Exploring the effect of auditory stimuli as environmental enrichment in a Holstein herd using glucocorticoids as biomarker

by

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Declaration

I, Lize-Mari Erasmus, hereby declare that this thesis, submitted for the MSc (Agric) Animal Science: Animal Breeding and Genetics degree at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at any other University.

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1 Corinthians 15:10 “Yet not I, but the Grace of God within me”.

Abstract

Health and welfare are inextricably linked within efficient and sustainable dairy production. There are several potential risk factors which may affect the well-being of dairy cows, including chronic stress. Even though environmental enrichment can be used as a tool to decrease the potential stress that cows might experience, it is seldom applied to livestock production systems outside of research purposes. The aim of this study was to explore the effect of auditory stimuli as environmental enrichment in a Holstein herd through the use of glucocorticoid concentrations. Non-invasive methods, namely faecal glucocorticoid metabolites (fGCM) and milk glucocorticoid (mGC) concentrations, were employed as stress-associated biomarkers. Activity level and milk yield formed additional parameters for the study. Nine cows in their second- and third-lactation were divided into three groups (A-C) and through the use of a Latin Square as experimental design, each group was exposed to three treatments, namely constant exposure (CE), limited exposure (LE), and no exposure (NE) to slow classical music. Both faecal and milk samples were analysed for fGCM and mGC concentrations using respective enzyme immunoassays (EIA). A comparison between generated hormone concentrations indicated that fGCM concentrations were more meaningful in its use as a non-invasive biomarker. Cows exposed to constant music had lower stress-related fGCM concentrations ($P = 0.012$), as well as higher milk yields ($P > 0.0001$) and lowered activity levels during the morning activity period (named activity 1) and the evening activity period (named activity 3) (Activity 1: $P = 0.005$; Activity 3: $P = 0.048$). During no exposure to music, the cows had higher fGCM concentrations, lower milk production and higher activity levels. These findings indicate that auditory stimuli as a form of environmental enrichment have economic benefits to the producer, by potentially reducing the number of cows needed for profitable production as well as the amount of agricultural land required for production. Environmental enrichment will also assist in improving the way that consumers think about dairy farms and dairy production.

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List of abbreviations

°C	Degrees Celsius
ACTH	Adrenocorticotropic hormone
ABMs	Animal-based measurements
ANOVA	Analysis of Variance
ANS	Autonomic Nervous System
BCS	Body Condition Score
CE	Constant exposure to music
CI	Confidence interval
CRF	Corticotropin-Releasing Factor
CRH	Corticotropin-Releasing Hormone
COP	Code of Practice
CV	Coefficient of Variance
DAFF	Department of Agriculture, Forestry and Fisheries
DALRRD	Department of Agriculture, Land Reform and Rural Development
dB	Decibel
DF	Degrees of Freedom
DIM	Days in Milk
DMI	Dry Matter Intake
DSA	Dairy Standard Agency
DW	Dry Weight
e.g.	For example
EIA	Enzyme Immunoassay
ERL	Endocrine Research Lab
et al.,	And others
EU	European Union
fGCM	Faecal Glucocorticoid Metabolite
Fig	Figure
FMI	Fluid Merit Index
g	Grams
GLM	General linear models
HPA-axis	Hypothalamic-Pituitary-Adrenal axis
Hz	Hertz
K.	Köchel catalogue
kHz	Kilohertz
LE	Limited exposure to music
LEVLO	Department of Agricultural Economics, Extension and Rural Development
m	meters
MANOVA	Multiple Analysis of Variance
mGC	Milk Glucocorticoid
mg	Milligrams
mL	Millilitres
MPO	Milk Producers Organisation
MRI	Mammal Research Institute
N	Sample size

N-ABM	Non-Animal-Based Measurement
NAS	Natural and Agricultural Sciences
NE	No exposure to music
ng	Nanogram
No.	Number
NSPCA	National Council of Societies for the Prevention of Cruelty to Animals
OIE	World Organisation for Animal Health
pg	Picogram
r	Correlation coefficient
R	Regulation
SA	South Africa
SAS	Statistical Analysis System
SABS	South African Bureau of Standards
SANS	South African National Standard
SAMPRO	South Africa Milk Processors’ Organisation
SCC	Somatic cell count
SD	Standard deviation
SPCA	Society for the Prevention of Cruelty to Animals
TMR	Total mixed ration
UP	University of Pretoria
USB	Universal Serial Bus
WQ	Welfare Quality

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Chapter 1: Introduction

1.1 Introduction to dairy cow welfare

Animal welfare is a continuously growing topic of discussion in both science and society (Dunston-Clarke *et al.*, 2020; Mulder & Zomer, 2017). Historically, the focus of dairy production was solely on increasing milk yields, but this had unintended negative consequences on reproduction, health and longevity (Oltenacu & Broom, 2010). Since then, the production criteria have been changing to include functional and welfare traits into the breeding objectives of cows (Miglior *et al.*, 2005). The World Organisation for Animal Health (OIE) describes animal welfare as a human responsibility, which includes all aspects of animal life, including proper management, housing, disease prevention and treatment, humane handling and responsible care (OIE, 2019). There is, however, no globally accepted definition of animal welfare (Alonso e*t al.*, 2020; Mellor, 2016). For the purposes of this study, the term animal welfare refers not only to the proper treatment and care of an animal, but also ensuring that its physical and mental needs are addressed.

Due to the continuous increase in world population, it is vital to ensure efficient livestock production (Capper *et al.*, 2009). Consequently, most dairy cattle are housed indoors allowing various benefits, including being shielded from harsh climates, protection against parasites and the provision of a nutritionally balanced diet throughout the year (Ritter *et al.*, 2019; Cook, 2008). Indoor housing is, however, associated with modification to the behaviour of cows due to confinement in an impoverished environment that hinders them to satisfy their behavioural needs (Chen & Ogura, 2017; Crouch *et al.*, 2019). Intensive farming systems are also characterized by increased herd sizes, restrictive environments, inadequate rest, and an increased risk for lameness and mastitis (Llanos *et al.*, 2018; Mee & Boyle, 2020). The global increase in the number of cows that are raised in zero-grazing systems defend the need to explore different methods to meet the cows' needs and thereby improve their welfare (Mandel *et al.*, 2016). The concern of animal welfare is not equally distributed among the different livestock species (Clark *et al.*, 2016). The welfare of dairy cows used to be perceived as adequate due to the association of dairy cows with rolling green pastures and, as a result, the need to enhance dairy cow welfare has been underestimated (Armbrecht *et al.*, 2019). Dairy cows are regarded as the second greatest welfare problem in the European Union (EU) due to current production practices, which may lead to discomfort and pain (Leliveld & Provolo, 2020; Sadiq *et al.*, 2017). Consequently, there is plenty of room for improving the welfare of dairy cows (De Vries *et al.*, 2015).

Cow welfare has a direct impact on the physiology, longevity, fertility and productivity of dairy cows (Grant, 2012). Involuntary culling occurs when welfare traits are compromised (Chiumia *et al.*, 2013; Schuster *et al.*, 2020), primarily due to claw disorders, lameness and mastitis - three of the primary contributors to cow comfort (Olechnowicz *et al.*, 2016). Impaired welfare directly affects the cost of production, leading to economic strain and therefore, producers may benefit from including positive welfare indicators into their dairy production system (Doyle & Moran, 2015; Pritchard *et al.*, 2013).

To ensure cows are comfortable in their current environment, it is necessary to extend to them the freedom to fulfil not only their physiological needs, but also their psychological needs (Curtis, 1987; Doyle & Moran, 2015). The physical needs of cows include rest, exercise, rumination and feeding activities (Cook, 2020), which can be monitored through the use of a pedometer. The activity levels of cows are often used to evaluate their health (Rutten *et al.*, 2013). Cows have a strong need to rest, and the general notion is that cows produce more milk when they spend more time resting (Schirmann *et al.* 2012; Temple *et al.*, 2016). Even though various studies report that rest is not a threshold event, consequences of inadequate rest include reduced production and longevity, increased risk for claw problems, lameness and lower milk yield (Gomez & Cook, 2010; Ito *et al.*, 2010).

Milk production occurs across South Africa (SA) with the Western Cape Province as the largest commercial milk producer with 30.3% and Gauteng contributing 2.0% (SA Department of Agriculture, 2021). The milk industry is the seventh-largest agricultural industry, providing a gross value of R16 579 million per annum and as a result, increased milk production through improved cow welfare holds great economic potential (SA Department of Agriculture, 2021; Grant, 2012). In experimental herds, such as the Holstein herd of the University of Pretoria (UP) Future Africa Experimental Farm, several factors affect the potential comfort that these cows may experience. Even though the Holstein herd is conducted as a commercial dairy farm, the UP Future Africa Experimental Farm is not exclusively used for farm animal production. Consequently, these cows are subjected to additional stressors that affect the overall well-being of the herd, including nutritional and/or production research, student training, frequent handling by humans and noise pollution from other sectors (Dorléans, 2019; Ebinghaus *et al.*, 2020).

Quantifying the possible stress that dairy cows are experiencing has become a focal point of improved welfare (Chen *et al.*, 2015; De Vries *et al.*, 2013; Rees *et al.*, 2016). Stressors are defined as factors that represent a threat to the state of an individual's homeostasis (Bombail, 2019). Stress has negative consequences on both conservation and economics (Millspaugh & Washburn, 2004), through the increased maintenance requirements of cows and associated increase in costs, reduction in efficiency and lower profitability of the animal (Chebel *et al.*, 2016; Collier *et al.*, 2017). If an animal perceives a stressor, it will experience a consequential physiological cascade, of which cortisol or corticosterone are the predominant measurable glucocorticoids in the bloodstream (Termeulen *et al.*, 1981; Verkerk *et al.*, 1998). Even though blood glucocorticoids were previously used to examine the effect of stress, blood sampling is an unfavourable process in modern dairy farming due to the inherent complication of having to handle and constrain the animal, which usually causes an increase in respective glucocorticoid concentrations (Comin *et al.*, 2013; Del Corvo *et al.*, 2020). The recent focus on animal welfare aims to reduce discomfort during sampling (Otovic & Hutchinson, 2015; Schwinn *et al.*, 2016; Termeulen *et al.*, 1981) and as a result, glucocorticoids and its metabolites can be measured in integuments and body excreta, including hair, faeces, urine and milk (Alhussien & Dang, 2018; Ito *et al.*, 2017).

Environmental enrichment has the potential to improve cow welfare through decreased stress levels (Mandel *et al.*, 2016). The greatest impact of improved cow welfare is seen in economically relevant responses of dairy cattle, such as feed intake, milk production and health (Cook, 2020; Grant, 2012). Studies have shown that cows that are less stressed have higher milk production and increased milk let-down, along with reduced incidences of disease and illness (Mandel *et al.*, 2016). The primary goals of an environmental enrichment program are to (1) enhance barren environments, (2) improve the cows' biological function, (3) provide the freedom to express normal behaviour and prevent frustration and boredom, and (4) increase the animal's ability to cope with physiological challenges and environmental stressors (Chen & Ogura, 2017; Crouch *et al.*, 2019; Matković *et al.*, 2020; Panchbhai & Thakur, 2016). Environmental enrichment is an important aspect of animal welfare - the addition of appropriate stimuli results in the improvement of animal well-being, where the absence of appropriate stimuli causes conflict behaviour, deprivation of motivation and negative affective states (Algers, 2011; De Azevedo *et al.*, 2007).

Five different categories of enrichment can be employed namely (1) social enrichment, (2) sensory enrichment, (3) physical enrichment, (4) occupational enrichment and (5) nutritional enrichment (Bloomsmith *et al.*, 1991; Panchbhai & Thakur, 2016). Each category of enrichment can have both short- and long-term effects and each enrichment method can contribute to the cow's welfare in different ways (Mandel *et al.*, 2016; Von Borell *et al.*, 2007). Sensory enrichment will be the focus of this study, the remaining enrichment types will not be included in the scope. Sensory enrichment, specifically auditory stimuli, can be applied with relative ease within various indoor cow housing systems (Mandel *et al.*, 2016; Wells, 2009). Various studies have reported that cows stimulated by music as a form of auditory stimuli during milking exhibited a faster milk release and also produced more milk (Mandel *et al.*, 2016; Uetake *et al.*, 1997).

Research has found that dairy cows prefer slow music to fast paced music and similarly, instrumental music (specifically classical music) is a more effective stimuli when compared to other musical genres such as rock or Latin (Donghai et al., 2018; Lemcke et al., 2021; North & Mackenzie, 2001; Wells, 2009). Cows stimulated by music with a slow tempo as a form of auditory stimuli during milking exhibited a faster milk release and also produced up to three percent more milk (Dhungana et al., 2018; Kenison, 2016; Mandel et al., 2016; Uetake et al., 1997).

According to Berry (2001), positive human-animal relationship forms the basis of positive animal welfare. Cows that are housed indoors rely on humans for many aspects of their everyday lives and this daily interaction with humans affects the cows' productivity and behaviour (Mandel et al., 2016). The stockpersons' attitudes and behaviours toward the cows have a significant impact on the level of fear cows experience (Ebinghaus et al., 2020). Consequently, increased fear of humans will be reflected in an increased avoidance distance (Bertocchi et al., 2018), reduced milk yield (Fernándes et al., 2021) and decreased success at first insemination (Ebinghaus et al., 2020). Fearful cows can experience an immediate release of cortisol in aversive situations (Gellrich et al., 2015, Möstel & Palme, 2002). Classical music affects humans in a positive way, specifically through reduced heart rates and stress levels, along with elevated endorphin levels (Yehuda, 2011). Consequently, there is an indirect effect on the welfare of cows through improved human handling (Mandel et al., 2016).

Music has the potential to decrease physiological parameters such as heart rate and blood pressure, which are believed to be indicators of stress (Dhungana *et al.*, 2018). Therefore, it can be assumed that music can assist in decreasing the potential stress that cows may be experiencing (Dorléans, 2019). Further research is essential in order to determine the effect of music on stress and welfare, which is what this study aims to explore.

1.2 Aim and objectives of the study

The overall aim of the study was to examine the effect of auditory stimuli as a form of environmental enrichment on milk yield and activity in a Holstein herd using glucocorticoid concentrations as biomarker.

The aim will be achieved through the following objectives:

1. Monitoring faecal glucocorticoid metabolites and milk glucocorticoid concentration of cows exposed to auditory stimuli during:
   1.1 Constant exposure to music
   1.2 Limited exposure to music - occurring only in the milking parlour
   1.3 No exposure to music
2. Evaluate the use of faecal glucocorticoid metabolites and milk cortisol concentration as biomarker
3. Compare milk yield and activity levels of cows when exposed to above mentioned stimuli regimes (1.1-1.3)
4. Assess the effect of music on the handlers in the milking parlour through the use of a questionnaire

Chapter 2: Literature review

2.1 Introduction

Health and welfare are inextricably linked within dairy production (Garcia, 2001; Pinillos, 2018) and the efficient production of cows is dependent on their health status. Improving dairy cow welfare has various benefits, including increased production and improved longevity (Cook, 2008). Animal welfare has a history spanning over 200 years (Rollin, 1990) and therefore, a short historic overview will be provided discussing the aspects which impacts dairy cow welfare directly - specifically in South Africa.

