: Milk samples were bacteriologically examined for mastitis pathogens using methods described by the National Mastitis Council (1987).

Milk composition and SCC: Samples of milk from all quarters were collected during morning milking and analysed for somatic cell count (SCC) and protein contents by a commercial milk analysis laboratory (Moned, Zutphen, The Netherlands). Samples were preserved with sodium azide/bronopole and refrigerated until analysis.

Miscellaneous

Clinical inspection: Clinical inspections of all animals on general health aspects and on udder health in particular were carried out by the same veterinarian.

Behavioural observations and heart rate recordings: Continuous behavioural recordings were video-taped between 0.5 and 8.5 hr PI and analysed for: numbers of vocalisations, defecations and urinations and lying-, standing- and feeding time and bouts. Heart rate was recorded at 1 minute intervals between -1.25 and 13.5 hr PI.

Rectal temperature: Temperature measurements were collected with a hand-held thermometer (Terumo®, Model C31, Terumo Corporation, Tokyo, Japan) between 0 and 13.5 hr PI. Milk temperatures during morning milking, averaged over days -2, -1 and 0 were used for reference.

Automatic recordings: Milk production and milk temperature were automatically recorded in the milking parlour at every milking. Milk production data were corrected for milking interval and are presented as $kg.12h^{-1}$.

Milk production data: Average milk yield (305 days) and milk solids were estimated from 3-weekly milk production records.

Disease incidence: Disease was diagnosed during both first and second lactation by the same veterinarian. Incidence and treatment-days were recorded.

Statistical analysis

In accordance with the lower detection limit of the assay, cortisol concentrations below this limit were fixed at 0.5 ng.ml^{-1}. Statistical comparisons between LC- and HC-cows were made at all time points using a two-sample Student's t-test. High autocorrelations between successive time-points within the day of treatment did not allow the use of a repeated measures analysis of variance and therefore the statistically more robust t-test was used. Where variances of groups were unequal, approximate t-statistics were calculated using the method of Scheffé (1959). Within groups, differences between pre- and postinjection time points were compared by using a Student's t-test for paired-samples. Effects on milk production and milk somatic cell counts were estimated by REML (eg Engel, 1990; Searle *et al.*, 1992, Ch. 6) with group (LC, HC), days before and after treatment (-5...+7) and milking time (morning, evening) as fixed effects and random cow effects. Overall tests for main effects and interactions were derived from the Wald test (Buist and Engel, 1992). Statistical significance was considered at $P \leq 0.05$. Values are expressed as means $\pm$ SEM.

RESULTS

Changes in peripheral blood leukocytes

As illustrated in Fig. 7.2A, the intra-group comparison revealed no significant differences in white blood cell counts between LC- and HC-cows at any of the sampling times. Within the group of LC-cows, however, a marked and significant decrease as compared to baseline was found in the number of white blood cells at the time of endotoxin-treatment. In LC-cows, the number of white blood cells remained reduced until 8 hr PI in contrast to a short-lived and slight but significant reduction in white bloodcell counts in HC-cows from 2 to 3 hr PI.

As compared to the pre-injection level within groups, the number of mature (segmented) neutrophils in the blood was significantly reduced in LC cows (0 to 4 hr PI) but not in HC-cows (Fig. 7.2B). Similar to what was found in white blood cell numbers, the number of segmented neutrophils was already decreased from 0 hr PI. At 3 (P = 0.056) and 4 (P = 0.068) hr PI the number tended to be lower in LC- than in HC-cows (Fig. 7.2B). In both groups the number of segmented neutrophils, compared with base-line values within groups, was significantly increased from 9 hr PI. In HC-cows the increase was more pronounced than in LC-cows.
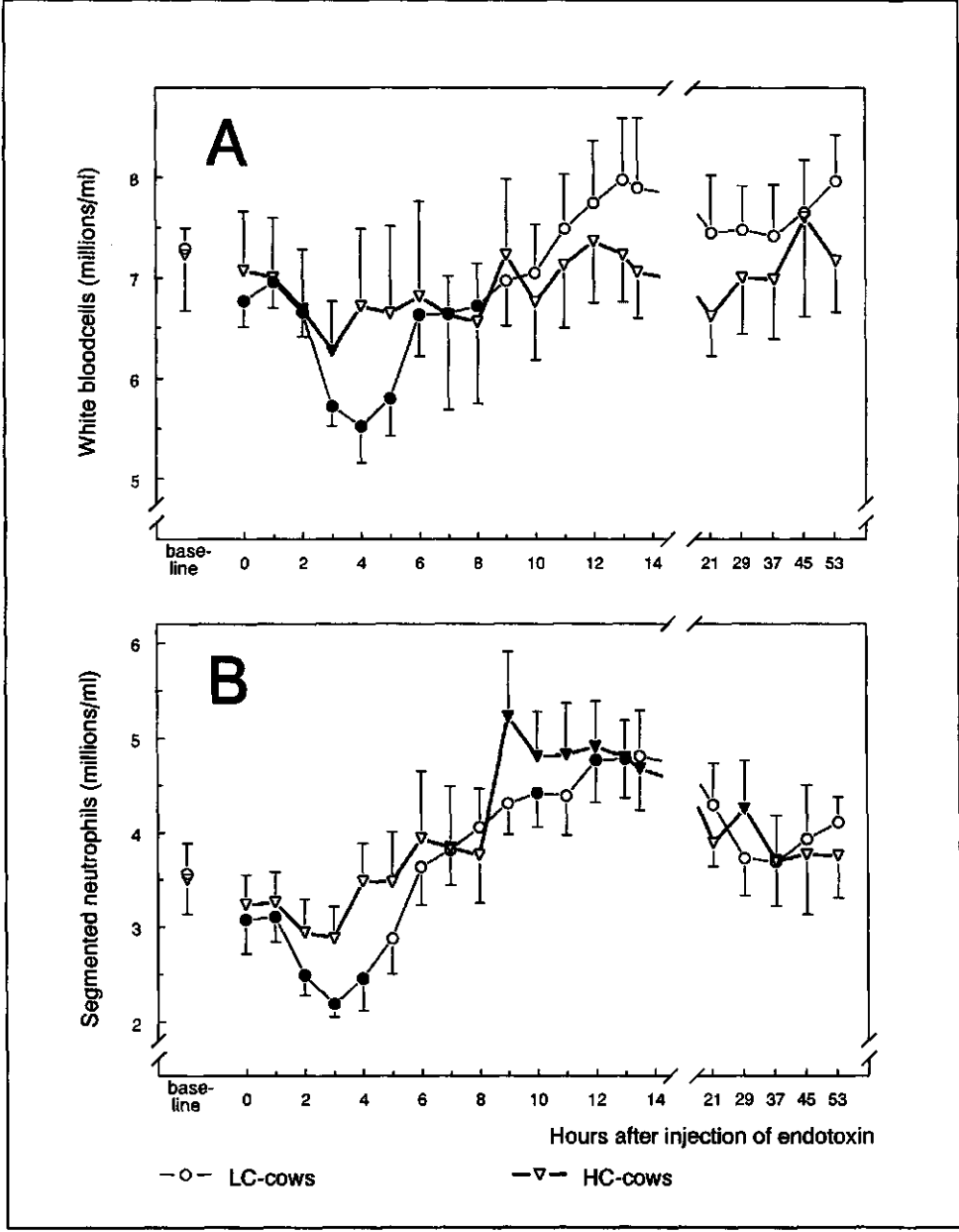
Figure 7.2. Total leukocyte counts (A) and segmented neutrophil counts (B) in blood of cows during endotoxin-induced mastitis. LC-cows (circles) had low- and HC-cows (triangles) had high plasma cortisol concentrations after having been exposed to a novel environment, one year prior to the present study. All cows were socially isolated at time = -1 hr. One gland of each cow was challenged with 10 µg E.coli-endotoxin at time = 0 hr. Data are expressed as means ± SEM from 8 HC-cows and 10 LC-cows. Circles and triangles are closed when values differed significant (P ≤ 0.05) from baseline values within groups.
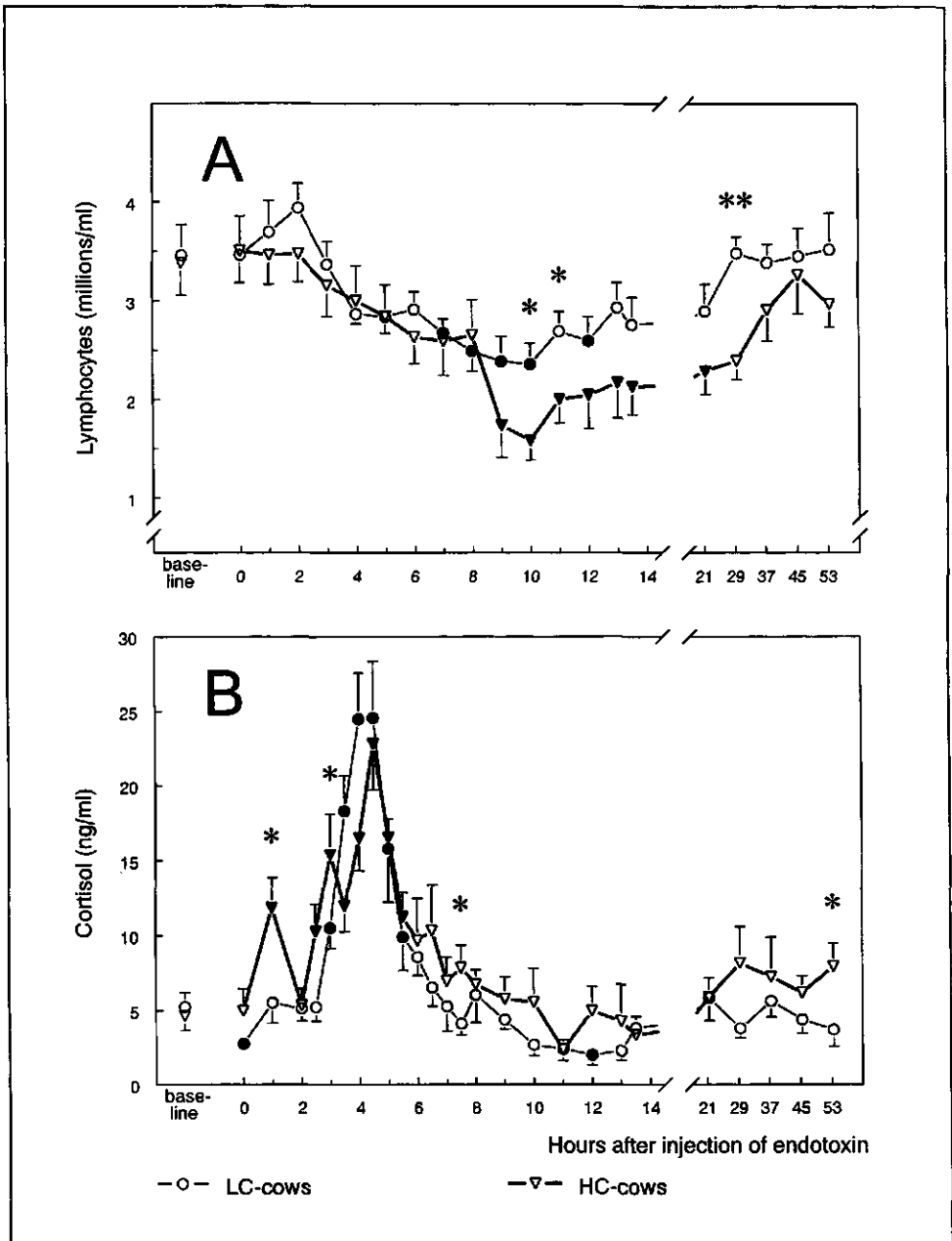
Figure 7.3. Total lymphocyte counts (A) and cortisol concentrations (B) in blood of cows during endotoxin-induced mastitis. Statistically significant differences between LC- and HC-cows are indicated: * P ≤ 0.05. Circles and triangles are closed when values differed significant (P ≤ 0.05) from baseline values within groups. See Fig. 7.2 for key.

