Master Thesis

The effects of stocking density on animal health and welfare of dairy cattle in the Netherlands

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Prefatory note

Within the study of Veterinary Medicine at the University of Utrecht all students have to fulfil a research internship during the master. This master thesis is the final report of the research internship carried out by G. van der Heijden at the Ruminant Department of GD Animal Health in Deventer. Research was executed to get to know more about the management factors on dairy farms, like stocking density, which possibly influence animal welfare of dairy cows. This study was carried out within the 1H4F Biomarkers Welfare Dairy Cattle project. The Biomarkers Welfare Dairy Cattle project is being carried out by GD Animal Health commissioned by ministry of Agriculture, Nature and Food Quality and ZuivelNL/DairyNL.

The 1H4F Biomarkers Welfare Dairy Cattle project is still in progress, therefor this master thesis is confidential.

Contents

Abstract

The space allocated for cows and stocking density differs considerably in free stall housing for dairy cows. There are no (exact) figures about the stocking density in dairy barns in the Netherlands. The objective of the present study was to investigate possible associations between stocking density and animal health and welfare. Fifty-five farms in the Netherlands, with a mean herd size of 107 ± 51 cows (mean ± SD), were included in this study. The farms were visited during the period December 2018 to March 2019. KoeKompas (= Cow Compass) (KK) was carried out at each farm to obtain parameters for animal health and welfare and Continue DiergezondheidsMonitoring score (Dutch for Continuous Animal Health Monitor) (CDM score) were requested. On each farm, ten randomly selected and up to five sick dairy cows were examined clinically and cortisol was investigated in hair samples (n=366). Data was analysed with Pearson correlation, t-test or Mann-Whitney U test and (multivariable) regression models on farm level or (multivariable) linear mixed models on animal level.

There were 19 (34,5%) overstocked (>100% stocking density) farms on the number of cubicles, 37 (67,3%) farms on the number of feeding places and 43 (78,2%) farms on water supply.

Overstocking of cubicles, feeding places and water supply was associated with a significant lower welfare monitor score (WMS) feed (15 vs 47; 30 vs 47; 30 vs 47) and overstocking of cubicles and feedings places was associated with a higher WMS behaviour (76 vs 37; 60 vs 30), compared to non-overstocked. Overstocking of cubicles was associated with a significant higher behaviour score (3,58 ± 0,84 vs 3,00 ± 1,04), overstocking of feeding places with a significant higher deviating body condition score (BCS) (%) (5,6 ± 4,5 vs 9,2 ± 6,6) and BCS <2 (%) (1 vs 1) and overstocking of water supply with a significant lower deviant cows score (3,7 ± 0,9 vs 4,3 ± 0,7) and higher somatic cell count (SCC) score KK (4 vs 3).

Stocking density of cubicles, feeding places and water supply was significantly associated with WMS feed (R^2= .12; .13; .20), stocking density of cubicles and water supply with WMS behaviour (R^2= .21; .10) and stocking density feedings places with milk production per day (R^2=.07) and heel score KK (R^2=.08). Stocking density of cubicles, feeding places and water supply was significantly associated with the WMS feed (R^2=.24) and WMS behaviour (R^2=.22) in a multivariable linear regression model.

Overstocking of cubicles, feeding places and water supply was significantly associated higher cortisol concentration in hair samples (63,1 vs 52,3; 66,8 vs 49,9; 67,6 vs 52,9) from individual dairy cows, compared to cows housed in non-overstocked farms.

The study gives an indication that overstocked housing can cause stress and has an impact on animal welfare, but more research is necessary.

Keywords: stocking density, overstocking, health, welfare, cortisol, dairy

Introduction

On the first of December 2018, the Netherlands housed 1.552.000 adult dairy cows (two years or older) (CBS, 2019). The number of dairy farms decreased in the Netherlands in recent years, but the average number of cows on a dairy farm increased (CBS, 2017). Dairy cattle are kept indoors for a considerable part of the year (even if grazing takes place). If the circumstances in the barn aren't optimal, it could affect animal health and welfare. Partly due to the increased attention of the general public to animal welfare in general, the housing of dairy cattle must be critically assessed.

Animal welfare

Animal welfare is a complex concept that can be defined at different levels, just like health, environment or safety (Stafleu et al., 1996). Despite all the attention that the subject animal welfare has recently received within the veterinary profession as well as within the social and political field of forces, the question of what welfare is actually remains a topic of discussion. There are different views on animal welfare and different definitions are used in literature. One of the first attempts to define scientifically proved welfare was made in 1965 by Brambell. They formulated freedoms with the assumption that animal welfare was guaranteed when an animal is free from these conditions. The five freedoms are (Brambell, 1965):

1. Animals are free from hunger and thirst
2. Animals are free from discomfort
3. Animals are free from pain, injury and diseases
4. Animals are free from fear and (chronic) distress
5. Animals are free to express normal behaviour

Developments and new knowledge have resulted in several welfare concepts over the years. Because of the social responsibility as the guardian of animal welfare, it is important that veterinary medicine has a clear position within the discussion about animal welfare. The veterinary welfare concept, from the Faculty of Veterinary Medicine (Utrecht University) approaches animal welfare from a (patho) biological perspective, in relation to ethical values and norms: "An individual is in a state of well-being when it is able to adapt to its living conditions and thereby achieves a positive state"(Ohl et al., 2009). As the animal approaches the limits of its own adaptability, its welfare can be compromised.

Stocking density

Cows are social herding animals by nature and show synchronous behaviour, particularly as far as eating and resting are concerned. Ethologists describe cattle as allelomimetic, meaning that they all like to perform the same activity at the same time. Therefore, it is considered important that there is at least one resting and one feeding place per housed animal (Cook & Nordlund, 2004). If there is a limited number of resting or eating places, the synchronism can be at stake and chronic stress can occur, particularly in low-ranking animals. Galindo & Broom (2000) observed three dairy herds through a five-month period. For the study they observed social rank, behaviour and lameness. In all three herds, the stocking density was one cow per cubicle in a free stall. Low-rank cows spent significant less time lying, standing significant more time still in the passageway and standing more time (partly) in the cubicles, compared to middle- and high-rank cows. With an increase in standing time, the risk to become lame increased: more than 60% of the low-rank cows became lame compared to 18% of the high-rank cows, at 25 weeks in lactation.

In dairy cattle, overstocking is when the number of cows exceeds the number of cubicles (Cook & Nordlund, 2004; Naess, Bøe & Østerås, 2010). Overstocking can also be defined in relation to the number of feeding places. There is overstocking when the number of cows exceeds the number of feeding places (Cook & Nordlund, 2009; Naess, Bøe & Østerås, 2010). In the years 1983 to 1986, the effects of overstocking in dairy cattle in the Netherlands were studied on the 'Waiboerhoeve'. The

overstocking was 30% (stocking density of 130%) for cubicles and 30% (stocking density if 130%) for feeding places. The control group had a feeding and resting place for each cow (stocking density of 100%). The total number of diseases over the four years was virtually the same for test and control groups. Pyometra and especially cows stepping on their teats were more common in the overstocked group compared to the control group. In addition, there was a decrease in the cubicle use of about 50 minutes per day in the overstocked group compared to the control group (Snoek, 1988).
Fregonesi, Tucker & Weary (2007) investigated the effect of overstocking on the lying and standing behaviour of dairy cattle. They created stocking density levels of 100, 109, 120, 133 and 150% for cubicles, with 12 cows per group, for a week. The results show that cows spent significant less time lying down, when there was overstocking (>100% stocking density) (table 1). Overstocking had no effect on time spent standing with only the front legs in the cubicle. When fewer cubicles were available, animals were more likely to be displaced from cubicles, so there is significant more (direct) competition. Cows lay down more quickly after milking at 150% stocking density, compared to 100% stocking density, due to increased (indirectly) competition for cubicles. The increased competition for cubicles in barns with overstocking, leads to more stress and affects animal welfare.

Table 1: Stocking density (relative to cubicles) and mean time spend on behaviour variables (Fregonesi, Tucker & Weary, 2007).

	Stocking level for cubicles						
Variable	100%	109%	120%	133%	150%	**SE**	***p* ≤**
Lying in cubicle (h/24 h)	12,9	12,1	12,0	11,5	11,2	0,26	0,001
Standing with front legs in cubicle (h/24 h)	1,4	1,4	1,4	1,4	1,5	0,18	0,722
Standing outside cubicle (h/24 h)	8,4	8,9	9,1	9,6	9,9	0,40	0,004
Latency to lie down after milking (min)	39	34	38	28	26	4,2	0,025
Displacements from cubicles (n/5 h)	0,7	0,9	1,6	2,1	1,9	0,21	0,001

Grant (2004) reviewed the influence of stocking density on eating, resting and ruminating behaviour. The results for different stocking densities were shown below in table 2. Especially the decreased resting time and increased standing time at higher stocking densities are notable.

Table 2: Stocking density (relative to cubicles) and relative time spend on behaviour to a 100% stocking density (stocking density 100 = 1,00) (Grant, 2004).

Citation	**Stocking density (%)**	**Resting**	**Eating**	**Ruminating**	**Standing**
Batchelder (2000)	100		1,00	1,00	
	130		0,95	0,75	
Winkler et al. (2003)	66	1,02			0,95
	100	1,00			1,00
	150	0,88			1,22
Fregonesi et al. (2004)	100	1,00			1,00
	110	0,92			1,12
	120	0,88			1,15
	135	0,84			1,19
	150	0,80			1,25
Wierenga & Hopster (1990)	100	1,00	1,00		1,00
	125	1,00	1,04		1,25
	133	0,98	0,95		1,52
	155	0,93	1,01		1,46
Matzke & Grant (2002)	85	0,95	1,02		0,95
	100	1,00	1,00		1,00
	120	0,73	1,02		1,20

Collings et al. (2010) aimed to determine the effects of spatial and temporal restrictions, on the feeding and competitive behaviour of group-housed cows. There were two levels of feeding places stocking density, two cows per feed bin (150% stocking density) and one cow per feed bin (100% stocking density), and two levels of feed access time, 14 (06.00-20.00h) versus 24 hours per day access. In total eight groups of each six cows were tested on each of the four treatment combinations for one week. The dry matter intake (DMI) and lying time was not affected by overstocking and did not decline when temporal access was restricted. The group with overstocking and temporally restriction had significant greater feeding rates during the day, and especially during the peak feeding period, compared with not restricted. During peak feeding period, the overstocked group had significant reduced DMI and feeding times and significant increased feeding rates. The conjunction of restricting temporal access with overstocking resulted in the greatest increase in daily displacements, particularly during peak feeding period. So, to minimize feed bunk competition, adequate space and time to access feed is essential.
So, there are several studies showing that stocking density influences the behaviour of cows, and therefore animal welfare.

Cortisol

Overstocking influences the behaviour of cows and causes more competition between cows, which could result in (more) stress. The hypothalamic pituitary adrenal (HPA) axis is the central stress response system. A stress stimulus stimulated the hypothalamus to release corticotrophin-releasing factor (CRF), also known as corticotrophin-releasing hormone (CRH). CRF binds to CRF receptors on the anterior pituitary gland and stimulate the synthesis of adrenocorticotropic hormone (ACTH). ACTH acts on the adrenal cortex to induce the secretion of glucocorticoids. Cortisol is the primary glucocorticoid released from the adrenal glands of cattle during periods of stress and can be measured in serum relatively easily through standard laboratory tests (Cruz-Topete & Cidlowski, 2015). Cortisol measurements in body fluids are not always a reflection of long-term cortisol exposure. Circulating steroids, like cortisol, seems to accumulate in hair throughout all of its growth period (Stalder & Kirschbaum, 2012). In contrast to other sample materials, cortisol concentrations in the hair are not influenced by circadian rhythmicity or other factors that induce short-term elevation of HPA axis activity. The analysis of cortisol in hair is a relatively new method to measure cumulative cortisol exposure over months. Although analysis of cortisol in hair is considered a relatively new method, several studies investigated the association between hair cortisol concentrations, as a biomarker of stress, and animal health (Burnett et al.,2015; Comin et al., 2013), stocking density (Silva et al., 2016), breed (Peric et al., 2013), reproduction (Burnett et al., 2015; Comin et al., 2013), cow's environmental conditions (Peric et al., 2017; Comin et al., 2011) and reproductive treatments (Biancucci et al., 2016). So, cortisol concentrations in hair could be a potential indicator for dairy cattle welfare by providing a useful and practical tool for long-term steroid monitoring (Tallo-Parra et al., 2018). Despite all the promising results, there are still gaps in the knowledge of hair steroid determination. Cortisol concentrations in hair present high variability between individual cows (Comin et al., 2013). Little is known about the incorporation of the hormones in the hair shaft or possible influencing factors on local cortisol production. External factors may have a significant influence on the cortisol concentration in hair (Salaberger et al., 2016).

Legislation and regulation

For cattle, the general welfare rules in "Wet dieren" (Dutch for Animals Act) and "Besluit houders van dieren" (Dutch for Decree containing rules for keepers of animals) apply. These mostly consist of target regulations; the animals must have a sufficient housing, adequate drinking water, suitable feed, sufficient hygienic conditions, etcetera. A handbook, called Handboek Keten Kwaliteit Melk (Dutch for Handbook Chain Quality Milk), has been written by the Dutch Dairy cooperation. This book contains an Animal Welfare chapter, which includes requirement for the dairy cows, such as they must be able to lie freely in the barn and that the stocking density may not exceed 110% (Qlip, 2017).

Welfare monitoring

In the Netherlands, there are three systems to score animal welfare on dairy farms: Welzijnswijzer (Dutch for Welfare Indicator Dairy cattle), Continue Welzijns Monitor (Dutch for Continuous Welfare monitor) and KoeKompas (Dutch for Cow-compass) (KK). The European standard is the Welfare Quality system. Commissioned by the Ministry of Economic Affairs and ZuivelNL (Dutch for Dairy of the Netherlands), the project "Measuring and improving animal welfare in the livestock chain (welfare monitor) of the dairy cattle sector" was carried out from 2012 to 2015. The aim of the project was to develop a more practical method to assess animal welfare in the current housing systems on Dutch dairy farms. In an applied project of Eerdenburg et al. (2018), the four welfare monitoring systems were compared on 60 dairy farms in the Netherlands, with the aim to develop one practical and feasible welfare monitor recognized in Europe. Based on this research, the new welfare monitor called Koe-Kompas Bèta is composed and integrated in the existing KK. This measurement method is calibrated to the Welfare Quality system.

A number of standards are included in KK to score a farm. These standards also include standards for the stocking density of the cubicles, the feeding places and drinking water supply. The standards are basically the same for conventional milking system (CMS) and automatic milking system (AMS) farms, except for the stocking density of the feeding places. An AMS farm has a standard of 70% for the number of feeding places per cow, and a CMS farm 100%. So, an AMS farm is allowed to have 30% overcrowding of the feeding places with the standard of KK.

Animal health monitoring

Dairy farmers can only supply milk from healthy cows in the Netherlands. In the Dutch dairy sector, there are three systems to monitor and provide insight of animal health on dairy farms: Periodiek BedrijfsBezoek (Dutch for Periodic Farm Visit), Continue DiergezondheidsMonitoring (Dutch for Continuous Animal Health Monitor) (CDM) and KK. These systems are based on European legislation and requirements of the dairy sector. Each dairy company provides access to one of these monitoring systems for their farmers. Every dairy farmer is obliged to use one of these monitoring systems (Remmelink et al., 2018).

Aim of the study

The aim of this study is to gain insight in the stocking density in dairy cattle barns in the Netherlands, and to investigate the effects of stocking density on animal health and welfare. In this study, the associations between stocking density, health, welfare and hair cortisol levels will be investigate.

Hypothesis

H0= There is no significant association between stocking density and animal health or welfare.

H1= There is a significant association between stocking density and animal health or welfare.

Material and methods

This study was carried out within the 1H4F Biomarkers Welfare Dairy Cattle project.

Farms and animals

For the project, 1H4F Biomarkers Welfare Dairy Cattle, 76 dairy farms in the Netherlands were visited during the indoor season (December 2018 until March 2019). The number of farms was a compromise between, the power needed for a meaningful epidemiological analysis and the amount of work to carry out all necessary observations on each farm. The sample size needed to commensurate with the available resources, such as cost, time and accessibility.
Fifty percent of the dairy farms were randomly selected by GD Animal Health and fifty percent were suggested by their Dairy cooperation based on a suspicion for reduced welfare. The suspicion for reduced welfare was based on, for example, CDM status C, a switch from CDM status C to PBB (Dutch for Periodical Farm Visit), bad score KK (housing conditions, animal welfare, disease incidence) or the observations of a field employee during a farm visit. In addition, a farm had to have at least 30 dairy cows. On the farm, the samples of ten randomly selected cows and five chronic sick cows were collected. The ten cows were randomly selected by asking how many cows were milked during the farm visit, for example 72 cows were milked, divided this number by ten and then selected each seventh cow that enters the milking parlor. Prior to the farm visit, the farmer was asked to select five chronic sick cows. The farmer got instructions on how to select these chronic sick cows. For example, it should not be cows that only have a milk production drop, but cows with chronic lameness, inflammation or disease history. If the farmer did not have five chronic sick cows, the veterinarian could still designate chronic sick cows in the herd during the farm visit. If less than five chronic sick cows were present in the herd, only these chronic sick animals were sampled.
These methods have been chosen to get most variation in welfare at farm and cow level.

