UNIVERSIDADE DE LISBOA

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TUTOR(A):
Dr. Dário Guerreiro

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Acknowledgments

"Where should I go?" – Alice. "That depends on where you want to end up" – said The Cheshire cat.

In the beginning of this journey, I was just like Alice. I did not know which way I should go, and I did not know where I wanted to end up. Throughout these six years I started to build my path, always with doubts, but with a lot of support, love, and friendship from amazing people that became part of this wonderful odyssey.

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Resumo

Efeito do stress crónico na incidência de doenças metabólicas e infecciosas em vacas leiteiras

O cortisol no pelo tem sido usado como uma ferramenta de diagnóstico para avaliar o stress crónico em vacas leiteiras, uma vez que oferece como vantagens ser não invasivo, fácil e rápido de colher e oferece uma visão retrospetiva de stress do animal ao longo de um período de meses. O objetivo deste estudo foi avaliar se a concentração de cortisol no pelo de vacas leiteiras no período seco é um preditor de doenças metabólicas ou infeciosas no primeiro mês pós-parto. Para além disso, temos como objetivos investigar a relação entre a concentração de cortisol no pelo de vacas leiteiras e a morbilidade dos seus vitelos durante o primeiro mês de vida. Outros objetivos foram estudar se a condição corporal, número de lactações, distância de fuga e produção leiteira aos 305 dias afetam os níveis de cortisol no pelo e/ou têm impacto no desenvolvimento de doença em vacas leiteiras. O pelo da cauda foi cortado a vacas do período seco em cinco explorações diferentes, no mesmo dia a distância de fuga foi medida e a condição corporal de cada animal foi avaliada. Após emparelhamento de vacas doentes e saudáveis tendo em consideração o número de lactações, produção leiteiras aos 305 dias e condição corporal, 18 vacas saudáveis e dezanove doentes foram incluídas na análise. Não foram encontradas diferenças significativas entre vacas saudáveis e doentes. Níveis elevados de cortisol no pelo das vacas do nosso estudo foram associados a vitelos mais saudáveis (p=0,039). Com base nestes dados, podemos concluir que a concentração de cortisol no pelo não foi um indicador útil para diferenciar o desenvolvimento de uma doença metabólica ou infeciosa em vacas leiteiras, no entanto, níveis de cortisol elevados no pelo em vacas leiteiras estão associados a melhores índices de saúde nos seus vitelos.

Palavras-chave: cortisol, pelo, stress crónico, doenças metabólicas, doenças infeciosas

Resumo alargado

Efeito do stress crónico na incidência de doenças metabólicas e infecciosas em vacas leiteiras

O eixo hipotálamo-pituitária-adrenal é um sistema neuro endócrino responsável pela resposta fisiológica do stress. Este eixo é responsável pela produção de glucocorticoides, nomeadamente o cortisol, que regula vários processos fisiológicos importantes. O cortisol está envolvido na regulação do sistema imunitário, cardiovascular e reprodutivo, influenciando também funções cognitivas e de crescimento.

No entanto, em situações de stress crónico, o cortisol em excesso é nefasto para o organismo. O stress crónico tem um impacto significativo na fertilidade de vacas leiteiras e enfraquece o sistema imunitário dos animais aumentando a sua suscetibilidade a doenças. Em humanos, o stress crónico tem impacto negativo na função cognitiva e poderá levar ao desenvolvimento de doenças cardiovasculares e metabólicas.

O cortisol no pelo tem sido usado como uma ferramenta inovadora de diagnóstico para avaliar o stress crónico em vacas leiteiras, uma vez que oferece como vantagens ser não invasivo, fácil e rápido de colher e oferece uma visão retrospetiva de stress do animal ao longo de um período de meses. Ainda existe algum desconhecimento relativamente aos mecanismos de deposição do cortisol no pelo, no entanto a maioria dos investigadores presume que o cortisol, sendo uma substância lipofílica, é incorporado na matriz de queratina do pelo através da corrente sanguínea.

Estudos realizados em humanos provaram uma relação entre elevados níveis de cortisol no cabelo e aumento da incidência de doenças cardiovasculares. Alguns estudos em bovinos, verificaram que animais doentes tinham um nível aumentado de cortisol no pelo comparativamente a animais saudáveis.

O objetivo deste estudo foi avaliar se a concentração de cortisol no pelo de vacas leiteiras no período seco pode ser usado como um preditor de doenças metabólicas ou infeciosas no primeiro mês pós-parto. Para além disso, temos como objetivo investigar a relação entre a concentração de cortisol no pelo de vacas leiteiras e a morbilidade dos seus vitelos durante o primeiro mês de vida. Outros objetivos foram estudar se a condição corporal, número de lactações, distância de fuga e produção leiteira aos 305 dias afetam os níveis de cortisol no pelo e/ou têm impacto no desenvolvimento de doença em vacas leiteiras. Cerca de 3 centímetros de pelo da cauda foi cortado a vacas do período seco em cinco explorações diferentes na região de Lisboa e vale do tejo, no mesmo dia a distância de fuga foi medida e a condição corporal de cada animal foi avaliada com base no método de Penn-State. Trinta dias após cada animal parir, informações acerca do seu estado de saúde (desenvolvimento de doenças metabólicas e infeciosas) e acerca da saúde dos seus

vitelos foi registado. Foram selecionadas, para análise laboratorial do pelo, 19 vacas doentes foram emparelhadas com 18 animais saudáveis tendo em consideração o número de lactações, produção leiteira aos 305 dias e condição corporal. O pelo dos 37 animais selecionados foi processado em laboratório e foi medida a concentração do cortisol através de um kit de ELISA. Não foram encontradas diferenças significativas entre vacas saudáveis e doentes. Níveis elevados de cortisol no pelo das vacas do nosso estudo foram associados a vitelos mais saudáveis (p=0,039). Com base nestes dados, podemos concluir que a concentração de cortisol no pelo não foi um indicador útil para diferenciar entre vacas saudáveis e vacas que vieram a desenvolver doença metabólica ou infeciosa, no entanto, níveis de cortisol elevados no pelo em vacas leiteiras estavam associados a melhores índices de saúde nos seus vitelos.