Fig. 7.3A shows a gradual decline in the number of lymphocytes in both groups during the first 8 hr after endotoxin-treatment. Between 8 and 10 hr PI, the number of lymphocytes in the HC-cows, but not in LC-cows, showed a drop of over 40%. Compared with the baseline level within groups, lymphocyte numbers in LC-cows were significantly reduced for a shorter time (7 to 12 hr PI) than in HC-cows (9 to 21 hr PI). Differences between groups were significant at 10, 11 and 29 hr PI. In the case that the original contrast between LC- and HC-cows was further accentuated by including only the 6 LC-cows that had the lowest and the 6 HC-cows that had the highest cortisol concentrations in response to the earlier novel environment test (Hopster et al., 1998), the differences in lymphocyte numbers would also have increased and become significant or would have tended to be different during the whole period from 10 to 53 hr PI.

Cortisol concentrations

At 1 hr PI, cortisol concentrations in plasma of HC- but not in LC-cows (P = 0.02; Fig. 7.3B) were significantly increased as compared to baseline concentrations. As expected, endotoxin strongly activated the HPA axis, illustrated by the significant increase between 3 and 5.5 hr PI in both groups and peak concentrations of plasma cortisol at 4-5 hr PI. Maximum plasma cortisol concentrations did not differ between LC-cows (24.5 ng. ml^{-1}) and HC-cows (22.8 ng.ml^{-1}) and plasma cortisol concentrations returned to baseline concentrations (2-6 ng.ml^{-1}) at 6 hr PI. Although significant differences in cortisol levels in plasma were incidentally found (ie at 2.5, 7.5 and 53 hr PI) they did not point to any prolonged period during which the adrenocortical response to endotoxin was different between the two groups. From 10.00 to 13.00 hr PI, plasma cortisol concentrations of LC-cows tended to be lower than baseline. Significancy was measured at 12 hr PI only. During the same period plasma cortisol concentrations of HC-cows only tended to be lower than baseline at 11 hr PI (Fig. 7.3B).

Zinc concentrations

The administration of endotoxin caused a marked but similar and steady decrease in plasma Zn concentrations in both groups (data not shown). From 6 to 21 hr PI, Zn concentrations in both LC- and HC-cows were significantly decreased as compared to the mean baseline level of 1.08±0.05 mg.l^{-1}. The lowest concentrations (0.58±0.05 mg.l^{-1}) were reached at 11 and 12 hr PI. This reduction in plasma Zn concentrations was followed by a significant increase between 29 (1.2±0.05 mg.l^{-1}) and 53 (1.35±0.07 mg.l^{-1}) hr PI.

Behaviour and heart rate

During the 8 hr observation time, HC-cows vocalised significantly more frequent (595±222) than LC-cows (81±24). All cows stood for over 90% of the 8 hr observation

period and spent about 9% of their time on feeding. Only 1 LC- and 1 HC-cow laid down for more than 3 hr. It should be noted that cows that were lying, were encouraged to get up every sampling time. This happened 2.8 times in LC-cows and 1.9 times in HC-cows. LC- and HC-cows defecated 7.2 and 6.9 and urinated 4.9 and 8.5 times per animal respectively. Variability between cows was large and, except for the number of vocalisations, none of the differences in behaviour were significant. Between 0.5 and 8.5 hr PI, heart rate increased significantly ($P \leq 0.01$) in both LC- and HC-cows with by 10%. Overall, LC-cows (91 ± 1.9 bpm) tended to have a higher heart rate ($P = 0.08$) than HC-cows (86 ± 2.0 bpm).

Rectal temperature

A significant ($P \leq 0.05$) relationship ($r_s - 0.75$) between rectal- and milk temperature, measured during milking at 9 hr PI and just before milking respectively, justified the use of mean milk temperature (days -2, -1 and 0) as a reference for rectal temperature. Reference temperatures of both groups were equal. Rectal temperature in HC- but not in LC-cows was significantly ($P \leq 0.05$) increased already at the time of endotoxin-treatment ($+0.47°C$) as well as 1 hr later ($+0.59°C$). In LC-cows rectal temperature only started to increase from 2 hr PI onwards. In LC-and HC-cows, maximum rectal temperatures were reached at nearly the same time (at 7.2 ± 0.97 and 6.5 ± 0.44 hr PI respectively). Fifty percent of all cows had a $> 1°C$ increase in temperature. Highest increase ($+3.2°C$) was shown by a LC-cow, reaching $41.4°C$.

Milk production, milk protein and milk somatic cell count

Although 12 hr milk production was consistently higher in LC- than in HC-cows both during morning (Fig. 7.4A) and evening milking (Fig. 7.4B) and before as well as after endotoxin-treatment, these differences were not significant at any time. On average, cows produced 11% less milk at the morning milking prior to the endotoxin-treatment (day - 0) up to the morning milking before their return to the cubicle house (day - 2).

In contrast to a higher milk production in LC-cows compared to HC-cows during the 12-day period around treatment, protein concentrations in the morning milk of the endotoxin-treated quarter of LC-cows (3.32%) were consistently lower ($P = 0.01$) in this period than in HC-cows (3.65%). Effects of the endotoxin-treatment on milk protein content were not found (data not shown).

The administration of 10 μg endotoxin caused an acute, significant but transient increase in the number of somatic cells in the infected quarter (Fig. 7.4C). Six days after treatment, SCC returned to pre-treatment level without any therapeutic intervention.
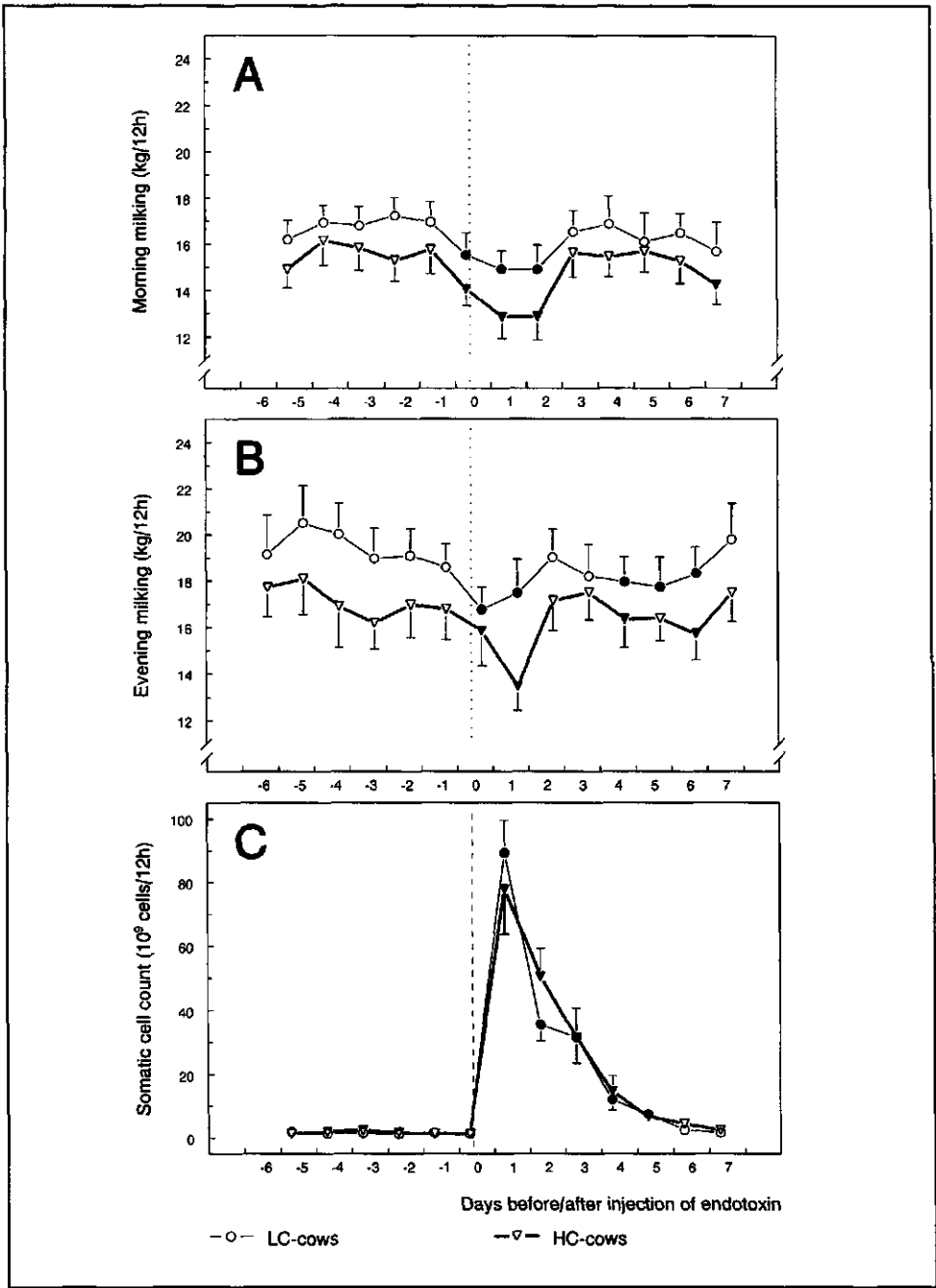
Figure 7.4. Mean (± SEM) milk yield during morning (A) and evening (B) milking as well as milk somatic cell counts (C) of LC-cows (circles) and HC-cows (triangles). Milk yield is corrected for milking interval and expressed as k.12h⁻¹. See Fig. 7.2 for key.