There are several potential risk factors which may affect the well-being of dairy cows, including chronic stress. Even though environmental enrichment can be used as a tool to decrease the potential stress that cows might experience (De Vries *et al.*, 2013), it is seldom applied to livestock production systems outside of research purposes (De Azevedo *et al.*, 2007). Young *et al.* (2003) argues that enrichment is rarely used on farms due to various economic constraints and because most animal scientists view environmental enrichment as a luxury.

The aim of this review is to discuss relevant literature and describe the current status of dairy cow welfare and the potential improvement through the use of environmental enrichment. Indicators that can be used to identify the state of dairy cow welfare will be discussed, limited specifically to the indicators used in dairy cow welfare. Further, a description of stress and the stress reaction will be provided and discussed, along with its impact on the welfare of dairy cows.

2.2 Historic overview of animal welfare

The past few decades have experienced a great uprising in social concerns of animal welfare (Alonso *et al.*, 2020; Rollin, 1990). *The Five Freedoms* (Table 2.1) were set forth for the first time in 1965 as part of the Brambell report (Broom, 2011; Richter & Hintze, 2019) and has since become the universal standard of suitable animal welfare (Bayvel & Cross, 2010). *The Five Provisions* (Table 2.1) were designed by Mellor & Reid (1994) as a response to *the Five Freedoms*, with the intention of providing a comprehensive and systematic manner of identifying and grading various forms of welfare compromises and/or enhancements. The combination of *the Five Freedoms* and *the Five Provisions* provided a framework for the assessment of welfare within the livestock industry (Mellor, 2016; Webster, 2016).

Table 2.1 *The Five Provisions* in reaction to *the Five Freedoms* (adapted from Mellor & Reid, 1994)

Freedoms	Provisions
Freedom from thirst, hunger and malnutrition	By providing ready access to fresh water and a diet to maintain full health and vigor
Freedom form discomfort and exposure	By providing an appropriate environment including shelter and a comfortable resting area
Freedom from pain, injury and disease	By prevention or rapid diagnosis and treatment
Freedom fom fear and distress	By ensuring conditions and treatment which avoid mental suffering
Freedom to express normal behaviour	By providing sufficient space, proper facilities and company of the animal's own kind

Both *the Five Freedoms* and *the Five Provisions* were, however, regarded to be too vague to be implemented into most livestock production systems, including dairy farms. As a result, seven primary and five secondary biological needs have been identified (Noordhuizen & Lievaart, 2005). These 12 biological needs (Table 2.2) are based on the key factors that impact the health, behaviour, production and fertility of dairy cows (Bracke *et al.*, 2001). The primary and secondary biological needs, in turn, formed the foundation of cow comfort - which has a direct impact on the physiology, longevity and productivity of dairy cows (Cook, 2008; Doyle & Moran, 2015; Grant, 2012).

Table 2.2 Primary and secondary biological needs of livestock

Primary biological needs	Secondary biological needs
Feed and feed-related behaviour	Excretion (Faeces and urine)
Water and drinking-related behaviour	Thermoregulation
Resting, lying and standing	Exploration and orientation
Locomotion (and claw/leg disorders)	Grooming, comfort behaviour
Social comfort (interactions)	Reproduction and rearing
Health status	
Safety (fear, flight behaviour, aggression)	

Even though South Africa is often regarded as industrialized, there is still a spectrum of development within the country itself - some areas are urbanised and others rural (Van der Hoeven *et al.*, 2012). Veterinarians are believed to be the driving force of animal welfare, due to the existing perception that welfare consists solely of the treatment and prevention of disease (Cornish *et al.*, 2016). Veterinary services are often focused on the main urbanized areas and rural sections are often regarded as remote, impoverished, and difficult to access (Wilkins *et al.*, 2005). As a result, the National Council of Societies for the Prevention of Cruelty to Animals (NSPCA) has been providing support and assistance to rural farmers (NSPCA, 2021). The NSPCA, governed by the *SPCA Act of 1993*, is the primary organization enforcing animal welfare in South Africa, managing over 90% of welfare-related problems (Subramanien, 2018; Wilkins *et al.*, 2005). Founded in 1955, the mission statement set forth by the NSPCA is the prevention of cruelty to animals through legal means, allowing the NSPCA to promote animal welfare (NSPCA, 2021). The NSPCA has a specialised Farm Animal Unit which operates on a national scale with the primary goal of uplifting the welfare of livestock (DSA, 2013).

In South Africa, the Department of Agriculture, Land Reform and Rural Development (DALRRD) is responsible for enforcing both the *Animal Protection Act, No. 71 of 1962* and the *Animal Improvement Act of 1998* (Bracke, 2009). In 1999, the Department of Agriculture (now part of the DALRRD) published the National Code of Practice (COP) - providing instruction on the use of animals for scientific and teaching means (DAFF, 1990). In 2008, this code was replaced by the South African National Standard (SANS), developed by the South African Bureau of Standards (SABS, 2008). Ever since the SANS has been established and implemented, animal care and standards of welfare has improved in South Africa (Mohr *et al.*, 2016). The Milk Producers Organisation (MPO), South Africa Milk Processors' Organisation (SAMPRO), Milk SA and the Dairy Standard Agency (DSA) are also at the frontline of dairy welfare in South Africa, focusing on empowerment, education, research and development, and quality improvement of livestock products (van Heerden, 2021).

The South African laws and standards are set forth clearly and is regarded as unambiguous (Mohr *et al.*, 2016). Animal welfare regulations are, however, time consuming and difficult to enforce (Bracke, 2009). Even though South Africa has a competitive welfare legislation, enforcement thereof is lacking and lawbreakers are seldom prosecuted (Bilchitz, 2014). The consumer holds the producer responsible for the health and well-being of dairy cows by expecting the producer to adhere to the standards and regulations set forth by the government (Rink *et al.*, 2019). Consumers often react positively toward products derived from animals with a good quality of life (Mellor, 2016; Vigors & Lawrence, 2019) and therefore, producers may benefit from implementing programs to support good welfare for dairy cows, such as environmental enrichment (Richter & Hintze, 2019).

2.3 Environmental Enrichment to improve cow welfare

The term enrichment implies an improvement to the benefit of the animal (Newberry, 1995). The primary goals of an environmental enrichment program are to (1) enhance barren environments, (2) improve the biological function of cows which includes health, inclusive fitness and reproductive success, (3) provide the freedom to express normal behaviour and prevent frustration and boredom, (4) increase the animal's ability to cope with physiological challenges and environmental stressors (Chen & Ogura, 2017; Crouch *et al.*, 2019; Mandel *et al.*, 2016; Matković *et al.*, 2020; Panchbhai & Thakur, 2016). Environmental enrichment is generally accomplished through an addition to the environment of the animal, which may range from an object, material or stimulus, to changing the housing of the animal or management style (Newberry, 1995). Five categories of environmental enrichment have been identified, namely: sensory, physical, occupational, social and nutritional (Bloomsmith *et al.*, 1991; Chen & Ogura, 2017; Panchbhai & Thakur, 2016). The impact of these enrichment programs can be evaluated through animal behaviour, neurological assessment and physiological measurements (De Azevedo *et al.*, 2007).

The interest in environmental enrichment can be traced back to Darwin (as far back as 1859) who observed an increase in the capabilities of animals exposed to enrichment (Zentall, 2021). The effect of enrichment on the brain and behaviour of animals was discovered due to pioneering research of the 1960s to 1970s, which reported that enrichment improved various sections of the brain including the visual and somatosensory cortex, increased cortical thickness and altered auditory cortex (Diamond, 2012; Zentall, 2021). As a result, environmental enrichment has been increasingly used, especially in field such as veterinary sciences, zoology, and applied ethology (De Azevedo *et al.*, 2007; Newberry, 1995).

Research pertaining to the welfare of livestock holds a great deal of potential, due to the great number of animals available (De Azevedo *et al.*, 2007). Environmental enrichment has already been applied to various animal husbandry systems, including broilers (Leone & Estevez, 2008), laying hens (Shimmura *et al.*, 2010), dairy cows (Uetake *et al.*, 1997), cattle (Ishiwata *et al.*, 2006) and pigs (Van de Weerd & Day, 2009). Environmental enrichment programs have also been implemented on farms as a way to improve the public opinions of livestock production systems (Mandel *et al.*, 2016; Newberry, 1995).

The recent increase in social concerns of animal welfare has accentuated the rearing conditions of cows, placing the focus on ways that can improve their surroundings (Wells, 2009). Application of environmental enrichment to livestock has been a significant breakthrough in the improvement of animal welfare (Ishiwata *et al.*, 2006; Matković *et al.*, 2020) and can be a viable tool to improve dairy cow welfare at an individual level (Newberry, 1995; Richter & Hintze, 2019; Vigors & Lawrence, 2019). The addition of sensory enrichment results in improved cow comfort and allows cows to cope with various challenges associated with indoor rearing (Chen & Ogura, 2017; Dorléans, 2019; Wells, 2009). The application of enrichment may also assist in the amelioration of aggressive behaviour and associated chronic stress to subordinate cows (Ninomiya, 2014). Therefore, employing measures of environmental enrichment encourages freedom from a state of suffering – which supports the school of *the Five Freedoms* (Duncan & Olsson, 2001).

It is necessary to investigate the need of the animal and select the form of enrichment accordingly (Van der Weerd & Day, 2009). It is imperative to remember that if the cow's needs are not addressed, the enrichment may not be successful in achieving the desired goal (Cook, 2008). An enrichment study by Ninomiya & Sato (2009) investigated the effect of physical enrichment by providing comfortable resting spaces and tools that can be used for self-grooming. The authors report that the method for welfare improvement is determined in terms of the added value to the livestock product, however this does not agree with the definition of enrichment from an animal welfare perspective. The study reported that the calves exposed to the enrichment did not gain weight any faster than the cows that did not receive the enrichment.

Enrichment does have its benefits, especially if used to improve the welfare of cows (Mandel *et al.*, 2016). A physical enrichment study on heifers that were exposed to brushing and human presence showed a decrease of nervous, agitated and fearful behaviour and an increase of calm and sociable behaviour (da Silva *et al.*, 2021). The change in behaviour showed an improved adaptation of the heifers to the milking system, which decreases the risk of injury to both heifer and handler. A prominent benefit of environmental enrichment is that the success of an enrichment program did not vary with environmental type, meaning that enrichment can be applied to livestock housed in different environments with the same expected level of success (De Azevedo *et al.*, 2007). However, the use of environmental enrichment in livestock is limited due to perceived financial constraints and additional demands on caretakers, making producers hesitant to employ enrichment programs (Newberry, 1995). Structural alterations as a form of physical enrichment have achieved high success rates, where cognitive, sensory, nutritional and social forms of enrichment have been less successful (De Azevedo *et al.*, 2007; Matković *et al.*, 2020).

Despite constrains, considering the sensory ability of animals in welfare programs may have various benefits, ranging from improved animal production and well-being, to ease of handling and improved conditions for the human caretakers (Nielsen, 2018). The auditory abilities of cattle are well developed, making the use of auditory stimuli a viable option in studies relating to dairy cow welfare (Uetake *et al.*, 1997). Researchers found that considering the sensory abilities of animal showed an improvement in the quality and validity of results (Nielsen, 2018), defending the notion that the sensory range of cows deserves increased attention (Newberry, 1995).

2.4 Music as auditory stimuli

The perception cows have of their environment has a significant impact on their welfare (Cook, 2008), but the sensory abilities of animals are often disregarded when investigating animal behaviour and welfare (Nielsen, 2018; Pšenka *et al.*, 2016). Auditory stimuli are a form of sensory enrichment that is less commonly used for cows and other forms of livestock (De Azevedo *et al.*, 2007). There are two broad categories of auditory stimuli, namely (1) sounds found in the species' natural habitat and (2) other types of auditory stimuli not commonly associated with the natural habitat (Wells, 2009). Domestic animals have been changed through human selection and they are vastly different to their ancestors (Price, 2002). As a result, natural habitat of the modern-day domestic cow is quite different compared to their predecessors and defining natural auditory stimuli has been challenging (Broom, 2010; Lawrence & Vigors, 2020).

Cows receive information on their environment through all five of their senses, namely hearing, touch, vision, smell and taste (Lemcke *et al.*, 2021; Veissier & Boissy, 2007). Cows are able to easily rotate their ears at the base of their heads which allows them to recognize sounds all around them. Sound is commonly described through the use of the terms Hertz (Hz) and Decibels (dB). Decibels refer to the loudness of the sound where Hertz is a measurement of the frequency of sound (Heffner, 1998; Lemcke *et al.*, 2021). Cows have excellent hearing, ranging from 23 Hz to 35 kilohertz (kHz), which is nearly double the frequency range of humans (Heffner & Heffner, 1983; Lemcke *et al.*, 2021; Manci, 1988). Low-frequency sounds are easily audible to cows, as well as high-frequency sounds that are inaudible to humans (Heffner, 1998). Sensitivity to sound varies across species (Heffner & Heffner, 1992; Rickard *et al.*, 2005). Cows have a sound threshold of 85 to 90 dBs (Dorléans, 2019), and physical damage to hearing occurs at a range exceeding 110 dB (Phillips, 2009; Pšenka *et al.*, 2016).

Different types of auditory stimuli have successfully been employed in various functions on dairy farms, including training cattle to approach a food source, alteration of cow behaviour, increased milk production and increased growth rates through improve feeding behaviour (Dorléans, 2019; Mandel *et al.*, 2016; Nielsen, 2018; Uetake *et al.*, 1997; Wisniewski, 1977). Contrasts exist between researchers as some believe that personality and musical preference can be disregarded in livestock and thereby reduce the within group variation (Newberry, 1995; Rickard *et al.,* 2005), where others state that animals, like humans, have individual reactions to auditory stimuli and music (Dorléans, 2019; Kemp, 2020; Lemcke *et al.*, 2021).

Music can be a useful tool to improve the welfare of cows (Lemcke *et al.*, 2021), but the effect of music on animals depends on a variety of musical factors, including tempo, frequency (Hz), intensity (dB), and duration of exposure (Dhungana *et al.*, 2018; Weeks *et al.*, 2009; Wells, 2009). Animal factors, such as sensory abilities, breed, age and previous exposure, may also play a role in how the stimuli is perceived (Alworth & Buerkle, 2013; Brouček, 2014). An animal's hearing ability is determined genetically, and dairy breeds have been identified by Lanier *et al.* (2000) as having superior responses to musical stimulation when compared to beef breeds.