Palavras-chave: cortisol, pelo, stress crónico, doenças metabólicas, doenças infeciosas

Abstract

Effect of chronic stress in the incidence of metabolic and infectious diseases in dairy cattle

Hair cortisol has been used as a tool to assess chronic stress in dairy cows as it offers the advantage of being non-invasive, easy, and fast to collect and gives a retrospective view of the animal's stress over months. The aim of this study was to evaluate if hair cortisol concentrations of dairy cows in the dry period could be used as a predictor of metabolic or infectious diseases during the first month post calving. Furthermore, we aimed to investigate the relation between hair cortisol concentration in the dairy cows and their calf's morbidity during the first month of life. Other objectives were to study if body condition score (BCS), parity, flight distance and previous lactations' milk production affects the hair cortisol levels and/or have an impact in the development of disease in dairy cows. Hair from the tail switch was clipped once from cows in the dry period on five different farms, and on the same day the flight distance was measured and BCS was assessed for each cow. After pair matching diseased and healthy cows considering number of lactations, previous 305-day milk yield and BCS, 18 healthy and 19 diseased cows were included in the analysis. We found no significant differences in any of the parameters analysed between healthy and diseased cows. We found that higher hair cortisol levels in cows from our study were associated with healthier calves ($p=0.039$). Based on this data, hair cortisol concentration was not considered a useful indicator to differentiate between healthy cows and cows that developed a metabolic or infectious disease, however, higher hair cortisol levels in dairy cows were associated with better health outcomes for their calves.

Keywords: cortisol, hair, chronic stress, metabolic diseases, infectious diseases

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List of abbreviations

ACTH – Adrenocorticotropic hormone / corticotropin

BCS – Body condition score

BVD – Bovine viral diarrhoea

CBG – Corticosteroid binding globulin

CRH - Corticotropin-releasing hormone

CV - Coefficient of variance

DIM – Days in milk

ECBHM – European College of Bovine Health Management

ELISA - Enzyme-linked immunoassay

HPA – Hypothalamic-pituitary-adrenal

IBR – Infectious bovine rhinotracheitis

POMC - Pro-opiomelanocortin

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I. Externship report

The final externship of the Integrated Master's Degree in Veterinary Medicine was divided in three parts:

- Extracurricular externship at the SLU – Swedish University of Agricultural Sciences in the Ruminant Clinic.
- Curricular internship at the Sá Guerreiro Vet, Lda.
- Extracurricular externship at the Kelso Galedin Vets and at the University of Glasgow School of Veterinary Medicine.

1.1. Ruminant Clinic – SLU

This externship was carried out under the supervision of Professor Jean-Francois Valarcher, from 6th of September 2021 until 8th of November 2021, accounting for a total of 392 hours.

The Ruminant Clinic (Figure 1) offers veterinary care (diagnostics, medical and surgical treatment) to cattle, sheep, and goats in Sweden. It is considered to be a referral centre for educational purposes as well as for supporting veterinarians with advisory at both individual and herd level.

The author had the opportunity to work with the ruminant clinic team comprising two professors, two interns, one ECBHM resident and two members of staff, participating actively in the daily clinical activities at the ruminant clinic.

Every morning the team would gather to make a round discussion about the cases admitted to the clinic. Afterwards, the morning was dedicated to the treatment and care of the hospitalized animals. Every friday afternoon the ruminant clinic team met to join the weekly journal club discussion were the author proactively participated. During the rest of the week, in the afternoons the author joined the veterinary pathology rounds to discuss the cases that were necropsied. The author also had the opportunity to practice surgeries, suturing and other technical procedures on cadavers along with the 5th year students.

Figure 1 – Animal housing and surgery facilities in the SLU Ruminant Clinic.

1.2. Sá Guerreiro Vet, Lda

The curricular internship was carried out under the supervision of Dr. Dário Guerreiro, between 4th of January 2022 to 29th of April 2022, accounting for a total of approximately 960 hours.

During this time, the author followed Dr. Dário Guerreiro and Dr. André Parada in the daily activities of a veterinary ambulatory practice. Sá Guerreiro Vet, Lda company provides veterinary services mainly to cattle, sheep, and goat herds, but also to wild, exotic and companion animals.

The author had the opportunity to develop clinical and professional skills by participating in various activities in the areas of animal health/prophylaxis, reproduction, surgery, and emergencies. A description of the activities is mentioned below:

- Participation in the official herd health program (tuberculosis and brucellosis testing).
- Involvement in prophylaxis programs (vaccination programs against Clostridium, BVD and IBR and deworming of cattle and small ruminants).
- Assistance in a variety of surgeries or procedures, such as left displaced abomasum, caesareans, reduction of uterine prolapses, orchiectomies and discussion of the main surgical techniques and different types of anaesthesia.
- Cooperation in the clinical examination, discussion of differential diagnosis and participation in the treatment plans for individual cases, including digestive diseases (neonatal diarrhoea, ruminal tympany, intestinal

parasitosis), respiratory (bovine respiratory disease), neurologic (clostridiosis), metabolic (ketosis) and infectious diseases (mastitis, retained placenta, metritis, infectious keratoconjunctivitis) and others such as lameness, abscesses, wounds and bone fractures, and heart disease.

- Reproduction visits to several dairy farms and suckler herds for pregnancy diagnosis, evaluation of the cow reproductive tract (diagnosis of metritis and endometritis), discussion of different hormonal protocols and their application in different contexts and adapted to different herds.
- Companion animals and wild animals consultations (vaccination of companion animals, orchiectomies in wild animals)
- Reflection of own strengths and limitations in the clinical, professional, and personal fields.

During this time, the author also worked for her thesis project. She visited five different farms for hair collection along with Professor Ricardo Bexiga and PhD student Luis Capela.

In the laboratory, the author processed the hair samples for cortisol extraction and analysis with the help of PhD student Luis Capela.

1.3. University of Glasgow School of Veterinary Medicine

This extracurricular externship was carried out under the supervision of Professor Lorenzo Viora, from 20th of June 2022 until 29th of July 2022, accounting to a total of approximately 240 hours.

The externship was divided into two parts: the first two weeks the author stayed at a mixed practice (Galedin Vets) in Kelso, Scotland and the following four weeks at the School of Veterinary Medicine in Glasgow, Scotland.

At the Galedin Vets the author had the opportunity to attend some of the companion animal consultations and surgeries. Twice a week, the author followed the farm veterinarian to join the routine reproduction visits of two big dairy farms in the area. After the visit, she participated in the advisory meeting along with the responsible veterinarian and the farmers to discuss the hormonal protocols and the fertility results.

At the School of Veterinary Medicine in Glasgow the author participated actively in diagnosis and treatment of the cases in the Galloway Building along with the clinicians and residents. She also engaged in the weekly journal club along with the professors and ECBHM residents.