Milk production records and disease incidence

Milk production and milk composition data are summarized for both groups for the first two lactation periods (ie standardized 305-day periods) in Table 7.1.

Table 7.1. Milk production and fat- and protein composition of milk from LC-cows and HC-cows in 1st and 2nd lactation. Data are expressed as means (standardized 305-day periods) ± SEM. P indicates the probability of the differences between LC- and HC-cows. See Fig. 7.2 for key.

Lactation number	Parameter	LC-cows			HC-cows			P
		Mean	±	SEM	Mean	±	SEM	
1	Milk (kg)	6990	±	258.7	6899	±	289.3	NS
	Fat (%)	4.90	±	0.07	4.97	±	0.08	NS
	Protein (%)	3.51	±	0.05	3.54	±	0.05	NS
2	Milk (kg)	8904	±	381.9	7912	±	433.1	0.086
	Fat (%)	4.64	±	0.10	4.84	±	0.11	NS
	Protein (%)	3.52	±	0.07	3.59	±	0.08	NS

No significant differences between LC- and HC-cows were found in any of the given parameters, although LC-cows tended ($P = 0.086$) to produce more milk in their second lactation. Likewise, no significant differences were found between LC- and HC-cows in disease incidence, the number of days treated and the interval between calvings (Table 7.2).

Table 7.2. Disease incidence, numbers of days treated and calving interval in LC-cows and HC-cows in 1st and 2nd lactation. Data are expressed as means ± SEM. P indicates the probability of the differences between LC- and HC-cows. See Fig. 7.2 for key.

Lactation number	Parameter	LC-cows			HC-cows			P
		Mean	±	SEM	Mean	±	SEM	
1	Disease incidence	1.20	±	0.21	1.25	±	0.27	NS
	Days treated	5.7	±	1.18	3.5	±	0.42	NS
2	Disease incidence	2.30	±	0.47	2.25	±	0.63	NS
	Days treated	14.6	±	5.22	13.7	±	5.31	NS
1-2	Days between calvings	405	±	15.4	376	±	10.0	NS

DISCUSSION AND CONCLUSIONS

General response to endotoxin

In accordance with previous studies, intra-mammary administration of *E. coli* endotoxin produced an acute and transient mastitic episode in all cows with only mild mastitic and systemic reactions. These reactions included a decrease followed by an increase in the number of circulating neutrophils (Paape et al., 1974; Lohuis et al., 1989) and in plasma zinc concentrations (Verheijden et al., 1982; Lohuis et al., 1989) and a temporary increase in rectal temperature (Paape et al., 1974; Lohuis et al., 1989; Jackson et al., 1990; Shuster et al., 1993) and in cortisol concentrations (Paape et al., 1974; Jackson et al., 1990; Shuster and Harmon, 1992; Shuster et al., 1993). Milk production decreased (Shuster and Harmon, 1992) and milk SCC increased (Paape et al., 1974; Shuster et al., 1993). The course of responses was also similar to those reported in previous studies, maximum changes occurred at similar time points after endotoxin-treatment.

The present results show that a different sensitivity of dairy cows to environmental stressors is associated with significant differences in the number of cells that may be activated in innate and adaptive immune responses. These differences involve peripheral blood neutrophil and lymphocyte numbers.

Reduction in blood neutrophil numbers

Effects of endotoxin. The marked reduction in the number of circulating neutrophils in LC- but not in HC-cows may be explained by differences in mechanisms that regulate the distribution of neutrophils. These mechanisms serve to either redistribute neutrophils in the body or modulate neutrophil turnover (production and/or destruction). As redistribution of neutrophils is seen as the most plausible explanation for the decrease in blood neutrophil numbers in stressed rats (Dhabhar et al., 1995), we assume that HC- and LC-cows differ in ability to recruit neutrophils from the circulating and marginal storage pools into inflamed mammary tissue. Since the half-life of neutrophils is short (6 hr) and, since they are incapable of cell division, they must have been supplied from the blood stream. In addition, lowest blood neutrophil numbers occurred at 3 hr PI in both groups. This 3 hr lag-phase between intra-mammary administration of endotoxin and the maximum decrease in neutrophil numbers is in agreement with the time which neutrophils need to exit the circulation and to reach the infected tissue in maximum numbers (Persson et al., 1992). We therefore assume that the differences in blood neutrophil numbers is primarily due to differences in migration of neutrophils to the endotoxin-infused quarter. Higher circulating neutrophil numbers at 3 hr PI in HC-cows may therefore be associated with less neutrophils present at the infection site, which in its turn, may increase the risk of developing mastitis (Burvenich et al., 1994; Kehrli and

Shuster, 1994). Further studies are required into factors (ie expression or activity of adhesion molecules on the surfaces of leukocytes and/or endothelial cells) which caused the assumed difference in neutrophil migration between LC- and HC-cows.

Effects of environmental stress. We found that the number of blood neutrophils was already decreased in HC- but not in LC-cows at the time of the endotoxin-treatment as compared to baseline values. This could be due to differences in response to the experimental procedure. Activation of the HPA axis is suggested to increase milk SCC (Giesecke, 1985; Berning et al., 1987). As neutrophils constitute 80-90% of cells in normal milk (Lee et al., 1980) these findings support the idea that stress-induced activation of the HPA axis could have induced migration of neutrophils from blood to milk in the present study. Consequently, the difference in pre-treatment neutrophil numbers between LC- and HC-cows could be attributed to the isolation and tethering procedure which may have induced different levels of stress in LC- and HC-cows. Differences in plasma cortisol concentrations however, were not significant in the 1 hr period prior to endotoxin-treatment and thus other factors must have contributed to the differences in neutrophil numbers at the time of the endotoxin-treatment.

Reduction in circulating lymphocyte numbers

Effects of endotoxin. Berzi et al. (1986) reported a delay of 4 to 6 hrs between an increase in glucocorticoid concentrations and a decrease in circulating lymphocyte numbers. In the current study, we found a similar delay between the endotoxin-induced increase in cortisol concentrations (maximum ca. 25 ng.ml^{-1} at 4 hr PI) and the highest depletion of lymphocytes from the circulation in both LC- and HC-cows (9-10 hr PI). GCs are generally considered to be involved in leukocyte redistribution in men (Cupps and Fauci, 1982) and rats (Dhabhar et al., 1995). In chickens (Gross and Siegel, 1983; Jones et al., 1988; Gross, 1992), in juvenile alligators (Morici et al., 1997) and in pigs (Salak-Johnson et al., 1996), GCs significantly reduced the number of peripheral blood lymphocytes. In rats, adrenalectomy significantly reduced the magnitude of stress-induced changes in lymphocyte numbers (Dhabhar et al., 1995). Circulating lymphocytes are sensitive to the effects of exogenous or endogenous GCs (Cupps and Fauci, 1982). This strongly suggests that the increase in cortisol concentrations indeed caused the observed reduction in lymphocyte numbers. As only slight differences were found in cortisol concentrations between LC- and HC-cows, however, the significant difference in reduction in lymphocyte numbers between the two groups that appeared from 9 hr PI is obviously not attributable to differences in cortisol concentrations. Therefore, other factors released during stress are likely to have contributed to the difference in circulating lymphocyte numbers.

Effects of environmental stress. After cows had been isolated, tethered and infected, HC-cows vocalised significantly more than LC-cows and had increased rectal temperatures already at the time of the endotoxin-treatment. These are indications that HC-cows were emotionally more disturbed by the changes in their environment. At 7.5 hr PI the herd was collected for milking and the milking machine started in a room next to the stanchion barn. These audible signals may have further intensified the stress of isolation in HC-cows by strengthening their motivation to join the herd. The inability to escape from such a situation is known to induce profound immunosuppression in rats (Laudenslager *et al.*, 1983).

The rate of lymphocytes disappearing from the circulation (*ie* 40% within 1 hr in HC-cows) before milking is in accordance with what has been found in rats that were restrained for 2 hr (Dhabhar *et al.*, 1995). This suggests that environmental stress may have been responsible for the sudden fall in lymphocyte numbers in HC-cows. It is not likely, however, that the rapid and large-magnitude reduction in lymphocyte numbers is attributable to increased plasma cortisol concentrations. Although cortisol concentrations in LC- and HC-cows differed significantly at 7.5 hr PI, concentrations were low and differences were small. Therefore, we assume that other factors, not dependent on HPA axis activity, must have also contributed to the drop in lymphocyte numbers in HC-cows. Neural catecholamines and endogenous opioids are considered possible candidates for these stress-induced changes in leukocyte distribution (Dhabhar *et al.*, 1995). Further research will be necessary to substantiate this hypothesis as well as to unravel the underlying mechanisms.

Clinical relevance of differences in stress-responsiveness

An important question is whether differences in stress-responsiveness will lead to increased susceptibility to infections in dairy cows. It is important to recognize that although psychological stress affects the numbers of peripheral leukocytes, leukocyte redistribution can rapidly reverse after cessation of stress (Dhabhar *et al.*, 1995). Moreover, the number of peripheral blood leukocytes is still an insensitive measure of the functional capacity of the immune system to free the animal of infectious substances. According to Ursin (1994), the answer to the question about the clinical relevance of stress-induced immunosuppression must come from sound and clinical trials and sound epidemiological research. The number of cows involved in the exploratory part of the present study is too low to draw final and valid conclusions on this point. The tendency however, that HC-cows produced less milk than LC-cows as well as previous findings which support the existence of an inverse relationship between the ease of being frightened or stressed on the one hand and growth (Hemsworth *et al.*, 1993) or milk production (Seabrook, 1994, Kovalčiková and Kovalčik, 1982) on the other are regarded

arguments to further study these relationships in larger groups of cows with genetically comparable milk production abilities.