Various species of animals, including dairy cows, prefer slow music to fast paced music and similarly, instrumental music (specifically classical music) is more effective than other musical genres such as rock or Latin (Donghai *et al.*, 2018; Lemcke *et al.*, 2021; North & Mackenzie, 2001; Wells, 2009). Most studies focused on auditory stimuli employed various genres in their experimental design (Donghai *et al.*, 2018; Kemp, 2020; Lemcke *et al.*, 2021). A study by Donghai *et al.* (2018) exposed three groups of 18 cows to a different genre of music, namely Latin, Rock and African percussion. A reduction in milk yield was observed for all three genres, along with an increase in serum globulin contents, which is often associated with an increased immune system function.

A recent study (Lemcke *et al.*, 2021) exposed 17 dairy cows to various genres of music, including Blues, Rock and Classical. All 74 selected musical pieces had a tempo of less than 100 beats per minute, which was played at random. The musical stimulus was used in intervals of two to four days with music and then no musical stimulus. The study found that cows exposed to music visited the Automatic Milking System (AMS) more often, but no effect was observed on the daily milk yield of the cows. The author deduced that the cows found the music attractive and therefore it may be a practical welfare tool.

Research has also reported that cows stimulated by music as a form of auditory stimuli during milking exhibited a faster milk release and also produced more milk (Kenison, 2016; Mandel *et al.*, 2016; Uetake *et al.*, 1997). More specifically, cows that were stimulated by music with a relaxed tempo showed a three percent increase in milk production, whereas fast rock music led to a two percent decrease in milk yield (Dhungana *et al.*, 2018).

Classical music can be employed as a method to improve animal welfare through drowning out potentially disturbing background noises and therefore, playing music in the milking parlour may have a particularly positive effect on cow welfare through drowning out the milking machine (Alworth & Buerkle, 2013). Dairy cows are believed to find listening to music as a pleasant activity and cows exposed to classical music were reported to be calmer and more docile when exposed to classical music during milking, however no behavioural or physiological measurements were employed to quantify the effect of classical music (Alworth & Buerkle, 2013; Lemcke *et al.*, 2021).

Classical music, originally for the benefit of the cows, can also have a positive effect on the human handlers and consequently, there is an indirect effect on the welfare of cows through improved human handling (Mandel *et al.*, 2016). The benefits of classical music on humans have been well documented, which include analgesic and anxiolytic properties, due to the ability of music to reduce stress, and alleviate pain and anxiety, respectfully (Alworth & Buerkle, 2013; Dhungana *et al.*, 2018). Similar responses are expected from dairy cows exposed to classical music, as music exposure induces a range of physiological changes in the cow, including altering cardiovascular homeostasis and hormonal secretion (Brouček, 2014). Cows exposed to classical music showed a slight decrease in average heart rate, along with a significant decrease in average respiration rate (Kemp, 2020; Wells, 2009). Heart rate and blood pressure are believed to be parameters of stress, both of which responded positively when cows were exposed to slow music (Dhungana *et al.*, 2018). Therefore, it can be assumed that music can assist in decreasing the stress that cows may be experiencing (Dorléans, 2019), but further research is essential in order to confirm the effect of classical music on the stress levels and welfare of dairy cows.

The ability of music to assist in improving the immune response of mammals has also been investigated (Dorléans, 2019). Serum proteins are used in dairy cows as indicators of health status which is imperative to welfare (Dhungana *et al.*, 2018). Cows with high functioning immune systems can ward off infections easier and be less of a financial burden to management (Bobbo *et al.*, 2017). Exposure to classical music increases the amount of serum globulins, thereby supporting an improved immune system (Donghai *et al.*, 2018).

Dairy cow housing features a spectrum of auditory stimuli resulting from human activities, which is classified as noise and regarded as a potential source of injury and discomfort to the cows (González-Grajales *et al.*, 2019). Chronic noise can have detrimental effects on the health status of cows as noise has a direct, negative impact on the feeding behaviour, energy consumption and reproductive physiology of cows (Brouček, 2014). Auditory stimuli should thus be applied with caution, as adding stimulus to an environment that is already loud may cause more harm than good (Newberry, 1995). In order to investigate the effect of music as a potential stressor or positive auditory stimulus for cows, suitable welfare parameters must be monitored (Dorléans, 2019).

2.5 Assessment of dairy cow welfare

Animal welfare science is a relatively new field - animal welfare was only acknowledged as both an ethical and a scientific discipline after 1965 (Carenzi & Verga, 2009; Marchant-Forde, 2015; Millman *et al.*, 2004). Development in subsequent fields of study like behaviour, nutrition and anatomy supported the notion that animal welfare forms an integral part of animal production (Krueger *et al.*, 2020; Lawrence & Vigors, 2020). Three primary concerns are expressed, namely: (1) the biological function of the animal, (2) the emotional state of the animal and (3) the ability of the animal to live a good and natural life (Fraser *et al.*, 1997; Von Keyserlingk & Hötzel, 2015).

Most research related to dairy cow welfare has focused on impaired welfare, due to the detrimental effects of poor cow welfare, such as lameness & claw disorders (Cook & Nordlund, 2009; Randall *et al.*, 2016), decreased feed intake (Botheras, 2007), reduction in milk yield (Oltenacu & Broom, 2010), decreased fertility and reproductive performance (Oltenacu & Algers, 2005), impaired longevity (De Vries, 2017) and increased incidences of mastitis (Olechnowicz *et al.*, 2016). High producing cows are most often susceptible to welfare issues, due to an increased risk of health problems (Rauw *et al.*, 1998; Von Keyserlingk *et al.*, 2009).

Despite the global belief that welfare is essential to all livestock animals, assessment of animal welfare remains a complex task due to the multi-faceted nature of welfare (Alonso *et al.*, 2020; Van Erp-van der Kooij & Rutter, 2020). Currently, there is no standardized method for assessing the overall welfare status of cows that is commonly agreed upon (De Graaf *et al.*, 2017; Spoolder *et al.*, 2003). Establishing a proper, site-specific welfare assessment protocol is the first step to improved cow welfare (De Vries *et al.*, 2015; Leliveld & Provolo, 2020). In order to identify existing and potential animal welfare problems, accurate evaluations and scientific protocols are needed (Arsoy, 2020). Welfare assessment programs have two primary goals: (1) to provide producers with a tool that allows them to evaluate the standard of welfare that is maintained on their farms and (2) to assure consumers that the animals were cared for and raised humanely (Vasseur *et al.*, 2010; Vasseur *et al.*, 2015). An indicator is defined as one or more measurements that can be used to evaluate the extent to which welfare goals are satisfied (Vasseur *et al.*, 2015). Most indicators can be used to assess welfare before the animals are clinically ill, suffering or dead (Von Keyserlingk *et al.*, 2009). Indicators may overlap and improving one aspect of animal welfare is likely to have a positive effect on the other factors (Vigors & Lawrence, 2019; Von Keyserlingk *et al.*, 2009). These indicators must be valid, reliable, accurate, sensitive and feasible in order to form part of an assessment program (Arsoy, 2020; De Graaf *et al.*, 2017; De Vries *et al.*, 2011; Doyle & Moran, 2015).

Indicators can be divided into three categories, namely (1) animal-based, (2) environment-based and (3) management-based measurements (Bertocchi *et al.*, 2018; Vasseur *et al.*, 2015). Animal-based measurements (ABMs) provide information directly from the animal, whereas environment-based measurements focus on the facilities where the cows are kept or milked (Doyle & Moran, 2015; Vasseur *et al.*, 2015). Evaluation protocols consisting only of environmental factors are insufficient (De Graaf *et al.*, 2017). ABMs are often regarded to be the most effective tool to assess the true state of welfare experienced by livestock animals and therefore, an animal-centred evaluation criterion is essential for valid and efficient results (Alonso *et al.*, 2020; Bertocchi *et al.*, 2018). Unfortunately, ABMs are costly and time consuming and can therefore not be applied with high frequency at farm level (De Vries *et al.*, 2013; Fernándes *et al.*, 2021). Resource-based and management-based measurements are used to identify possible risk factors that can cause impaired welfare (Blokhuis *et al.*, 2010; De Graaf *et al.*, 2017).

Animal measures of welfare can also be divided into direct and indirect measures. Direct animal measurements include body condition and locomotion scoring, lying behaviour, standing time, nasal discharge, vulvar discharge, behavioural tests and grooming, whereas indirect indicators refer to bulk milk, somatic cell count and on-farm mortality (Bertocchi *et al.*, 2018; Krueger *et al.*, 2020; Novak *et al.*, 2001; Whay *et al.*, 2003). Generally, there is a difference between indicators for short-term and long-term problems (Möstl & Palme, 2002). Short-term exposure to stressors normally employs indicators such as heart rate and plasma cortisol concentrations, whereas behaviour, immune system function and disease state are preferred for long-term exposure (Broom, 2011). The severity of the effects caused by a continuously perceived stressor increases with the length of time that the animal is exposed, as seen in figure 2.1 (Broom, 2011). A combination of welfare indicators is commonly used to assess the welfare of dairy cows, which is summarized in Table 2.3, according to the Welfare Quality's (WQ) four principles of animal welfare and the three primary concerns of welfare.

Figure 2.1 The severity of effect of a stressor against length of exposure (as explained by Broom (2011)).

In South Africa, dairy cow welfare audits are primarily conducted by the Dairy Standard Agency (DSA). The DSA developed a Code of Practice (COP) for milk producers to improve the welfare of dairy cows and ensure food safety (DSA, 2013). The dairy cow welfare audit is designed from the COP incorporating various legislations, including Regulation 961, Regulation 1555, SANS10049:2012, Animal Protection Act 1962 (Act 71 of 1962), Fertilizers, Farm Seeds, Seeds and Remedies Act 1947 (Act 36 of 1947) and SANS 1694: 2018 (Animal Welfare). The DSA welfare audit focuses on management- and environmental-based measurements spanning over 15 sections, including cow and calf care, management and treatment, as well as ensuring milking practices and animal handling is acceptable.

Management practices play an essential role in reducing the amount of stress that an animal may experience (Von Keyserlingk *et al.*, 2009). Many producers regard the reduction of stress and provision of basic needs as non-negotiable and therefore, focus remain on the reduction of poor welfare (Vigors & Lawrence, 2019). Indicators can be used to evaluate the duration, prevalence and severity of the stressors that the cows are exposed to (Oltenacu & Algers, 2005).

Table 2.3 Common indicators used to assess the welfare of dairy cows

Indicators relating to biological functioning			
Principle	**Criteria**	**Indicators**	**Type**
Good health	Health	(1) Disease incidence & Metabolic disorders	(1) ABM
		(2) Mastitis incidence & Somatic cell count	(2) ABM
		(3) Lame cows (%)	(3) ABM
		(4) Integument alterations (including swelling & lesions)	(4) ABM
		(5) Longevity & Morality	(5) ABM
	Performance	(1) Body Condition Score (BCS)	(1) ABM
		(2) Milk yield	
		(3) Composition of milk	(2) ABM
		(4) Growth rates	(3) ABM
		(5) Reproductive performance	(4) ABM
			(5) ABM
Indicators relating to natural living			
Good feeding	Absence of hunger and thirst	(1) Feeding behaviour	(1) ABM
		(2) Malnutrition	(2) ABM
		(3) BCS	
Good housing		(1) Cows with dirty legs, flanks, udders	(1) ABM
		(2) Cows outside lying area	(2) Non-Animal-Based Measure (N-ABM)
		(3) Duration of lying bouts	(3) N-ABM
		(4) Pen measurements	(4) N-ABM
		(5) Stall Design and Bedding	(5) N-ABM
Appropriate behaviour	Natural behaviour	(1) Movement	(1) ABM
		(2) Lying behaviour	(2) ABM
Indicators relating to affective state			
	Human-animal relationship	(1) Avoidance distance (>100cm)	(1) ABM
	Social behaviour	(1) Play behaviour	(1) ABM
		(2) Social interaction	(2) ABM
		(3) Allogrooming	(3) ABM
	Absence of pain, fear and distress	(1) Glucocorticoid concentration	(1) ABM
		(2) Heart rate	(2) ABM
		(3) Ruminating	(3) ABM

Information from the following references were used to design Table 2.3: (Armbrecht *et al.*, 2019; Cornish *et al.*, 2016; De Graaf *et al.*, 2017; De Vries *et al.*, 2011; Fregonesi & Leaver, 2001; Gieseke *et al.*, 2020; Leliveld & Provolo, 2020; Mee & Boyle, 2020; Napolitano *et al.*, 2009; Oltenacu & Algers, 2005; Rushen *et al.*, 2011; Sadiq *et al.*, 2017; Vasseur *et al.*, 2015; Vigors & Lawrence, 2019; Von Keyserlingk *et al.*, 2012; Whaytt *et al.*, 2003).

2.6 Stress and stressors

The term stress refers to a biological response that is triggered by an internal or external threat which places strain on the biological system of an animal (Collier *et al.*, 2017). Commonly used nomenclature defines a stressor specifically as an environmental stimulus which acts as a threat to homeostasis (Möstl & Palme, 2002). Stressors include, but are not limited to; management, thermal environment, social interaction, disease, and environmental factors including housing types (Ebinghaus *et al.*, 2020; Fernandes-Novo *et al.*, 2020). Environmental factors are not static and, as a result, livestock have adapted to situations through physiological, behavioural and morphological modifications (Amadori *et al.*, 2009; Sgorlon *et al.*, 2015). Minimizing exposure to stressors through improved management has recently become priority within the dairy industry, due to the detrimental effect of stress on the health, well-being and productivity of the dairy cow (Chen *et al.*, 2015; Grant, 2012).

Animals have an inherent reaction when exposed to any stressors, namely the stress response (Möstl & Palme, 2002; Palme, 2012). The stress response of animals is a complex mechanism (Chen *et al.*, 2015). From a physiological standpoint, stress is not inherently negative as the primary function of the stress response is to protect the animal and to restore homeostasis (Broom, 2001; Ganswindt *et al.*, 2010; Möstl & Palme, 2002). However, if the stress reaction is activated too often or for extended periods of time, it becomes chronic stress (Nedić *et al.*, 2017). The stress response varies in range and complexity between species, individuals and prior exposure to stressors (Cook *et al.*, 2004; Fernandez-Novo *et al.*, 2020). Activation of the stress response depends on both length of exposure and type of stimulus, along with the age, sex and physiological stage of the cow (Palme, 2012). Various systems are involved in the stress response, resulting in immune system alterations and behavioural changes of the cow (Von Borell *et al.*, 2007).

The stress response is divided into two phases, namely acute and chronic (Friend, 1991). The acute stress response is activated through the initiation of several receptors that responds to environmental changes (Collier *et al.*, 2017). These receptors may be activated anywhere from within a few minutes to a few days after the initial exposure (Collier & Gebremedhin, 2015). Sensory information is sent to the amygdala, which transmits a distress signal to the hypothalamus (Reeder & Kramer, 2005). The hypothalamus acts as the command centre, communicating with the rest of the body through the Autonomic Nervous System (ANS) (Veissier & Boissy, 2007). The acute response is driven primarily by the ANS, which stimulates the release of catecholamines and glucocorticoids and thereby activates the necessary transcription factors (Chen *et al.*, 2015; Möstl *et al.*, 1999). Acute stressors stimulate the release of adrenaline and noradrenaline, enabling the animal to respond rapidly through activation of their fight or flight response (MohanKumar *et al.*, 2012). Subsequently, numerous efferent pathways, such as the sympathetic nervous system and the neuroendocrine system, are activated to emit a response to the environment (Collier *et al.*, 2017; Wiepkema & Koolhaas, 1993).