The selection of the farms by the Dairy cooperation was based on, among other things, CDM score. For the power calculation it was assumed that the CDM score in the overstocked group amounts to an average of 70 and in the non-overstocked group 80, with a standard deviation of 10. This power calculation resulted in a sample size of 16 per group. A sample size of 16 farms gave approximately 80% power (alpha = 0.05, two-tail) to reject the null hypothesis. A total of at least 32 farms were therefore be required.
Youngstock is excluded from this study.

Continue Diergezondheidsmonitoring

CDM is based on available routine data, such as information of individual milk samples from individual cows at test day (CRV) and the health status of the farm (GD). The score indicates the status of the animal health within the dairy farm. Quarter scores and annually moving average are calculated every quarter. CDM contains 11 aspects: cattle mortality, calf mortality, new udder infections, bulk milk somatic cell count, decrease in BSK (Dutch for farm standard cow), closed farming system, leptospirosis, Bovine Viral Diarrhea (BVD), Infectious Bovine Rhinotracheitis (IBR), paratuberculosis and salmonella. The norm, points and source of the data were shown in table 3. The scores of the farm are compared to the national average (NZO, 2019; Remmelink et al., 2018). The CDM score results in an animal health status A (≥ 70 points), B (60-70 points or <50 points in last quarter) or C (<60 points). Status A means healthy cattle, status B animal health deserves attention and status C insufficient animal health. The aim is to use one system by 2020 for the management and guarantee of animal health, animal welfare and food safety at the dairy farm (NZO, 2019; Brouwer et al., 2015).

Table 3: The parameters from CDM score with norm, points and source of data (NZO, 2019).

Parameter	Norm	Points	Source data
Cattle mortality	≤2,08% [1]	25	Rendac, I&R
Young stock mortality	≤20% [1]	10	Rendac, I&R
New udder infections	≤12,5% [1]	20	Test-day/dairy company
Bulk milk somatic cell count	≤290.000	15	Qlip
Difference in BSK (Dutch for farm standard cow)	≥-10 [2]	10	Test-day/dairy company
Closed farming system	No cattle moved on-farm [3]	10	I&R
Leptospirosis	Certified free	2	GD
BVD	Certified free	2	GD
IBR	Certified free	2	GD
Paratuberculosis	Status A or 6	2	GD
Salmonella	Certified unsuspected and/or level 1	2	Dairy company

[1] Per quarter; [2] relative to last quarter; [3] current quarter. I&R = identification & registration.

KoeKompas

KK is a visualization of the risk profile of a dairy farm. Using around 40 measuring points (risk control points) divided into seven categories, management points are assessed that possibly influence the quality of milk and/or quality of the way milk is produced. The seven categories are: milking, feeding and water, housing, animal welfare, work routines, animal health and youngstock. The farmer receives a questionnaire, with questions about milking, disease incidence, grazing and dehorning. KK must be performed by a certified veterinarian. The vet scores all items on the score list which consists of 7 Critical Success Factors (KSF), 40 Performance Indicators (PI) and more than 100 Management Control Points (MCP). The veterinarian determines a score for each PI. One PI can consist of several MCPs. The score is between 1 and 5, 1 is high risk and 5 low risk. The scores for the KSFs are determined by the scores of the PIs. Not all PIs count equally, because certain PIs have more impact on a KSF than other PIs. If the score for a particular part is unknown, the score will be 3. The combination of the questionnaire and the score list results in a report: KoeKompas figure, score list and a short report with explanation of scores and recommendations (KoeKompas, 2018).

The animal welfare monitor (WM), is based on the following principles: good feed, good housing, good health and normal behaviour. Each principle is calculated from measured parameters, see table 4. The score for each principle is between 0 and 100, 0 is very poor and 100 is very good. So, the higher the score, the better the welfare of the cattle on that farm. Every indicator has its own weight in the calculation of the score for each principle (Eerdenburg et al., 2018; KoeKompas, 2018). The exact calculation is not described.

KK was carried out at each dairy farm by a KoeKompas certified veterinarian in collaboration with students. In total, there were four veterinarians and four students (one veterinary medicine student and three HAS students) involved in the farm visits. Before the start of the study, the four veterinarians trained together on two farms with KK to minimise the differences in scoring.

Production and clinical data

Data for milk production and composition of individual cows were obtained from the most recent test-day, including days in milk (DIM), date of last calving, age, number of lactation, 305-days production, 305-days fat percentages and 305-days protein percentages. The farm overview for milk production and composition on farm level, was also obtained from the most recent test-day, including day production, fat percentages, protein percentages, 305-days production, 305-days fat percentages, 305-days protein percentages and “BedrijfsStandaardKoe” (Dutch for farm standard cow) (BSK). The data concerning udder health were obtained from the somatic cell count (SCC) overview of the farm and the SCC of individual cows from the most recent test day.

The ten randomly selected and up to five sick dairy cows were examined clinically by a veterinarian. The clinical examination includes Body Condition Score (BCS) (range from 1 to 5), rumen filling (range from 1 to 5), heel score (range from 1 to 5), number of thickened joints, hygiene score (range from 1 to 5), faeces score (range from 1 to 5), number of wounds, hair coat abnormality (yes or no), behaviour (calm, alert, anxious), clinical abnormality and lameness score (range from 1 to 5). In addition, the length of the hair is determined.

Table 4: Criteria from Welfare Quality system and indicators in KoeKompas Bèta per principle (KoeKompas, 2018).

Principle	Criteria (WQ)	Indicators KoeKompas Bèta
Good feed	1. Absence of prolonged hunger	BCS <1,5
	2. Absence of prolonged thirst	Sufficient drink water
		Clean drink water
Good housing	3. Lying comfort	Width cubicles
		Diagonal
		Hygiene
	4. Temperature (not measured)	-
	5. Freedom of movement	Grazing
Good health	6. Injuries	Lameness (locomotion score)
		Skin disorders
	7. Diseases	Diseases
	8. Pain by interventions	% dehorning cows
		Dehorning with painkillers
Normal behaviour	9. Normal social behaviour	-
	10. Normal species behaviour	Grazing
	11. Good men-animal relation	Avoidance test
	12. Positive emotional state	-

Animal health and welfare data

The parameters for animal health and welfare were obtained from KK, CDM, hair sample analysis and a questionnaire. Information about animal health was extracted from KK, chapter disease incidence, and CDM. For animal welfare, welfare parameters were obtained from KK and the hormone cortisol was investigated in hair samples. In KK, there is a chapter called animal welfare and the welfare monitor for animal welfare. KK pays attention to the following parameters (also called performance indicators by KK) in the chapter animal welfare: dairy cattle activity (behaviour, number of inactive cows), body condition score, locomotion score, heel swelling, hygiene of the cows, abnormal cows (ruminating, faeces score, rumen score) and general impression (hair coat, hair damage). The performance indicators body condition score and locomotion score weighs stronger, because these two indicators largely influence the animal welfare. In addition, the information about food and water (BCS, feed and water supply), housing conditions ("damslapers", number of cubicles, size of the cubicles, softness of the cubicles) and animal health was used. The WM indicators were shown in table 4. For the stocking density, the number of cows, cubicles, eating spaces and drinking spaces were obtained from KK.
Besides this data, a questionnaire was made to obtain more information about animal health and cows that lay on the floors rather than in the cubicles, "damslapers". The questionnaire is added in appendix 1.

Samples

During farm visits, blood, faeces, hair and milk samples of individual dairy cows, ten randomly selected and up to five sick cows were taken for further diagnostic analysis. Samples were collected by a qualified veterinarian and students. During the sampling, animals were head locked individually

at the feeder. Hair samples were taken with an electric hair clipper in the region between scapula and tuber coxae. The length of the hair (cm) was also noted.
The samples were analysed in the laboratory of GD Animal Health by qualified laboratory analysts. Several biomarkers were determined in the samples, such as neuroendocrine, immune and metabolic markers. Only the neuroendocrine marker cortisol, determined in hair samples, was used for this study.

Biomarker: Cortisol

The hair cortisol concentrations were determined according GD Animal Health guidelines. The validated protocol is confidential. In short, the hairs were washed, dried and then weighed and ground. The grounded hair was then weighed and methanol was added. Sonification then took place and the tubes were placed in the shaker, in a stove, for 24 hours. The sample then went through a number of other steps and finally the cortisol was measured with the Immulite, in nanomole per milliliter (nmol/mL).

Statistical analysis

The study design is an observational cross-sectional study. Statistical analysis was performed using Statistical Package for the Social Sciences 25 (SPSS). The analyses were carried out at farm level and animal level. Descriptive statistics were used to describe the basic features of the data in this study, including scatterplots, boxplots and histograms. Normality of continuous data was tested using P-P plots and the Shapiro-Wilk test. All data, including herd and cow factors, was analysed using Pearson correlation coefficients bivariate on farm level and partial on animal level, with specific farm number as random effect. The stocking density was analysed as continuous data and also converted into binary values, overstocking (>100% stocking density) or no overstocking (≤100 stocking density). If the dependent variables were normally distributed a t-test was carried out for overstocking versus no overstocking. In case the normality test failed, a Mann-Whitney U test (Wilcoxon rank sum test) was performed. Statistical significance was defined as p <0,05. Results were presented as mean ± SD. The animal health data were not recorded on every farm, so the occurrence of diseases is often an estimation of the farmer and has low reliability. The animal disease incidence and the final score for animal health from KK were therefore excluded from the statistical analysis.

Farm level

Each farm (n= 55) was considered an experimental unit on farm level. The data was analysed using linear regression models (with simple scatter) to find associations between stocking density and animal health and welfare parameters. The stocking density of cubicles, feeding places and water supply were the independent or explanatory variable. First, all relevant variables from CDM and KK (CDM score, final score animal welfare, WM (feed, housing, health, behaviour), milk production) were analysed univariable. Second, all other variable (activity, BCS, ruminating, locomotion, heel, hygiene, deviant cows, general impression, damslapers, DD/DY, SCC, bulk milk somatic cell count (BMSCC), bald spots, wounds, swelling, bald heels) significant correlated with stocking density were analysed in a univariable linear regression model. In the multivariable analysis, the three independent variables (stocking density of cubicles, feeding places and water supply) were forced into one model, and then possible herd factors that could influence animal health and welfare were added to the model. The significant dependent variables (one at a time) and the three dependent variable were also forced into one model. The lowest predictor (and not significant) was removed from the multivariable models, one at a time. If the Estimate of the other variables which remain in the model, changed more than 10%, then there is confounding and the removed variable must remain in the model. The residuals were tested for normally distribution and constant variance, with scatter of the residuals (RESID) and the predicted values (PRED), histograms and Q-Q plots.

Animal level

Each animal (n= 366) was considered an experimental unit on animal level. The chronic sick animals (n=110) were a separate group and only used to test if there was a difference in cortisol level between randomly selected and chronic sick cows. The randomly selected animals (n= 256) were forced into a mixed model, with specific farm number as random effect. The stocking density of cubicles, feeding places and water supply were the independent variable and the cortisol concentration the dependent variable. In the multivariable analysis, the three independent variables (stocking density of cubicles, feeding places and water supply) were first separately forced into one model with the cortisol concentration, and then altogether into one model with cortisol concentration. All possible cow factors (parity, DIM, milk production) that could influence animal health and welfare were also added to the model. The residuals were tested for normally distribution and constant variance, with scatter of the residuals (RESID) and the predicted values (PRED), histograms and Q-Q plots.

Definitions

For this study, overstocking was defined as the situation where the number of dairy cows exceeds the number of cubicles or feeding places at a single farm, so if there is not one cubicle or feeding place for each cow. There was overstocking when the stocking density is more than 100%. There was overstocking for drinking water supply when there is less than 7 centimetre of drinking space per cow. There was no standard for drinking water supply for dairy cows, so the norm of KK was used. Because there were no exact numbers available of farms with overstocking in the Netherlands the final classification based on stocking density was made when the results were known. With the current “Phosphate regulation” it was also possible that there were fewer / few farms with overstocking.
Animal welfare on a farm is poor when the score for animal welfare in KK is three or below three (on the scale of 1 to 5) and animal health then the score for CDM is below 60 (on the scale of 0 to 100).

The aim of the analysis of cortisol in hair samples was to find out if there was an association between overstocked conditions on farms and the suspected higher cortisol level in hair samples of dairy cows. Finally; the association between animal health, welfare, cortisol levels and stocking density were determined.

Results

Farm level

At the time of the analysis for this study, 61 of the 76 selected farms were visited. The KK results from 55 farms were available for this study. KK was not yet completed for the other six farms, therefore these farms were excluded from this study. A sample size of 55 farms will give approximately 99,9% power (alpha = 0.05, two-tail) to reject the null hypothesis.

Thirty-five farms had a conventional milking system (CMS) (64%) and twenty farms an automatic milking system (AMS) (36%). In the Netherlands, 24% of the dairy farms have an AMS (KOM, 2019). The mean number of cows on the farms and the mean production results were shown in table 5. The production data were missing from one farm (n= 54 for production results). The mean daily milk production per cow from the most recent test day was 26,4 kg (± 4,4), with respectively a minimum and maximum of 15,9 and 36,7 kg milk per day. The mean 305 days milk yield was 8.709 kg (± 1279), with respectively a minimum and maximum of 5.735 and 11.920 kg milk per 305-days.

Table 5: Mean figures of this study and in The Netherlands.

	Study	**The Netherlands**
Total number of farms	55	17.123 [1]
Conventional milking system (% of total number of farms)	64 %	76% [1]
Automatic milking system (% of total number of farms)	36 %	24% [1]
Total number of cows	107 (± 51)	100 [2]
Total number of lactating cows*	96 (± 45)	-
Total number of dry cows*	11 (± 8)	-
Milk production, daily (kg)	26,4 (± 4,4)	28,1 [2]
Milk production, 305-d (kg)	8.709 (±1279)	9.123 [3]

[1] Milking parlors / robot systems registered by Stichting Kwaliteitszorg Onderhoud Melkinstallaties (Dutch for Quality Assurance Maintenance of Milk Installations) (KOM, 2019).
[2] Life production of Dutch studbook cows per year of culling, in the Netherlands 2018 (n= 318.712 cows) (CRV, 2019)
[3] MPR-statistics annually moving average in the Netherlands 2018 (n= 14.122 farms) (CRV, 2019)
*In various herds, dry cows were housed in the lactating group. Dry cows were then included in the number of lactating cows.

Stocking density

The descriptive statistics from the stocking density of the cubicles, feeding places and for water supply on the farms were shown in table 6. Boxplots were added in appendix 2.1, figure 2.1.1-2.1.3. The farm with maximum stocking density of cubicles was not the same farm as the farm with maximum stocking density of feeding places or water supply. The farm with maximum stocking density of feeding places was also not the same farm as the farm with maximum stocking density of water supply.

Table 6: Descriptive statistics from stocking density of cubicles, feeding places and water supply (%).

	Stocking density cubicles	**Stocking density feeding places**	**Stocking density water supply**
Mean	95,4	118,1	135,9
Standard deviation	16,9	32,6	47,4
Minimum	58,5	64	55,2
Maximum	125,5	242,4	262,9

The number of overstocked farms was shown in table 7. There were 19 (34,5%) overstocked (>100% stocking density) farms on the number of cubicles, 37 (67,3%) farms on the number of feeding places and 43 (78,2%) farms on water supply. Boxplots were added in appendix 2.1, figure 2.1.4-2.1.6.

Table 7: Overstocking (>100%) or no overstocking (≤100%) on the farms for the number of cubicles, feeding places and water supply (overall and for CMS and AMS separately). N = 55 dairy farms

	Stocking density ≤100%	Stocking density >100%	Overstocked farms, >100 % stocking density (%)
Overall			
Cubicles	36	19	35
Feeding places	18	37	67
Water supply	12	43	78
CMS			
Cubicles	21	14	40
Feeding places	9	26	74
Water supply	7	28	80
AMS			
Cubicles	15	5	25
Feeding places	9	11	55
Water supply	5	15	75

There was a moderate significant positive correlation between stocking density of cubicles and feeding places r=0,483, p<.000, between stocking density of cubicles and water supply r=0,492, p<.000 and between stocking density of feeding places and water supply r=0,425, p<.001. For overstocked versus non-overstocked there was a moderate positive correlation between cubicles and feeding places r=0,507, p<.000, cubicles and water supply r=0,384, p<.004 and feeding places and water supply r=0,382, p<.004.

Animal health and welfare

The descriptive statistics from the CDM score, the final score (FS) for chapter animal welfare from KK and the welfare monitor score (WMS) categories were shown in table 8. The descriptive statistics for these animal health and welfare parameters for overstocked (>100% stocking density) versus non-overstocked (≤100% stocking density) farms on cubicles, feeding places and water supply were added in appendix 2.2, table 2.2.1. Scatterplots were added in appendix 2.3, figure 2.3.1-2.3.6.

Table 8: Descriptive statistics from the CDM score and animal welfare score from KoeKompas on the farms.