General discussion

8.1. Introduction

The central aim of this thesis is to investigate whether individual dairy cows display different and coherent patterns of physiological and behavioural stress responses. Such responses may enable them to successfully maintain homeostasis in a frequently changing environment. On the basis of human (Ursin, 1980; Henry and Stephens, 1977) and animal (Bohus et al., 1987; Corson and Corson, 1976; Koolhaas et al., 1997, Hessing et al., 1994) literature it was hypothesized, that individual cows consistently differ in the way they cope with disturbances in their social or physical environment. Different ways of coping are characterized by a specific, integrated pattern of cognitive, emotional, behavioural and neuroendocrine responses. Consequently, similar environmental conditions (housing, management) may have different consequences for the welfare and health of different individual animals. On the other hand, taking account of individual characteristics in the daily management of groups of cows could offer possibilities to further improve the welfare and health of the individual dairy cow.

To objectively quantify physiological stress responses of individual dairy cows, methods for heart rate measurement and blood sampling were validated. Further, tests of controlled severity and reproducibility over time were developed for triggering behavioural and physiological stress responses well within biological limits. Finally, cows with strong and weak stress responses were selected and tested for differences in immune response and for consistency in stress responses over years.

First, current theories about the control of animal behaviour and the generation of emotional responses will be briefly introduced. These two topics, together with the current concept of stress (Chapter 1), will provide a basis for discussing the present findings in an integrated way. Secondly, the issue of individual differences in coping will be discussed. Finally, the question will be addressed how results from this study could contribute to the development of future management practices and breeding strategies that safeguard the welfare of high yielding individual dairy cows, also when the efficiency of quality milk production needs further improvement.

8.2. Control of behaviour

The behaviour of animals is characterized by both anticipatory actions and responses. In order to anticipate the occurrence (or non-occurrence) of events in their living environment, animals have to possess some knowledge of the relationships between current events (including their own actions) and future events. As far as this knowledge is not genetically programmed in the animal's nervous system, it must be acquired by tracking relevant events in the environment and learning about the causal relationships between them (Dickinson, 1980). This way of learning is termed associative learning. By this, and

by learning about spatial relationships, animals develop a neural representation of their living environment and of the consequences of their behaviour. Consultation of this information, called cognitive processing, allows the animal to extrapolate beyond the current state of the environment to a future time or a distant location and to utilize this extrapolation in flexible behaviour directed to the remote event.

Not all information, however, is equally reliable. This is determined by the correlation between the occurrence of events and by the variability of the position of relevant objects in the living environment. Maximum reliability is easily achieved when one event (E2) follows (or does not follow) the other (E1) in a highly predictable order or when relevant objects do not change their positions (Dickinson, 1980). On the contrary, when E2 follows E1 in a nearly random order or relevant objects change place frequently, temporal and spatial relationships will be detected only with great difficulty and the reliability of the corresponding neural information will be low. According to Dickinson (1980), behaviour is not just controlled by these cognitive or declarative processes, but also by routine-like or procedural processes. If an animal experiences that events follow each other in a predictable order and/or objects do not change their positions, the corresponding neural information will become more reliable. To improve the efficiency (cost, speed) of their behaviour, animals will then tend to shift from declarative control (action) to a more automaton like response (habit); ie procedural control. On the contrary, when events follow one another in a nearly random order, the predictability of events remains low and intensive cognitive processing is required all the time. Declarative control of behaviour offers the animal a great deal of flexibility and is therefore particularly adaptive in a changeable environment. Procedural control, in contrast, allows the animal to respond rapidly according to a fixed and regular way which may be appropriate in predictable surroundings.

8.3. Emotional expressions

When an individual cannot reliably predict relevant events in its physical or social environment, stress responses may be activated (Wiepkema and Koolhaas, 1992). These responses can be observed both as emotional expressions and measured as physiological changes at various regulatory levels. Emotional expressions will be particularly apparent in cases of a novel experience. Then, the individual does not possess a neural representation of the environment to rely on and will experience difficulties in adjusting its behaviour to what is going to occur. Novelty per se thus causes a high degree of uncertainty and is therefore seen as a potent frightening element of many stressors. This uncertainty, rather than the quality of the future event (negative or positive), determines to what extent emotional expressions and concomitant physiological responses will be activated (Wiepkema and Koolhaas, 1993). The existence of these latter responses is

well illustrated by the classical experiments of Weiss (1970, 1972). In rats, electric shock anticipated by a signal rendered less bodily harm (eg decrease in appetite, body weight loss, gastric ulceration, rise in body temperature, central catecholamine depletion) than unsignaled shock.

In cattle, emotional expressions have been observed during both play and fear. An upright tail posture, for example, gambolling, bucking, kicking and specific vocalisations ('baaocks') may be considered expressions of pleasure (Schloeth, 1961; Brownlee, 1954). Increased locomotor activity, eliminations (defecations and urinations) and vocalisations ('menh' calls; Kiley, 1972) may represent fearful expressions to social isolation (De Passilé et al., 1995). Conversely, immobility and silence are also considered indicators of fear (Boissy and Bouissou, 1995). These apparently inconsistent fear responses clearly illustrate that the degree to which specific emotional expressions emerge, eg either silence and behavioural inhibition or vocalisations and locomotor activity, depends also on the extent to which different fear-inducing stimuli (isolation, novelty) counteract in evoking specific emotional expressions. Although it is likely that dairy cows also use their body (posture, head, face, ears, eyes) for expressing their emotions, a reliable definition of these emotional expressions is still lacking.

When both the associative learning theory and the principle of emotional expression are applied to the present study, it is likely that the first exposure of cows to the novel environment (Chapter 6) evoked an emotional response (uncertainty). Many cows eliminated on their way to or during their stay in the starting box. In the box they looked backward or forward with their heads in a low or high position. Once in the novel arena, cows walked around actively and vocalised. Walking around, particularly observed during the first exposure to the novel environment, provided the cow with information about spatial details of the environment. During the second exposure to the novel environment, a decline in these expressions indicated that, on average, cows became readily acquainted with the situation. Probably, cows had experienced consistent temporal relationships between different events during the novel environment test. Some cows, for example, immediately turned around after they had entered the novel arena and moved to the swing doors, seemingly waiting to get out.

In contrast to the emotional expressions during the novel environment test, a sudden shift from the habitual side of the milking parlour to the non-habitual side did not elicit any of such expressions (Chapter 5). This was most surprising, because these cows showed a consistent preference for one of the parlour sides in almost all lactation months. It suggested that the side of the milking parlour was a significant factor for them. The absence of eliminations, the normal feeding and milking behaviour and the lack of signs of milk retention, however, indicated that this assumption was wrong. In section 8.6, I will further elaborate on possible explanations for the existence of side preference.

In contrast to what was expected, none of the cows was emotionally aroused when prevented from visiting her habitual side. Possibly, cows perceived the shift from one side of the milking parlour to the other as an irrelevant change in a highly rigid cascade of events that represented the milking routine. In other words, the side of the parlour was only of minor importance for the predictive value of the cow's neural information about the milking routine.

Another explanation for the absence of stress during enforced parlour side choice may be that cows already have learned the insignificance of parlour side, either by experience or by observation. Although some cows consistently visited one side of the parlour, many of them were also milked more than once on the other side and thus may have experienced that sides were identical as far as milking and feeding procedures are concerned. The possibility that cattle have learned the insignificance of parlour side by observing cows at the opposite side of the parlour being fed and milked in a similar way, is rather speculative but can not be excluded. Preliminary findings indicate that naïve cows allowed to observe animals which were trained to select one out of four passages to successfully reach a feeder, made fewer errors than 'non-informed' controls (Stewart et al., 1992). In this experiment, however, it is not clear whether cows used also olfactory cues to track the right passage.

Compared with the deprivation of parlour side choice, a similar unexpected lack of emotional expressions was found in dairy cows, after their calves were removed after bonding. In addition to the explanation given in Chapter 4 and since all cows had calved at least once before, these cows may have learned that the disappearance of their calf has hardly any disadvantages for themselves. Another possibility may be related to the general effects of domestication and will be discussed in section 8.7.

So far, the results of different tests can be explained from the current associative learning theory (Dickinson, 1980) and support the hypothesis that locomotor activity, elimination and vocalisation in a novel environment can be considered emotional expressions.

8.4. Physiological indicators of emotion

Stress responses originate from both the autonomic nervous system (ANS) and the hypothalamus-pituitary-adrenocortical (HPA) system. The ANS is composed of two sub-systems; the para-sympathetic and the sympathetic branch. In the sympathetic branch, a distinction can be made between the adrenomedullary system and the sympathetic nerve terminal, using adrenalin and noradrenaline as key neurotransmitters respectively. In the HPA system, corticotrophin releasing hormone, vasopressin, adrenocorticotropic hormone and corticosteroids are key regulators (Stratakis and Chrousos, 1995).

Many stressors have in common that they evoke not just emotional but also physiological responses, as indicated by changes in adrenalin, noradrenaline and corticosteroids. These physiological responses support the redirection of oxygen and nutrients to the muscles and the brain. Because of their important role in the energy metabolism, these physiological mechanisms are also activated during intensive muscular activity, albeit in combinations and at levels that are different from those situations in which physical exercise is accompanied by stress. Stress, for example, will shift the sympathetic response from neuronal outflow of noradrenaline to a more general adrenomedullary release of adrenalin (De Boer et al., 1990; Scheurink et al., 1996). This was illustrated by naïve (first swim) rats that, compared to trained swimmers, showed an exaggerated outflow of adrenalin and a reduction of the exercise-induced increase in noradrenaline (Scheurink et al., 1989). Another example of such a stress-induced increase in adrenalin comes from rats which had learned to cover an electric probe with sawdust. When the probe was presented again, but now in the absence of sawdust, a shift emerged from noradrenaline to adrenalin (Korte et al., 1992). It has been hypothesized that this shift originates from a stress-induced outflow of endogenous serotonin in the paraventricular nuclei of the hypothalamic brain area (Scheurink et al., 1996). Although the limited physiological measurements in our study do not justify far reaching conclusions about underlying mechanisms, it is tempting to speculate that such mechanisms are also involved in both cardiac and adrenocortical activation during the first exposure of cows to a novel environment (Chapter 6).