Chronic stress can be defined as prolonged periods of elevated glucocorticoid concentration due to the continuous perception of a stressor (Bombail, 2019; Möstl & Palme, 2002; Verkerk *et al.*, 1998; Wiepkema & Koolhaas, 1993). Briefly after adrenaline and noradrenaline concentrations are elevated, the HPA-axis is activated leading to increased glucocorticoid output facilitating temporal hyperglycaemia (Herdt & Emery, 1992; Herman *et al.*, 2016; Von Borell *et al.*, 2007). The HPA-axis consists of the hypothalamus, the pituitary gland and the adrenal glands (Brown & Vosloo, 2017). The HPA-axis may be activated during either beneficial or detrimental circumstances and therefore, it is essential to include a combination of parameters to evaluate stress (Kemp, 2020; Palme, 2012; Rushen *et al.*, 2011). Challenging situations like disease, environmental changes and negative energy balance result in the activation of the HPA-axis (Comin *et al.*, 2013; Huzzey e*t al.*, 2011; Mormède *et al.*, 2007). Individual activation of the HPA-axis is dependent on genetic and environmental factors (Sgorlon *et al.*, 2015).

Rapid activation of the HPA-axis forms a vital part of the animal's defence mechanism (Hein *et al.*, 2020). A continued threat causes the hypothalamus to release corticotropin-releasing factor (CRF), also called corticotropin-releasing hormone (CRH) and vasopressin, which mediates the stress response (Manteca *et al.*, 2016). CRH travels to the pituitary gland, triggering the release of adrenocorticotropic hormone (ACTH) (Möstl *et al.*, 1999; Veissier & Boissy, 2007). ACTH travels to the adrenal glands prompting them to produce glucocorticoids, with cortisol as primary hormone in most mammals (Di Francesco *et al.*, 2021). Cortisol is released from the adrenal cortex into the blood where it circulates through the body of the cow to various sites, including the liver and the udders (Brown & Vosloo, 2017; Hein *et al.*, 2020). The interaction of the HPA-axis is depicted in figure 2.2.

The HPA-axis has a key role in the regulation of various biological processes, including reproduction and the immune response (Comin *et al.*, 2013; Ebinghaus *et al.*, 2020; Manteca *et al.*, 2016). High producing cows require recruitment of the HPA-axis to re-establish homeostasis, together with the autonomic nervous system and the immune system (Nedić *et al.*, 2017; Sgorlon e*t al.*, 2015). In dairy cows, specifically, adrenocortical and pituitary activity are linked to energy regulation, milk yield and glucose concentrations in plasma (Gellrich *et al.*, 2015; Giesecke, 1985). Chronic stress has detrimental effects on the health of animals, including (1) decreased individual fitness through immunosuppression and reduced self-curing capacity (Ivemeyer *et al.*, 2018; Nedić *et al.*, 2017), (2) atrophy of tissue (Möstl & Palme, 2002), (3) decreased reproductive success (Fernandez-Novo *et al.*, 2020), (4) reduced growth (MohanKumar *et al.*, 2012) and as a result, (5) overall welfare (Chen *et al.*, 2015). Circulating glucocorticoids may, however, improve the fitness of an animal through mobilising energy and inducing behavioural and physiological changes (Ganswindt *et al.*, 2010; Morrow *et al.*, 2002; Möstl & Palme, 2002; Touma & Palme, 2005).

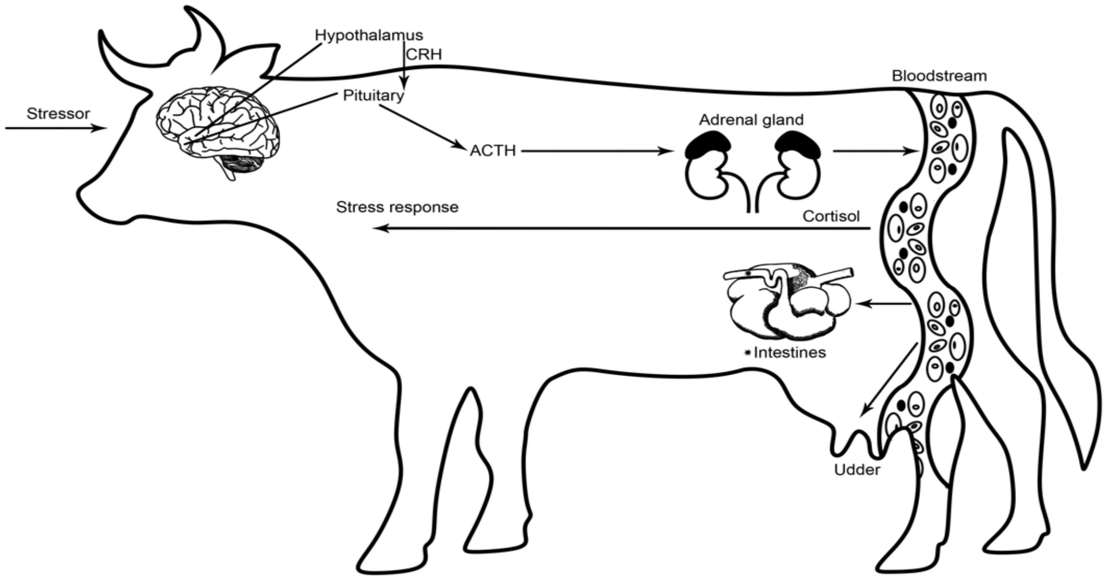

Figure 2.2 A simplified visual representation of the HPA-axis interaction
A stressor activates the hypothalamus to release CRH which travels to the pituitary gland and triggers the release of ACTH. ACTH travels to the adrenal glands prompting them to produce glucocorticoids, like cortisol, which are then released from the adrenal glands into the bloodstream and travel (e.g.,) to the udder and is present in the milk. Once unfolding its effect at the target tissues, glucocorticoids get metabolised in the intestines and are excreted via urine or faeces.

2.7 Glucocorticoids and its metabolites as an indicator for physiological stress

Reliable diagnostic (bio)markers serve as a means to investigate the stress perceived by individual cows (Gellrich *et al.*, 2015). Glucocorticoids have the responsibility to assist the maintenance of homeostasis through aiding immune response (Fernandez-Novo *et al.*, 2020), energy metabolism (Chen e*t al.*, 2015), inflammatory processes (Nedić *et al.*, 2017), reproduction efficiency (Von Borell *et al.*, 2007), growth (MohanKumar *et al.*, 2012) and brain function (Lefcourt *et al.*, 1993). The quantification of glucocorticoids, like cortisol, has proven to be a valuable tool to employ when evaluating stress in livestock through its function as the endpoint of HPA-activity (Alhussien & Dang, 2018; Manteca *et al.*, 2016).

Variations in hormone levels have been extensively used as indicators of pain in cows (Anil *et al.*, 2005), meat quality (Bozzo *et al.*, 2018), chronic lameness (Fischer-Tenhagen *et al.*, 2018) milk quality (Sgorlon *et al.*, 2015) and distress (Shi *et al.*, 2021). Elevations in glucocorticoid concentrations are often used as a physiological indicator for the activation of the HPA-axis (Moya *et al.*, 2013) and can therefore be used as an indirect welfare indicator to measure possible adverse effects that the cow may be experiencing (Chen *et al.*, 2015; Hein *et al.*, 2020; Narayan *et al.*, 2018).

Even though blood cortisol was traditionally quantified to examine the effect of stress, blood sampling is an unfavourable process in modern dairy farming due to the inherent complication of having to handle and constrain the animal, causing an increase in glucocorticoid concentrations (Comin *et al.*, 2013; Del Corvo *et al.*, 2020). The recent focus on animal welfare aims to reduce discomfort during sampling (Hein *et al.*, 2020; Otovic & Hutchinson, 2015; Schwinn *et al.*, 2016; Termeulen *et al.*, 1981) and as a result, glucocorticoids and its metabolites can be measured in integuments and body excreta including hair, faeces, urine and milk (Alhussien & Dang, 2018; Narayan *et al.*, 2018; Ito *et al.*, 2017). Concentrations of glucocorticoids in the blood, saliva, milk and urine of cows reflect the function of the HPA-axis shortly after its activation (Sgorlon *et al.*, 2015). In contrast, faecal cortisol reflects HPA-axis activity from up to two days prior to measurement due to the passage of gut contents and metabolism time (Nedić *et al.*, 2017).

A significant correlation exists between plasma and milk cortisol concentration, due to the ability of steroid hormones in blood to diffuse across blood-milk barriers (Alhussien & Dang, 2018; Rushen *et al.*, 2008). The close association of alveolar milk to the vasculature of the udder permits rapid exchange of free glucocorticoids between blood and milk until milk let-down (Del Corvo *et al.*, 2020; Verkerk *et al.*, 1998). As a result, milk glucocorticoid (mGC) concentration reflects the glucocorticoid concentration of blood plasma and can be used to evaluate stress in the place of blood (Nedić *et al.*, 2017).

Changes in mGC concentration can effectively be used as a measurement to identify alternations of HPA-axis activity in lactating cows (Bremel & Gangwer, 1978; Nedić *et al.*, 2017). However, glucocorticoids have a limited timeframe to be evident in the milk (Sgorlon *et al.*, 2015). Blood glucocorticoids are rapidly transferred into milk and consequently, respective concentrations will only be reflected in milk for two to four hours after exposure to a stressor (Termeulen *et al.*, 1981; Verkerk *et al.*, 1998). The transfer rate of glucocorticoids steadily declines due to the absence of sustained HPA-axis activation and consequently, cortisol becomes diluted as a function of both milking interval and milk yield (Sgorlon *et al.*, 2015).

Quantifying glucocorticoids in milk is regarded beneficial as it can provide information on individual level, as well as for within and between herd comparisons (Sgorlon *et al.*, 2015). MGC concentrations vary between farms due to the impact of environmental conditions, including stocking rate, cubicle design and the number of cows per productive area (Del Corvo *et al.*, 2020; Ito *et al.*, 2017). Milk is regarded as the primary matrix for sampling of dairy cows, due to the non-invasive nature, ease of collection, and repeatability (Del Corvo *et al.*, 2020). Milk sampling requires no manipulation of animals as it forms part of the daily milking routine - providing a less stressful procedure than blood collection (Alhussien & Dang, 2018; Fukasawa & Tsukada, 2010). As a result, milk collection satisfies the animal welfare recommendations through its minimal disturbance of cows (Ito *et al.*, 2017).

Glucocorticoid concentrations of Holstein cows are generally observed to be higher when compared to other breeds - presumably due to the intensive breeding history of the Holstein herd and the consequential physiological adaptation to high milk production (Higashiyama *et al.*, 2014; Nedić *et al.*, 2017). These high producing Holsteins are particularly susceptible to various stressors, including heat and handling (Comin *et al.*, 2013). Glucocorticoid concentrations have an indirect effect on milk yield, as milk yield is influenced by the health status of cows, along with genetic potential, physiological state, lactation stage and level of feeding (Chen *et al.*, 2015; Hernandez *et al.*, 2002; Macdonald *et al.*, 2005; Moorby *et al.*, 2009; Sgorlon *et al.*, 2015).

Assessment of adrenocortical activity through quantification of faecal glucocorticoid metabolites (fGCM) is a well-established non-invasive method to evaluate the stress levels of cows (Bertulat *et al.*, 2013; Millspaugh & Washburn, 2004). There is a correlation between fGCM and blood glucocorticoid concentrations, and the adrenal activity of cows (Bertulat *et al.*, 2013; Morrow *et al.*, 2002). However, a time delay exists before the increased plasma glucocorticoid concentrations are reflected in the excreted fGCM, approximately 10 to 12 hours, due to gut passage time from the duodenum to the rectum (Huzzey *et al.*, 2011; Morrow *et al.*, 2002). The measurement of fGCM concentration reflects the glucocorticoid concentration that is present in the blood prior to excretion (Narayan *et al.*, 2018). Glucocorticoids are metabolised in the liver, excreting their metabolites via bile into the intestines (Di Francesco *et al.*, 2021; Hein *et al.*, 2020). The stress response allows adequate quantities of metabolites to be analysed, defending fGCM use as a trusted method of analysis (Hein *et al.*, 2020; Möstl & Palme, 2002; Touma & Palme, 2005).

As a result, fGCM concentrations represent the cumulative hormone secretion which are less affected by short periodic fluctuations or pulsative changes (Palme, 2012; Touma & Palme, 2005). Faecal samples are, therefore, valuable to research for the evaluation of stress over prolonged periods, as hormone levels are accumulated over a certain period of time (Ebinghaus *et al.*, 2020; Di Francesco *et al.*, 2021; Millspaugh & Washburn, 2004). The time delay varies between species and can also be influenced by the individual animal and feed intake (Morrow *et al.*, 2002; Palme, 2012). Faecal samples thereby offer the advantage of a *post hoc* evaluation (Touma & Palme, 2005). FGCM monitoring has the added benefit of being a feedback-free sampling method, which is particularly useful for on-farm monitoring as neither the animals nor the daily activities are disturbed (Di Francesco *et al.*, 2021; Morrow *et al.*, 2002).

Throughout the transition period of dairy cows, changes in dry matter intake (DMI) are common and may cause alterations in manure output (Nennich *et al.*, 2005). Even though manure output may be altered, research has shown that level of intake does not significantly influence the concentration of glucocorticoid metabolites in the faeces (Huzzey *et al.*, 2011; Rabiee *et al.*, 2001). Elevated fGCM concentrations cannot be immediately associated with a certain stressor, due to other scenarios that may also cause an increase in fGCM levels, such as mating and feed restriction. As a result, no absolute threshold value can be defined (Ebinghaus *et al.*, 2020; Gellrich *et al.*, 2015). Welfare analysis requires assessment of animal stress at farm level (Morrow *et al.*, 2002). Quantifying a stress biomarker will assist in determining the best management practices, improved herd productivity and animal well-being (Morrow *et al.*, 2002).

2.8 Factors affecting glucocorticoid concentration in dairy cows

Natural variation

Secretion of glucocorticoids is pulsative and has a mean pulse interval of roughly 120min (Gellrich *et al.*, 2015). Cortisol concentrations may fluctuate due to natural circadian rhythms, along with the influence of extrinsic factors like climate, diet and disturbances prior to sampling (Chen *et al.*, 2015; Lefcourt *et al.*, 1993). This pulsating nature of cortisol secretion may alter the HPA-axis activity (Comin *et al.*, 2013). Lefcourt *et al.* (1993) established that glucocorticoid concentrations were naturally at their peak between midnight and midmorning, and lowest in the afternoon.