The Galloway building receives sick animals (cattle and small ruminants) from different farms around Scotland. The animals are mostly used for teaching purposes.

Twice a week there was a visit to two dairy farms. These visits were divided into two parts. Early in the morning the author would join the routine herd visits carried out by the residents, for pregnancy diagnosis and post-partum checks, afterwards she attended and participated in the on-farm herd health report discussions along with the farmers and residents to discuss a variety of topics such as fertility, nutrition, infectious diseases, calf's health and neonatal care, and others.

II. Literature review

1. The HPA axis

The hypothalamic-pituitary-adrenal (HPA) axis is one of the major neuroendocrine systems involved in the physiological stress response (Aguilera 2012). It is stimulated every time the animal perceives an event as stressful (Brown et al. 2017) and aids the adaptation to the surrounding environment. This can be accomplished by releasing a cascade of stress-related hormones involving the hypothalamus, the pituitary gland, and the adrenal cortex in an effort to regulate homeostasis (Brown et al. 2017).

For a successful adaptation to stress, a coordinated activation of neuroendocrine responses is necessary, including the activation of behavioural, endocrine, immune, and autonomic responses to preserve homeostasis (Aguilera 2012).

The behavioural responses include alertness, improved cognition and increased awareness, and are essential for avoidance and protective responses. Autonomic and endocrine responses are crucial for physiological adaptive changes (Aguilera 2012).

The activation of the stress response starts when the animal perceives an environmental change or potential injury and signals a peripheral receptor. Subsequently the information travels through the sensory nerves of the spinothalamic tract (afferent pathways) and transmits it to the central nervous system, including the thalamus and hypothalamus (Collier et al. 2017).

Afterwards, in the paraventricular nucleus of the hypothalamus, corticotropin-releasing hormone (CRH) and vasopressin are synthesized (Aguilera 2012). These hormones trigger the endocrine cells in the pituitary gland (corticotropes) into synthesizing and secreting pro-opiomelanocortin (POMC) (Salak-Johnson and McGlone 2007).

POMC is a precursor protein that can be expressed in various tissues, but its main sites of expression are corticotrope cells in the pituitary, melanotropes in the intermediate lobe and neurons in the arcuate nucleus of the hypothalamus. The POMC molecule produces a variety of biologically active peptides according to the cell type. Corticotropin (ACTH), β-lipotropin and β-endorphin are peptides of the corticotrope cells in the pituitary (Aguilera 2012).

ACTH will stimulate the production of glucocorticoids such as cortisol in the *zona fasciculata* of the adrenal gland cortex. Subsequently, glucocorticoids are released into the bloodstream, transported by corticosteroid binding globulin (CBG) to be finally diffused through cellular membranes by binding to mineralocorticoid and glucocorticoid receptors (Aguilera 2012).

Glucocorticoids are essential for life and regulate numerous of physiologic processes such as immune function, skeletal growth, cardiovascular function, reproduction, and cognition (Oakley and Cidlowski 2013). Furthermore, circulating glucocorticoids are crucial for the metabolic adaptation to stress, and also to apply negative feedback on the HPA axis (Aguilera 2012). Additionally, CRH synthetized in the hypothalamus binds to CRHR1 in the brain and promotes the behavioural and autonomic adaptation to stress (Aguilera 2012) (Figure 2). However, blood cortisol levels will rise and become chronically elevated when the

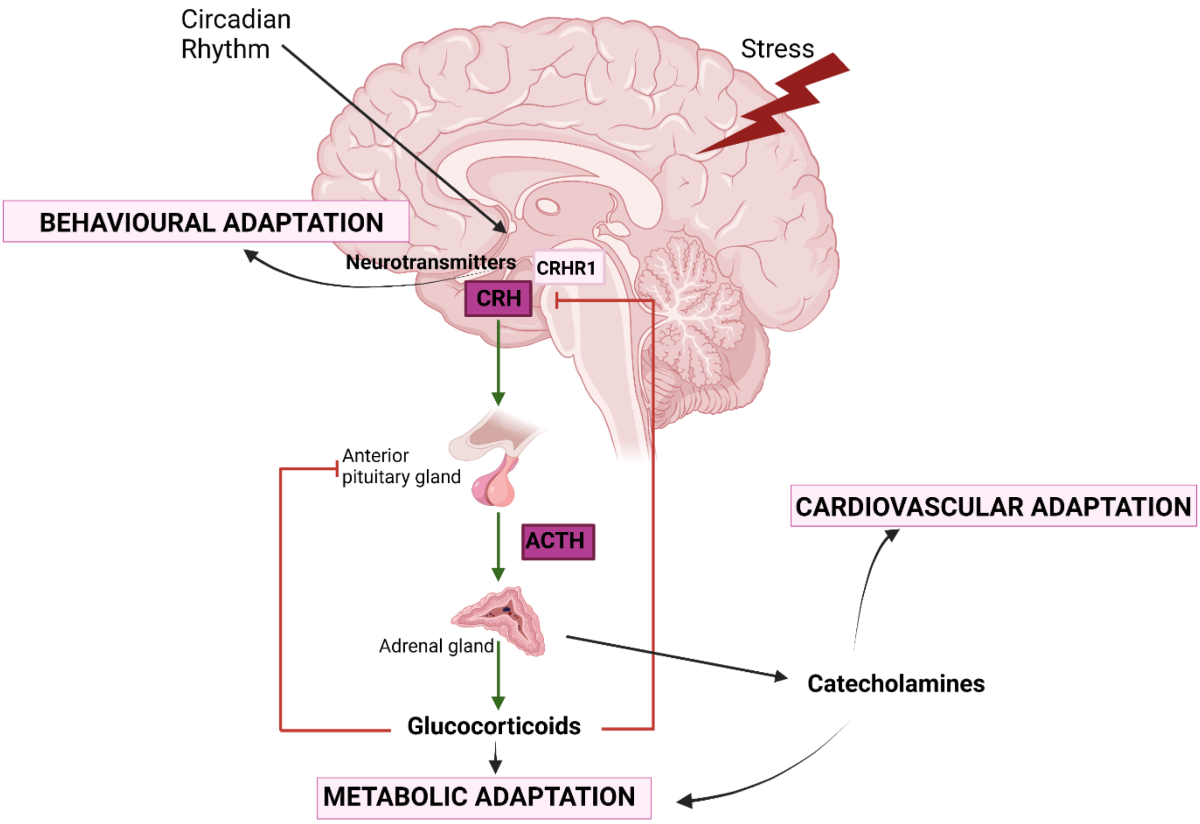


Figure 2 - Interactions between activation of the hypothalamic-pituitary-adrenal axis (HPA) and major adaptive responses to stress (created by the author in BioRender®).

individual is challenged with several and repeated stressors (Zijlmans et al. 2015).