8.5. Individual differences in coping

In agreement with numerous studies in many animal species (Fokkema, 1985; Korte, 1991 in rats; Benus, 1988 in mice; Lyons et al., 1988; in goats; Hessing, 1994 in pigs), the present study indicates that dairy cows show consistent individual differences in their behavioural and physiological stress response patterns. Some cows, for example, showed a marked increase in plasma cortisol concentrations in response to repeated venepuncture whereas others did not respond or responded only slightly (Chapter 3). Likewise, profound and consistent differences were found between cows in their response (heart rate, cortisol, locomotor activity, vocalisations) to a novel environment test (Chapter 6). Coherent response patterns were found among heart rate and behavioural and adrenocortical activity. Heart rate increase, for example, as recorded when cows were on their way to the novel environment, reliably predicted walking time in the novel arena as well as the concentration of plasma cortisol at the end of the test. Furthermore, cows, classified during their first lactation as cows with either high (HC) or low (LC) cortisol responses to the novel environment test, still responded in the same way one year later. In their second lactation, HC-cows showed higher heart rate effects

and stronger adrenocortical responses than LC-cows (Chapter 6). In the second lactation, HC-cows also showed higher body temperature and vocalised more than LC-cows after they had been socially isolated. In addition, handling, related to intra-mammary injection of endotoxin, induced higher concentrations of cortisol in HC- than in LC-cows. (Chapter 7). These differences between HC- and LC-cows, one year after the original classification, strongly suggest that individual dairy cows respond consistently differently to various changes in their living environment. The strong decrease in the number of circulating blood lymphocytes, found in HC-cows but not in LC-cows (Chapter 7), further supports the existence of fundamental differences in stress response and indicates that such individual traits may also affect the dairy cows' immune system.

The question arises how these findings fit into current theoretical concepts of individual differences in coping behaviour (Koolhaas et al., 1997). When an organism is challenged, one of two basically different responses (fight-flight or conservation-withdrawal) may emerge each with its own behavioural and physiological characteristics (Chapter 1). In rats and mice, a large body of evidence has been produced that supports the existence of two general behavioural strategies or coping styles; ie active and passive (Bohus et al., 1987; Koolhaas and Bohus, 1991). Recently, Koolhaas et al. (1997) put forward that one of the most fundamental differences between active and passive coping animals seems to be the degree in which their behaviour is guided by environmental stimuli. By using this formulation, they facilitated a less rigid definition of coping style, ie a continuum of coping behaviour with quite distinct coping styles at either end. Moreover, the difference in degree in which individual animals interact with their environment could point to underlying differences in the extent their behaviour is under either declarative or procedural control.

8.6. Declarative versus procedural control of coping behaviour

This basic difference in coping behaviour, ie declarative versus procedural control of behaviour, is best illustrated with experiments by Benus et al. (1987) in male house mice. Mice were selected for either short- or long latency to attack a conspecific. After they were trained to walk through a closed-field maze, slow-attackers appeared to spend more time than fast-attackers in exploring the maze, once it had been turned 90° or after a piece of tape had been stuck on the floor of the maze. In another experiment, mice had learned to walk a specific pathway through the maze to find a food reward. When the configuration of the maze was changed, fast-attackers made more errors than slow-attackers. The authors suggest that the slow-attacking, ie passive coping animals, spent more time on exploring environmental stimuli than fast-attackers, ie active coping animals. The latter animals readily developed behavioural routines.

In the experiment described in Chapter 5, strong evidence was found that dairy cows also differ in propensity to develop behavioural routines. Differences between dairy cows in consistency of parlour side choice, may be regarded as differences in routine behaviour. Despite some cows showing a distinct individuality in preference for a particular parlour side during the whole lactation period, the parlour side itself seemed to be of minor significance for them. The question arises why cows persist in visiting a particular side of the milking parlour when parlour side as such is apparently irrelevant?

Imagine heifers which are to be milked in a two sided milking parlour for the first time in their life. They may visit a particular parlour side because it has some advantage for them (eg less competition). Other possibilities are that they visit a side completely by chance or that they are forced by conspecifics or man to choose a particular parlour side. During milking, animals are fed concentrates. Apart from which motivation under-lies their choice, heifers are able to quickly learn associations between a specific place (parlour side) and a food reward (Kovalčik and Kovalčiková, 1986). According to Olton's theory of optimal foraging behaviour (Olton et al., 1981), animals may either return to the location where they were last successful in obtaining food (win-stay strat-egy), provided that they did not experience food depletion, or may shift to a new forag-ing site at the start of a new foraging expedition. A win-stay strategy is efficient when food is of high quality and clumped; a win-shift strategy is appropriate when food is variable in quality and is dispersed in the environment (see also Thomas et al., 1985). Rats in different habitats adopt different foraging strategies, although they seem to have an inherent tendency to shift after a successful foraging bout (Olton et al., ref. cit.).

Assuming that cattle under primeval conditions used similar strategies, it is likely that during migration in open, prairie-like country with low quality vegetation the win-shift strategy appeared to be appropriate, whereas a win-stay strategy was more efficient in sedentary populations in fertile, bushed or wooded habitats. It is tempting to specu-late that such differences in foraging strategy have been conserved during domestication and are now reflected in differences in side preference. Hosoi et al. (1995) studied this phenomenon in an artificial setting. They offered a feeding trough with concentrates in either arm of a Y-maze. In six sessions each including three trials, cows were repeatedly given access to either arm of the maze to select one arm for feeding. Cows were re-moved before the food was depleted. It appeared that some cows adopted the win-stay strategy already after their first trial. These cows probably associated a particular arm of the Y-maze with food. Other cows, however, visited the two arms of the maze in a random order. Although only four cows were used in this study, the results at least support the idea that cows may adopt different foraging strategies.

When cows are milked in a two-sided milking parlour, individual animals that tend to adopt a win-stay strategy may still differ in ability to persist in visiting their preferred side. This will lead to a further divergence in the degree in which successive

choices will be consistent. Cows that adopted a win-stay strategy and were able to effectively practise this strategy as well, thus maximized the predictability of their choice. According to Dickinson (1980), it is likely that these cows will tend to procedural control of parlour side choice to further reduce (cognitive) processing capacity. Cows, however, which are less able to control their social environment, will often be prevented by herd members from realizing their initial choice. These cows will thus enter a particular parlour side according to circumstances and require more flexible, declarative processes to control their behaviour. Based on this reasoning, it is possible that differences in consistency in parlour side choice are influenced by differences in foraging strategy, in coping behaviour or in social rank. The fact that two beef heifers which displayed escape behaviour and agitation (flight) were also the most resistant to spatial changes in a Y-maze, whereas calmer animals tended to vacillate more at the decision-point (Grandin et al., 1994) may be considered circumstantial evidence for the existence of a relationship between consistency of side choice and coping style in dairy cows. A controlled study of the early development of parlour side preference in dairy cows, in combination with additional tests for coping style, foraging strategy and social rank may further substantiate this hypothesis. In addition, it may be interesting to also include the position of the hair whorl on the forehead of a cow in this study because of its relationship with both temperament (Grandin et al., 1995) and parlour side preference (Tanner et al., 1994).

8.7. Coping strategies in Holstein Friesian dairy cows

In contrast to the removal of the calf (Chapter 4) and the deprivation of parlour side choice (Chapter 5), the novel environment test appeared provocative enough for triggering a stress response. Therefore, it could be used for distinguishing cows with different coping behaviour (Chapter 6). We should, however, be cautious when interpreting these differences in terms of distinct coping styles as reported in rats and mice. Genetic selection for certain traits may actually mean co-selection for a specific coping style. Differences in selection traits between rodents and Holstein Friesian dairy cows may thus have disturbed an original frequency of active and passive coping individuals in the population.

Distinct coping styles were found in territorial male rats and mice between genetic lines or individuals that differ remarkably in species-specific offense reactions (attack latency). The Tryon Maze Dull S3 rat, for example, used in many of the social defeat tests (Fokkema, 1985; Koolhaas et al., 1990; Fokkema et al., 1995), is known as a highly aggressive animal. Dairy cows, however, have been selected against vigorous defence (fight/flight) reactions for thousands of years. According to studies of behavioural differences between wild and domestic animals, domestication implies the following general-

izations (Hemmer, 1990). Compared to their wild ancestors, domestic animals, unless selected for aggression, are less ready to flee or fight and have weaker alarm reactions. Their behaviour is less controlled by chronobiological stimuli, they have looser social bonds and have intensified sexual behaviour. It is obvious that these changes are associated with changes in brain volume and function. Domesticated ducks (Ebinger, 1995) and pigs (Plogmann and Kruska, 1990), for example, have smaller brain volumes than their wild conspecifics. As to brain function, a reduced density of $5-HT_{1A}$ receptors in the brains of domesticated rats (Hammer et al., 1992) and silver foxes (Popova et al., 1991) suggest the involvement of central serotonin in the mechanism of domestication. In the domesticated silver fox, this change is associated with a dramatic decline in flight distance. A similar relationship between $5-HT_{1A}$ receptor density and behaviour has been found in mice. Animals, classified as non-aggressive (passive), showed lower postsynaptic $5-HT_{1A}$ receptor numbers in limbic and cortical regions than aggressive (active) animals (Korte et al., 1996b). It is likely that similar mechanisms favoured a shift to the less intense, passive and more flexible coping behaviour in domesticated cattle. These basic changes are illustrated in Fig. 8.1. The vertical axis represents the strength of behavioural and physiological stress responses; the horizontal axis represents the degree of the environmental change. Due to domestication, similar changes in the social or physical environment (A in Fig. 8.1) induce different behavioural and physiological stress responses in wild and domesticated animals.

Domestication, however, probably affected the behaviour of different cattle breeds to different degrees. In juvenile (< 1 yr) Limousin cattle, for example, 2-3 % of the animals reacted violently to human approach when separated from their conspecifics (Bonnet, 1992; Le Neindre et al., 1995). In addition, social isolation induced more struggling and stronger cardiac and adrenocortical responses in Aubrac than in Holstein heifers (Boissy and Le Neindre, 1997). And finally, in contrast to what we observed in Holstein Friesian dairy cows, active attempts to jump out of a starting box were reported on beef crossbreds by Grandin et al. (1994). Besides differences in behavioural responses, physiological stress responses may also differ remarkably between breeds. The average adrenocortical response to a 5-min novel environment test, for example, was about 5 times higher in Spanish fighting breed cows (Sánchez et al., 1996) than in Holstein Friesian dairy cows (Chapter 6). The reactivity of beef cattle, compared with dairy cattle, thus may be closer to that of their ancestors (Fig. 8.1).