Heat stress

Skin thermoreceptors are activated on hot days which stimulate the HPA-axis for the release of adrenocorticotropin (Gaughan *et al.*, 2008). This process is an adaptive function that occurs whenever the weather conditions exceed the cow's thermal comfort zone (Alhussien & Dang, 2018; Veissier *et al.*, 2018). As a result, glucocorticoids can be used as an accurate indicator of cows experiencing thermal stress (Alhussien & Dang, 2018). Heat stress has detrimental effects on milk production (West, 2003), DMI and growth (O'Brien *et al.*, 2010), fertility and the welfare of cows (Pryce & Haile-Mariam, 2020).

Disease

The immune response stimulates production of regulatory cytokines which, in turn, stimulates the release of glucocorticoids from the pituitary-adrenal axis (Charmandari *et al.*, 2005; Sgorlon *et al.*, 2015). Subsequent long-term alterations of the basal cortisol concentration have a negative effect on the homeostasis of the animal, leading to increased susceptibility of numerous diseases (Comin *et al.*, 2013). Painful diseases, such as lameness, may cause an increase in cortisol concentrations (Gellrich *et al.*, 2015). Increased concentrations of fGCM have been used as indicator to detect chronic stress and was found to be positively correlated with welfare impairments, like the prevalence of hock lesions (Ivemeyer *et al.*, 2018; Rouha-Mülleder *et al.*, 2010). The effect of lameness on glucocorticoid concentrations are, however, still debated due to contrasting result by researchers (Gellrich *et al.*, 2015). O'Driscoll *et al.* (2015), found that lameness specifically caused by sole ulcers displayed elevated blood cortisol levels when compared to healthy cows. Conversely, Almeida *et al.* (2008) and Walker *et al.* (2010) did not detect any difference in either blood or milk cortisol concentrations when comparing lame cows to healthy ones. Consequently, detecting a significant increase of mGC in lame cows depend on the underlying cause as well as the duration of the experienced distress (Gellrich *et al.*, 2015).

Somatic Cell Count (SCC) are commonly used as an indicator of subclinical mastitis (Sharma *et al.*, 2011). SCC influences mGC concentration, but only when the SCC is higher than 400 000 cells/ml (Sgorlon *et al.*, 2015). Extended periods of increased glucocorticoid concentrations result in a suppressive effect on the immune system of the cows which can be linked to potential udder infections (Ebinghaus *et al.*, 2020). A significant correlation has become evident between the HPA-axis activity and subclinical mastitis; cows suffering from metritis also portrayed elevated cortisol concentrations (Galvão *et al.*, 2010; Kulcsár *et al.*, 2005). Ivemeyer *et al.* (2018) correlated an increase curing rate of mastitis to a lower physiological stress response of lactating cows. FGCM concentrations were found to be positively correlated to somatic cell scores, portraying the detrimental effect of cortisol levels on the health of cows (Ebinghaus *et al.*, 2020).

Genetics and environmental factors

Combined with the physiological demands of high milk production, cows are challenged in their attempts to maintain homeostasis which may lead to a variety of reproductive and metabolic disorders (Chen *et al.*, 2015). The high production of milk in the Holstein breed is normally associated with a modified endocrine status, specifically increased concentrations of somatotropin and decreased concentrations of insulin, which support increased production without the associated metabolic disorders (Higashiyama *et al.*, 2014). In other words, increased milk yield is generally associated with elevated levels of plasma cortisol (Negrão & Marnet, 2006; Sgorlon *et al.*, 2015).

Glucocorticoid concentrations may vary between farms due to the impact of environmental conditions, including stocking rate, cubicle design and the number of cows per productive area (Del Corvo *et al.*, 2020; Ito *et al.*, 2017). The environmental effects may superimpose the genetic background (Verkerk *et al.*, 1998). Situations such as restricted feed intake, altered behaviour, overstocking and regrouping may cause an increase in cortisol concentrations (Gellrich *et al.*, 2015; Huzzey *et al.*, 2011).

Human-animal relationship

Cows that are housed indoors rely on humans for many aspects of their everyday lives and this daily interaction with humans affects the cows' productivity and behaviour (Mandel *et al.*, 2016). Contact with humans are inevitable, but varies with management, the size of the herd and the level of automation on the farm (Cornish *et al.*, 2016). According to Berry (2001), positive human-animal relationship forms the basis of positive animal welfare. The stockpersons' attitudes and behaviours toward the cows have a significant impact on the level of fear cows experience (Ebinghaus *et al.*, 2020). Consequently, increased fear of humans will be reflected in an increased avoidance distance (Bertocchi *et al.*, 2018), reduced milk yield (Fernándes *et al.*, 2021) and decreased success at first insemination (Ebinghaus *et al.*, 2020). Fearful cows can experience an immediate release of cortisol in aversive situations (Gellrich *et al.*, 2015, Möstel & Palme, 2002). The cow-human interaction affects the welfare and comfort of cows directly, as Kielland *et al.* (2010) found that farmers with greater levels of empathy for their dairy cows had fewer incidences of hock lesions (Grant, 2012).

2.9 Conclusion

The need to improve dairy cow welfare has been underestimated in the past, but the health and welfare of dairy cows has become essential to both the dairy industry and consumer acceptance. Research has been able to identify the detrimental effects of stress and poor welfare on dairy cows, but few attempts have been made to ameliorate these factors through the use of environmental enrichment. Dairy cows have exceptional hearing and therefore, auditory stimuli have great potential to decrease stress and improve welfare.

Chapter 3: Materials and methods

3.1 Ethics approval

The study was conducted on the UP Future Africa Experimental Farm with ethical approval (Ethics number: NAS243/2020) from both the Faculty of Natural and Agricultural Sciences, as well as the Animal Ethics Committee of the University of Pretoria.

3.2 Study site

The UP Future Africa Experimental Farm (-25.7515068, 28.2485752) is situated in Pretoria, one of South Africa's capital cities. The farm forms part of the University's research grounds and is in close proximity to the University's main campus in the Hatfield suburb.

In order to ensure that the UP Future Africa Experimental Farm was suitable for an enrichment study, a welfare evaluation was conducted in collaboration with the Dairy Standard Agency (DSA) under guidance from Dr Mark Chimes, a welfare specialist. The audit criteria consisted of 10 sections, namely (1) Regulatory requirements, (2) Environment and milking shed premises, (3) Milking shed, (4) Udder preparation and good milking practices, (5) Training, (6) Herd health and animal welfare, (7) Animal feeding, (8) Animal housing, (9) Dairy management, contingency planning and preventative actions, and (10) Documentation.

The UP Future Africa Experimental Farm received a score of 87.49% (green status) and was thus suitable for conducting the enrichment study.

3.3 Study animals

The Holstein cows used in this study were kept under similar conditions in paddocks with shade netting. The North facing side of the paddock was used for the study to minimize external variables and expose the cows to the same environmental challenges, similar noise pollution and feeding schedule. Second- and third-lactation cows were housed separately from first-lactation cows, due to the potential negative effect between cow hierarchies. The paddocks provided access to a shaded feed bunk filled with a total mixed ration (TMR) three times per day (07:00, 12:00 and 18:30) and shaded water troughs were available *ad libitum*. Cows were milked three times per day (07:00, 12:00 and 18:00) through the use of a herringbone milking system. The milk yield of cows was automatically recorded with the Afifarm milking system version 3.076 (SAE Afikim) installed in the milking parlour. Three sets of milk production data were recorded as follows: Milk 1 was recorded during the morning milking, followed by Milk 2 during the afternoon milking and Milk 3 was recorded during the evening milking.

3.4 Experimental design

The lactating cows at the UP Future Africa Farm were ranked based on the Fluid Merit Index (FMI), which is the genetic merit of the cows including milk volume, milk components and functional herd life (Gresse, 2018). Nine adult lactating Holstein cows, in their second- or third-lactation, were identified based on their (1) production potential, (2) age and (3) days in milk (DIM). High producing cows are believed to be more susceptible to stress and therefore, three high, three average and three low producing cows were selected for the study. These cows were of similar age and DIM. The selected cows were divided into three groups (A-C), each group consisting of a high, an average and a low producing cow.

The trial period was divided into three different treatments, namely constant exposure (CE), limited exposure (LE), and no exposure (NE) to music. CE occurred without interruptions for the full trial period, LE occurred only in the milking parlour (cows were milked three times per day) and during NE the cows experienced no exposure to music. The combination of the three groups of cows and three treatments allowed the use of a Latin Square (3x3) as experimental design - as indicated in Table 3.1 - with each letter only occurring once within each column and within each row.

Table 3.1 A 3x3 Latin Square illustrating treatments and groups.

	CE	**LE**	**NE**
Period 1	A	B	C
Period 2	B	C	A
Period 3	C	A	B

By using the Latin Square, each group of cows experienced every treatment. Each treatment had a duration of 28 days, which included 12 sampling events. In order to prevent any potential stress of moving cows between groups and locations, the sound system was moved between the groups. The three groups were each exposed to their allocated treatment at the same time - whilst group A was receiving constant exposure, group B was receiving limited exposure and group C was receiving no exposure to music. After 28 days, the groups moved on to their next treatment as allocated in the Latin Square design until all three groups received all three treatments.

3.5 Sound system

Constant exposure to auditory stimuli was done through a sound system (consisting of a Sony MEX-N5300BT stereo and two pairs of Sony XS-GTF1639 speakers) installed at the paddock. The exterior of the sound system was designed from materials made to withstand numerous challenges, including environmental factors such as rain and wind. The system (fig. 3.1) was mounted to the roof of the barn to minimalize the risk of theft and to allow the music to be focused on the cows without the cows being able to cause any damage to it. The sound system was designed with an alternating current, switching from electricity to battery power, which allowed continuous exposure to music without any interruptions.

Figure 3.1 Four speakers in paddock area, two facing the feeding area and two facing the freestall section.

Two speakers were angled towards the feeding area of the cows, due to the amount of time the cows spend in this area. The remaining two speakers were angled to the freestall section of the paddock area, but these speakers were faded for reduced volume in order to prevent the adjacent camps from hearing the music. These speakers provided continues exposure to classical music, 24 hours per day for the full trial period. Classical music spanning over more than eight hours were played on a continuous shuffle to avoid un-intended conditioning (Uetake *et al.*,1997).

Limited exposure to classical music occurred only in the milking parlour. A JBL Speaker was mounted at the height of the cows' ears, 1.2m away from the cows - according to recommendations by Heffner & Heffner (1992). The classical music was played via Bluetooth through a mobile phone. The playlist consisted of recommendations from previous studies, including the *Pastoral Symphony No. 6* by Beethoven, *Claire de Lune* by Debussy, Bach's *Cello Suite No. 1 in G major* and Mozart's *Adagio from Divertimento No. 1, K. 205* (Alworth & Buerkle, 2013; Crouch *et al.*, 2019; Kenison, 2016). The complete playlist is provided in appendix A.

3.6 Activity level

Activity data was recorded for the nine focal animals through individual pedometers, Pedo Plus (SAE Afikim), attached to the hock joint of the cow and collected during each milking session through the Afifarm management program. Three sets of activity data were recorded as follows: Activity 1 reflected the activity of the period between the evening milking and the morning milking of the following day, while Activity 2 reflected the activity between the morning milking and the noon milking and Activity 3 reflected the activity between the noon milking and the evening milking.

3.7 Sample collection and preparation

3.7.1 Faeces

Faecal samples were collected three times per week for each of the nine focal cows - every Monday, Wednesday, and Friday during the evening milking session. A laboratory number was assigned to each of the faecal and milk samples, which was correlated with the cow number, and combined into a single excel sheet.

Between 30 to 50g of faecal material was collected from a well-mixed dropping and placed in a 30 ml plastic tube (fig 3.2), immediately placed in a cooler box, and stored at -20°C within 30 minutes of collection. All samples remained frozen until further processing.

At the Endocrine Research Lab (ERL) of the University of Pretoria, frozen faecal samples were lyophilised, pulverised and sifted through a mesh strainer in order to remove fibrous material (Ganswindt *et al.*, 2012). Between 0.100 to 0.110g of faecal powder was mixed with 3 ml 80% ethanol in water. The suspensions were vortexed for 15 minutes and then centrifuged for 10 minutes at 3300 x g. Afterwards, the supernatants were transferred into microcentrifuge tubes and kept at -20°C until analysis.

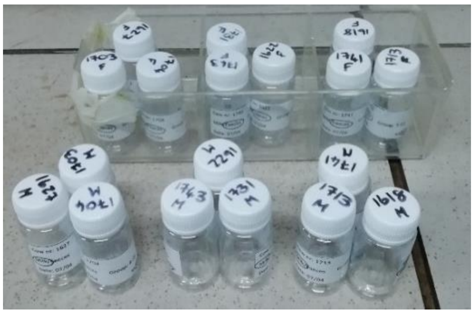

Figure 3.2 30 ml sampling tubes used for the collection of milk and faecal samples.

Figure 3.3 Milk containers used to gather homogenized samples.

3.7.2 Milk

About 20 ml of milk was collected between 18:00 to 19:30 during the evening milking sessions every Monday, Wednesday and Friday. Collected samples were shaken three times, transferred to a new tube (fig 3.2), placed in a cooler box and stored at -20°C within 20 minutes after collection. The initial collection containers (fig. 3.3) were rinsed with warm water between each sampling to prevent contamination. At the ERL, the milk samples were centrifuged at 3000 x g at 4°C for 15 minutes to separate the fat and the skim milk, with the latter being used for hormone analysis (Gellrich *et al.*, 2015).

3.8 EIA analyses

The analyses were performed at the ERL of the University of Pretoria, South Africa, following established protocols (Ganswindt *et al.*, 2002). The faecal extracts were analysed for immunoreactive glucocorticoid metabolite concentrations using an Oxoaetiocholanolone enzyme immunoassay (EIA) measuring 11,17dioxoandrostanes. The milk samples were analysed for immunoreactive glucocorticoid concentrations using a Cortisol EIA with Cortisol (4-pregnene-11β,17α,21-triol-3,20-dione) used as the standard and Cortisol-3-CMO-DADOO-biotin used as the label. Palme & Möstl (1997) give details of the utilized assays, including antibody cross-reactivities.

The sensitivity of the two EIAs were 25 pg/ml milk and 1.2 ng/g dry faecal weight (DW), respectively. The intra- and inter-assay Coefficient of Variation (Table 3.2) were determined by repeated measurements of high- and low-value quality controls.

Table 3.2 Intra- and Inter-assay Coefficient of Variation values for fGCM and mGC concentrations

Coefficient of Variation (%)		
fGCM concentration	Intra-assay	5.33 and 6.14
	Inter-assay	10.54 and 13.55
mGC concentration	Intra-assay	4.42 and 6.24
	Inter-assay	13.96 and 14.28

3.9 Assessment of the effect of music on the handlers in the milking parlour

The six handlers assigned to the Holstein herd of the UP Future Africa Experimental Farm operate in two teams of three people each. The handlers ranged from 34 to 42 years of age and are employed at the dairy section for four years or more. The work schedule is portrayed in table 3.3.