While acute HPA axis responses to stress are rapid and short-term, ACTH responses during chronic or repeated stress differ according to the intensity, frequency, and the capacity to control the stress. In certain circumstances responses are sustained, whereas in other cases a desensitization or adaptation occurs despite the persistence of the stressor. Generally, in chronic stress conditions there is an overproduction of ACTH and glucocorticoid in response to a novel stressor, even if there is habituation to the primary persistent stimulus (Figure 3) (Aguilera 2012).

Transitory activation of the HPA axis facilitates the animal's adaptation to stress by triggering several physiological reactions in different target tissues, such as increased vascular tone, lipolytic and proteolytic activity - energy mobilization, stimulation of gluconeogenesis and glycolysis, and suppression of the inflammatory response (Greco and

Stabenfeldt 2012; Aguilera 2012). A repeated activation of the HPA axis can lead to a prolonged and maladaptive cortisol exposure causing a disruption of the cardiovascular, metabolic, immune, and nervous systems (McEwen 1998).

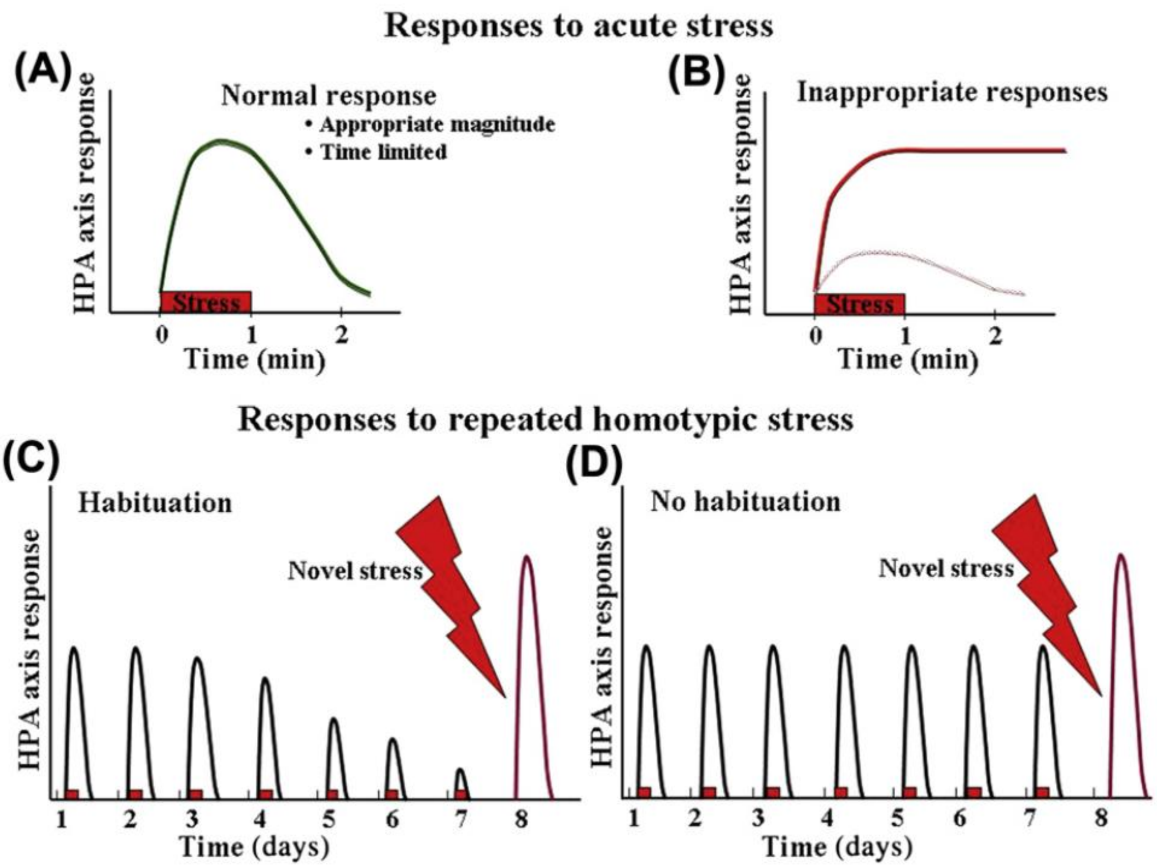


Figure 3 - Simplified illustration of the HPA axis responses to acute and repeated stress. In a normal response to acute stress, ACTH and glucocorticoid return to basal levels within a time limit (A). An inappropriate response can occur depending on the type and context of the stressor and the individual's genetic background (B). During repeated stress, and depending on the nature and magnitude of the stressor, a desensitization of the HPA axis responses develops, but there is a hyperresponsiveness to a new stress (red arrow) (C). Chronic stressors such as intense pain, blood loss and hypoglycemia do not cause acclimatization and also exhibit hyperresponsiveness to a novel stimulus (D). Adapted from Aguilera 2012.

Increased levels of glucocorticoids in response to stress affects the growth hormone secretion in the pituitary, having an impact on cattle growth rates and performance (Kumar et al. 2012). In addition, excessive secretion of stress mediators might impair the reproductive function and predispose to the development of cardiovascular diseases, diabetes, and hypertension in humans (McEwen 2006; Chrousos 2009).

A review by Zijlmans et al. (2015) concluded that higher levels of maternal cortisol in pregnant women were related to altered/negative child outcomes such as lower birth weight, shorter birth length, more respiratory and skin illnesses, larger systemic vascular resistance, and lower artery elasticity. Moreover, elevated maternal cortisol during pregnancy is associated with higher child cortisol concentrations.

2. Stress

Stress can be defined as an external event or condition that poses or is perceived as a threat to the body's homeostasis. A stressor is an element of the environment that places a pressure on a biological system (Collier et al. 2017). However, it is important to keep in mind that not all types of stress are harmful or damaging for the animal. In some cases, stress, or eustress, is considered beneficial for the animal (Trevisi and Bertoni 2009). Eustress refers to a correct or optimal stress level that is adaptive (e.g., reproduction and survival) and therefore improves the animal welfare (Villalba and Manteca 2019). Conversely, the stress that causes negative effects and compromises the animal's welfare is defined as distress (Trevisi and Bertoni 2009).