	N	Mean	Standard deviation	Minimum	Maximum
CDM score (recent quarter)	38	80	16	36	100
CDM score (annually moving average)	39	69	15	24	100
Final score welfare KoeKompas	55	3,06	0,36	2,20	3,8
Welfare monitor score feed	55	42	27	7	100
Welfare monitor score housing	55	54	7	34	67
Welfare monitor score health	55	51	12	28	75
Welfare monitor score behaviour	55	48	24	19	89

N = sample size

Stocking density of the cubicles

The stocking density of cubicles was moderate negative correlated with WMS feed (r=-0,34) and moderate positive correlated with WMS behaviour (r=0,46), increased somatic cell count (SCC) after calving (%) from the most recent test-day (r=0,56) and increased SCC after calving (%) in the last quarter (r=0,46) (table 9). The significant correlated variables with no overstocking versus overstocking of cubicles were shown in table 10a. When comparing overstocking (>100% stocking density) and no overstocking (≤100% stocking density) of cubicles, with an independent-samples t-test, there was a significant difference in behaviour score herd KK (3,6 vs. 3) (table 11). WMS feed (15 vs. 47) and WMS behaviour (76 vs. 37) differs also significantly between overstocked versus non-overstocked, with a Mann-Whitney U test (table 12).

A simple linear regression was used to find associations between stocking density of cubicles and WMS feed. Stocking density of cubicles was significantly associated with WMS feed, F(1, 53)= 6,98, *p* <.011, R^2=.116 (table 13a). The R^2 value of .116 means that 11.6 percent of the variation in WMS feed is due to variation in stocking density of cubicles. The variable stocking density of cubicles was also significantly associated with WMS behaviour (R^2=.21) (table 13a). Overstocking versus no overstocking of cubicles was significantly associated with WMS feed (R^2=.21), WMS behaviour (R^2=.20) and behaviour score KK (R^2=.08), in a simple linear regression model (table 14a). The residuals were approximately normally distributed and the variability was constant.

Stocking density of the feeding places

The stocking density of feeding places (%) was moderate negative correlated with WMS feed (r=-0,37) and daily milk production (r=-0,27), and weak positive correlated with heel score KK (r=0,29) (table 9). The significant correlated variables with non-overstocking and overstocking of feeding places were shown in table 10b. When comparing overstocking (>100% stocking density) and no overstocking (≤100% stocking density) on feeding places, there was a significant difference in percentages deviating body condition score (BCS) (number of cows with BCS <2 and >4 of the total number of scored cows for BCS in percentages) between overstocking and no overstocking (9,2 vs. 5,6%), with an independent-samples t-test (table 11). WMS feed (30 vs. 47), WMS behaviour (60 vs. 30) and percentage cows with BCS <2 (1 vs. 1%) differ also significantly between overstocked versus non-overstocked, with a Mann-Whitney U test (table 12).

Stocking density of feeding places was significantly associated with milk production per day (R^2=.07), WMS feed (R^2=.13) and heel score KK (R^2=.08), in a simple linear regression model (table 13b). Overstocking versus no overstocking of feeding places was significantly associated with WMS feed (R^2=.15), WMS behaviour (R^2=.09) and deviating BCS(%) (R^2=.07), in a simple linear regression model (table 14a). The residuals were approximately normally distributed and the variability was constant.

Stocking density of water supply

The stocking density of water supply was moderate negative correlated with WMS feed (r=-0,44), and moderate positive correlated with WMS behaviour (r=0,32), increased SCC (%) in the last half year (r=0,53) and increased SCC (%) in the last year (r=0,43) (table 9). The significant correlated variables with non-overstocking and overstocking of water supply were shown in table 10b. When comparing overstocking (>100% stocking density) and no overstocking (≤100% stocking density) on water supply, there was a significant difference in deviant cows score (based on rumen filling, faeces score and ruminating of the herd) KK between overstocking and no overstocking (3,7 vs. 4,3), with an independent-samples t-test (table 11). WMS feed (30 vs 47) and SCC score KK (4 vs. 3) differ also significantly between overstocked versus non-overstocked, with a Mann-Whitney U test (table 12).

Stocking density of water supply was significantly associated with WMS feed (R^2=.20) and WMS behaviour (R^2=.10), in a simple linear regression model (table 13b). Overstocking versus no overstocking of water supply was significantly associated with WMS feed (R^2=.14), heel score KK (R^2=.08), deviant cow score KK (R^2=.08) and SCC score KK (R^2=.09), in a simple linear regression model (table 14b). The residuals were approximately normally distributed and the variability was constant.

Non-significant variables

The following variables were non-significant correlated for all three stocking density variables: CDM score current quarter, CDM score annually moving average, FS chapter animal welfare KK, WMS housing, WMS health, milk production 305-days (kg), BSK , activity score KK, non-active cows (%), BCS score KK, ruminating (%), locomotion score (%) (1, 2-3 and 4-5), hygiene score KK, general impression score KK, number of damslapers, DD/DY, SCC (cells/mL), increased SCC (%) recent/last quarter, new increased SCC(%) recent/last quarter/half year/year, increased SCC after calving (%) half year/year, bulk milk somatic cell count (BMSCC), total number of bald spots and total number of wounds/swelling/bald heels.

Stocking density of cubicles and feeding places were non-significant correlated with the variable daily milk production (kg), deviating BCS (%), BCS <2 (%) and heel score KK. Stocking density of feeding places and water supply not with the behaviour herd score KK and increased SCC after calving (%) recent/last quarter. Stocking density of cubicles and feeding places not with deviant cow score KK, SCC score KK and increased SCC (%) half year/year. Only the significant variables were discussed.

Table 9: Bivariate Pearson correlation for dependent variables and stocking density of cubicles, feedings places and water supply (%).

Variables	**N**	**r**	***P***
Stocking density of cubicles (%)			
Welfare monitor score feed	55	-0,341	.011
Welfare monitor score behaviour	55	0,457	.000
Increased SCC after calving, recent test-day (%)	19	0,558	.013
Increased SCC after calving, last quarter (%)	20	0,455	.049
Stocking density of feeding places (%)			
Welfare monitor score feed	55	-0,365	.006
Daily milk production	55	-0,270	.049
Heel score KoeKompas	55	0,290	.032
Stocking density of water supply (%)			
Welfare monitor score feed	55	-0,443	.001
Welfare monitor score behaviour	55	0,320	.017
Increased SCC, last half year (%)	24	0,527	.008
Increased SCC, last year (%)	23	0,433	.039

N = sample size; R = Pearson's product moment correlation coefficient; *p* = p value.

Table 10a: Bivariate Pearson correlation for dependent variables and overstocking (>100% stocking density) versus no overstocking (≤100% stocking density) of cubicles.

Variables	**N**	**r**	***P***
Overstocking of cubicles (yes vs. no)			
Welfare monitor score feed	55	-0,455	.000
Welfare monitor score behaviour	55	0,451	.001
Behaviour score KoeKompas	55	0,276	.042
Increased SCC after calving, recent test-day (%)	19	0,466	.044
Increased SCC after calving, last quarter (%)	20	0,562	.010
Increased SCC, last half year (%)	24	0,408	.048
New increased SCC, last half year (%)	24	0,415	.044
Increased SCC after calving, last half year (%)	20	0,597	.005
Increased SCC, last year (%)	23	0,416	.048
New increased SCC, last year (%)	23	0,455	.029
Increased SCC after calving, last year (%)	23	0,514	.024

N = sample size; R = Pearson's product moment correlation coefficient; *p* = p value.

Table 10b: Bivariate Pearson correlation for dependent variables and overstocking (>100% stocking density) versus no overstocking (≤100% stocking density) of feeding places and water supply.

Variables	**N**	**r**	***P***
Overstocking of feeding places (yes vs. no)			
Welfare monitor score feed	55	-0,391	.003
Welfare monitor score behaviour	55	0,294	.030
Deviating body condition score (%)	55	0,270	.047
Overstocking of water supply (yes vs. no)			
Welfare monitor score feed	55	-0,382	.004
Heel score KoeKompas	55	0,280	.039
Deviant cows score KoeKompas	55	-0,280	.038
SCC score KoeKompas	55	0,298	.027

N = sample size; R = Pearson's product moment correlation coefficient; *p* = p value.

Table 11: Independent-samples t-test for parametric variables with overstocking (>100% stocking density) or no overstocking (≤100% stocking density) of cubicles, feeding places and water supply.

	Overstocking		**No overstocking**			
	Mean	SD	Mean	SD	**t**	***p***
Overstocking of cubicles (yes vs. no)						
Behaviour score KoeKompas	3,58	0,84	3,00	1,04	-2,089	.042
Overstocking of feeding places (yes vs. no)						
Deviating body condition (%)	9,15	6,60	5,60	4,45	-2,038	.047
Overstocking of water supply (yes vs. no)						
Deviant cows score KoeKompas	3,72	0,93	4,33	0,65	2,124	.038

SD = standard deviation; N = sample size; t = t value; *p* = p value.

Table 12: Mann-Whitney U test for nonparametric variables with overstocking (>100% stocking density) or no overstocking (≤100% stocking density) of cubicles, feeding places and water supply.

	Overstocking median	**No overstocking median**	**U**	**Z**	***p***
Overstocking of cubicles (yes vs. no)					
Welfare monitor score feed	15	46,5	148,5	-3,428	.001
Welfare monitor score behaviour	76	36,5	149,0	-3,435	.001
Overstocking of feeding places (yes no)					
Welfare monitor score feed	30	47	153,0	-3,232	.001
Welfare monitor score behaviour	60	30	202,0	-2,362	.018
Body condition score <2 (%)	1	1	211,5	-2,119	.034
Overstocking of water supply (yes vs.no)					
Welfare monitor score feed	30	47	126,5	-2,682	.007
SCC score KoeKompas	4	3	147,5	-2,324	.020

U = Mann-Whitney U; Z = Z value; *p* = p value.

Table 13a: Univariable linear regression model for health and welfare indicators with stocking density of cubicles (%).

Variables	**Estimate**	**SE**	***p***	**R^2**
Welfare monitor score feed			.011	.116
Constant	93,24	19,66		
Stocking density of cubicles (%)	-0,54	0,20		
Welfare monitor score behaviour			.000	.209
Constant	-13,89	16,89		
Stocking density of cubicles (%)	0,65	0,17		

SE = standard error; *p* = p value; R^2 = coefficient of determination.

Table 13b: Univariable linear regression model for health and welfare indicators with stocking density of feedings places and water supply (%).

Variables	Estimate	SE	*p*	R^2
Daily milk production (kg)			.049	.072
Constant	30,77	2,23		
Stocking density of feeding places (%)	-0,04	0,02		
Welfare monitor score feed			.006	.134
Constant	77,27	12,77		
Stocking density of feeding places (%)	-0,30	0,10		
Heel score KoeKompas			.032	.084
Constant	3,03	0,36		
Stocking density of feeding places (%)	0,01	0,003		
Welfare monitor score feed			.001	.196
Constant	75,79	9,93		
Stocking density of water supply (%)	-0,25	0,07		
Welfare monitor score behaviour			.017	.102
Constant	26,23	9,52		
Stocking density of water supply (%)	0,16	0,07		

SE = standard error; *p* = p value; R^2 = coefficient of determination.

Table 14a: Univariable linear regression model for health and welfare indicators with overstocking (>100% stocking density) versus no overstocking (≤100% stocking density) of cubicles and feeding places.

Variables	Estimate	SE	*p*	R^2
Welfare monitor score feed			.000	.207
Constant	50,78	3,98		
Overstocking of cubicles (yes vs. no)	-25,20	6,77		
Welfare monitor score behaviour			.001	.204
Constant	40,50	3,62		
Overstocking of cubicles (yes vs. no)	22,66	6,16		
Behaviour score KoeKompas			.042	.076
Constant	3,00	0,16		
Overstocking of cubicles (yes vs. no)	0,58	0,28		
Welfare monitor score feed			.003	.153
Constant	56,83	5,81		
Overstocking of feeding places (yes vs. no)	-21,94	7,09		
Welfare monitor score behaviour			.030	.086
Constant	38,28	5,48		
Overstocking of feeding places (yes vs. no)	14,94	6,68		
Deviating body condition score (%)			.047	.073
Constant	5,65	1,41		
Overstocking of feeding places (yes vs. no)	3,51	1,72		

SE = standard error; *p* = p value; R^2 = coefficient of determination.

Table 14b: Univariable linear regression model for health and welfare indicators with overstocking (>100% stocking density) versus no overstocking (≤100% stocking density) of water supply.

Variables	Estimate	SE	*p*	R^2
Welfare monitor score feed			.004	.144
Constant	61,00	7,16		
Overstocking of water supply (yes vs. no)	-24,21	8,09		
Heel score KoeKompas			.039	.078
Constant	3,4	0,20		
Overstocking of water supply (yes vs. no)	0,49	0,23		
Deviant cow score KoeKompas			.038	.078
Constant	4,33	0,26		
Overstocking of water supply (yes vs. no)	-0,61	0,29		
SCC score KoeKompas			.027	.089
Constant	2,75	0,34		
Overstocking of water supply (yes vs. no)	0,88	0,39		

SE = standard error; *p* = p value; R^2 = coefficient of determination.

Stocking density of cubicles, feeding places and water supply

A multivariable linear regression model was used to associate WMS feed with stocking density of cubicles, feeding places and water supply. These variables were significantly associated with WMS feed (R^2=.24). One variable, stocking density of water supply (*p*=.033), added statistically significantly to the model (table 15). The variables stocking density of cubicles, feeding places and water supply were also significantly associate with WMS behaviour (R^2=.22). One variable, stocking density of cubicles (*p*<.012), added statistically significantly to the model.
When stocking density of cubicles, as lowest predictor (and not significant) was removed from the multivariable model with WMS feed, the Estimates of the other variables which remain in the model, changed more than 10%. So, there was confounding and the stocking density of cubicles must remain in the model. The significant multivariable linear regression models were shown in table 15. The multivariable linear regression for overstocking versus no overstocking of cubicles, feeding places and water supply were shown in table 16. The residuals were approximately normally distributed and the variability was constant. The multivariable linear regression models with herd factors were non-significant.

Non-significant in multivariable models

The following variables were non-significant in multivariable linear regression models with stocking density of cubicles, feeding places and water supply (%): daily milk production (kg), heel score KK. In the multivariable models with overstocked versus non-overstocked of cubicles, feeding places and water supply, the following variables were non-significant: deviating BCS (%), deviant cow score KK, SCC score KK.
The possible herd factors were non-significant when added to the multivariable linear regression models, with stocking density (%) and no overstocking versus overstocking of cubicles, feeding places and water supply: milk system (AMS/CMS), residual feed (good/moderate/insufficient), width feeding place (cm), height feed bunk (cm), width walkway behind feed bunk (cm), width walkway between cubicle rows (cm), quality floor (good/moderate/insufficient), width cubicle (cm), length cubicle (cm), diagonal cubicle (cm), height obstacle head space (cm), softness cubicles (good/moderate/insufficient). The lowest predictor (and not significant) was removed from the multivariable models, one at a time. But still there was no significant herd factor as predictor in the multivariable mixed model.

Table 15: Multivariable linear regression model for health and welfare indicators with stocking density of cubicles, feedings places and/or water supply (%).

Variable	**Estimate**	**SE**	***p***	***p (Anova)***	**R^2**
Welfare monitor score feed				.003	.240
Constant	98,39	18,89	.000		
Stocking density of cubicles (%)	-0,15	0,24	.522		
Stocking density of feeding places (%)	-0,15	0,12	.206		
Stocking density of water supply (%)	-0,18	0,08	.033		
Welfare monitor score behaviour				.005	.221
Constant	-14,09	17,36	.421		
Stocking density of cubicles (%)	0,57	0,22	.012		
Stocking density of feeding places (%)	-0,002	0,11	.982		
Stocking density of water supply (%)	0,06	0,08	.395		
Welfare monitor score behaviour				.002	.221
Constant	-14,16	16,92	.407		
Stocking density of cubicles (%)	0,56	0,20	.007		
Stocking density of water supply (%)	0,06	0,07	.379		

SE = standard error; *p* = p value; R^2 = coefficient of determination.

Table 16: Multivariable linear regression model for health and welfare indicators with overstocking (>100% stocking density) or no overstocking (≤100% stocking density) of cubicles, feeding places and water supply.

Variable	**Estimate**	**SE**	***p***	***p (Anova)***	**R^2**
Welfare monitor score feed				.001	.276
Constant	64,07	7,22	.000		
Overstocking of cubicles (yes vs. no)	-16,25	7,88	.044		
Overstocking of feeding places (yes vs. no)	-9,22	7,98	.253		
Overstocking of water supply (yes vs. no)	-13,03	8,46	.130		
Welfare monitor score behaviour				.005	.222
Constant	42,31	6,79			
Overstocking of cubicles (yes vs. no)	22,05	7,41			
Overstocking of feeding places (yes vs. no)	6,06	7,50			
Overstocking of water supply (yes vs. no)	-7,27	7,96			

SE = standard error; *p* = p value; R^2 = coefficient of determination.