A questionnaire (Appendix B) was developed to investigate the effect of music on the handlers. The questionnaire was compiled in collaboration with Dr D Jordaan from Department of Agricultural Economics, Extension and Rural Development (LEVLO) using the Likert scale – a five-point scale system often used to assess different opinions (Habibi *et al.*, 2014). Five of the six handlers responsible for the cows in the milking parlour completed the questionnaire at the end of the trial period. The sixth handler was unable to participate.

Table 3.3 Monthly work schedule of handlers in the dairy parlour

	Morning milking (weekdays)	**Afternoon milking (weekdays)**	**Evening milking (weekdays)**	**Weekend**
Week 1	Team 1	Team 1	Team 2	Team 2
Week 2	Team 2	Team 2	Team 1	Team 1
Week 3	Team 1	Team 1	Team 2	Team 2
Week 4	Team 2	Team 2	Team 1	Team 1

3.10 Statistical analysis

All data available for the activity levels and milk yield of each cow were added to a spreadsheet containing the results of the EIA performed on the milk and faecal samples**.** Quality control was done on the data set and extreme values identified as outliers were removed from the analysis. Results obtained for the dependent variables are reported as P-values, Sample size (N), Degrees of Freedom (DF) and F-value. For all statistical analyses, a P value of ≤ 0.05 was considered significant.

The statistical analyses were performed using SAS statistical software version 9.4. (SAS Institute Inc). Regression, analysis of variance (ANOVA), and analysis of covariance (ANCOVA) are all included into the General Linear Model (GLM), allowing performance of all three statistical tests in a single procedure. The GLM is able to predict more than one dependent variable through the use of a multivariate analysis of variance (MANOVA) from one or more independent variables. The treatments (constant, limited and no exposure) were the independent variables, whereas fGCM and mGC concentrations, milk yield and activity were the dependent variables. Cow age, lactation number and production potential (low, medium high) were included as fixed effects.

The PROC GLM procedure was conducted using the formula (Kelley & Bolin, 2013):

$Y = \alpha + \beta_1 X_1 + \beta_2 X_2 + \beta_k X_k + \mathcal{E}$
Where:
Y = dependent variable
α = constant
β = beta coefficients
X = Independent variables (Constant, limited and no exposure)
$\mathcal{E}$ = Error term

Subsequently, the Tukey-Kramer test was used as a post hoc test because of its ability to evaluate the significance of the difference between each pair of means that are based on different sample sizes by using the formula (Salkind, 2006):

$$\mathrm{CD} = q_{1-\alpha}(k, df_E)\sqrt{\left[\frac{MSE}{2}\left[\frac{1}{Ni} + \frac{1}{Nj}\right]\right]}$$

Where:
$q_{1-\alpha}(k, df_E)$ = the Studentized range statistic at Level A for k means and dfE,
dfE = Error degrees of freedom,
MSE = Error mean square,
Ni = group mean i (i = 1,…, k)
Nj = group mean j (j = 1,…, k), but j ≠ i.

The independent variables used in the GLM were not normally distributed, therefore the non-parametric test called Spearman's rho was performed to measure the strength and direction of correlations between two variables by using the formula (Salkind, 2006):

$$r_s = 1 - \frac{6\sum d_i^2}{n(n^2-1)}$$

Where:
r_s = Spearman's rho correlation coefficient
d_i = Difference between the two ranks of each observation
n = number of observations

The strength of the correlation was indicated as described by Asuero *et al.*, 2006 (Table 3.4). The correlations for this study were described as significant at the 0.01 and 0.05 level (2-tailed) for all three exposure treatments.

Table 3.4 The size of the correlation coefficient (r) and its meaning (adapted from Asuero et al., 2006)

Size of r	Interpretation
0.00 to 0.29	Little if any correlation
0.30 to 0.49	Low correlation
0.50 to 0.69	Moderate correlation
0.70 to 0.89	High correlation
0.90 to 1.00	Very high correlation

Chapter 4: Results

4.1 Overview of key effects

The effect of the three treatments on the dependent variables are summarised in table 4.1. The pooled means combine all three group (A-C) averages into a single value. Cow age, lactation number and production potential, all significant, were accounted for in the analysis. Constant exposure to music had the lowest mean fGCM concentration and the lowest mean activity level (Activity 1 & 3), and limited exposure to music had the highest milk production (Milk 1 to 3).

Table 4.1 Effects of constant, limited and no exposure to music on milk and faecal glucocorticoid concentration, milk yield and activity level through the comparison of pooled least square means

Variable	Units	Constant exposure	Limited exposure	No exposure
fGCM concentration*	µg/g DW	0.16^{a}	0.20^{b}	0.20^{b}
mGC concentration	ng/ml	1.19^{a}	1.40^{a}	1.32^{a}
Milk 1***	L/cow	12.34^{a}	13.05^{a}	10.72^{b}
Milk 2***	L/cow	5.46^{a}	5.49^{a}	4.86^{b}
Milk 3***	L/cow	6.18^{a}	6.81^{b}	5.54^{c}
Activity 1**	Steps/cow	85.08^{a}	93.92^{b}	87.61^{ab}
Activity 2	Steps/cow	198.54^{a}	194.03^{a}	192.17^{a}
Activity 3*	Steps/cow	172.03^{a}	180.68^{b}	175.58^{b}

*****P < 0.001; **P < 0.01; *P < 0.05**

^{ab} Within a row, LS Means without a common superscript differ (α = 0.05)

4.2 FGCM concentrations of cows exposed to auditory stimuli

Exposure to classical music had an effect on the fGCM concentrations of the cows (P = 0.012; N = 324; DF = 2; F = 4.50). Cows (fig. 4.1) showed an overall 19.6% decrease in fGCM concentrations when constantly exposed to classical music (overall individual mean 0.16 µg/g DW), compared to no exposure (overall individual mean 0.20 µg/g DW). In contrast, a 2.1% increase in fGCM concentrations was observed during limited exposure to music (overall individual mean 0.20 µg/g DW) compared to no exposure. The coefficient of variation for fGCM concentrations of all groups together was 74.22%.

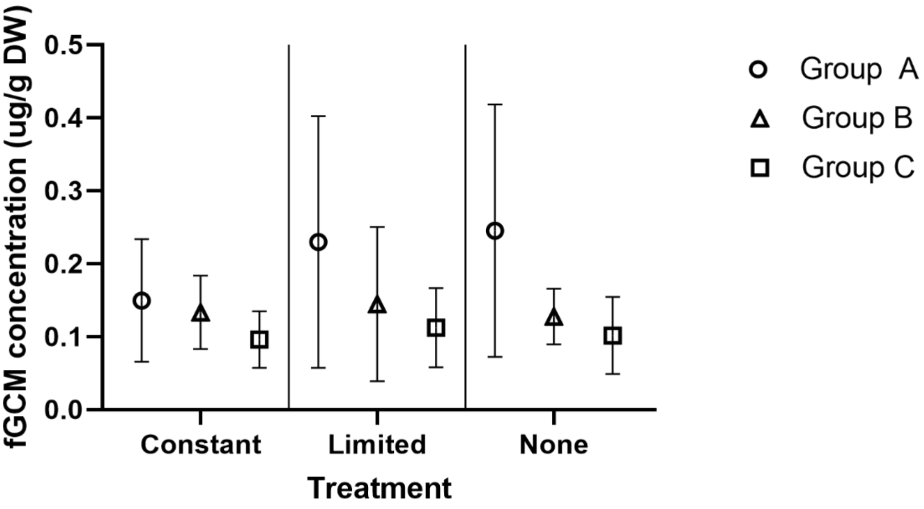

Figure 4.1 FGCM concentrations (mean (symbols) ± SD (error bars)) of 3 groups (A, B, and C) of Holstein cows exposed to constant, limited and no exposure of classical music.

4.3 MGC concentrations of cows exposed to auditory stimuli

Exposure to classical music did not have an effect on the mGC concentrations of cows (P = 0.492; N = 322; DF = 2; F = 0.71). Cows (fig. 4.2) showed no significant differences when constantly exposed to classical music (overall individual mean 1.19 ng/ml), compared to limited exposure (overall individual mean 1.40 ng/ml) or no exposure (overall individual mean 1.32 ng/ml). The coefficient of variation for mGC concentration of all groups together was 130.70%.

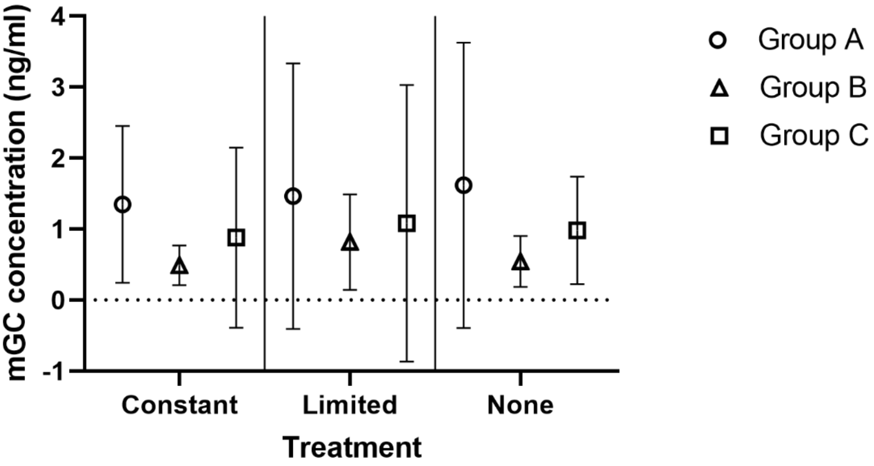

Figure 4.2 MGC concentrations (mean (symbols) ± SD (error bars)) of 3 groups (A, B, and C) of Holstein cows exposed to constant, limited and no exposure of classical music.

4.4 Correlations between dependent variables

No correlation was found between fGCM concentrations and milk yield, or between fGCM concentrations and activity level. Similarly, no correlation was found between mGC concentrations and milk yield, or between mGC concentrations and activity level. Little to no correlation was found between fGCM and mGC concentrations during constant exposure (+0.288) and no exposure (+0.283), with a low correlation found during limited exposure: (+0.445).

4.5 Activity level of cows exposed to auditory stimuli

Significant changes in activity level (P = 0.005; N = 727; DF = 2; F = 5.42) were observed during the first activity period (18:00 – 06:00). Cows showed an overall decrease of 3% in mean activity level when constantly exposed to music (overall individual mean 85.07 steps/cow) compared to when not exposed (overall individual mean 87.62 steps/cow) (fig. 4.3). Further, cows showed an overall increase of 7.2% in mean activity level during limited exposure to music (overall individual mean 93.92 steps/cow) compared to when no exposure.

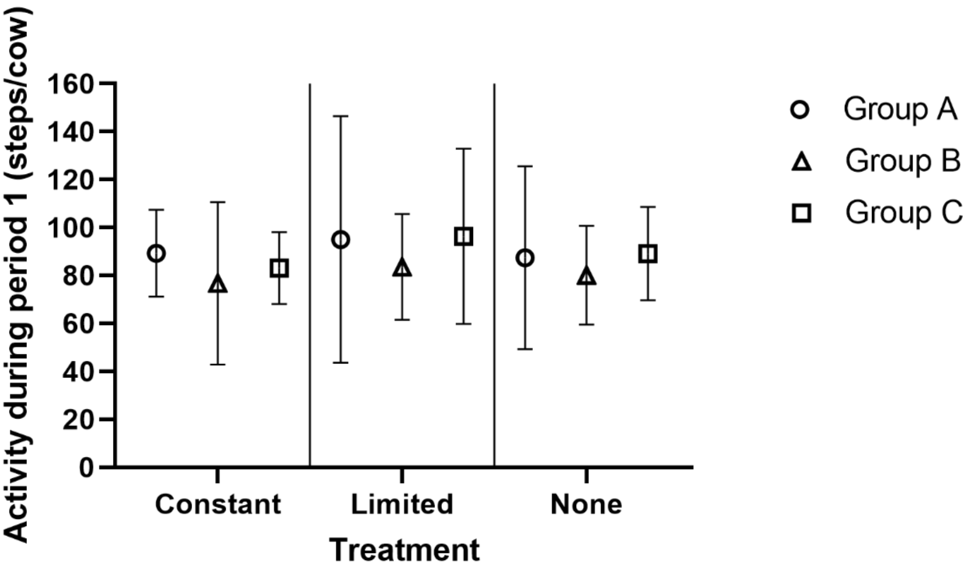

Figure 4.3 Activity level during period 1 (mean (symbols) ± SD (error bars)) of 3 groups (A, B, and C) of Holstein cows exposed to constant, limited and no exposure of classical music.

No significant changes (P = 0.249, N = 707, DF = 2; F = 1.39) in activity level were observed between constant exposure (overall individual mean 198.54 steps/cow), limited exposure (overall individual mean 194.03 steps/cow) or no exposure (overall individual mean 192.17 steps/cow) for the groups during activity level 2 (06:00 – 12:00) (fig. 4.4).

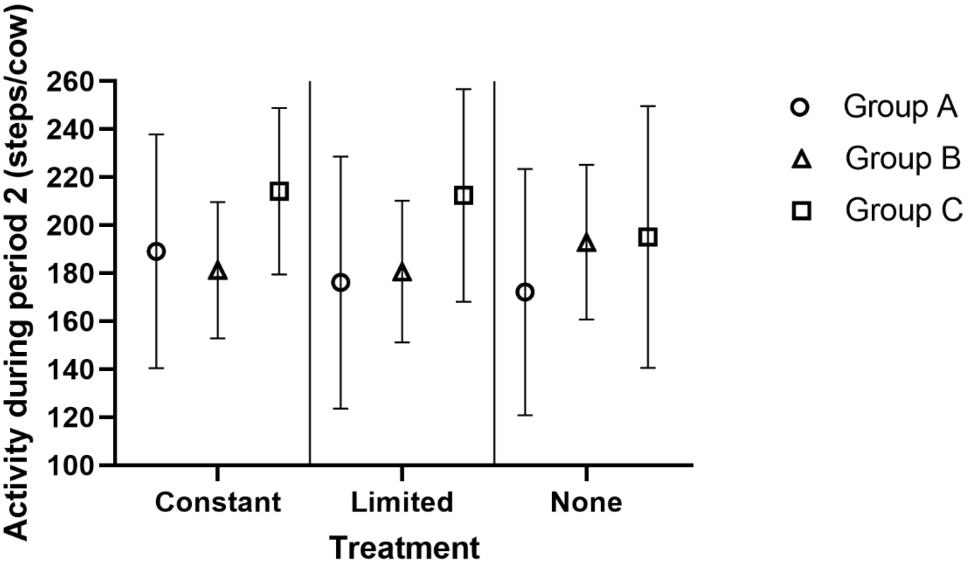

Figure 4.4 Activity level during period 2 (mean (symbols) ± SD (error bars)) of 3 groups (A, B, and C) of Holstein cows exposed to constant, limited and no exposure of classical music.