The animal's environment is unstable since it is subjected to continuous unpredictable changes (Brown et al. 2017). Once the animal's central nervous system perceives a threat, it develops a behavioural, autonomic, neuroendocrine, or immunological response (Kumar et al. 2012) that will help the individual to cope with the stressor (Brown et al. 2017). Therefore, the animal's response to stress involves consumption of energy to remove or reduce the impact of a certain stressor (Collier et al. 2017).

The animal's perception of stress poses a threat to the body's homeostasis since it always increases the animal maintenance requirements (Collier et al. 2017). When a farm animal faces a certain stressor, the energy once used for production will be redirected in an attempt to regulate homeostasis. Invariably this has consequences to the animal production system as it will decrease the animal performance and productivity, increasing costs to the farm (Collier et al. 2017).

The biological reaction to stress is divided into acute and chronic stress phases. Acute stress emerges when an animal is exposed to a stressful event for a short period of time, which can range from a couple of hours up to a few days and is associated with the fight or flight response (Brown et al. 2017). Facing a stressor, the afferent pathways will transmit the information to the thalamus and hypothalamus in the central nervous system and to the cortex, where the information is perceived. Subsequently, efferent pathways are activated to respond to the stressor in the animal's environment. The acute stress response is induced by the autonomic nervous system. Catecholamines and glucocorticoids are released; transcription factors involved in the acute response are activated and the metabolism modifies (Collier et al. 2017). Acute stress is considered to be short term and therefore insignificant when it comes to welfare issues (Trevisi and Bertoni 2009).

Chronic stress persists for weeks, and it can be described as a continuous stress response with excessive secretion of stress mediators (Burnett et al. 2015) by the endocrine system (Collier et al. 2017) resulting in a prolonged disruption of the body's homeostasis (Brown et al. 2017) and in a new physiological state (Collier et al. 2017). In humans, chronic stress may lead to several disorders such as anxiety, depression, cognitive dysfunction, osteoporosis, hypertension, type 2 diabetes mellitus, and other metabolic disorders (Chrousos 2009).

Trevisi and Bertoni (2009) mentioned that the exposure to chronic stress leads to ovarian dysfunction in dairy cows since it reduces the frequency of LH (luteinizing hormone) pulses. Moreover, heat stress in Holstein cows decreases the conception rate (Cavestany et al. 1985) having a significant impact on fertility.

Under stressful conditions the animal’s immune system is supressed, thus the susceptibility to diseases increases. Stress transport is an important factor for the development of bovine respiratory disease. Filion et al. (1984) have shown that transport stress increased pneumonia and mortality in calves challenged with bovine herpesvirus. In addition, stress can increase the susceptibility to mycobacterial diseases (Sheridan et al. 1994).

3. Fear, stress and the fight or flight response

Fear is frequently referred as a behavioural reactivity or agitation. Exposure to a novelty, startle reactions and hiding from predators are examples of fear reactions in animals (Grandin and Deesing 2014). Novelty is also perceived as a very strong stressor (Grandin 1997). In farm animals, intense fear can cause chronic stress which may jeopardize the expression of essential behaviours (social, sexual, parental), compromising the productivity of the animal and product quality (Grandin and Deesing 2014).

According to Grandin and Deesing (2014) fear can be described as an emotion induced by a perceived threat that causes the animal to move away from the area of that perceived danger. However, fear related behaviours differ based on the nature of the threat. These behaviours include active and passive strategies such as active defence (attack), active avoidance (flight, hiding or escape) and passive avoidance (immobility or freezing).

In the wild, when a predator is distant, the animal’s initial reaction is freezing. Immobility behaviour lowers the chances of detection. When the predator approaches, flight is the prevalent reaction (Grandin and Deesing 2014).

The intensity of reaction in response to a stressor is usually used to assess the strength of the underlying emotion. The typical example is the fight/flight response that presumes that a greater level of activity indicates a higher level of fear. However, one must keep in mind that genetic factors are also involved in the intensity of fear reactions, and also, that an absence of activity (immobility) can also indicate fear (Grandin and Deesing 2014).

4. Stress and the immune system

The production of stress-related hormones has an impact on the immune system both in humans (Herbert and Cohen 1993) and in animals (Salak-Johnson and McGlone 2007).

Acute and chronic stress both affect immune function, but chronic stress most often suppresses the immune system (Salak-Johnson and McGlone 2007). As reviewed by Salak-Johnson and McGlone (2007), bovine and porcine immune cells incubated with cortisol have inhibited lymphocyte proliferation, IL-2 production, and neutrophil function.

With chronically elevated levels of cortisol in the bloodstream, there is a decrease in the number of circulating lymphocytes and antibody production and an increased number of neutrophils (Trevisi and Bertoni 2009). The synthesis of interleukin-1 by macrophages and interleukin-2 by T helper cells is compromised causing poorer B-lymphocyte and cytotoxic T-cell activity.

Increased levels of cortisol also decrease interleukin-beta, impairing T-helper cell -1 and -2 balance and action (Broom 2006). In humans, stress is associated with lower levels of total serum IgM and salivary IgA (Herbert and Cohen 1993).

Lavon et al. (2008) observed that Holstein cows who were administered *E. coli* endotoxins had higher cortisol levels in plasma, suggesting that an inflammatory response increases levels of cortisol. Hence, not only prolonged stress in dairy cattle rises the levels of cortisol, but also inflammatory responses and endotoxemia.

Since stressful events are commonly associated with an increase of the HPA axis' activity, cortisol measurement can be an indicator for stress and useful to monitor the activity of the HPA axis (Dallman et al. 1987).

5. Methods for assessing cortisol

Cortisol, which is known to be a marker of stress response in animals, can be analysed in the blood, saliva, urine, and faeces. However, these methods only give a retrospective interval of a few minutes up to a maximum of two days (Meyer and Novak 2012). Because some of these methods require invasive procedures and close handling of dairy cattle, there can be rapid increases in blood cortisol and contradicting results might occur (Burnett et al. 2014; Heimbürge et al. 2019).

Moreover, plasma and saliva cortisol levels follow a circadian rhythm being higher in the morning and can vary throughout the day within a ultradian rhythm with hourly pulses. This will lead to individual discrepancies depending on the time of the day (Aguilera 2012; Kumar et al. 2012; Meyer and Novak 2012).