Individual Animals

At the time of the analysis for this study, the cortisol levels were determined in 366 hair samples. The hair samples were from 256 random selected and 110 chronic sick cows, housed in 27 different farms (20 CMS and 7 AMS). The production results were not available of 338 individual animals and the SCC of 328 cows. A reason for the lack of data was, for example, a heifer or multiparous cow that just calved and was not yet milked during the test day. The distribution over the parturitions was shown in table 17 and the descriptive statistics of the production results from the most recent test-day were shown in table 18.

Table 17: Number of parturitions (for all cows and for randomly selected and chronic sick cows separately).

Number of parturitions	**All cows**		**Random cows**		**Chronic sick cows**	
	N	%	N	%	N	%
Primiparous	82	22,4	61	23,8	21	19,1
Multiparous	270	73,8	185	72,3	85	87,3
Unknown	14	3,8	10	3,9	4	3,6

N = sample size

Table 18: *Descriptive statistics production results from the most recent test-day (for all cows and for randomly selected and chronic sick cows separately).*

	N	Mean	Standard deviation	Minimum	Maximum
All cows					
Days in milk (DIM)	338	189	132	6	827
Milk yield 305d (kg)	338	8.324	2.173	1.144	15.147
SCC (x1000/mL)	328	185	474	2	4.482
Random cows					
Days in milk (DIM)	235	198	137	6	827
Milk yield 305d (kg)	235	8.432	2.103	4.232	14.131
SCC (x1000/mL)	227	175	438	2	4.479
Chronic sick cows					
Days in milk (DIM)	103	170	119	8	560
Milk yield 305d (kg)	103	8.077	2.316	1.144	15.147
SCC (x1000/mL)	101	207	547	5	4.482

N = sample size

Cortisol

The descriptive statistics of the cortisol concentration in the hair samples were shown in table 19 and figure 1 - 3. The boxplots of the cortisol concentrations in hair samples of cows housed in overstocked versus non-overstocked conditions, were added in appendix 2.4. The cows with the higher cortisol concentrations were not housed in one farm.
The cortisol concentration differ not significantly between the random selected (Mdn=59,5) and chronic sick cows (Mdn=57,6) with a Mann-Whitney U test; U=13725,5, Z=-0,382, *p*=.702. It was not possible to do this analysis with the random effect of specific farm number. The dataset shows a large variation in cortisol concentration between random and chronic sick animals on one farm. The descriptive statistics on farm level, for random and chronic sick cows, were added in appendix 2.5.

Table 19: Descriptive statistics of cortisol concentration (nmol/L)

	N	Mean	Standard deviation	Minimum	Maximum
All cows	366	67,9	37,6	16,1	369,2
Random cows	256	66,4	33,3	16,1	248,9
Chronic sick cows	110	71,6	46,2	28,6	369,2

N = sample size

Figure 1: Histogram of cortisol concentrations (nmol/L) of all cows (n=366).

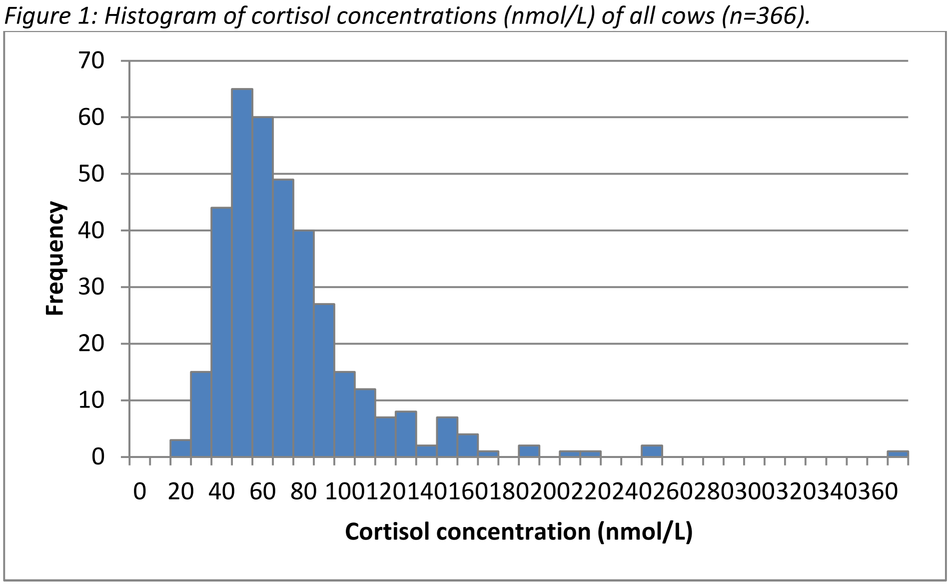


Figure 2: Histogram of cortisol concentrations (nmol/L) of all randomly selected cows (n=256).

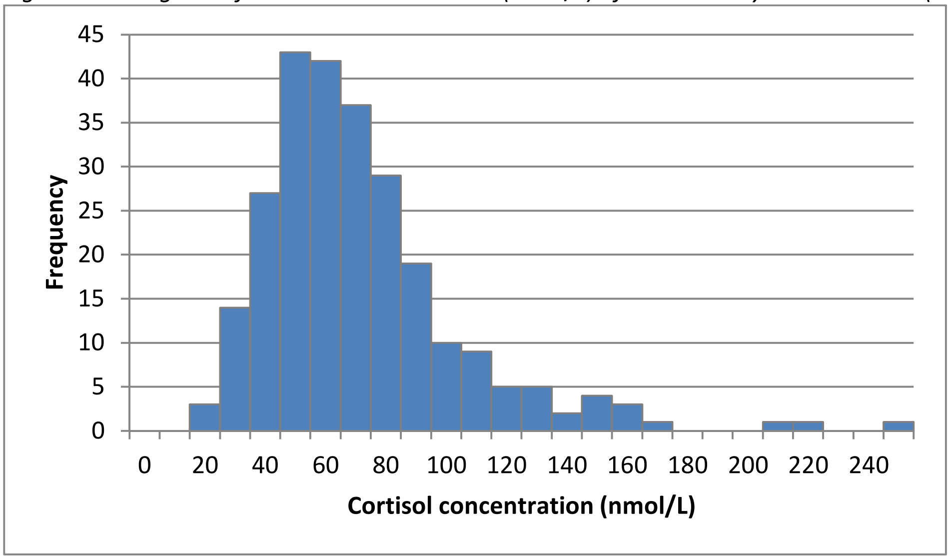
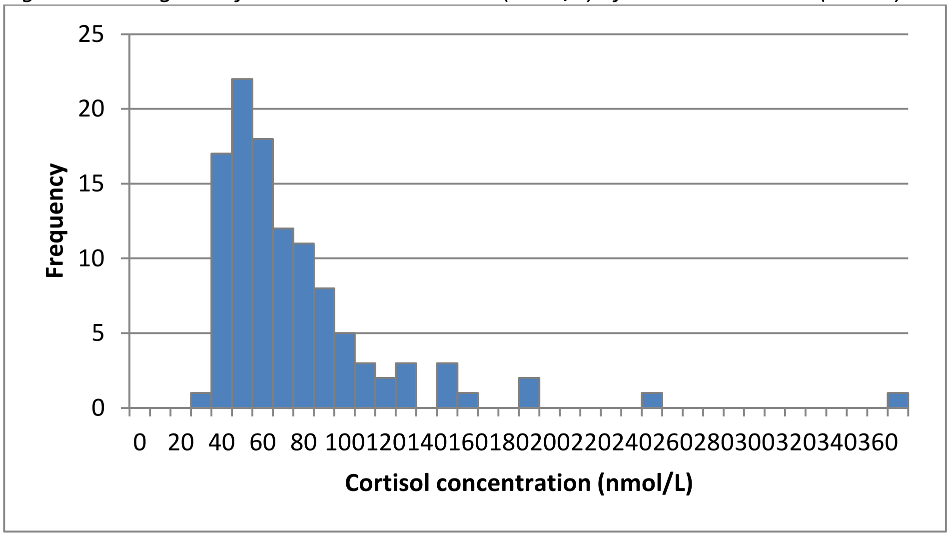


Figure 3: Histogram of cortisol concentrations (nmol/L) of chronic sick cows (n=110).



Randomly selected cows

The stocking density of cubicles, feeding places and water supply (%) were weak positive correlated with the cortisol concentration in hair samples (r=0,17; r=0,22; r=0,16) (table 20). Overstocking versus no overstocking of cubicles, feeding places and water supply were also weak positive correlated with the cortisol concentration in hair samples (r=0,21; r=0,27; r=0,17) (table 20).

The cortisol concentration in hair samples differ significantly between overstocked versus non-overstocked farms on cubicles, feeding places and water supply (68 vs. 53; 67 vs. 50; 63 vs. 52) (table 21). It was not possible to do this analysis with the random effect of specific farm number.

Univariable mixed models

Stocking density of cubicles, feeding places and water supply (%) were significantly associated with cortisol concentration in hair, in a mixed model, with the random effect specific farm number. The results were shown in table 22.

Overstocking and no overstocking of cubicles, feeding places and water supply were also significantly associated with cortisol concentration in hair, in a mixed model, with the random effect specific farm number. The results were shown in table 23. The residuals were approximately normally distributed and the variability was constant.

Multivariable mixed models

Stocking density of cubicles, feeding places and water supply (%) were, separately and all together, significantly associated with cortisol concentration in hair samples from dairy cows, in a multivariable mixed model, with the random effect specific farm number (table 24). With possible cow factors added to the model, stocking density of cubicles, feeding places or water supply were significantly associated with cortisol concentration in hair samples (table 24). The lowest predictor (and not significant) was removed from the mixed models and confounders were kept in the models. The results were shown in table 24. Parity, primiparous versus multiparous, was a significant predictor in de models. The multivariable mixed model with all three stocking density variable (%), cortisol concentration and possible cow factors was non-significant (table 24). The residuals were approximately normally distributed and the variability was constant.

Overstocking versus no overstocking of cubicles, feeding places and water supply were, separately and all together, significantly associated with cortisol concentration in hair samples, in a multivariable mixed model (table 25). The lowest predictor (and not significant) was removed from the mixed models and confounders were kept in the models. The results were shown in table 25. The multivariable mixed models were still significant when possible cow factors were added to the models (table 25). The residuals were approximately normally distributed and the variability was constant. Parity, primiparous versus multiparous, and overstocking versus no overstocking of feeding places was a significant predictor in the models.

Table 20: Partial Pearson correlation (random effect specific farm number) for cortisol concentration (nmol/L) in hair samples of random cows (n=256) and stocking density of cubicles, feedings places and water supply.

Variables	**r**	***p***
Cortisol concentration (nmol/L)		
Stocking density of cubicles (%)	0,173	.006
Stocking density of feeding places (%)	0,222	.000
Stocking density of water supply (%)	0,162	.010
Cortisol concentration (nmol/L)		
Overstocking of cubicles (yes vs. no)	0,209	.001
Overstocking of feeding places (yes vs. no)	0,273	.000
Overstocking of water supply (yes vs. no)	0,173	.006

N = sample size; R = Pearson's product moment correlation coefficient; *p* = p value.

Table 21: Mann-Whitney U test for cortisol concentration (nmol/L) in hair samples with overstocking (>100% stocking density) or no overstocking (≤100% stocking density) of cubicles, feeding places and water supply.

	Overstocking median	**No overstocking median**	**U**	**Z**	***p***
Cortisol concentration (nmol/L)					
Stocking density of cubicles	67,6	52,9	5148,0	-4,665	.000
Stocking density of feeding places	66,8	49,9	5139,5	-5,119	.000
Stocking density of water supply	63,1	52,3	5410,0	-3,522	.000

Mdn = median; U = Mann-Whitney U; Z = Z value; *p* = p value.

Table 22: Univariable mixed model for cortisol concentration (nmol/L) in hair samples with stocking density of cubicles, feedings places and water supply (%), with the random effect specific farm number.

Variables	Estimate	SE	*p*
Cortisol concentration (nmol/L)			
Intercept	38,43	10,44	.000
Stocking density of cubicles (%)	0,29	0,11	.007
Cortisol concentration (nmol/L)			
Intercept	38,14	8,03	.000
Stocking density of feeding places (%)	0,26	0,07	.000
Cortisol concentration (nmol/L)			
Intercept	49,84	6,67	.000
Stocking density of water supply (%)	0,13	0,05	.010

SE = standard error; *p* = p value; R^2 = coefficient of determination.

Table 23: Univariable mixed model for cortisol concentration (nmol/L) in hair samples with overstocking (>100% stocking density) or no overstocking (≤100% stocking density) of cubicles, feeding places and water supply, with the random effect specific farm number.

Variables	Estimate	SE	*p*
Cortisol concentration (nmol/L)			
Intercept	74,73	3,23	.000
Overstocking of cubicles (yes vs. no)	13,88	4,17	.001
Cortisol concentration (nmol/L)			
Intercept	74,86	2,76	.000
Overstocking of feeding places (yes vs. no)	17,95	4,02	.000
Cortisol concentration (nmol/L)			
Intercept	70,53	2,53	.000
Overstocking of water supply (yes vs. no)	12,07	4,32	.006

SE = standard error; *p* = p value; R^2 = coefficient of determination.

Table 24: Multivariable mixed model for cortisol concentration (nmol/L) in hair samples with stocking density of cubicles, feedings places and water supply (%), with the random effect specific farm number.

Variable	**Estimate**	**SE**	***p***
Cortisol concentration (nmol/L)			
Intercept	35,34	10,49	.001
Stocking density of cubicles (%)	0,04	0,15	.775
Stocking density of feeding places (%)	0,22	0,11	.056
Stocking density of water supply (%)	0,03	0,07	.636
Cortisol concentration (nmol/L)			
Intercept	31,56	15,59	.044
Stocking density of cubicles (%)	0,35	0,11	.002
Parity (primiparous vs. multiparous)	11,89	5,22	.024
DIM (days)	-0,03	0,02	.080
Milk production 305days (kg)	0,00	0,00	.655
Cortisol concentration (nmol/L)			
Intercept	36,36	11,28	.001
Stocking density of cubicles (%)	0,34	0,11	.002
Parity (primiparous vs. multiparous)	11,89	5,22	.024
DIM (days)	-0,03	0,02	.086
Cortisol concentration (nmol/L)			
Intercept	33,44	10,62	.002
Stocking density of cubicles (%)	0,31	0,11	.004
Parity (primiparous vs. multiparous)	11,06	4,82	.023
Cortisol concentration (nmol/L)			
Intercept	32,99	13,93	.019
Stocking density of feeding places (%)	0,27	0,07	.000
Parity (primiparous vs. multiparous)	12,46	5,19	.017
DIM (days)	-0,03	0,02	.084
Milk production 305days (kg)	0,00	0,00	.517
Cortisol concentration (nmol/L)			
Intercept	41,38	13,54	.003
Stocking density of water supply (%)	0,17	0,06	.003
Parity (primiparous vs. multiparous)	12,72	5,26	.016
DIM (days)	-0,03	0,02	.034
Milk production 305days (kg)	0,00	0,00	.419
Cortisol concentration (nmol/L)			
Intercept	23,70	15,98	.139*
Stocking density of cubicles (%)	0,12	0,15	.433
Stocking density of feeding places (%)	0,16	0,11	.152
Stocking density of water supply (%)	0,07	0,07	.330
Parity (primiparous vs. multiparous)	12,93	5,21	.014
DIM (days)	-0,03	0,02	.064
Milk production 305days (kg)	0,00	0,00	.404

SE = standard error; *p* = p value; R^2 = coefficient of determination.
*Non-significant!

Table 25: Multivariable mixed model for cortisol concentration (nmol/L) in hair samples with overstocking (>100% stocking density) or no overstocking (≤100% stocking density) of cubicles, feeding places and water supply, with the random effect specific farm number.

Variable	**Estimate**	**SE**	***p***
Cortisol concentration (nmol/L)			
Intercept	74,73	3,19	.000
Overstocking of cubicles (yes vs. no)	-0,62	6,59	.926
Overstocking of feeding places (yes vs. no)	18,21	6,75	.007
Overstocking of water supply (yes vs. no)	0,34	5,60	.952
Cortisol concentration (nmol/L)			
Intercept	73,13	10,14	.000
Overstocking of cubicles (yes vs. no)	16,87	4,36	.000
Parity (primiparous vs. multiparous)	12,32	5,17	.018
DIM (days)	-0,03	0,02	.044
Milk production 305days (kg)	0,00	0,00	.484
Cortisol concentration (nmol/L)			
Intercept	65,92	9,88	.000
Overstocking of feeding places (yes vs. no)	21,36	4,26	.000
Parity (primiparous vs. multiparous)	14,26	5,10	.006
DIM (days)	-0,03	0,02	.057
Milk production 305days (kg)	0,00	0,00	.176
Cortisol concentration (nmol/L)			
Intercept	70,63	10,16	.000
Overstocking of water supply (yes vs. no)	15,31	4,47	.001
Parity (primiparous vs. multiparous)	13,65	5,26	.010
DIM (days)	-0,03	0,02	.051
Milk production 305days (kg)	0,00	0,00	.702
Cortisol concentration (nmol/L)			
Intercept	74,18	4,17	.000
Overstocking of water supply (yes vs. no)	15,16	4,45	.001
Parity (primiparous vs. multiparous)	12,87	4,85	.008
DIM (days)	-0,03	0,02	.054
Cortisol concentration (nmol/L)			
Intercept	66,81	10,23	.000
Overstocking of cubicles (yes vs. no)	1,12	6,67	.867
Overstocking of feeding places (yes vs. no)	19,14	6,97	.006
Overstocking of water supply (yes vs. no)	2,23	5,78	.700
Parity (primiparous vs. multiparous)	14,41	5,15	.006
DIM (days)	-0,03	0,02	.055
Milk production 305days (kg)	0,00	0,00	.195

SE = standard error; *p* = p value; R^2 = coefficient of determination.