Significant changes in activity level (P = 0.048; N = 702; DF = 2; F = 3.05) were observed during the third activity period (12:00 – 18:00). Cows showed an overall decrease of 2.1% in mean activity level when constantly exposed to music (overall individual mean 172.03 steps) compared to when not exposed (overall individual mean 175.58 steps) (fig. 4.5). Further, cows showed an overall increase of 2.9% in mean activity level during limited exposure to music (overall individual mean 180.68 steps) compared to when not exposed.

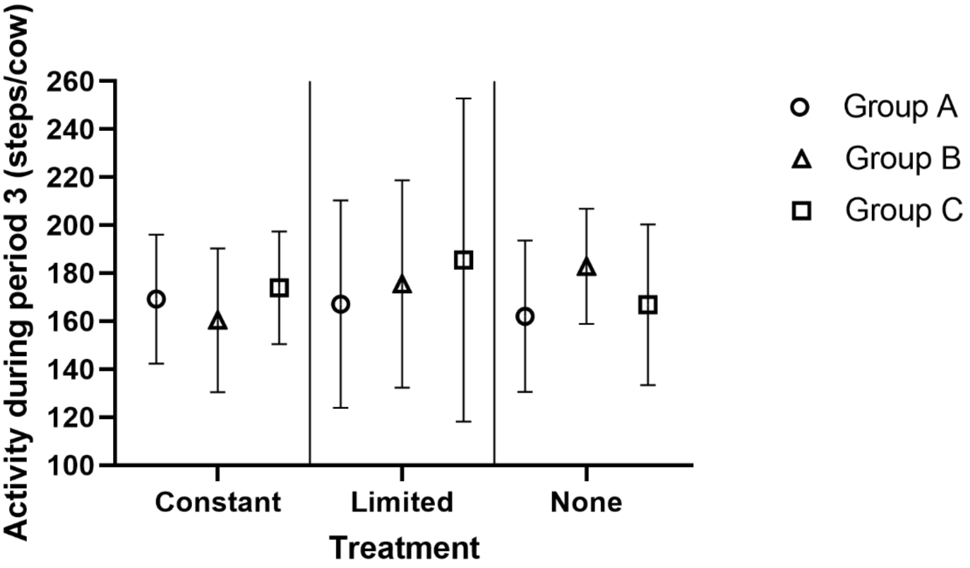

Figure 4.5 Activity level during activity period 3 (mean (symbols) ± SD (error bars)) of 3 groups (A, B, and C) of Holstein cows exposed to constant, limited and no exposure of classical music.

4.6 Milk production of cows exposed to auditory stimuli

A significant increase in milk yield was observed for all three milking sessions, tested at 06:00 (milk 1: $P < 0.0001$; $N = 699$; $DF = 2$; $F = 22.95$), 12:00 (milk 2: $P < 0.0001$; $N = 686$; $DF = 2$; $F = 10.12$) and 18:00 (milk 3: $P < 0.0001$; $N = 674$; $DF = 2$; $F = 22.04$), when exposed to musical treatments.

During the morning milking session (06:00), cows showed an overall increase of 21.7% and 15.1% in mean milk yield during limited exposure (overall individual mean 13.05 L/cow) and constant exposed to music (overall individual mean 12.34 L/cow), respectively, when compared to not exposed (overall individual mean 10.72 L/cow) (fig. 4.6).

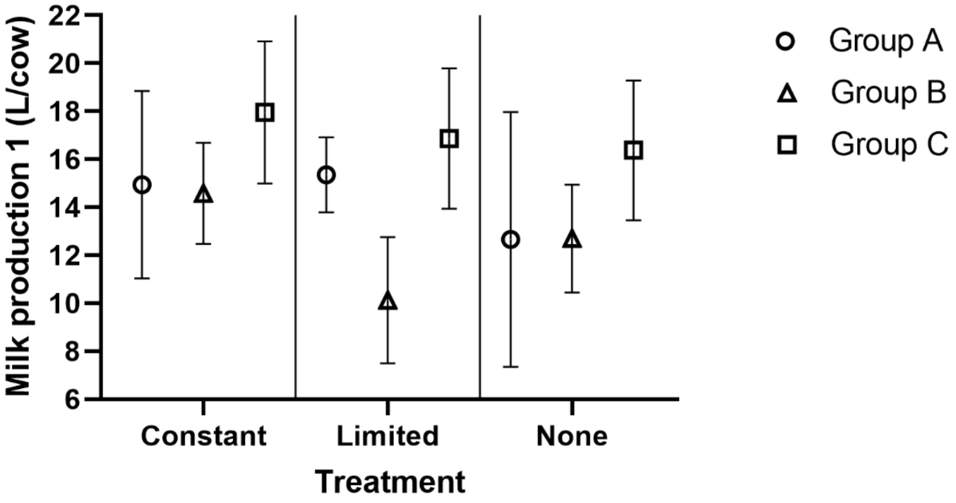

Figure 4.6 Morning milk yield (mean (symbols) ± SD (error bars) of 3 groups (A, B, and C) of Holstein cows exposed to constant, limited and no exposure of classical music.

During the afternoon milking session (12:00), cows showed an overall increase of 13% and 12.4% in mean milk yield during limited exposure (overall individual mean 5.49 L/cow) and constant exposed to music (overall individual mean 5.46 L/cow), respectively, when compared to not exposed (overall individual mean 4.86 L/cow) (fig. 4.7).

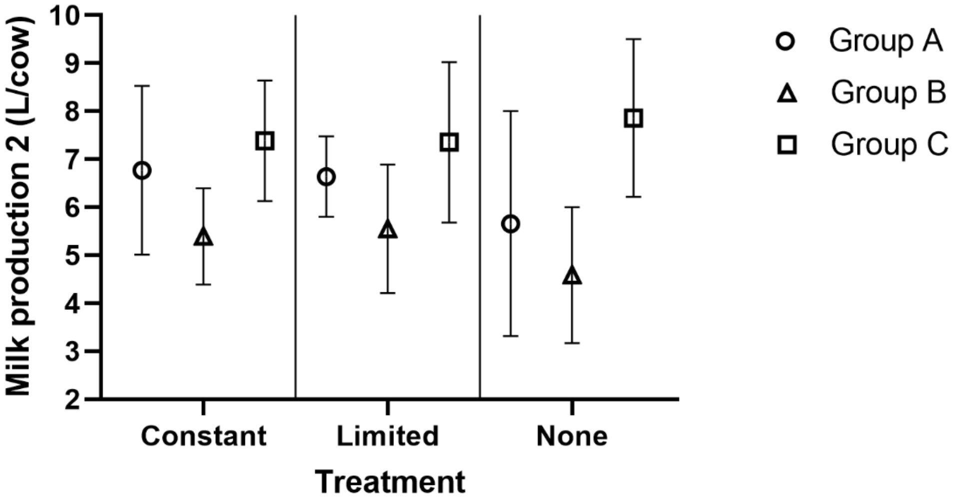

Figure 4.7 Afternoon milk yield (mean (symbols) ± SD (error bars)) of 3 groups (A, B, and C) of Holstein cows exposed to constant, limited and no exposure of classical music.

During the evening milking session (18:00), cows showed an overall increase of 22.9% and 11.6% in mean milk yield during limited exposure (overall individual mean 6.81 L/cow) and constant exposed to music (overall individual mean 6.18 L/cow), respectively, when compared to not exposed (overall individual mean 5.54 L/cow) (fig. 4.8).

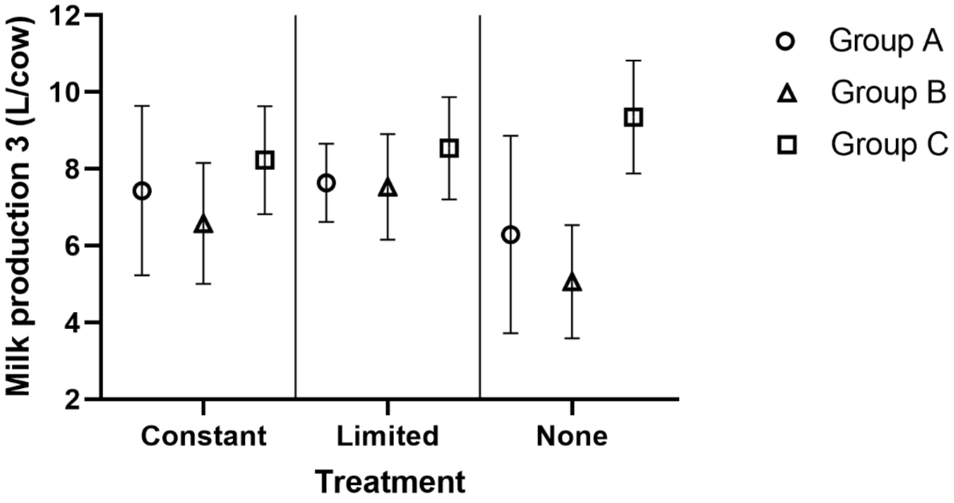

Figure 4.8 Evening milk yield (mean (symbols) ± SD (error bars)) of 3 groups (A, B, and C) of Holstein cows exposed to constant, limited and no exposure of classical music.

4.7 The effect of music on the handlers in the milking parlour

The handlers all indicated that they enjoy working with the cows. Three of the handlers indicated that they always enjoy listening to music while working, while the remaining two handlers indicated that they occasionally enjoy listening to music when they are busy working. There was also a positive response to the exposure of classical music in the milking parlour, as four of the handlers indicated that they enjoy listening to the classical music in the parlour and only one of the handlers felt neutral about it.

Chapter 5: Discussion

5.1 Discussion

Sensory enrichment in the form of auditory stimuli (exposure to classical music) generated contrasting results between constant exposure and limited exposure to music. Key findings of this study include a decrease in fGCM concentrations of Holstein cows during constant exposure to classical music and a respective increase in fGCM concentrations during limited exposure to music. Milk production increased, for all three milking sessions, during both constant and limited exposure to classical music compared to no exposure. Activity levels during activity period 1 and 3 decreased during constant exposure to music and increased during limited exposure to music.

Even though glucocorticoid quantification is frequently used to evaluate a stress response, almost no studies have focused on fGCM concentrations of dairy cows when exposed to sensory enrichment. A study by Ebinghaus *et al.* (2020) focused on the fGCM concentrations of dairy cows during increased contact time with humans. Although the study is not defined as an enrichment study by the author, increased contact time with humans is regarded as a form of social enrichment. The study showed an association between increased contact time with humans and lower fGCM concentrations. The decrease in fGCM concentrations during constant exposure to music observed in this study may be an indication that the cows found the music relaxing.

Limited exposure to classical music occurred only in the milking parlour where the milking machine is used to remove the milk from the cow's udder. The noise omitted from the milking machine can be overwhelming, ranging from 39.6 to 70.2 dBs (average of 54dBs). The classical music had to compete with the noise generated by the milking machine, and the combination of the two may have been interpreted as noise by the cows. The erratic nature of limiting the classical music to the milking session may have functioned as a stressor rather than a relaxing stimulus. Noise can increase HPA activity and glucocorticoid levels, which can have detrimental effects on the health status, feeding behaviour, energy consumption and reproductive physiology of cows (Brouček, 2014). Newberry (1995) issued a warning that the addition of an auditory stimulus to an already noisy environment may do more harm than good.

The tendencies observed, namely decreased average fGCM concentrations during constant exposure and a slightly increased average fGCM during limited exposure, suggests that sensory enrichment does have potential as an enrichment program when applied correctly – otherwise it can be interpreted as a stressor. To validate these tendencies, a larger sample size will be required. Various studies observed a substantial variation in fGCM concentrations between individuals and between farms (Rees *et al.*, 2016). As a result, it may be beneficial for future studies to determine a baseline prior to employing a form of environmental enrichment. In order for the results to be comparable, collection (method and time) and analysis should be kept the same.

No significant changes in milk glucocorticoid (mGC) concentrations were observed in this study. MGC concentration as a biomarker is seen as an efficient tool to evaluate the stress perceived by cows due to its ease of collection through routine milking sessions (Del Corvo *et al.*, 2020). Very few, if any, studies have investigated the effect of sensory enrichment on the mGC concentrations of cows. Research has been more focused on the effect of lactation stage and weather conditions on mGC concentrations; a study by Alhussien & Dang (2018) found that mGC concentrations vary over seasons and the highest mGC concentrations were observed during hot, humid weather. Previous research has also reported higher mGC concentrations during the early lactation stages compared to later stages (Gellrich *et al.*, 2015; Schwalm & Tucker, 1978). The analysis on mGC was done differently to the current study, as the authors separated the hormone from the milk prior to storage. Cortisol is able to diffuse across the blood-milk barrier, and Gygax *et al.* (2006) warns that a leak of cortisol back from milk to plasma should be considered. Studies report that respective mGC concentrations will only be reflected in milk for two to four hours after exposure to a stressor (Termeulen *et al.*, 1981; Verkerk *et al.*, 1998). Milk samples may need to be taken more regularly to account for this restriction.

A comparison between generated hormone concentrations, indicated that fGCM concentrations were more meaningful in its use as a non-invasive biomarker. A low positive correlation was observed between fGCM and mGC concentrations during limited exposure to music, indicating that if fGCM concentrations increased, mGC concentrations would also increase. A strong correlation between mGC and blood glucocorticoid concentration has been reported (Gygax *et al.*, 2006) and similarly, a strong positive correlation between fGCM and blood glucocorticoid concentrations has been reported (Huzzey *et al.*, 2011). However, the relationship between fGCM and mGC concentrations require more research.

Activity levels are often used as a behavioural tool to investigate the health status, period of oestrus or the affective state of cows (Rutten *et al.*, 2013). Cows exposed to auditory stimuli in the current study showed no significant changes in activity level during activity period 2 (06:00 to 12:00), which may be due to environmental sounds such as handlers, birds, other cows, cars, and feeding machine, overpowering the music.

Activity period 1 (18:00 to 06:00) is defined as the primary resting period of cows (Beauchemin, 2018). Rumination has been reported to peak approximately four hours after feeding, which is associated with an increase in time spent lying (Schirmann *et al.*, 2012). The Holstein herd housed at the UP Future Africa Experimental Farm are fed at 18:30 after milking and are provided with enough feed to sustain them until morning. The peak resting period of these cows are during the evening hours and therefore, a decrease in activity (as seen during constant exposure to music) will be beneficial and allow proper rest and rumination.

The behaviour of cows measured through their activity levels (specifically activity period 1 and 3) suggest that the cows did find constant exposure to classical music soothing, which is supported by studies reporting a decrease in aggression of cows when exposed to slow music (Dorléans, 2019; Ninomiya, 2014). Cows have a strong need to rest, and the general notion is that cows produce more milk when they spend more time resting (Schirmann *et al.* 2012; Temple *et al.*, 2016). Even though various studies report that rest is not a threshold event, a noted consequence of inadequate rest is lower milk yield (Gomez & Cook, 2010; Ito *et al.*, 2010).