Different breeds, however, are mostly kept for different purposes (beef, milk) and under different management conditions (extensive, intensive). Intensive rearing conditions (indoor housing, regular handling, feeding) lead to docile animals showing little aggression (Le Neindre et al., 1996).
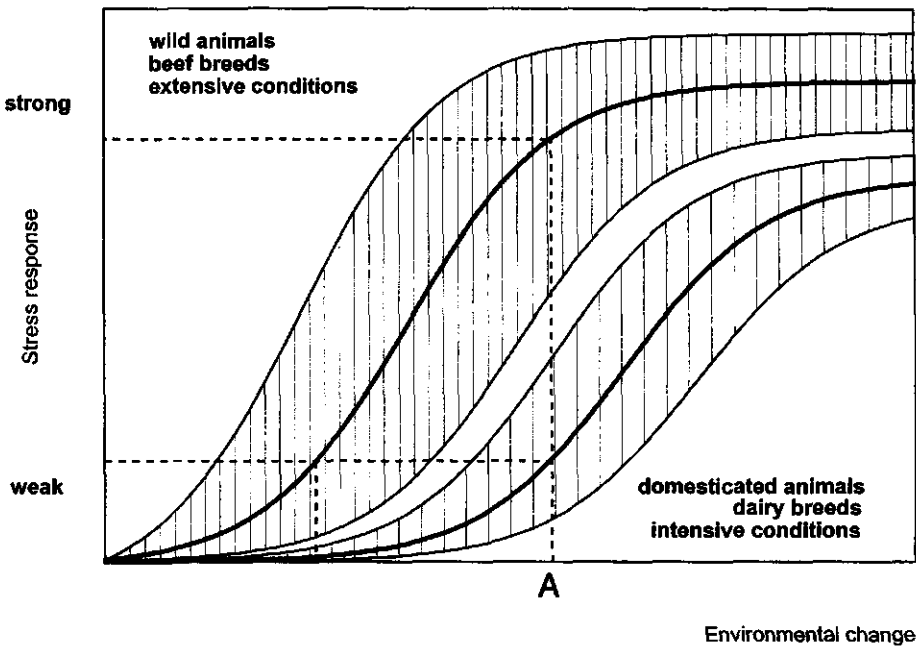
Figure 8.1. Representation of the strength of the stress response of cattle in relation to the extent of environmental change, according to differences in domestication, breed and husbandry conditions between cattle populations. The upper line represents beef cattle under extensive conditions; the lower line represents dairy cattle under intensive conditions. Dashed areas indicate population variance.

In addition to the effects of domestication, intensive rearing conditions and one-sided selection for milk production may therefore have enhanced the decline in emotional reactivity in Holstein Friesian dairy cows (Fig. 8.1). This will probably be associated with corresponding ontogenetic and genetic changes in neural and endocrine mechanisms.

As to genetic changes, prolactin is critical to the establishment of lactation and milk production (Ostrom, 1990). It is not surprising therefore, that milk production level is positively correlated with plasma concentrations of prolactin (Klindt, 1988). On the other hand, prolactin levels appeared to be negatively correlated with activity and exploration in rats (Marti-Carbonell et al., 1993). Similar relationships may thus argue for a further weakening of the stress responses in Holstein Friesian dairy cows because of selection for milk production.

Finally, it is likely that the exclusive use of female animals in our experiments whereas only male animals were used in the majority of rodent studies of coping styles, may also have been involved in the degree to which distinct coping styles emerge. Gonadal steroids underlie the anatomical, morphological and physiological differentiation of an indifferent embryo into a male or female phenotype. According to Leshner

(1978, 1979), androgens modulate behaviour in two distinct ways, ie permanently and/or transiently. Permanent and irreversible effects, ie organizational effects, result from the early androgen state during a critical period early in maturation (perinatal period).

Organizational effects alter the anatomical characteristics of the brain, change the sensitivity of specific neuronal sites to hormones circulating in adulthood and produce permanent changes in the functioning of the adult endocrine system. Transient effects, ie activational effects, are due to increased hormone concentrations at the time a behaviour occurs. It can thus not be excluded that differences in both organizing and activating effects of androgens may have important consequences for the way individuals cope with specific difficulties in their environment. Testosterone, for example, is related to the initiation of behaviour (Ursin, 1980) and may thus be associated with active coping (Koolhaas et al., 1997). Effects of testosterone as well as differences between male and female, however, depend largely on the experimental paradigm. In a social defeat test, for example, plasma testosterone levels were positively correlated with offensive aggression (Schuurman, 1980). Likewise, in an operant learning experiment, male rats contacted the lever earlier and more frequently than females (Van Hest and Van Haaren, 1989). In an open-field test, on the other hand, female rats have been observed to be more active and to explore more than male rats (Beatty, 1979; Van Hest et al., 1987; Gray, 1991).

The tendency that female dairy calves spent more time than males in completing a maze when intra-maze cues were faced for the first time, may also be explained by relatively more exploration in the female animal. Likewise, the fact that females took two-thirds the time of males to complete the maze during their second exposure, may have resulted from increased behavioural activity in female calves (Arave et al., 1992). Further evidence for fundamental differences in behaviour between the two sexes comes from studies in sheep and poultry and is related to differences in fearfulness. During a novel environment test in sheep, females showed higher locomotor activity than males, they ate for a shorter time, vocalised more often and sooner and spent more time away from a novel stimulus (Vandenheede and Bouissou, 1993). Similar findings were reported in seven-day-old domestic chicks. Female chicks were markedly more active in a novel environment than males and the frequency of distress calls was also far higher in females (Jones, 1977). These results are interpreted as males being less fearful than females.

In pigs, distinct coping styles were reported by Hessing et al. (1994). In this study, both female piglets and castrates were used, but differences in coping style between these groups were not analysed. Evidence for organizational influences of testosterone on coping style in piglets was produced by Van Erp-van der Kooy et al. (1997). They used Hessing's 'back-test' for determining coping styles in 826 piglets. It appeared that

females (1/2) were more often classified than males (1/3) as high resisting *ie* active coping piglets. As to the activating effects of testosterone, treatment of heifers with testosterone reduced their behavioural activation and adrenocortical response in social and non-social situations (Bouissou, 1990; Boissy and Bouissou, 1994).

Based on all these findings, it is questionable how active and passive coping behaviour relate to fundamental differences in feminine and masculine brain, behaviour and physiology. In a population of females, the passive coping style is likely to dominate whereas in males, active coping behaviour may be the dominating style. These differences between males and females are associated with differences in fearfulness, *ie* males being less fearful than females. If this assumption is right, passive coping behaviour may thus be the dominating style in Holstein Friesian dairy cows.

To sum up, cumulative effects of domestication, intensive rearing and handling, one-sided selection for milk production and a feminine brain may have weakened the stress response of Holstein Friesian dairy cows. It is most likely that such forces have shifted the coping behaviour of Holstein Friesian dairy cows to a more passive style. More research, however, is necessary to substantiate this hypothesis and I therefore consider it premature to draw final conclusions about the existence of different coping styles in dairy cows. Compared with studies in rodents (Koolhaas *et al.*, 1997), the number of parameters as well as the diversity of experimental paradigms in this thesis is limited. To further investigate the existence of coping styles in Holstein Friesian dairy cows, tests in which animals are enticed to explore novel objects or unknown conspecifics in the relative absence of fear (eg in their home environment), may be particularly useful. In addition, in different tests various behavioural, physiological and neurochemical characteristics of individual animals need to be studied in an integrated way.

8.8. Implications for dairy management and breeding.

Stress responses in cattle have been associated with fear (Boissy, 1995). In threatening situations, therefore, differences in stress response may reflect differences in fearfulness. Fear in dairy cows can seriously impair their welfare and performance, especially when restrictions imposed by dairy farming systems do not allow the cow to express appropriate and adaptive responses to fear-inducing stimuli (see for review Boissy, 1995; Jones, 1996). Moreover, the freedom from fear is considered one of the five requirements for good animal welfare (Farm Animal Welfare Council, 1992). Although fear responses are of vital importance for cattle to survive in their natural environment, they may be extremely self-damaging when the animals are kept in an artificial, restrictive environment. Besides, in large and heavy animals like dairy cows, fear-reactions can easily lead to handling problems that may also endanger the stock person's safety (Grandin, 1993).

As shown in Chapter 7, stress in cows is associated with changes in their immune system. HC-cows showed a dramatic and persistent decrease in peripheral lymphocyte numbers in contrast to LC-cows. At the same time, HC-cows vocalized more than LC-cows and showed a higher body temperature. At this moment, it is not known how this relates to disease susceptibility. In this context, it is important to know what mechanisms underlie the reduction in circulating lymphocyte numbers. Which sub-populations of lymphocytes are most affected during stress and to what extent do these lymphocyte sub-types from HC- and LC-cows differ in expression of specific stress hormone receptors and/or adhesion molecules? Moreover, it is necessary to study if such an immunological state implies a compromise of functional qualities of the immune system. The model presented here may offer new possibilities for studying the relationship between stress and disease in dairy cows. At this moment, however, it seems premature to conclude that dairy cows will be rendered susceptible for infectious diseases by stress.

The tendency that milk production is negatively correlated with the strength of the cow's response to stress, is also an intriguing finding (Chapter 7). It is likely that such relationships may become particularly apparent in environments that lack social and physical stability. As long as environmental changes are within a certain range (Fig. 8.1), stress responses will hardly emerge in any of the cows. Beyond this range, however, similar changes in the environment will affect different cows differently. In highly reactive (fearful, nervous) cows in changeable environments, a wide variety of physical and social factors may frequently activate the stress response. This means that in such cows, anabolic processes may be regularly dominated by catabolic processes thus impairing growth, reproductive function, immunologic function and milk production (Stratakis and Chrousos, 1995). A possible relationship between fearfulness and performance traits may offer new perspectives for studying interactions between genotype and environment and argues for further studies into these relationships in larger groups of cows in a wide variety of environments.

Handling treatments that evoke strong stress responses may markedly impair productive and reproductive performance in farm animals and influence manageability as well (Hemsworth, 1997). Dairy cows will frequently encounter man (milker, stockmen, AI-men, veterinarian) who apply some kind of painful or nasty treatment eg hoof trimming, ear-tagging, intramuscular injection, jugular venepuncture or palpation of the reproductive tract. Depending on the individual's fearfulness, such manipulations may activate their stress response to different degrees (Nakao et al., 1994; Alam and Dobson, 1986). As has been indicated by a lack of adrenocortical response in Chapter 3, repetitive handling and habituation of cows to such treatments can help them to adapt, at least to human contact. Moreover, adverse treatments carried out in a special place appeared to be less likely to lead to a generalized stress response to human contact

which will be apparent in other places (Munksgaard et al., 1997; De Passilé et al., 1996). Cows apparently link aversion to a specific location when such a place is a better (ie more reliable) predictor of the treatment than human contact. This argues for a special treatment box on dairy farms where cows can be treated individually, but still in contact with other cows.