6. Hair Cortisol

Some recent studies indicate that hair cortisol can be a potential tool for measuring the long-term activity of the HPA axis (Heimbürge et al. 2019). Hair cortisol is not affected by circadian variations or factors that induce short-term stress in animals such as close handling or transport (Meyer and Novak 2012; Comin et al. 2013). Thus, the measurement of cortisol levels in hair seems to be a promising non-invasive technique for assessing chronic stress in animals and humans as it gives a wide retrospective view over a period of several months (Van Uum et al. 2008; Comin et al. 2011; Heimbürge et al. 2019; Vesel et al. 2020).

Hair growth cycle encloses three phases: anagen (active growth), catagen (transitional stage) and telogen (resting phase) (Barroso et al. 2011).

The mechanisms of cortisol incorporation into the hair shaft are still poorly understood (Meyer and Novak 2012). In the forensic field, it is known that the major incorporation mechanism of lipophilic drugs into the hair shaft is through the bloodstream (Barroso et al. 2011). Because cortisol is a lipophilic substance, it is thought that the main source of cortisol incorporation is via vascular supply into the keratin matrix of the hair shaft during the anagen phase per passive diffusion (Cook 2012; Meyer and Novak 2012). Additionally, it is possible that cortisol is incorporated into the hair via local glandular secretions (sweat and sebaceous glands) that permeate into the follicle (Cook 2012; Meyer and Novak 2012) (Figure 4).

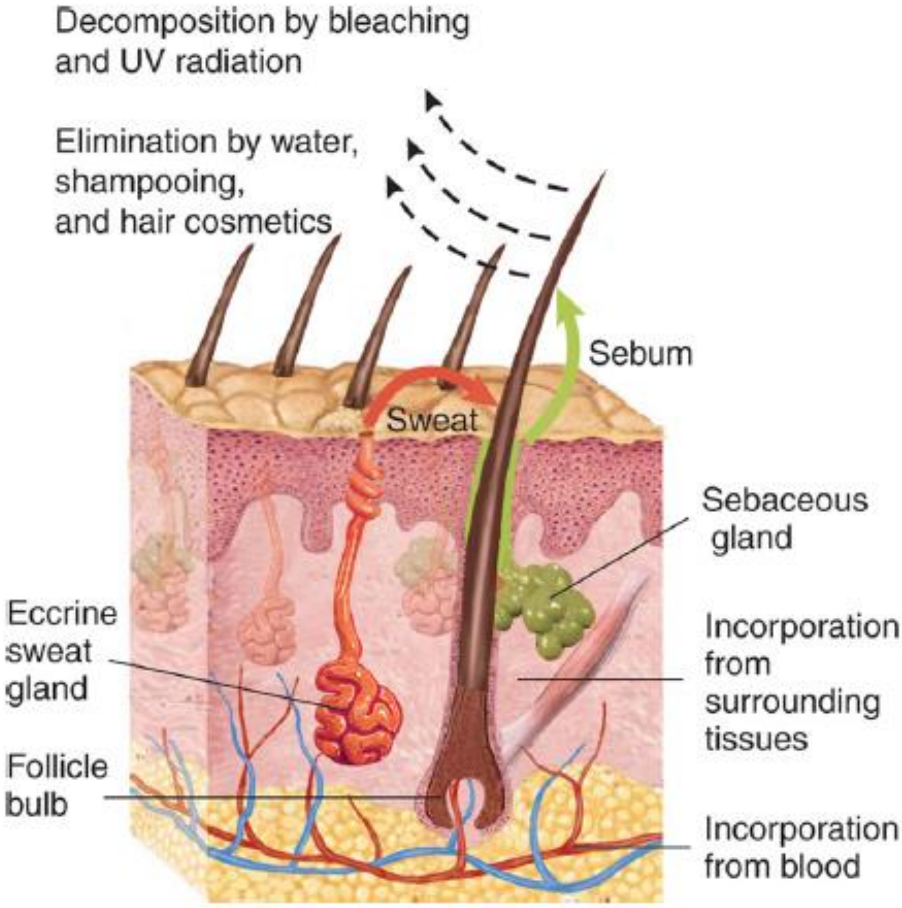


Figure 4 - Mechanisms of incorporation and elimination of lipophilic substances in human hair. Adapted from (Meyer and Novak 2012)

Some studies indicate that the human hair follicle itself might synthetise cortisol (Meyer and Novak 2012). Cirillo and Prime (2011) described an autonomous production of cortisol by keratinocytes. However, most authors believe that hair cortisol levels correlate with systemic cortisol levels.

The measurement of hair cortisol concentrations is emerging as a diagnostic tool for many psychological and physical diseases in human studies.

Studies in humans demonstrate that increased hair cortisol levels are associated with higher incidence of cardiovascular diseases, and also, with poorer recovery and response to treatment (Iob and Steptoe 2019), whereas Bautista et al. (2019) suggest that elevated hair cortisol is a risk factor for hypertension.

Van Uum et al. (2008) found a positive correlation between hair cortisol concentration and severe chronic pain in adults. Moreover, hair cortisol measurement can be used as a diagnostic tool for Cushing's syndrome both in humans and domestic animals (Thomson et al. 2010; Ouschan et al. 2013).

Studies performed in health workers at the onset of the COVID-19 pandemic proved higher levels of hair cortisol after the start of the pandemic when compared to hair corresponding to an earlier period (Ibar et al. 2021; Marcil et al. 2022 Dec; Rajcani et al. 2021). Hair cortisol may be used as an important non-invasive biomarker to assess and control burnout in healthcare professionals.

A meta-analysis by Stalder et al. (2017) recognized several covariates of human hair cortisol concentrations such as age, sex and hair washing frequency that should be considered for future investigations. However, those covariates had a small effect size and under most situations, their influences were considered virtually negligible.

In the veterinary medicine field, research with hair cortisol measurement to assess welfare conditions, chronic stress and some diseases with economic relevance in dairy cows are becoming a matter of interest in the academic community.

A study carried out by Comin et al. (2013) has demonstrated a significant positive correlation between hair cortisol and dairy cows suffering from a disease (e.g., subclinical and clinical mastitis, metritis, laminitis) or physiologically compromised (cows that calved one month before hair sampling, initiated a new lactation and were subjected to changes in the group and diet) when compared with clinically normal lactating cows.