Discussion

The stocking density of cubicles, feeding places and water supply varies on dairy farms. On average, the studied herds had low stocking density of cubicles and (slightly) higher-than-recommended space availability at the feed bunk and on water supply. Although, herdsize decreased as a result of the current "Phosphate regulation", there is still overstocking on farms in the Netherlands, both on CMS and AMS farms.

KoeKompas has a different standard for the stocking density of feeding places for CMS and AMS farms; 100% for CMS, so 1 feeding place per cow and 70% for AMS, 0,7 feeding place per cow. There is no evidence for this standard described in KK. DeVries, Keyserlingk & Beauchemin (2003) observed when cows, milked in a parlor, are managed under industry standard of 0,6 meter of feed bunk space per cow, fewer than 70% of the animals feed simultaneously at feeding peak times. Cows housed intensively indoors express less synchronization of behaviour than cows kept on pasture (Miller & Wood-Gush, 1991). Synchronization of behaviour does still occur on farms with CMS, particularly around milking and feed delivery. With an AMS the milking events are spread over 24 hours and cause possibly less synchrony behaviour. Wagner-Storch & Palmer (2003) observed a more consistent flow of animals to the feed bunk during the day on AMS farms, when comparing CMS and AMS, what could indicate that less feed bunk space per cow is required on AMS farms. However, in a more recent study increased space at the feed bunk on AMS farms was positively associated with the milk yield (Deming et al., 2013). There are more factors that influence feeding patterns, including management factors like fresh feed availability (DeVries, Keyserlingk & Beauchemin, 2005). When these factors were included in the multivariable regression models in this study, there was no significant association.

The stocking density of water supply shows large variation in this study. Only, twelve (21,8%) farms were up to the standard of seven centimeter drinking space per cow. Cattle require water for physiological processes associated with maintenance, growth, fattening, pregnancy and lactation. Restriction (25 and 50%) of drinking water relative to ad libitum intake, result in rapid decreased feed intake and milk yield in dairy cows (Burgos et al., 2001). Which mechanisms or other compensatory changes in digestion and metabolism are exactly activated by dehydration in lactating dairy cows is unknown. A sufficient supply of water is essential to avoid negative effects on animal health, performance and welfare (Murphy, 1992; Meyer et al., 2004).

Animal health and welfare

The multivariable linear regression models for WMS (feed or behaviour), and stocking density of cubicles, feeding places and water supply were significant. However, when the herd factors were added to the model, the model was not significant. There was not even a significant association when the lowest (non-significant) predictor was removed from the model. There was confounding, so the predictor needs to keep in the model. The herd factors were not correlated with WMS (feed or behaviour) or stocking density of cubicles, feeding places or water supply. There is no explanation why these herd factors were not significant associated and this was also in contrast with literature and/or logical reasoning.

There were no associations found between stocking density and animal health, the variable CDM score current quarter and annually moving average. However, the CDM score was not known from all farms (38 versus 39 farms). There was an association between feeding places and milk production which could be an indication of reduced animal health. More research is needed to investigate the association between stocking density and animal health. No association was found between final score animal welfare from KoeKompas and stocking density. The results of this score shows small variation in this study (3,06 ± 0,36).

Overstocked farms (on cubicles, feedings places and water supply) had significant lower scores for WMS feed and a significant linear association was found between WMS feed and stocking density of the cubicles, feeding places and water supply. The WMS feed contains the indicators BCS < 1,5, sufficient drink water (seven centimetre drinking space per cow and maximal 15 cows per quick drinker with a water flow of more than 15 litre per minute, more than two drink locations and a drinking angle less than 60 degrees) and clean drink water (water quality brightness and colour) (Eerdenburg et al., 2018; KoeKompas, 2018). The exact calculation of the WMS is not described, but it is noted if a component weighs stronger in the calculation (KoeKompas, 2018). Eerdenburg et al. (2018) described the scoring method of the WM, but the parameters included in the calculation do not fully correspond with the parameters for the WM described in KK (KoeKompas, 2018).
No literature could be found with respect to the association between WMS feed and the stocking density of cubicles. On farms with overstocking of cubicles cows stand longer and spend less time lying down/resting (Fregonesi, Tucker & Weary, 2007; Winkler et al., 2003; Matzke & Grant, 2002; Wieringa & Hopster, 1990) and less ruminating activity (Batchelder, 2002). Standing costs energy and can result in more lameness and less ruminating activity results in less feed utilization, which could result in skinny cows and therefore a lower body condition score. In this study there was no significant association between BCS and stocking density of cubicles.

For the WM, the number of deviating BCS, BCS <2 and BCS >4, must be noted. The percentage deviating BCS, calculated by the number of cows with a deviating BCS, compared to the total number of scored cows for BCS, is significant higher on overstocked farms on feeding places, compared to non-overstocked farms on feeding places. Only the percentage cows with BCS <2 is significant higher on overstocked farms on feedings places, compared to non-overstocked farms on feeding places. No research has been found investigating the associations between housing conditions, stocking density, and BCS. An explanation for the significant higher percentage of cows with BCS <2, housed in overstocked farms on feeding places, could be that low-ranking animals have reduced or no access to the feed bunk and spend less time at the feed bunk, which could result in skinny cows.
The variation in WMS feed is for 19,6% due to variation in the stocking density of water supply. However, the stocking density of water supply is part of the calculation of the WMS feed.

WMS behaviour is significant higher, so better, on overstocked farms (on cubicles, feedings places and water supply), than on non-overstocked farms. WMS behaviour contains the indicators grazing and avoidance test (Eerdenburg et al., 2018; KoeKompas, 2018). The farms with overstocking of cubicles or feeding places have on average significant (Mann-Whitney U test, $p < .05$) more days grazing per year (138 and 124 days), compared to farms without overstocking of cubicles or feeding places (88 and 67 days). This could be the explanation for the higher score for overstocked farms on cubicles or feeding places. The difference in grazing days between overstocked and non-overstocked of water supply was not significant.
The avoidance test is performed by looking at which distance the cows avoid human contact. The distance is equal to a score: score one avoidance at 0 cm (can be touched), score five avoidance from 10 or more meters (KoeKompas, 2018). There is only a significant difference in avoidance test, also called behaviour of the herd in KK, between overstocked and non-overstocked farms on cubicles. On overstocked farms on cubicles the cows scored better on the avoidance test, than the cows on non-overstocked farms. But the rounded final score for both is 3, which is equivalent to normal behaviour.
The animal welfare chapter of KK describes that the number of non-active animals is also recorded for the WMS behaviour (in contrast to the indicators for the WM behaviour described in the introduction of KK) (KoeKompas, 2018). There was no significant association between non-active animals and stocking density or overstocked versus non-overstocked of cubicles, feeding places or water supply.

There was a significant association between stocking density of the feeding places and milk production per day; the variation in milk daily production is for 7% due to variation in the stocking density of feeding places. Higher stocking density of feeding places is associated with lower daily milk production. To compare farms the 305-day production is more reliable, but no significant relation or difference is found between the 305-d production and stocking density or overstocked versus non-overstocked of cubicles, feeding places or water supply.

Overstocked farms on water supply scored significant lower on deviant cow score than non-overstocked farms on water supply. The deviant cows score is based on the overall rumen filling score, manure score and ruminate activity score of the herd (KoeKompas, 2018). Water is necessary for a proper rumination and digestion. Senn et al. (1996) reported decrease in energy intake in lactating cows with water deprivation for 48 hours. Intake of grass and corn pellets was reduced significantly. The rapid reduction in feed intake during dehydration was clearly due to a decrease in meal size, whereas meal frequency can even be increased at the same time. Unlike non-ruminants, lactating cows do not compensate during rehydration phase for the energy intake reduction during water deprivation. A close relation between eating and drinking was observed with ad libitum feed and water supply. The hypothesis is that an enhanced prandial increase in ruminal fluid osmolality contributes to dehydration-induced hypophagia (Burgos et al., 2001). A possible explanation for a higher number of deviating cows on farms with overstocking of water supply is that less water intake causes less feed intake and therefore moderate rumen filling. Moderate rumen filling, can also cause less ruminate activity.

The SCC score from KK is higher, so better, on overstocked farms on water supply compared with farms without overstocking of water supply. The SCC score is a score for the BMSCC and the percentage of increased SCC cows (SCC is >250 for multiparous cows and SCC is >150 for primiparous cows). These two data combined are equal to a score: score 1 if BMSCC is below 150 and less than 15% increased SCC cows, score 5 if BMSCC is above 300 and more than 30% increased SCC cows (KoeKompas, 2018). Separately, there was no significant difference in SCC or increased SCC, between overstocked farms versus non-overstocked farms on water supply. This is possibly because the exact numbers of increased SCC cows were not available from all farms (35 farms unknown). There is no explanation for a relation between less water supply and less cows with increased SCC.
The SCC data from the test-day (increased SCC (%), new increased SCC(%), increased SCC after calving (%) recent/last quarter/half year/year), were analysed with Pearson correlation and excluded from further analysis, because the data were not available from all farms. With these numbers the power was too low.

The heel score from KK is higher, so better, on farms with higher stocking density of feeding places. The heel score is also higher on overstocked farms on water supply compared with non-overstocked farms on water supply. Heel score 1 is a thick, damaged heel and score 5 a soft heel with no bald spots. There is no explanation for the association between heel score and stocking density of feeding places or water supply.

Cortisol

There was no significant difference in cortisol level in hair samples between random and chronic sick animals. It was not possible to do the Mann-Whitney U analysis with the random effect of specific farm number. The dataset shows a large variation in cortisol concentration between random and chronic sick animals housed in one farm. The randomly selected group also includes cows that may be chronic sick. An example is a random cow with a somatic cell count of 4.479.000 cells/mL.
Housing in overstocked conditions (on cubicles, feeding places and water supply) results in significant higher cortisol concentrations in hair samples. It was not possible to do this analysis with the random effect of specific farm number. Cortisol concentrations in hair samples of dairy cows was also

significantly associated with the stocking density and overstocking versus no overstocking of cubicles, water supply and water supply, with the random effect of specific farm number.

Parity, primiparous versus multiparous, was a significant predictor in the multivariable mixed models. These models suggest that primiparous animals have a higher cortisol concentration in hair samples, compared to multiparous cows. The mean cortisol concentration was higher for primiparous cows, compared to multiparous (74,41 ± 37,08 nmol/L versus 63,30 ± 31,25) in this study. These results were in contrast with the results of Burnett et al. (2015). Burnett et al. (2015) reported also a significant parity effect on cortisol concentration in hair samples of cows absent of clinical disease, but the exact opposite: multiparous animals had consistently higher cortisol concentrations than primiparous animals (9,2 versus 7,8 pg/mg). A possible explanation for this difference was not discussed in the study. Wierenga (1990) reported that younger cows were more frequently displaced from cubicles and the feed bin. Displacements could lead to stress, which could lead to higher cortisol levels and higher cortisol concentration in hair samples. More research is necessary for the possible association between parity and cortisol concentration in hair samples of dairy cows.

Overstocked versus non-overstocked of feeding places was also a significant predictor in the multivariable mixed models. The higher cortisol concentrations in hair samples from dairy cows housed in overstocked farms on feedings places is in contrast with the results of Silva et al. (2016). However, Silva et al. (2016) investigated only two stocking densities, 80 or 100% for feeding places, so without overstocking. At increased stocking densities, cows increase direct competitive behaviour through increased displacements at the feed bunk (Collings et al. 2011; Proudfoot et al., 2009). More feed bunk competition leads to stress and could result in higher cortisol concentration in hair.

No literature could be found investigating the association between cortisol concentrations in hair samples from (dairy) cows and stocking density of cubicles or water supply, or housed with overstocking of cubicles or water supply. Cows housed in overstocked conditions on cubicles spent less time lying down, so less time for resting and more time standing (Fregonesi, Tucker & Weary, 2007; Winkler et al, 2003; Wierenga & Hopster, 1990; Matzke & Grant, 2002), and have more (direct) competition for cubicles (Fregonesi, Tucker & Weary, 2007). Low-rank cows spent less time lying, standing more time in the passageway or (partly) in the cubicles, compared to middle- and high-rank cows (Galindo & Broom, 2000). So, overstocked conditions on cubicles will affect cows, but lower-rank cows certainly. The increased competition for cubicles, leads to more stress and could result in higher cortisol concentration in hair. A possible explanation for the association between cortisol concentration in hair samples and stocking density of water supply, is more competition at the water basin, which causes more displacements and more stress. Benatallah, Ghozlane & Marie (2019) found increased cortisol levels in serum of cows during a water restriction of eight days. But, Burgos et al. (2001) found decreased plasma cortisol concentrations during water restriction of eight days. Cortisol plays an important role in maintaining fluid balance and plasma electrolytes (Parker et al., 2003), but the exact role of cortisol in water restriction or less water supply is unknown.

Cortisol concentrations in hair could be a potential indicator for dairy cattle welfare by providing a useful and practical tool for long-term steroid monitoring (Tallo-Parra et al., 2018). Despite all the promising results, there are still gaps in the knowledge of hair steroid determination; high variability between individual cows (Comin et al., 2013), higher concentrations of cortisol in white hair than black hair (Burnett et al., 2014; del Rosario et al. , 2011). More research is necessary.

Study design

Selection of the farms was not random, because there is no normal distribution of the farms with regards to animal welfare. On average, animal welfare is fairly good on Dutch dairy farms. There are only a few farms with really poor welfare in the “tail” of the welfare distribution. So, it was necessary

to get some farms with the suspicion of poor welfare from the Dairy cooperation and they are overrepresented in the study population. This study population is therefore not a represented population of the Dutch Dairy farms. But there are still overstocked farms in the Netherlands.
The animal health data was not useful for this study, only the CDM score. The animal health data were not recorded on every farm, so the occurrence of diseases was often an estimation of the farmer and therefore not reliable.

Before the start of the study the four veterinarians trained together with KK on two farms to minimise the differences in scoring. In addition, there have been several meetings and brainstorming sessions to discuss the differences in scoring. During the farm visits some differences in the way of scoring between the veterinarians appeared, but in general the scoring was identical. Sufficient attention has been paid to score in the same way. Difference in score will always remain.

There was no other study that used KK or CDM score to score animal health or welfare in relation to stocking density, so it was sometimes difficult to compare results. Researchers investigated most of the time the effect of overstocking on the lying and standing behaviour of dairy cattle (Fregonesi, Tucker & Weary, 2007; Collings et al., 2013). For KK only the non-active animals were scored, no other behavioural parameters.

Conclusion

The aim of this study was to look for associations between stocking density and animal health or welfare. The outcome of this study shows that there are still farms with overstocking in the Netherlands, in particular on the number of feeding places and on water supply. Housing under overstocked conditions (on cubicles, feeding places and water supply) results in significant higher cortisol concentrations in hair samples, compared to non-overstocked conditions. These results give an indication that overstocked housing can cause stress and therefore has impact on animal welfare, but more research is necessary. A few significant weak associations were found between stocking density and certain welfare components from KoeKompas. No significant association was found between the CDM score and stocking density.

Recommendations

This thesis studies the association between stocking density and animal health and welfare. Determining the hormone cortisol in hair samples from dairy cows was part of this thesis. The results give an indication that overstocked housing can cause significant higher cortisol concentration in hair samples, so more stress. Prior to this study, it was expected that there would be a difference in cortisol concentration in hair samples, between randomly selected and chronic sick cows on farms. However, no association was demonstrated in this study. A possible explanation for this could be the selection of random and sick cows. The randomly selected group also included animals with abnormal clinical observations, for example SCC and locomotion score. For a follow-up study, it is recommended to further analyse the data and to convert random animals with for example high SCC (> 800,000 cells / mL), to chronic sick cows.

In the multivariable mixed models for cortisol concentrations, stocking density and cow factors, parity was a significant predictor in this study. This model suggests a difference in cortisol concentration in hair samples for primiparous and multiparous cows. The results of this study were in contrast with the results of Burnett et al. (2015). Overall, the differences in cortisol concentration in hair samples between primiparous and multiparous cows indicate that parity should be considered in cortisol studies in hair samples of dairy cows. For a follow-up study, it is recommended to further research the possible association between parity and cortisol concentration in hair samples of dairy cows.

The Pearson correlation performed in this study, suggests an association between the SCC data and the occupancy of the cubicles. It was not possible to further investigate this association, because of the small sample size. A suggestion for further research is the possible association between stocking density of cubicles and SCC data (SCC, increased SCC, new increased SCC, increased SCC after calving.

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Appendix

Appendix 1: Questionnaire

Vragenlijst voor de veehouder onderzoek Gaby
Onderstaande vragen hebben alleen betrekking op het melkvee koppel (niet op het jongvee!)