Cattle may become more fearful because of lack of human contact, particularly at a young age (Le Neindre et al., 1996). When herd size is growing and cows are fed and milked by automatons, close contact with man will be easily limited to situations that involve some kind of adverse treatment. As a result, cows will develop negative associations with human contact and will show larger flight distances. To prevent such situations developing, herdsman should spend time specifically handling their cows in a positive way eg by touching, stroking, scratching and feeding them and by talking to them. In this way, cows will also develop positive associations with human contact (Albright and Arave, 1997). Beef cattle studies indicate that these contacts are much more effective when they occur at a young age in connection with feeding (Boivin et al., 1992). Further studies are needed in Holstein Friesian dairy cattle to determine both the frequency and intensity of handling experience which may be required during specific phases of the rearing period to establish good human-animal relationships. Such relationships between dairy cows and human caretakers are important and more needs to be known about the factors influencing this.

Apart from improvements in management, selective breeding against vigorous stress responses is also important to consider. Stress responses are, at least partly, genetically programmed. It is not surprising, therefore, that there is a strong demand for inclusion of measures of fearfulness in breeding programmes in countries with relatively extensive dairy production systems (Morris et al., 1994; Bowman et al., 1996). The treatment presented in Chapter 6 offers promising opportunities for the objective assessment of an underlying characteristic or psychobiological profile, perhaps fearfulness. By developing breeding strategies, rearing conditions or management procedures that further reduce the fearfulness of farm animals, not only productivity but also the health and welfare of both animals and man can be further improved.

Summary

Stress, coping and individual differences

The central aim of this thesis is to investigate whether individual dairy cows display different and coherent patterns of physiological and behavioural stress responses. Such responses enable them to successful adapt in a changing environment.

In Chapter 1, current concepts of adaptation and stress are introduced. Adaptation is necessary when the individual's need to perform specific behaviour, does not match the current or anticipated perceptions of the internal or external environment. Such a condition is termed *stress*. Physical and/or psychological factors that cause, support or magnify such a mismatch are called *stressors*. The behavioural and physiological responses that compensate this discrepancy are termed *stress responses*. Adaptation can be measured as the fade out of these responses.

The degree in which adaptation is accompanied by stress, is primarily determined by uncertainty, perceived by the organism, when it is not clear <u>how</u> and <u>if</u> adaptive changes can be realized. Individuals may differ remarkably in the way they cope with this problem. In such a situation, broadly speaking, their behaviour ranges between actively avoiding or tackling the problem and passively undergoing it. These two behavioural patterns strongly resemble the classical stress responses, *ie* fight/flight versus conservation/withdrawal, and are characterized in rodents and man by a specific, integrated pattern of cognitive, emotional, behavioural and physiological responses, termed *coping strategies* or *coping styles*.

Measuring stress responses in dairy cows

The active coping style is characterized by active behavioural responses as well as by dominating sympathetic activity. Increased concentrations of primarily noradrenaline and to a lesser extent adrenalin and glucocorticoid accompany active coping responses. Behavioural inhibition and activation of both the adrenomedullary and the hypothalamus-pituitary-adrenocortical systems is typical of the passive coping style. Passive coping is associated with increased concentration of adrenalin and corticosteroids and to a lesser degree also of noradrenaline.

Increase in heart rate is suitable for measuring dominating sympathetic activity. Plasma concentrations of cortisol are used for estimating adrenocortical activity. To reliably measure these two parameters in dairy cows, methods were developed for both the recording of heart rate and the 'stress-free' collection of blood samples.

For heart rate measurements in dairy cows, the Polar® Sport Tester has been modified and validated (Chapter 2). Simultaneous heart rate recordings with both the Polar® and classical ECG-equipment indicated significant correlations between the measurements when cows were quietly standing (0.88) or walking on a treadmill (0.72).

Artefacts, caused by muscle contraction, could be easily recognized by their characteristic heart rate patterns. Accordingly, missing values instead of erroneous measurements were produced.

A method for collecting only a few blood samples from many cows is reported in Chapter 3. Evidence is produced that baseline cortisol concentrations can be measured in single blood samples that are collected by jugular puncture within 1 min of first approaching the cow. To prevent handling from confounding cortisol concentrations, it is necessary that cows are accustomed to handling and to being restrained. When blood samples need to be collected repeatedly, however, jugular puncture may induce an increase in cortisol concentrations which seems to depend on the handling experience of the animal and on individual differences.

Individual differences in stress response

The separation of cow and calf, 2-3 days after calving, evoked only a slight increase in heart rate in cows during the first minute after separation (Chapter 4). During the first 10 min after separation, no other behavioural (activity, vocalisations) or physiological (heart rate, cortisol) signs of stress could be detected. This indicated that the removal of the calf after bonding could not be used for triggering an acute stress response in dairy cows in further experiments.

In Chapter 5, the preference of dairy cows for visiting a particular side of the milking parlour has been studied in the light of evidence in mice that active coping animals easily develop behavioural routines. Marked differences were found between individual cows in consistency of parlour side choice. Some cows systematically visited one side of the parlour for a longer time, whereas others alternated randomly. Social factors hardly influenced this individual trait. It was surprising, however, that in cows which consistently visited one side of the parlour, deprivation of choice hardly elicited any stress responses (behaviour, heart rate, milk production). Side preference of dairy cows in the milking parlour thus seemed to be a consistent behavioural routine with only unimportant implications for the welfare of cows if it were to be interrupted.

In Chapter 6, the short- and long-term consistency of behavioural and physiological responses of dairy cows which were repeatedly tested in a 'novel environment' test is described. Individual cows showed consistent and individual-specific stress responses. Consistency appeared in behaviour, in heart rate and in plasma cortisol concentrations within one week. Consistency of individual responses was also found for heart rate and plasma cortisol concentrations when tests were spaced 1 yr apart. Handling prior to the exposure to the novel arena, besides the exposure itself, appeared to be an important stress-inducing element in the novel environment test. The study produced clear evidence that individual dairy cows differ consistently in the degree to which they respond

to environmental challenge, ie a combination of novelty, isolation and handling. The treatment offers exciting opportunities for the objective assessment of an underlying characteristic or psychobiological profile, perhaps fearfulness.

Individual immunological characteristics

Ten cows with low and eight cows with high plasma cortisol concentrations in response to the short stay in novel environment, were selected out of the group of 58 heifers. Low- and high responders were labelled LC- and HC-cows respectively. After one year, while in second parity, these cows were separated from herd-mates one after another and isolated and tethered for 55 hr in a stanchion barn (Chapter 7). Intra-mammary administration of E. coli endotoxin produced an acute and transient mastitic episode in all cows with only mild mastitic and systemic reactions. As far as their response to endotoxin is concerned, HC- and LC-cows responded similarly. In response to isolation, however, HC-cows showed stronger stress responses than LC-cows, as indicated by a higher increase in rectal temperature, in cortisol concentration after injection of endotoxin and in the number of vocalisations. Between 8 and 10 h post injection (PI) the number of circulating lymphocytes in HC- but not in LC-cows decreased markedly (40%) to 1.58×10^6 cells.ml^{-1} and remained so until 21 h PI. These results show that the stress response of dairy cows during social isolation is associated with the number of peripheral blood leukocytes after intra-mammary administration of endotoxin. Because plasma cortisol concentrations hardly differed between HC- and LC-cows, noncorticosteroid factors are likely to be involved.

Discussion

In chapter 8, current theories about the control of animal behaviour and the generation of emotional responses will be briefly introduced. These two topics, together with the current concept of adaptation and stress, provide a basis for discussing the findings of this study in an integrated way. The question is addressed why the dichotomy between active and passive coping animals, as reported in rats and mice, is likely to be different in dairy cows. Cumulative effects of domestication, intensive rearing and handling, one-sided selection for milk production and a feminine brain may have weakened the stress response of dairy cows. Therefore, distinct coping styles may be distinguished, although it is likely that such forces have shifted the coping behaviour of dairy cows to a more passive style. Finally the question is addressed how results from this study could contribute to the development of future management practices and breeding strategies.

Samenvatting

Stress, coping en individuele verschillen

Onderzoek bij gewervelde dieren heeft aangetoond dat individuen sterk kunnen ver-
schillen in de wijze waarop zij zich aanpassen aan plotselinge veranderingen in hun
sociale en/of fysieke omgeving. Een aanpassing is vereist als de behoefte van het indivi-
du om een bepaald gedrag uit te voeren niet overeenstemt met de mogelijkheden die
het heeft (of denkt te hebben) om dit in de gegeven of verwachte omstandigheden te
realiseren. In een dergelijke toestand is er sprake van **stress** of spanning. Fysieke en/of
psychologische factoren die deze toestand veroorzaken noemen we **stressoren**. Stress
kan worden opgeheven door veranderingen in de omgeving of door structurele aanpas-
singen in het gedrag en/of de fysiologie van het individu.

De mate waarin een aanpassing gepaard gaat met stress, wordt vooral bepaald
door de onzekerheid die een organisme ervaart als niet duidelijk is <u>hoe</u> en <u>of</u> de nood-
zakelijke aanpassing kan worden bereikt. Het gedrag, dat een individu in zo'n situatie
laat zien, varieert globaal tussen actief aanpakken of ontwijken van het probleem dan
wel passief de problemen ondergaan. Deze twee gedragspatronen vertonen sterke
overeenkomsten met de klassieke stressresponsen i.c. vechten/vluchten versus afwach-
ten/terugtrekken en worden gekenmerkt door een specifiek, geïntegreerd patroon van
cognitieve, emotionele, gedragsmatige en fysiologische reacties. Men noemt dit **'coping'**
strategieën. De neiging van het individu om in belastende omstandigheden in meer of
mindere mate te kiezen voor één van beide strategieën is afhankelijk van erfelijke
factoren, en van ervaring. De feitelijke keuze hangt daarnaast samen met de
keuzemogelijkheden die de situatie biedt.

Inmiddels is duidelijk dat niet alleen mensen er een eigen manier van omgaan met
stress op na houden, maar dat ook bij o.a. ratten, muizen en varkens individuele ver-
schillen in stressreactie kunnen worden aangetoond. Bij melkkoeien is echter weinig
bekend over het bestaan van dergelijke, aan het individu gebonden gedrags- en
fysiologische kenmerken. Inzicht in het bestaan ervan biedt wellicht mogelijkheden om
de omstandigheden op het melkveehouderijbedrijf beter af te stemmen op de indivi-
duele eigenschappen van melkkoeien, waardoor het welzijn van het individu kan
worden verbeterd.