Burnett et al. (2015) concluded that hair cortisol concentrations were positively associated with clinical diseases in dairy cows, however this method might not be useful to differentiate situations of minor stress, such as the development of a subclinical endometritis. The authors found that when comparing pregnant and non-pregnant dairy cows at 100 DIM (days in milk), animals with a positive diagnosis had significantly lower hair cortisol levels at 42 and 84 DIM when compared with cows that were not pregnant.

In contrast, a study performed by Fischer-Tenhagen et al. (2018) found no difference in hair cortisol concentrations between dairy cows affected with lameness and non-lame animals, suggesting that hair cortisol is not a good biomarker for stress caused by chronic lameness in dairy cows. Burnett et al. (2015) did not establish a relation between hair cortisol in dairy cows and important blood biomarkers associated with metabolic status and acute inflammation (e.g., β-hydroxybutyrate, haptoglobin and ceruloplasmin).

Not only stress-related disorders influence hair cortisol concentrations. Hair-specific features and external causes may affect hair cortisol levels and interfere with the interpretation of results (Burnett et al. 2014; Salaberger et al. 2016; Otten et al. 2021). Burnett et al. (2014) described a variation in cortisol concentration of different hair colours. White hair had approximately double the cortisol concentration than black hair in dairy cows. Mechanisms related with melanocyte development or differentiation could be implicated in these findings.

External sources, such as corticosteroid medication, extensive hair brushing (Salaberger et al. 2016) and washing or chemically dyeing the hair can influence hair cortisol concentrations (Cook 2012). Otten et al. (2021) demonstrated that artificial light (both visible and UV) can degrade hair cortisol from pigs and cattle in an *in vitro* experiment. There are also differences in the sampled area of the body. In dairy cows, hair from the tail switch had greater concentrations of cortisol when compared to hair sampled from the shoulder, but there were no statistically significant differences between the tail switch and the hip or top line (Burnett et al. 2014). A study by Moya et al. (2013) reported similar results in beef cattle, as the concentrations of cortisol from the tail were higher compared with the head and shoulder.

The tail switch has a growth rate of 0.51 $\pm$ 0.05 mm/day (Burnett et al. 2014). The growth rate of the tail switch hair is over 10 times faster when compared with the hair growth rate from the hips and shoulder (Burnett et al. 2014). It is possible that hair from the tail switch needs more blood supply to grow, hence is more exposed to systemic cortisol that can be incorporated into the hair shaft (Vesel et al. 2020). Moya et al. (2013) found a significant positive association between hair cortisol concentrations assessed from the tail switch and the hip region of Angus cross bulls, and its level in saliva samples. Because of this, the tail switch seems to be the preferred sampling region to access the HPA axis activity.

Studies demonstrate a parity effect on hair cortisol concentrations, where multiparous cows have greater levels of hair cortisol when compared to primiparous cows (Burnett et al. 2014; Burnett et al. 2015; Endo et al. 2017).

Milk production during the first 30 DIM tended to have a negative relation with hair cortisol levels, where cows with greater milk yield had lower levels of cortisol in the hair (Burnett et al. 2015).

Braun et al. (2017) found significant differences in hair cortisol throughout pregnancy. Hair cortisol concentrations reached a maximum concentration in the month of parturition and decreased significantly in the following month.

Also, when comparing multiparous dairy cows at calving, higher hair cortisol levels were detected in animals with BCS (body condition score) equal or superior to 2.75 when compared to animals with BCS lower than 2.75, in a 1 to 5 scale (Burnett et al. 2015).

Higher concentrations of cortisol were found in the hair of Holstein-Friesian heifers compared to crossbreed F1 heifers with Swedish Red and Montbéliarde breeds (Peric et al. 2013), meaning that the breed could have an impact in hair cortisol levels. Conversely, Endo et al. (2017) found no statistically significant differences in hair cortisol concentrations between Brown Swiss crossbred and Holstein cows.

The processing method affects the amount of cortisol extracted from the sampled hair. Samples processed with a laboratory ball mill had significantly greater cortisol concentrations than samples minced with surgical scissors (Burnett et al. 2014).

Table 1 summarizes the different factors affecting hair cortisol concentration in cattle.

Table 1. Factors affecting hair cortisol concentrations in cattle.

Factor	Hair cortisol concentrations	References
Parity	Tendency to be higher in multiparous cows compared to primiparous.	Burnett et al. (2014); Burnett et al. (2015); Endo et al. (2017)
Breed	Contraditory results.	Peric et al. (2013); Endo et al. (2017)
Body region	Higher from the tail switch.	Moya et al. (2013); Burnett et al. (2014)
Pregnancy	Higher in the last month of gestation.	Braun et al. (2017)
BCS	Higher in animals with BCS ≥ 2.75 (1 to 5 scale).	Burnett et al. (2015)
Hair colour	Higher levels in white hair when compared with black hair (dairy cows).	Burnett et al. (2014)
Diseases	Contraditory results.	Comin et al. (2013); Burnett et al. (2015); Fischer-Tenhagen et al. (2018)
Processing method	Higher concentrations in samples processed with a ball mill.	Burnett et al. (2014)
External sources	Higher when corticosteroid medication is administered. Higher concentrations with extensive hair brushing. Lower levels when hair is exposed to artificial light (*in vitro*).	Salaberger et al. (2016); Otten et al. (2021)

7. Common/important metabolic diseases in cattle

Over the past decades, the incidence of diseases in dairy cattle has increased and consequently higher culling rates have been reported in European countries and in the US (Ametaj 2017).

Diseases impact the animal's performance which is a major problem in dairy industries as it affects productivity with severe economic consequences (Liang et al. 2017). Furthermore, when a disease impacts a farm, animal welfare and well-being are unequivocally compromised (Ametaj 2017).

There are seven frequent and important diseases of the modern dairy cow: mastitis, hypocalcaemia, lameness, ketosis, metritis, retained placenta, and displaced abomasum (Liang et al. 2017). A brief review of each one of them will be described below.

Mastitis is an inflammatory response to a certain microorganism that invades and multiplies in the mammary gland and induces distinct lesions on the mammary tissue (Bianchi et al. 2019). It is considered to be one of the most important diseases in the dairy animal industry.

Hypocalcaemia occurs due to low levels of calcium in the plasma at the time of parturition or within the first 48 hours after calving. Approximately 50% of multiparous dairy cows suffer from subclinical hypocalcaemia. Milk fever occurs when the animal presents clinical signs such as muscle weakness, mental depression, hypothermia and recumbency. The incidence of milk fever is about 4% to 9% (Constable et al. 2016).