A recent study focused on the effect of audiobooks, country music and classical music as auditory stimuli on the behaviour of cows (Crouch *et al.*, 2019). The Holstein-Friesian cows showed increased locomotive behaviour and decreased resting behaviour during constant exposure to the auditory stimuli. The author suggested that providing periods of no stimulation may increase resting and rumination time in cows. In the current study, limited exposure to classical music resulted in an increased activity level. A higher activity level is associated with reduced production and longevity, increased risk for claw problems, lameness and lower milk yield (Gomez & Cook, 2010; Ito *et al.*, 2010).

Ninomiya (2014) stated that expressing behaviour aids an animal in coping with a stressor, which is an essential aspect of animal welfare. Kicking and stomping are behavioural indicators of stressed cows, which may explain why the pedometer registered a higher activity level during limited exposure to music. Lying and standing times of cows have been reported to increase during exposure to classical music (Lemcke *et al.*, 2021), but Dorléans (2019) argues that the intensity of behavioural changes may be breed specific. It is, therefore, needed to establish a breed-specific baseline of normal behaviour on the specific farm which can be used for comparison, such as periods of no exposure to music (used as the baseline levels in the current study). However, the current study was limited to activity level, but future studies can explore other factors of cow behaviour, such as resting bouts and length of resting bouts.

The cows at the UP Future Africa Experimental Farm are milked 3 times per day (Milk 1 to 3), providing three opportunities to investigate the milk production of cows exposed to classical music as a form of sensory enrichment. Milk yield of cows increased during constant exposure to music, but the highest mean for all three sessions was observed during limited exposure to music.

A recent study used various genres of music as sensory enrichment to investigate the effect thereof on the milk production of cows (Kemp, 2020). No music had a significantly higher milk production compared to all other genres. However, the study exposed cows to a new musical genre every day. The results may be due to the effect of various musical genres on the cows, or it may come across as a stressor due to the lack of adaptation, which explains why Kemp (2020) saw an increase in milk production after removing the musical stimuli. Kemp (2020) advised that the stimuli should be played more often for improved results, which agrees with the results observed in this study. North & McKenzie (2001) reported that cows exposed to slow music portrayed a higher average milk production, which supports our findings. The classical music used in this study was specifically chosen because of its slow tempo.

The highest production averages were seen during limited exposure to music. A study by Kiyici *et al.* (2013) reported that cows exposed to auditory stimuli had an increased milk let down speed (Mandel *et al.*, 2016). Studies have reported a correlation between milk let down speed and milk yield (Erskine *et al.*, 2019; Singh *et al.*, 2010). It is possible that cows exposed to auditory stimuli during limited exposure in the milking parlour had an increased milk let down speed and an associated increase in milk yield. Milk let down speed was, unfortunately, outside of the scope for this study. Future studies may incorporate milk let down speed as a parameter for cows exposed to classical music.

Cows headed to the milking parlour have full udders and wait in a que to enter the parlour. There are various activities happening in the milking parlour and it is also the main contact point with the handlers. Even though the music had a positive effect on the handlers, the other factors in the parlour may have resulted in increased fGCM concentrations. An increase level of activity and increased milk yield was observed during limited exposure to music.

An increase in energy (seen through increase activity level captured by the pedometer) is associated with increased milk yield (Coppock, 1985). However, an increase in energy due to increase feed intake is also associated with a decrease in digestive efficiency and an increase in heat production, which can increase susceptibility of cows to heat stress (VandeHaar & St-Pierre, 2006). Heat stress may, in turn, increase fGCM concentrations – as seen in this study. Music with a slow tempo appears to increase milk production, but the influence of feed intake requires investigation. Future studies can focus on the association between stress parameters (such as fGCM concentrations, heart rate and respiratory effect), feed intake and milk production of cows exposed to auditory stimuli.

The trend observed in the current study is that constant exposure to classical music resulted in a significant decrease in fGCM concentrations, along with a decrease in activity level and an increase in milk production. Comparisons at group level showed that group A had the highest mean fGCM concentration and consistently (Milk 1 to 3) had the lowest mean milk production. Group C had the lowest mean fGCM concentration and consistently (Milk 1 to 3) had the highest mean milk production. The study indicates that constant exposure to classical music may have a beneficial effect on cows which, in turn, has a positive effect on the behaviour and production of cows.

5.2 Outcomes of the study

Chronic stress of cows is believed to be identified through prolonged periods of elevated glucocorticoid concentrations (Möstl & Palme, 2002). These extended periods of elevated GC concentrations can result in immunosuppression and reduced curing rates, which means that the cows require extended periods to recover from a disease and/or illness (Ivemeyer *et al.*, 2018). Higher GC concentrations are also associated with tissue atrophy, reduced growth, reduced energy and decreased reproductive success (Fernandes-Novo *et al.*, 2020; Nedić *et al.*, 2017).

Cows that are ill/diseased cows require more frequent veterinary intervention and medication, which oftentimes include antibiotics (Anika *et al.*, 2019). As a result, the milk from the diseased cow cannot be sold for human consumption (Priyanka *et al.*, 2017). The profitability of the cow is reduced due to increased cost and increased maintenance requirements. The longer the cow remains ill, the longer her milk cannot be sold, and the farmer loses a larger income (Loo *et al.*, 2019).

Lower stress levels in dairy cows could assist in relieving the immune system from constant pressure, allowing the cows to be less susceptible to illness and disease (Comin *et al.*, 2013; Garcia, 2001). The production manager would benefit from healthy cows through fewer visits from the veterinarian and medication costs and no period of milk withdrawal. Classical music as auditory stimuli can thus assist in the health and welfare management of the cows.

Constant exposure to classical music resulted in a 15% increase in milk production during the morning milking session. The UP Future Africa Experimental Farm produced an average of 13.9 L/cow during the morning milking session when cows were not exposed to classical music. A 15% increase on 13.9 L/cow is 2 L/cow. The UP Future Africa Experimental Farm sells their milk to the Dairy Shop for R5/L. An addition 2L/cow on our nine sampling cows is an additional 18L per month, providing an additional R97.2 per month for the farm. The South African dairy industry has an average herd size of 459 cows producing an average of 18.6L/cow (van Heerden, 2021), to which a 2L/cow increase can result in an additional 918L per month.

Auditory stimuli as a form of environmental enrichment, therefore, also have economic benefits to the producer. This can reduce the number of cows needed for profitable production and reduce the amount of agricultural land required for production. Environmental enrichment will also assist in improving the way that consumers think about dairy farms and dairy production.

Chapter 6: Conclusion

The study found that cows constantly exposed to classical music had decreased fGCM concentrations, lower activity levels, and higher milk production. Limited exposure to music did not have the same stimulating affect, but instead may have acted as a stressor. The biggest limitation of the study was the small sampling size. Despite the restriction, significant results were observed and can be used for future studies. Many factors impact the fGCM concentration of cows, and consequently fGCM concentrations cannot be linked to stress levels and supporting parameters are required. Future studies can include additional stress parameters (heart rate, respiratory rate, etc), along with feed intake and milk let down to investigate the effect of auditory stimuli on the cow. Future studies can also benefit from increased focus on the human-animal relationship and the impact thereof on the glucocorticoid concentrations of cows.

This study validated the potential positive effects of environmental enrichment on lactating cows and exposure to auditory stimuli has the potential to improve the health and welfare of cows, as well as economic benefits through improved milk production.

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Appendix A

Name of composition	Composer
6 Cello Sonatas: No. 4 in G Major: I. Andante	Jean-Baptiste Barrière
6 Cello Sonatas: No. 4 in G Major: II. Adagio	Jean-Baptiste Barrière
Oboe Concerto in D minor, S.Z799	Alessandro Marcello
Nocturne in E flat major, Op. 9, No. 2	Frédéric Chopin
Ballade	Luke Faulkner
The Symphony No. 6 in F major, Op. 68 (Pastoral)	Ludwig van Beethoven
Piano Concerto No 3 in C minor, Op. 37: II. Largo	Ludwig van Beethoven
Piano Concerto No. 14 in C-sharp Minor, Op. 27, No. 2 (Moonlight)	Ludwig van Beethoven
Bach: Brandenburg Concerto No. 4 in G major, BWV 1049: I. Allegro	Johann Sebastian Bach
Nocturne No. 20 in C♯ minor, Op. posth.	Frédéric Chopin
Concerto Grosso in G major, Op. 6, No. 1: III. Adagio	George Frideric Handel
Concerto Grosso, Op. 3, No. 3 in G major: III. Allegro	George Frideric Handel
Concerto Grosso No. 1 in D major: I. Largo II. Allegro III. Largo – Allegro IV. Largo – Allegro V. Allegro	Arcangelo Corelli
Divertimento in D, K. 251: II. Minuetto III. Andantino	Wolfgang Amadeus Mozart
Duo for Two Cellos, Op. 51, No. 1: I. Allegro	Jacques Offenbach
Duo in D Major, Hob. X11: I. Moderato	Franz Joseph Haydn
Peer Gynt Suite No. 1, Op. 46: I. Morning Mood	Edvard Grieg
Edvard Grieg - Piano Concerto in A minor, Op. 16: II. Adagio	Edvard Grieg

Symphony No. 7 in C major ("Le midi"), H. 1/7: 1. Adagio - Allegro 2. Recitativo. Adagio 3. Adagio - Allegro - Adagio 4. Menuetto & Trio 5. Finale. Allegro	Franz Joseph Haydn
Concerto for Flute, Harp, and Orchestra in C Major, K. 299: II. Andantino III. Rondeau, Allegro	Wolfgang Amadeus Mozart
Flute Concerto No. 1 in G Major, K. 313: II. Adagio - Allegro ma non troppo	Wolfgang Amadeus Mozart
Flute Concerto No. 2 in D major, K.314: I. Allegro aperto	Wolfgang Amadeus Mozart
Piano Concerto No. 1 in E minor, Op. 11: II. Romance - Larghetto	Frédéric Chopin
The Élégie, Op. 24	Gabriel Fauré
"Ombra mai fu", also known as "Largo from Xerxes”	George Frideric Handel
Orch. Ducros: Gymnopédie No. 1	Erik Satie
Water Music, Suite No. 1 in F major, HWV 348	George Frideric Handel
Water Music: Suite No. 2 in D major, HWV 349	George Frideric Handel
Concerto grosso in B-Flat Major, Op. 3, No. 2, HWV 313: II. Largo	George Frideric Handel
Concerto Grosso in E Minor, Op. 6, No. 3, HWV 321: V. Allegro, ma non troppo	George Frideric Handel
Concerto Grosso in F Major, Op. 6, No. 2, HWV 320: III. Largo - Adagio - Larghetto andante	George Frideric Handel
Was mir behagt, ist nur die muntre Jagd, BWV 208, "Hunt Cantata": Aria: Schafe konnen sicher weiden	Johann Sebastian Bach
Cello Suite No. 1 in G major, BWV 1007 (for solo cello): I. Prelude II. Allemande III. Courante	Johann Sebastian Bach

IV. Sarabande V. Menuet I - Menuet II VI. Gigue	
Symphony No. 3 in F major, Op. 90: III. Poco Allegretto	Johannes Brahms
Violin Concerto in D major, Op. 77: II. Adagio	Johannes Brahms
Piano Sonata No. 16 in C major, K. 545: II. Andante	Wolfgang Amadeus Mozart
La finta giardiniera, K. 196: Ouverture. Allegro molto	Wolfgang Amadeus Mozart
Le grand cahier (Suite for String Orchestra): I. La forêt et la rivière IV. Nos études VI. Le bain VII. Les pommes de grand-mère VIII. Théâtre IX. Accusations XI. La fin de la guerre	Alexander Litvinovsky
Symphony No. 5 in C-Sharp Minor: IV. Adagietto (Sehr langsam)	Gustav Mahler
Clarinet Concerto in A major, K. 622, II. Adagio	Wolfgang Amadeus Mozart
Eine Kleine Nachtmusik, K. 525: II. Romanze Andante III. Menuetto - Allegretto	Wolfgang Amadeus Mozart
Concerto in C major for flute and harp, K 299: I. Allegro II. Andantino III. Rondeau (Allegro)	Wolfgang Amadeus Mozart
Piano Concerto No. 21 in C major, K. 467: II. Andante	Wolfgang Amadeus Mozart
Divertimento No.2 in B-Flat Major, K.137: I. Andante III. Allegro assai	Wolfgang Amadeus Mozart
Piano Concerto No. 23 in A, K. 488: II. Adagio	Wolfgang Amadeus Mozart

Lucio Silla, K. 135. Ouverture: II. Andante	Wolfgang Amadeus Mozart
Canon in D major	Johann Pachelbel
Piano Concerto No. 21 in C major, K. 467: II. Andante	Wolfgang Amadeus Mozart
Symphony No. 5 in B flat major, D. 485: II. Andante con moto	Franz Peter Schubert
Sinfonia in G Major, RV 149: I. Allegro molto III. Allegro	Antonio Vivaldi
My Fatherland (Symphonic Poems): The Moldau	Bedřich Smetana
Suite Bergamasque, L. 75: III. Clair De Lune	Claude Debussy
Sinfonia No. 6 in Si minore, Op. 74 "Patetica": II. Allegro con grazia	Pyotr Ilyich Tchaikovsky
Serenade for Strings in C major, Op. 48: II. Valse. Moderato. Tempo di Valse III. Elegia. Larghetto elegiaco	Pyotr Ilyich Tchaikovsky
Viola Concerto in G major, TWV 51:G9: III. Andante	Georg Philipp Telemann
Carnival of the Animals, 13th movement: Le cygnet (The Swan) in G major	Camille Saint-Saëns

Appendix B

Date:
Milker: 1 2 3 4 5 6 7
Age: ___________
Gender: ___________________
Race/ethnicity: ___________

The aim of this questionnaire is to investigate the existing relationship between the human handlers and the cows on the Hillcrest Experimental Farm. The answers are anonymous and will be treated as confidential by the principal investigator. According to ethical bylaws, participants are under no obligation to complete this questionnaire. There are no incorrect answers, and no harm will come to participants by answering this questionnaire.

1. **How many years have you been working at the Experimental farm?**

Less than 2 years	**2 years**	**3 years**	**4 years**	**More than 4 years**

2. **How many years have you been working in the dairy unit?**

Less than 2 years	**2 years**	**3 years**	**4 years**	**More than 4 years**

3. **Which sections of the Experimental Farm do you work at?**
   Please tick all the appropriate sections

Dairy sector	**Sheep sector**	**Feeding & feed mill**

4. **Please indicate the most appropriate answer:**
   a. **I enjoy listening to the music in the parlour**

Strongly disagree	**Disagree**	**Neutral**	**Agree**	**Strongly agree**

   b. **I like working while listening to the music**

Never	**Rarely**	**Occasionally**	**Frequently**	**Always**

   c. **I enjoy working with the cows**

Strongly disagree	**Disagree**	**Neutral**	**Agree**	**Strongly agree**

5. **What type of music do you like?**

   ____

6. **Please indicate your level of education/training:**
   (e.g. National certificate, diploma, courses etc.)

   ____