Meten van stressreacties bij melkkoeien

Belangrijke fysiologische systemen die worden geactiveerd tijdens stress zijn het auto-
nome zenuwstelsel en het hypothalamus-hypofyse-bijnierschors-systeem. Het autonome
zenuwstelsel bestaat uit twee takken, de parasympatische en de sympatische tak. De
sympatische tak kan op zijn beurt weer worden verdeeld in het bijniermergsysteem en
de sympatische zenuwen, met respectievelijk adrenaline en noradrenaline als belang-

rijkste neurotransmitters. In het hypothalamus-hypofyse-bijnierschors-systeem spelen 'corticotropin releasing'-hormoon, adrenocorticotropine, vasopressine en corticosteroïden een sleutelrol.

Een overheersende sympatische activatie is kenmerkend voor een actieve stressrespons en gaat gepaard met een verhoging van vooral de concentratie van noradrenaline en in mindere mate adrenaline en glucocorticoïden. Bij een passieve respons is vooral sprake van een geactiveerd hypothalamus-hypofyse-bijnierschors-systeem, te onderscheiden als een verhoging van vooral adrenaline en corticosteroïden en in mindere mate noradrenaline.

Als maat voor de sympatische activiteit is de verhoging van de hartslag geschikt. Activiteit van de bijnierschors wordt geschat aan de hand van de concentratie van cortisol in het plasma. Teneinde beide parameters bij koeien betrouwbaar te kunnen vaststellen zijn technieken ontwikkeld voor het meten van de hartslag en het 'stressvrij' verzamelen van bloedmonsters.

Voor het meten van de hartslag bij melkkoeien is een voor humaan gebruik ontwikkelde hartslagmeter, i.c. de Polar® Sporttester, aangepast en gevalideerd (Hoofdstuk 2). Hiervoor werden bij 10 melkkoeien hartslagmetingen verricht met de Polar® en tegelijkertijd met klassieke ECG-apparatuur. Bij stilstaande dieren was de correlatie tussen beide metingen 0.88; tijdens lopen op een tredmolen daalde deze tot 0.72. Met name bewegingsartefacten bleken de metingen te kunnen verstoren. Situaties die, op basis van het karakteristieke meetpatroon, duidelijk herkenbaar waren en als zodanig dus wel ontbrekende maar geen foutieve waarnemingen opleverden. De apparatuur was dus bruikbaar en koeien in een ligboxenstal bleken er bovendien geen hinder van te ondervinden.

Om na te gaan in welke mate de stress of het verwachte ongerief van bloedafname via punctie van de halsader bij melkkoeien activatie van de bijnierschors tot gevolg zou kunnen hebben, zijn verschillende experimenten uitgevoerd (Hoofdstuk 3). Bij 20 melkkoeien die eraan gewend waren om tijdens de opname van ruwvoer enige tijd vast te staan in het voerhek had het snel (< 60 sec) en éénmalig afnemen van bloed geen significant effect op de cortisol concentratie, 18 minuten later. Na zo'n interval zou bij melkkoeien logischerwijs een stijging te verwachten zijn. Evenmin waren er aanwijzingen dat deze koeien bij herhaald puncteren (éénmaal daags, 6-12 dagen achtereen) een geconditioneerde cortisolrespons ontwikkelden. Bij 10 vaarzen die minder gewend waren aan dit soort handelingen bleken daarentegen grote individuele verschillen te bestaan in plasmacortisolreacties onder invloed van herhaald (5 maal in één uur gedurende 3 dagen) afnemen van bloed via venapunctie.

Individuele verschillen in stressrespons

Om de stressrespons te activeren werd bij 8 koeien die 2-3 dagen eerder hadden afgekalfd, en waar het kalf sindsdien bij was gebleven, het kalf weggehaald (Hoofdstuk 4). Bij geen van de koeien was er in de 10 minuten na de verwijdering van het kalf sprake van duidelijke tekenen (gedrag, hartslag, cortisol) van stress. De hartslagverhoging die er was, was gering en duurde slechts 1 minuut. Voor het opwekken van een acute stressrespons bij de melkkoe bleek het verwijderen van het kalf dus niet geschikt.

Geïnspireerd door het routinematige gedrag van muizen met een actieve copingstrategie is bij melkkoeien nagegaan in hoeverre individuele koeien voortdurend dezelfde kant van de melkstal bezochten, dan wel dit deden volgens een willekeurig patroon (Hoofdstuk 5). Drieëntwintig van de 89 onderzochte koeien in de koppel bleken gedurende langere tijd stelselmatig één van de beide kanten van de melkstal op te zoeken. Andere dieren daarentegen wisselden frequent. Sociale factoren bleken deze individuele eigenschap nauwelijks te beïnvloeden. Koeien met een duidelijke voorkeur die gedwongen werden om zich aan de andere kant van de stal te laten melken, reageerden hier nauwelijks op (gedrag, hartslag, melkproduktie). De keuze van de melkstalzijde lijkt bij melkkoeien dus vooral een (onbelangrijke) gewoonte; verstoring ervan heeft niet tot gevolg dat een stressrespons wordt geactiveerd.

Een derde test bestond uit het verplaatsen van een koe vanuit de koppel naar een onbekende omgeving, gevolgd door een kort, geïsoleerd verblijf (Hoofdstuk 6). Deze test wordt de 'novel environment'-test genoemd. In totaal werden 20 koeien en 58 vaarzen op deze manier getest. Bij elk van de koeien werd de test drie keer uitgevoerd, bij iedere vaars tweemaal. De test had een acute verhoging van de hartslag tot gevolg en een toename in cortisolconcentraties en in bewegingsactiviteit. Tussen individuele dieren bleken consistente verschillen te bestaan in reactie op de test zowel op korte (één week) als op lange termijn (één jaar). Sommige koeien vertoonden een grote toename in hartslag en plasmacortisolconcentraties en waren bijzonder actief, terwijl andere koeien slechts in geringe mate reageerden op de test. Ook bij melkkoeien heeft eenzelfde situatie dus voor verschillende dieren sterk uiteenlopende gevolgen. Vooral het nauw contact met de mens bleek een belangrijke stress-inducerende stimulus.

Individuele immunologische kenmerken

Op basis van de cortisolreactie tijdens de eerste 'novel environment'-test zijn uit de genoemde 58 vaarzen twee groepen samengesteld van dieren met een lage (LC) dan wel met een hoge (HC) cortisol concentratie. Één jaar later zijn deze, inmiddels tweedekalfs dieren, gedurende iets minder dan twee-en-een-halve dag opgestald in een grupstal, afgezonderd van soortgenoten (Hoofdstuk 7). Door middel van toediening van

endotoxine van de E-coli-bacterie in één van de uierkwartieren werd hier bij de dieren een afweerreactie opgewekt. Alle koeien, ongeacht de groep, vertoonden een respons die karakteristiek is voor de afweerreactie op endotoxine. HC-koeien hadden na opstallen een verhoogde lichaamstemperatuur (0.59°C) en loeiden gemiddeld 595 keer gedurende de 8 uur dat dit werd waargenomen. LC-koeien lieten daarentegen geen temperatuursstijging zien en loeiden in de waarnemingsperiode slechts 81 keer. Rondom melkenstijd vertoonden HC-koeien bovendien een plotselinge (binnen 1 uur) en sterke (> 40%) daling van het aantal lymfocyten in het bloed. Deze hield aan gedurende meer dan 12 uur. LC-koeien vertoonden deze daling niet. Dit bevestigt niet alleen dat tijdens stress HC-koeien consistent anders reageren dan LC-koeien, maar suggereert bovendien dat ze andere neuro-endocriene mechanismen activeren waardoor ook het immuunsysteem verschillend wordt beïnvloed. Het ontbreken van verschillen in cortisolconcentraties tussen HC- en LC-koeien doet bovendien vermoeden dat mechanismen, die niet aan bijnierschorsactiviteit zijn gerelateerd hierin een rol spelen.

Discussie

In de algemene discussie wordt kort uitleg gegeven over huidige theorieën met betrekking tot de regulatie van gedrag en het ontstaan van emoties. Deze vormen de achtergrond waartegen de resultaten van de verschillende hoofdstukken in dit proefschrift worden besproken. Vervolgens wordt besproken waardoor het beeld van een tweedeling in stressreakties, zoals dit voor ratten en muizen wordt geschetst, voor melkkoeien anders zou kunnen zijn. Domesticatie, selectie op melkproduktie en het feit dat koeien behoren tot het vrouwelijk geslacht hebben er waarschijnlijk belangrijk aan bijgedragen dat onze huidige melkkoeien over het algemeen niet erg sterk reageren op veranderingen in hun omgeving. Eventuele verschillen in copingstrategie zijn daardoor lastig te onderscheiden. Tenslotte wordt kort stilgestaan bij verwachte ontwikkelingen in de melkveehouderijsector en de gevolgen daarvan voor de hanteerbaarheid van melkkoeien.

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Curriculum Vitae

Hans Hopster werd geboren op 25 februari 1955 te Hoogland (Utr.) onder de rook van het CLO-Instituut voor de Veevoeding 'De Schothorst'. In 1971 behaalde hij het MULO-B diploma aan de Chr. Andreas MAVO in Amersfoort. Daarna doorliep hij het middelbaar technisch onderwijs aan de MTS voor de Autotechniek in Apeldoorn, waar hij in 1975 zijn vakdiploma verkreeg. Vervolgens volgde hij het hoger agrarisch onderwijs, eerst aan de Bijzondere Hogere Landbouwschool in Leeuwarden, daarna aan de Christelijke Hogere Landbouwschool in Dronten. In 1979 behaalde hij daar zijn diploma in de differentiatierichting Veehouderij. Op 1 augustus 1980 werd hij aangesteld bij het landbouwkundig onderzoek. Tot 1993 was hij werkzaam als technisch onderzoeksmedewerker bij het voormalig Instituut voor Veeteeltkundig Onderzoek (IVO-DLO) in Zeist. Een periode die in 1986 tijdelijk werd onderbroken door een aanstelling als bedrijfsleider van het melkgeitenbedrijf HVA-Almeerderhout bv te Almere. Vanaf 1993 werkte hij als wetenschappelijk onderzoeker bij de afdeling Huisvesting en Verzorging van het IVO-DLO. Sinds 1996 is hij werkzaam als senior wetenschappelijk onderzoeker rundveehouderij bij de afdeling Gedrag, Stressfysiologie en Management van het Instituut voor Dierhouderij en Diergezondheid (ID-DLO) in Lelystad. Het in dit proefschrift beschreven onderzoek werd deels bij het IVO-DLO, deels bij het ID-DLO uitgevoerd.