Damslapers

1) Hoeveel damslapers zijn er in het melkvee koppel?
2) Heeft u inzicht welke pariteit de damslapers zijn? NEE / JA:
   (aantal, eventueel diernummer/oornummer noteren)

	Totaal aantal	**Vaarzen**	**2e kalfs**	**3e kalfs en ouder**
Damslapers				

Klauwproblemen

1) Maakt u gebruik van Digiklauw? NEE / JA
   Zo ja, geeft u toestemming om dit in te zien? NEE / JA
2) Welke methode past u toe wat betreft klauwbekappen? (Hoe vaak?)
   a. Strategisch bekappen ……………………………….
   b. Koppelbekappen ……………………………………
3) Hoe vaak zijn onderstaande klauwaandoeningen voorgekomen afgelopen half jaar?
4) Heeft u inzicht bij welke pariteit u de klauwproblemen ziet? NEE / JA:

Klauwaandoeningen	**Totaal aantal afgelopen half jaar**	**Vaarzen**	**2e kalfs**	**3e kalfs en ouder**
Zoolbloedingen				
Witte lijndefecten				
Zoolzweren				
Stinkpoot				
Mortellaro				
Tussenklauwontsteking				
Tyloom				

Stofwisselingsstoornissen

1) Hoe vaak zijn onderstaande stofwisselingsstoornisssen voorgekomen afgelopen half jaar?
2) Heeft u inzicht bij welke pariteit u de stofwisselingsziekten ziet? NEE / JA:

Stofwisselingsstoornissen	**Totaal aantal afgelopen half jaar**	**Vaarzen**	**2^{e} kalfs**	**3^{e} kalfs en ouder**
Lebmaag				
Melkziekte				
Slepende melkziekte / ketose				

Hartelijk dank voor het invullen van bovenstaande vragenlijst!

Appendix 2: Statistics

2.1 Boxplots of stocking density of cubicles, feeding places and water supply

Figure 2.1.1: Boxplot of stocking density of cubicles (%) on non-overstocked (n=36) and overstocked (n=19) farms.

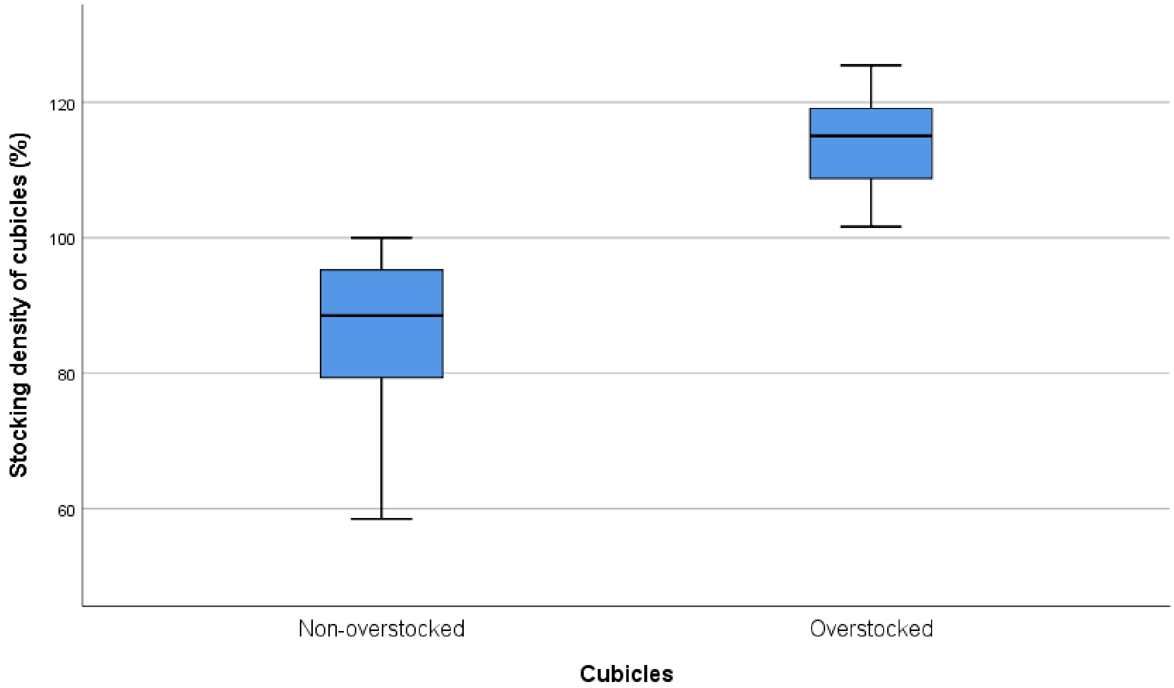


Figure 2.1.2: Boxplot of stocking density of feeding places (%) on non-overstocked (n=18) and overstocked (n=37) farms.

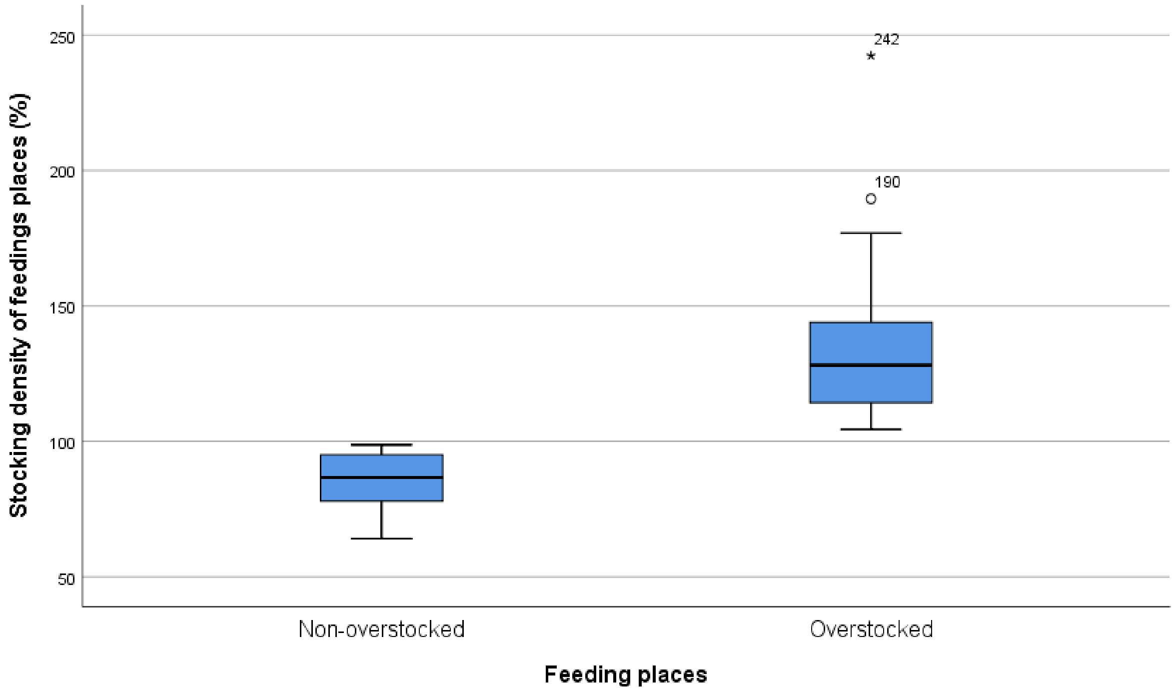


Figure 2.1.3: Boxplot of stocking density of water supply (%) on non-overstocked (n=12) and overstocked (n=43) farms.

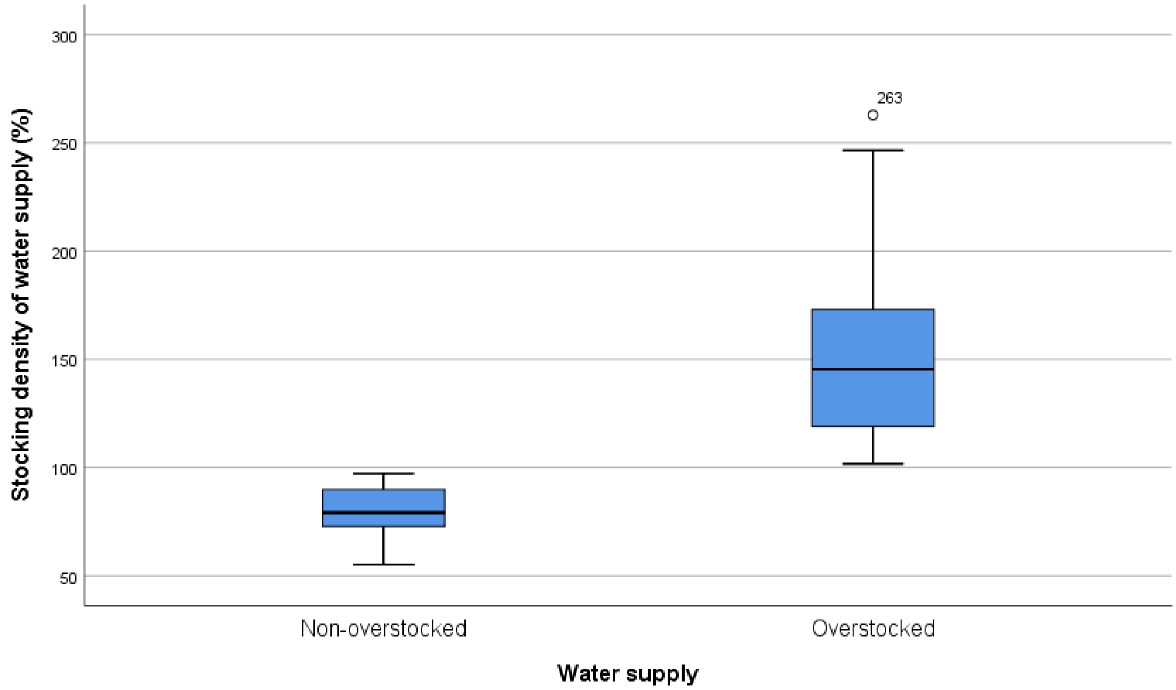


Figure 2.1.4: Boxplot of stocking density of cubicles (%) on farms with a conventional (n=35) and automatic (n=20) milking system.

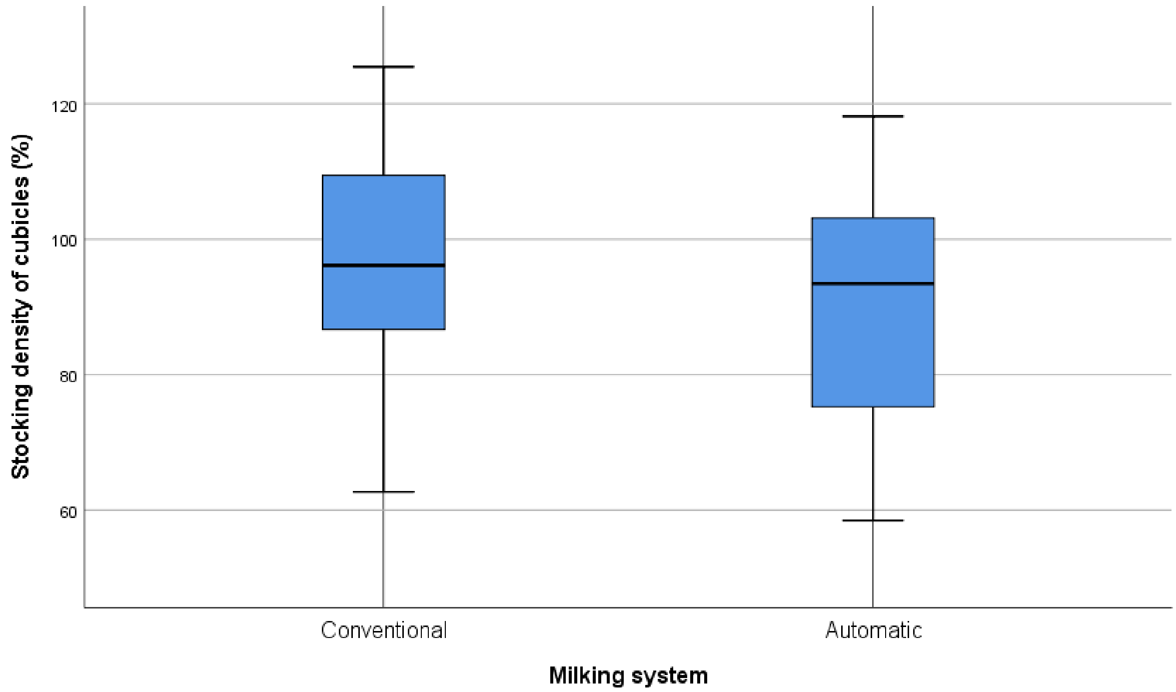


Figure 2.1.5: Boxplot of stocking density of feeding places (%) on farms with a conventional (n=35) and automatic (n=20) milking system.

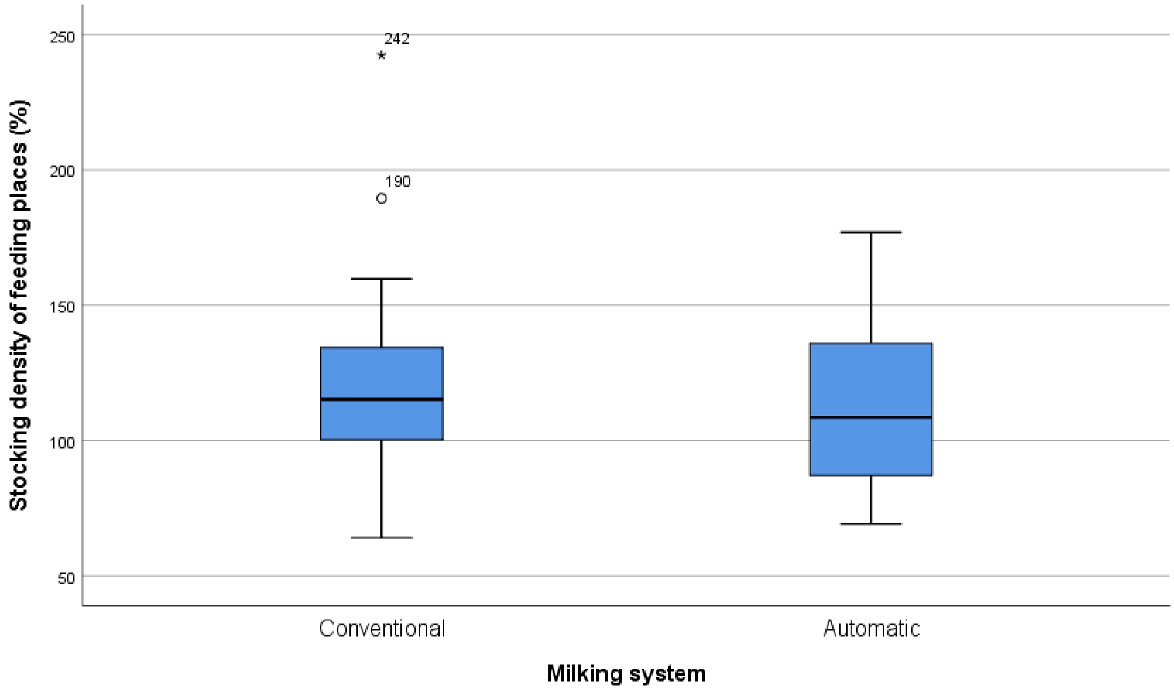
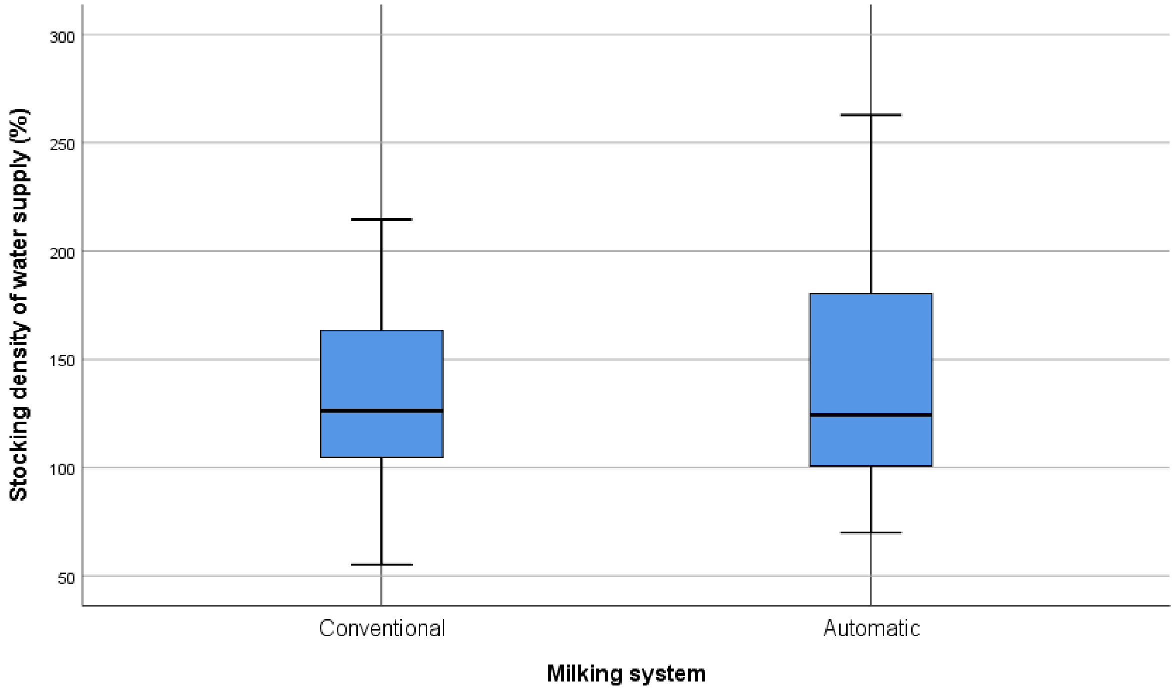


Figure 2.1.6: Boxplot of stocking density of water supply (%) on farms with a conventional (n=35) and automatic (n=20) milking system.



2.2 Descriptive statistics CDM score and animal welfare scores

Table 2.2.1 Descriptive statistics for CDM and animal welfare scores from KK overall and for overstocked (YES) versus non-overstocked (NO) farms on cubicles, feeding places and water supply.

	Overall	Overstocking cubicles		Overstocking feeding places		Overstocking water supply	
		NO	YES	NO	YES	NO	YES
CDM score per quarter							
N	38	24	14	13	25	7	31
Mean	80	80	82	78	82	81	80
Standard deviation	16	16	17	18	15	23	15
Minimum	36	36	39	36	39	36	39
Maximum	100	100	100	100	100	100	100
CDM score annually moving average							
N	39	24	15	13	26	7	32
Mean	69	70	69	68	70	65	70
Standard deviation	15	16	15	18	14	19	14
Minimum	24	24	46	24	46	24	41
Maximum	100	100	100	79	100	78	100
Final score welfare KoeKompas							
N	55	36	19	18	37	12	43
Mean	3,06	3,1	3,0	3,1	3,0	3,1	3,0
Standard deviation	0,36	0,34	0,42	0,38	0,36	0,32	0,38
Minimum	2,20	2,5	2,2	2,6	2,2	2,7	2,2
Maximum	3,80	3,8	3,8	3,8	3,8	3,8	3,8
Welfare monitor score feed							
N	55	36	19	18	37	12	43
Mean	42	51	26	57	35	61	37
Standard deviation	27	27	16	25	25	27	24
Minimum	7	12	7	28	7	30	7
Maximum	100	100	54	100	100	100	100
Welfare monitor score housing							
N	55	36	19	18	37	12	43
Mean	54	54	53	54	53	53	54
Standard deviation	7	7	7	7	7	8	7
Minimum	34	38	34	38	34	42	34
Maximum	67	67	61	63	67	67	63
Welfare monitor score health							
N	55	36	19	18	37	12	43
Mean	51	50	52	50	51	51	51
Standard deviation	12	12	12	12	12	13	12
Minimum	28	28	29	29	28	29	28
Maximum	75	75	73	75	73	75	73
Welfare monitor score behaviour							
N	55	36	19	18	37	12	43
Mean	48	41	63	38	54	44	49
Standard deviation	24	23	19	22	24	22	25
Minimum	19	19	21	19	19	19	19
Maximum	89	89	80	77	89	78	89

2.3 Scatterplots

Figure 2.3.1: Scatterplot between cubicles stocking density and WM feed score with the regression line.

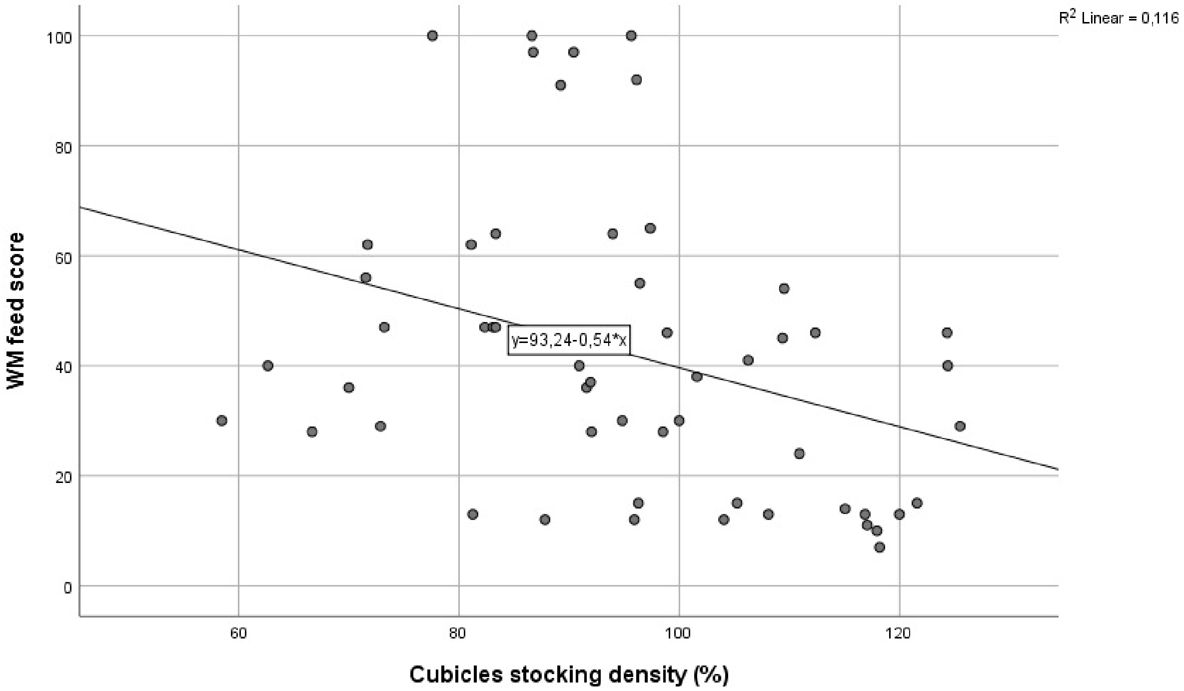


Figure 2.3.2: Scatterplot between cubicles stocking density and WM behaviour score with the regression line

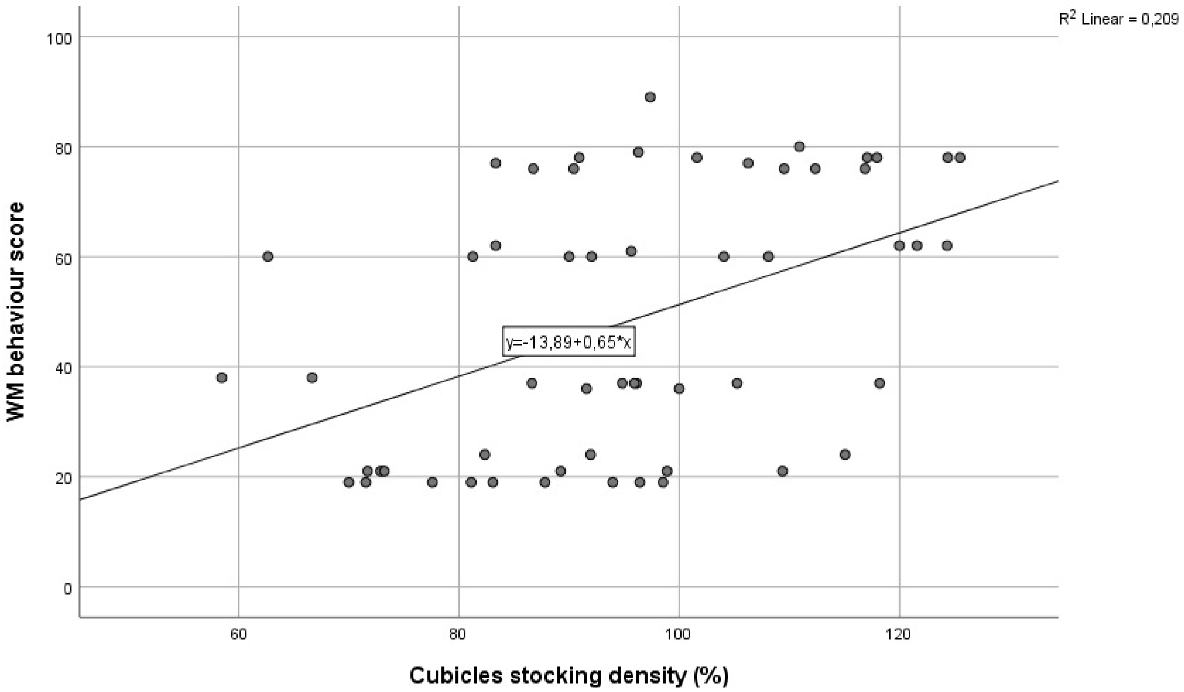


Figure 2.3.3: Scatterplot between feeding places stocking density and milk production per day with the regression line

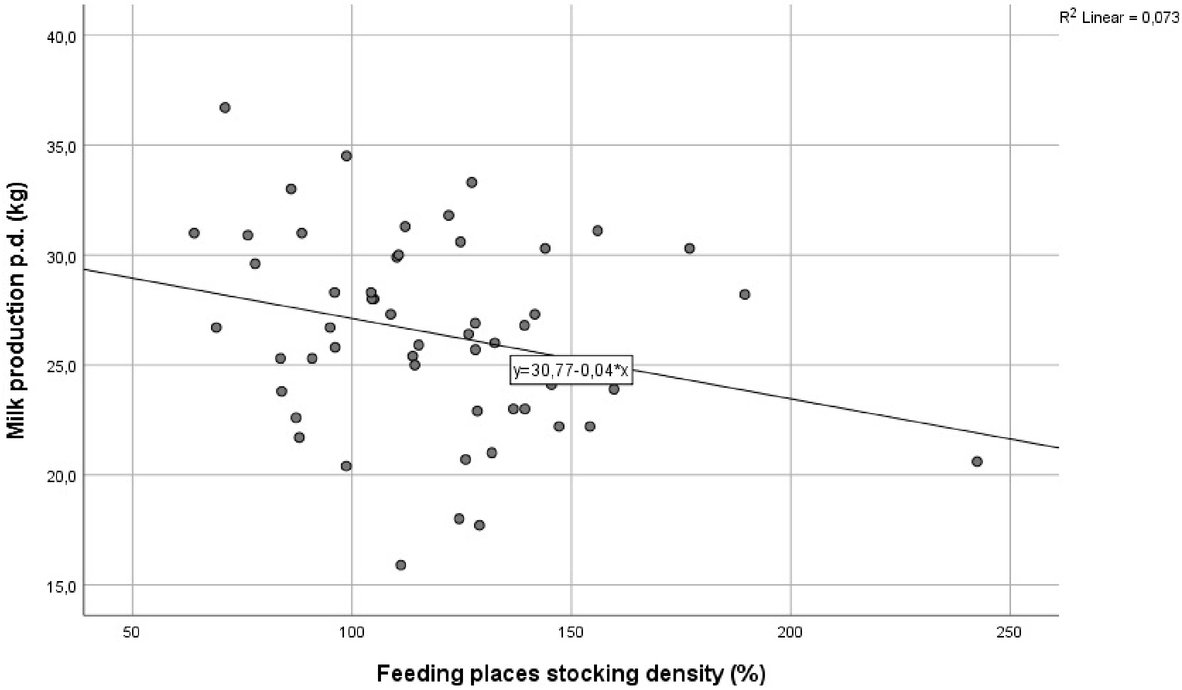


Figure 2.3.4: Scatterplot between feeding places stocking density and WM feed score with the regression line

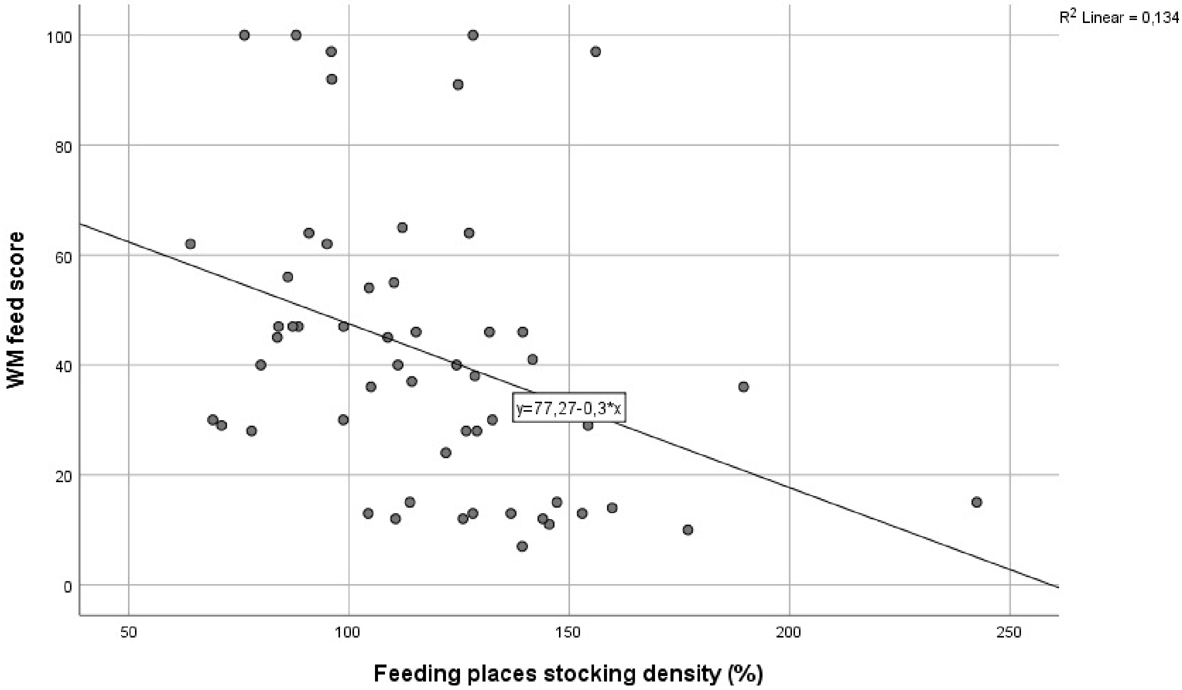


Figure 2.3.5: Scatterplot between water supply stocking density and WM feed score with the regression line

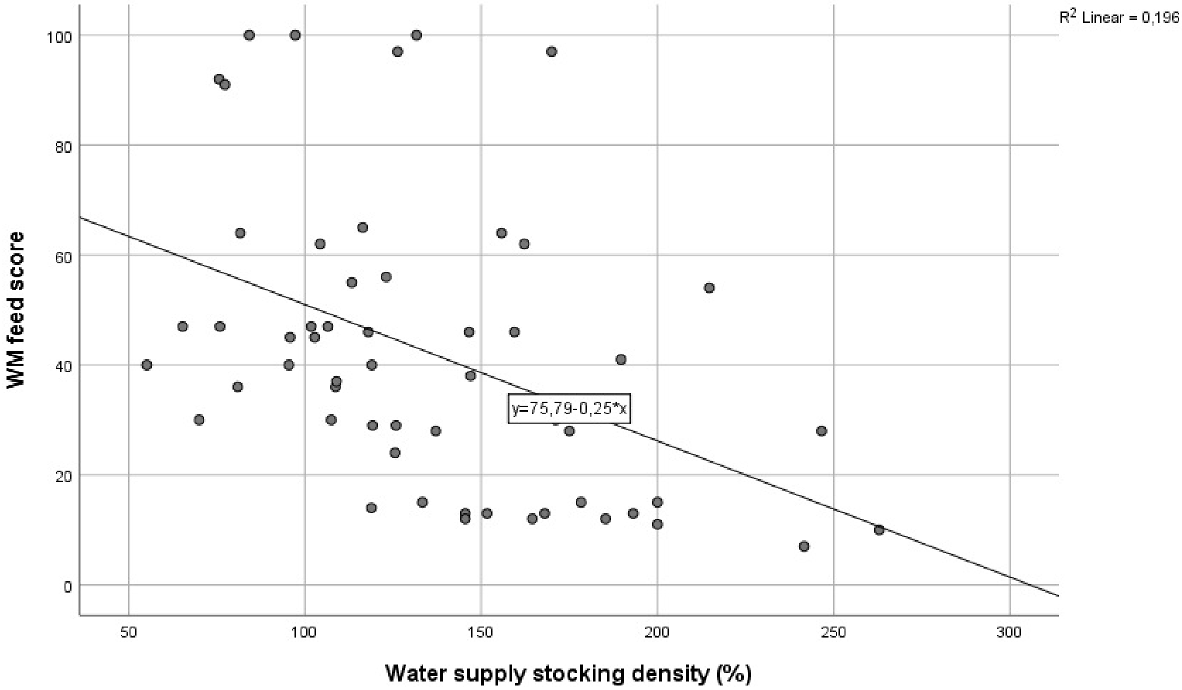
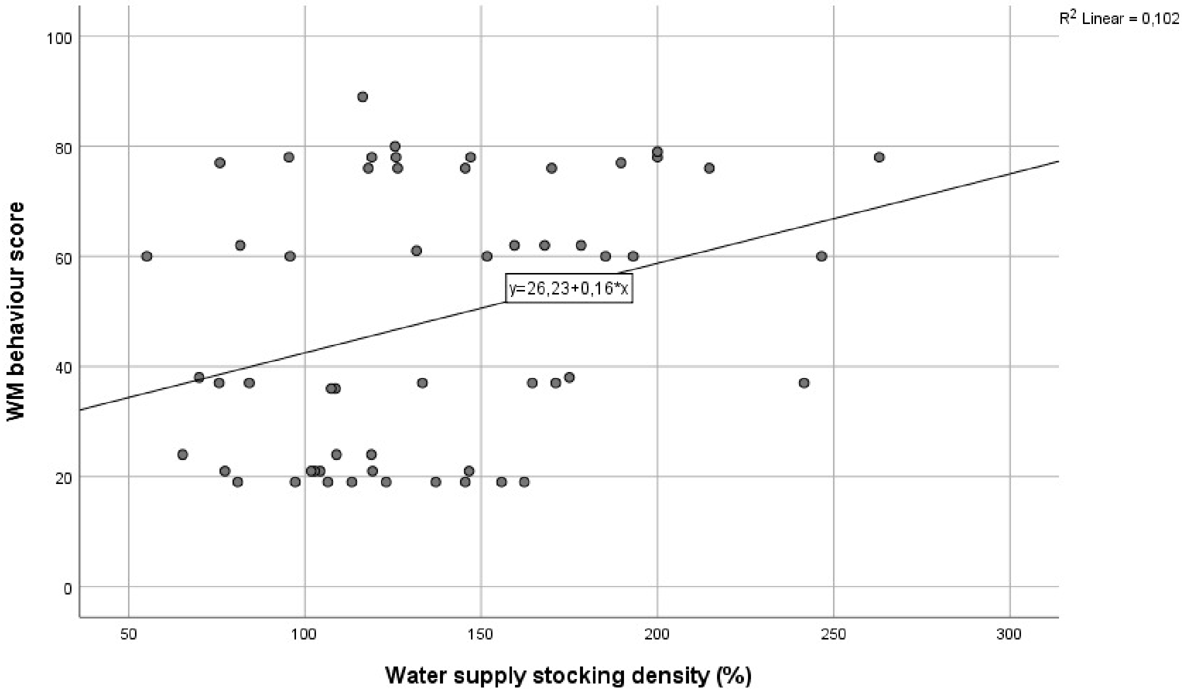


Figure 2.3.6: Scatterplot between water supply stocking density and WM behaviour score with the regression line



2.4 Boxplots of cortisol concentrations in hair samples for overstocked versus non-overstocked of cubicles, feeding places and water supply

Figure 2.4.1: Boxplot of cortisol concentration (nmol/L) in hair samples of dairy cows on farms with non-overstocking (n=154) and overstocking (n=102) of cubicles.

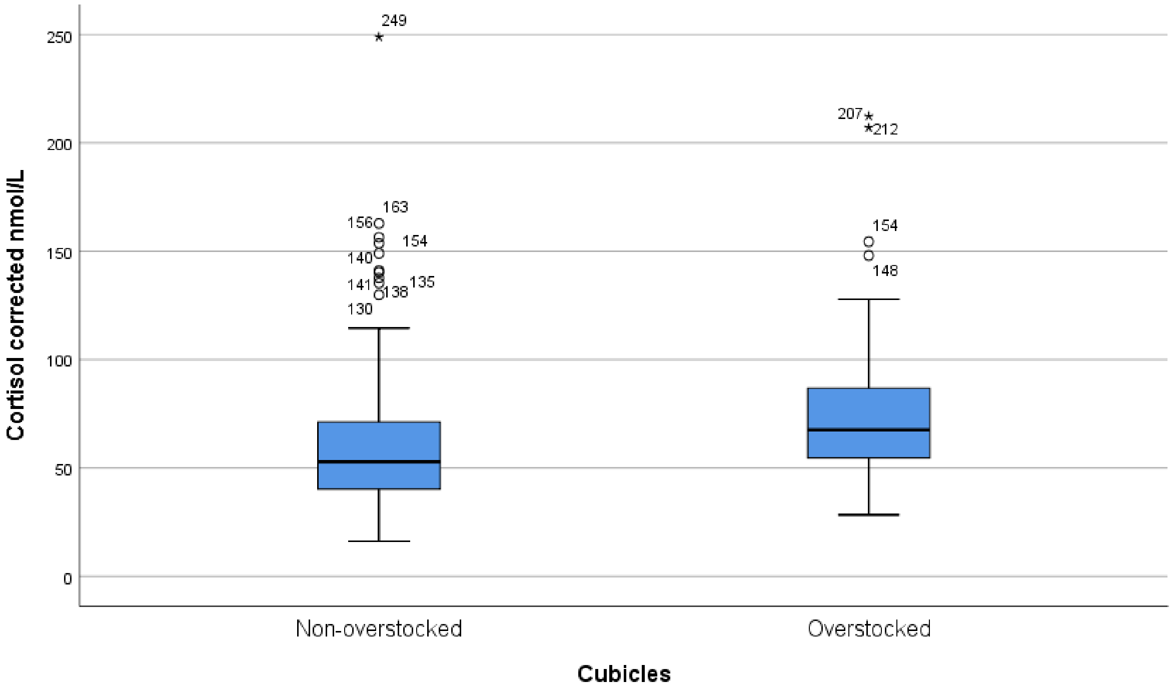


Figure 2.4.2: Boxplot of cortisol concentration (nmol/L) in hair samples of dairy cows on farms with non-overstocking (n=121) and overstocking (n=135) of feeding places.

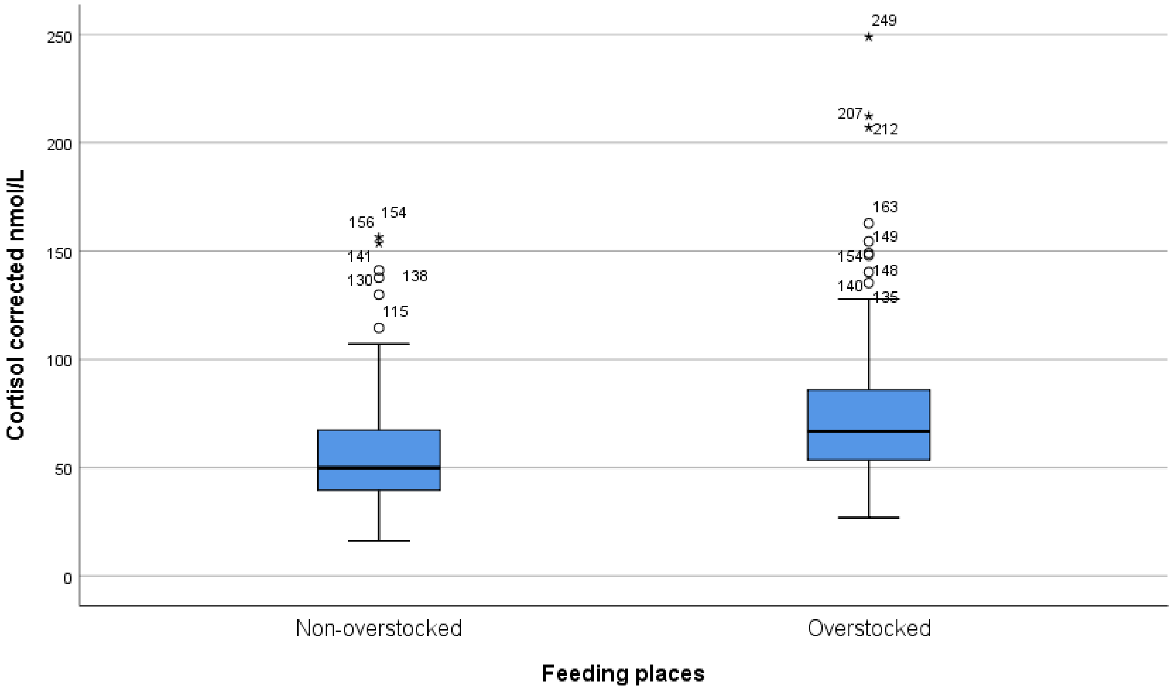
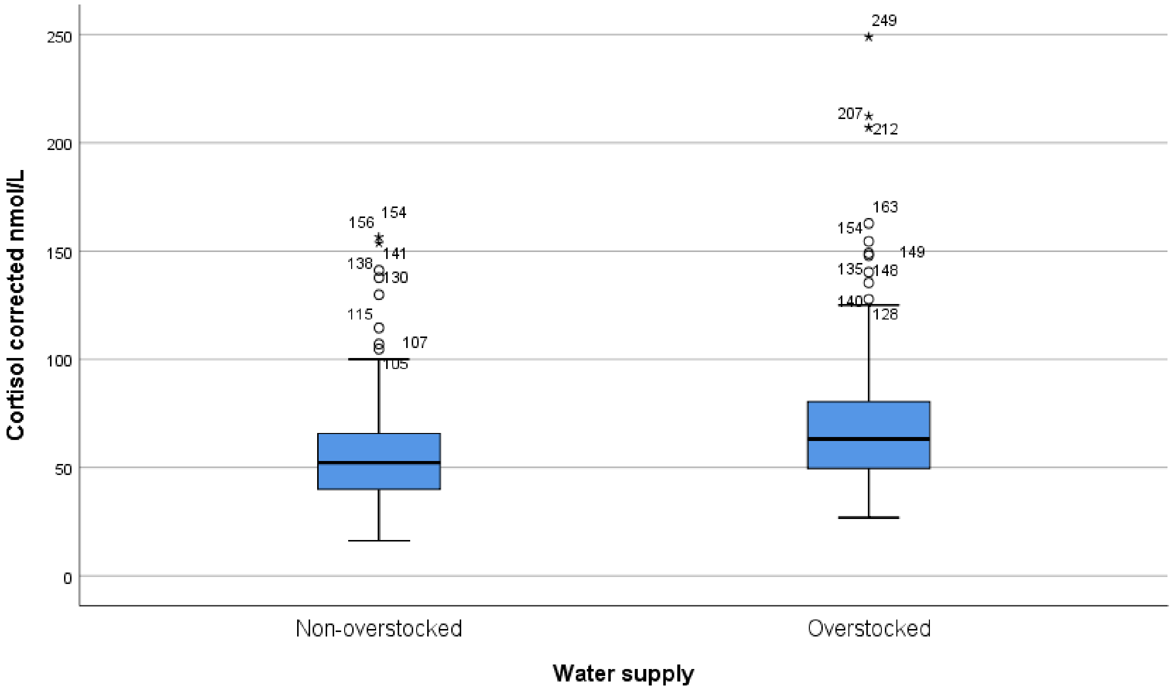


Figure 2.4.3: Boxplot of cortisol concentration (nmol/L) in hair samples of dairy cows on farms with non-overstocking (n=88) and overstocking (n=168) of water supply.



2.5 Descriptive statistics of cortisol concentration in hair samples on farm level

Descriptive statistics of cortisol concentration (nmol/L) on farm level, and for random and chronic sick cows.

	N	Mean	SD	Minimum	Maximum	Overstocking		
						Cubicles	Feeding places	Water supply
Farm number 22	13	50,13	20,44	28,21	102,00	No	No	Yes
Random cows	9	50,75	23,05	28,21	102,00			
Chronic sick cows	4	48,76	15,83	31,06	63,06			
Farm number 26	15	71,67	34,70	28,69	153,70	No	No	No
Random cows	10	71,02	40,74	28,69	153,70			
Chronic sick cows	5	72,98	21,79	51,19	102,06			
Farm number 28	17	104,56	43,48	62,11	212,37	Yes	Yes	Yes
Random cows	13	95,93	40,89	62,11	212,37			
Chronic sick cows	4	132,59	45,00	74,95	183,33			
Farm number 36	10	50,41	9,66	29,27	61,20	No	Yes	No
Random cows	8	50,07	10,63	29,27	61,20			
Chronic sick cows	2	51,84	6,63	47,15	56,52			
Farm number 37	15	53,93	19,93	29,90	89,04	No	No	Yes
Random cows	8	45,39	14,68	29,90	72,02			
Chronic sick cows	7	63,69	19,40	42,34	89,04			
Farm number 40	12	63,13	26,86	40,29	140,28	No	Yes	Yes
Random cows	8	69,67	31,22	40,29	140,28			
Chronic sick cows	4	50,04	5,45	44,04	56,60			
Farm number 46	15	48,68	10,82	29,06	69,58	No	No	Yes
Random cows	10	48,35	13,08	29,06	69,58			
Chronic sick cows	5	49,33	4,91	45,31	57,56			
Farm number 49	15	48,27	13,97	28,38	71,94	No	No	No
Random cows	10	46,85	11,61	29,38	67,33			
Chronic sick cows	5	51,10	19,09	31,54	71,94			
Farm number 52	15	80,33	24,55	46,17	115,63	Yes	Yes	Yes
Random cows	11	82,60	25,38	46,17	115,63			
Chronic sick cows	4	74,09	24,40	50,63	99,59			
Farm number 53	15	79,81	47,79	32,96	241,41	Yes	Yes	Yes
Random cows	10	70,51	14,56	54,88	99,04			
Chronic sick cows	5	98,41	82,88	32,96	241,41			
Farm number 54	11	66,92	40,74	19,04	141,12	No	No	No
Random cows	9	69,02	44,29	19,04	141,12			
Chronic sick cows	2	57,49	26,16	38,99	75,98			
Farm number 55	13	48,99	21,00	24,95	104,67	No	No	No
Random cows	10	50,19	23,49	24,95	104,67			
Chronic sick cows	3	44,99	11,48	33,10	56,02			
Farm number 56	15	59,49	15,97	33,16	85,15	No	No	Yes
Random cows	14	59,98	16,45	33,16	85,15			
Chronic sick cows	1	-	-	-	-			
Farm number 57	16	40,13	14,66	25,88	89,47	No	No	No
Random cows	10	36,18	7,05	25,88	49,95			
Chronic sick cows	6	46,71	21,72	32,02	89,47			
Farm number 58	8	126,63	59,06	67,94	248,96	No	Yes	Yes
Random cows	7	126,37	63,78	67,94	248,96			
Chronic sick cows	1	-	-	-	-			

N = sample size; SD= standard deviation

Descriptive statistics of cortisol concentration (nmol/L) on farm level, and for random and chronic sick cows.

						Overstocking		
	N	**Mean**	**SD**	**Minimum**	**Maximum**	Cubicles	Feeding places	Water supply
Farm number 60	13	94,99	48,20	41,79	207,07	Yes	Yes	Yes
Random cows	9	105,33	51,01	41,79	207,07			
Chronic sick cows	4	71,73	36,24	42,15	121,47			
Farm number 61	15	74,07	31,96	32,16	147,52	Yes	Yes	Yes
Random cows	10	67,15	28,10	32,16	125,00			
Chronic sick cows	5	87,91	37,94	49,65	147,52			
Farm number 62	14	66,19	31,65	28,63	148,00	Yes	Yes	Yes
Random cows	10	69,18	31,66	42,76	148,00			
Chronic sick cows	4	58,73	35,08	28,63	108,92			
Farm number 64	13	89,68	35,30	43,35	156,48	No	No	No
Random cows	10	86,58	33,77	43,35	156,48			
Chronic sick cows	3	99,99	46,23	50,36	141,84			
Farm number 65	13	88,90	90,68	29,95	369,23	Yes	Yes	Yes
Random cows	9	58,20	24,32	29,95	106,36			
Chronic sick cows	4	157,96	148,74	40,42	369,23			
Farm number 66	9	64,36	10,43	48,84	82,67	Yes	Yes	Yes
Random cows	6	60,27	8,41	48,84	70,90			
Chronic sick cows	3	72,55	10,36	61,96	82,66			
Farm number 67	4	52,95	24,93	34,26	86,94	No	Yes	Yes
Random cows	1	-	-	-	-			
Chronic sick cows	3	41,63	12,72	34,26	56,32			
Farm number 68	14	55,90	14,13	34,43	80,09	Yes	Yes	Yes
Random cows	9	59,40	14,49	34,43	80,09			
Chronic sick cows	5	49,61	12,31	37,81	64,42			
Farm number 69	3	74,54	15,36	57,57	87,49	No	No	Yes
Random cows	0	-	-	-	-			
Chronic sick cows	3	74,54	15,36	57,57	87,49			
Farm number 72	11	53,78	17,29	28,29	79,90	Yes	Yes	Yes
Random cows	8	53,87	18,29	28,29	79,90			
Chronic sick cows	3	53,55	17,99	38,46	73,47			
Farm number 76	12	62,14	32,29	26,80	149,00	No	Yes	Yes
Random cows	9	61,61	37,73	26,80	149,00			
Chronic sick cows	3	63,75	5,82	57,73	69,35			
Farm number 77	13	86,19	36,80	41,67	189,90	Yes	Yes	Yes
Random cows	7	85,70	15,10	60,88	107,14			
Chronic sick cows	6	86,77	54,55	41,67	189,90			
Farm number 78	12	48,68	26,39	16,13	107,07	No	No	No
Random cows	11	50,09	27,20	16,13	107,07			
Chronic sick cows	1	-	-	-	-			
Farm number 79	15	72,34	23,93	42,68	116,10	No	No	No
Random cows	10	66,37	19,81	42,68	100,00			
Chronic sick cows	5	84,26	29,22	53,51	116,10			

N = sample size; SD= standard deviation