Lameness in dairy cattle is a complex and multifactorial disease. The animal's health, genetics, and its environment are implicated in the progression and severity of the illness (Ametaj 2017). The housing and flooring systems of modern farms have a big impact on the percentage of lameness in dairy cows (Shearer et al. 2017). This disease has a huge influence on the animal's welfare and productivity (Liang et al. 2017).

Ketosis is a disorder in energy metabolism and involves various factors. The negative energy balance results in fat mobilization, hypoglycaemia, ketonemia and ketonuria (Constable et al. 2016). This disease leads to decreased milk production and higher culling in dairy cattle (Liang et al. 2017)

Retained placenta is frequently associated with abortion, dystocia, multiple births (Constable et al. 2016), hypocalcemia, several nutritional factors and certain stressors (Ametaj 2017). A common definition for retained placenta is the presence of foetal membranes 12 hours or more after parturition (Constable et al. 2016). The retention of foetal membranes is considered an important risk factor for the development of metritis; thus, the two diseases have a close relationship (Liang et al. 2017).

Metritis is a multifactorial disease defined as an inflammation of the uterus due to a mixed bacterial invasion. Puerperal metritis usually occurs within the first 10 days after parturition, however it can go up to 21 days after parturition and is clinically characterized by an enlarged uterus with a fetid-red brown watery uterine discharge with or without retention of foetal membranes, systemic signs such as inappetence and fever, and in severe cases anorexia, low milk production, and tachycardia (Sheldon et al. 2006; Constable et al. 2016)

Displaced abomasum occurs when the abomasum is displaced from its original position, in the floor of the abdomen, to the left or right due to accumulation and dilatation with gas. Left-displaced abomasum is the more common type of abomasal displacement, and although several predisposing factors are implied in the disease, it is primarily associated with low feed intake before and after calving (Constable et al. 2016).

Liang et al. (2017) described the incidence of each disease in the US according to parity (Table 2) and presented the total disease costs considering seven different categories: veterinary and treatment cost, milk production decrease, culling cost, discarded milk due to antibiotic use, death loss, cost of extended days open and labour costs.

Table 2. Incidence of postpartum diseases in dairy cows according to parity (adapted from Liang et al. 2017).

	Incidence (%) per parity		
Disease	**First**	**Second**	**Third+**
Mastitis	12.14	20.39	20.39
Milk fever	NA*	5.20	5.20
Lameness	33.20	30.90	30.90
Ketosis	12.30	12.60	12.60
Retained placenta	7.20	12.20	12.20
Metritis	13.90	4.40	4.40
Left-displaced abomasum	2.20	2.90	2.90

*Not applicable

According to Liang et al. (2017), left displaced abomasum was the most expensive disease having the higher veterinary fee, treatment cost and labour costs. The increased cost in veterinary treatment is explained by the necessity of surgery. The total cost for LDA was 432.48 $\pm$ 101.94 dollars for primiparous cows and 639.51 $\pm$ 114.10 dollars for multiparous cows. In comparison, ketosis was the least expensive disease for the farm having an average cost of 77.00 $\pm$ 24.00 dollars for primiparous cows and 180.91 $\pm$ 63.74 dollars for multiparous cows.

According to this study, the treatment for multiparous cows is pricier than for primiparous cows in every disease (except for hypocalcaemia). This is due to higher average daily milk yield (and consequently higher discarded milk), elevated body weight and more extended days open in multiparous cows (Liang et al. 2017).

Preventing diseases in dairy cows instead of treating sick individuals is becoming a more present reality in most dairy farms. Treating a disease has an enormous economic impact for the farm and, in addition, compromises animal welfare. It is very important for the farmer to recognize the real financial impact of having a disease on farm and have a more preventative approach. Creating awareness for the real costs of having a diseased animal in the farm and recognizing the financial impact is crucial as it will help farmers to create a more profitable and sustainable business, promoting animal welfare at the same time.

Chronic stress supresses the immune system in humans and animals, thus the susceptibility to infectious diseases is increased. Due to this, hair cortisol is becoming a tool of interest to assess the HPA axis activity and therefore chronic stress in animals. As reviewed above, several studies have shown an association between elevated hair cortisol levels and the incidence of diseases in dairy cows.

Hair cortisol is a new and non-invasive tool that could potentially help predict metabolic and infectious diseases in dairy cattle before any signs of clinical disease, by assessing chronic stress.

III. Effect of chronic stress on the incidence of metabolic and infectious diseases in dairy cattle

1. Objectives

The primary aim of this study was to determine if hair cortisol sampled from cows during the dry period could be a predictor of metabolic and infectious diseases occurring in the 30 days post-parturition period.

Additionally, secondary aims were to investigate the relation between hair cortisol in cows and morbidity in their calves during the first month of life; the relation between hair cortisol concentration and the flight distance and also, if BCS, parity and milk yield in the previous lactation affected hair cortisol concentrations and/or had an impact in the development of an infectious or metabolic disease in dairy cattle.

2. Materials and Methods

2.1. Field experiment

The study was conducted between December 2021 and February 2022 on five different farms in the Lisbon and Tagus valley region. Farms were selected based on availability and reliability of health records.

Hair samples were collected once from the tail switch of 116 dairy multiparous cows in the dry cow pen. All the cows that in the farm records had an expected calving date within 21 days of the first visit to each farm were included in the list of animals to sample. Cows that were visibly lame at the time of the experiment were excluded from the study.

Approximately three centimetres of hair was cut with a scissors from the tip of the tail switch, as close to the skin as possible and stored in a plastic tube marked with the respective cow number (Figure 5). Simultaneously the BCS of each animal was registered on a five-point scale with 0.25 point increments using the Penn-State assessment method (PennState Extension 2016). On the same day, the hair samples were frozen at -18ºC and kept in the dark until further processing. A total of 116 hair samples were obtained.

Figure 5 - Hair sample from the tail switch (original image).

The flight distance of every animal in the study was measured according to Uetake et al. (2002). The flight distance or flight zone can be defined as the distance between an individual and an animal before the animal starts to move away (Grandin and Deesing 2014).

A single measurement was made on the same day as the hair sample collection. All animals from the five different farms were housed in a straw yard and could move freely (Figure 6). The experimenter was 1.56 meters tall, was wearing a yellow shirt during the measurements and approached the animal's flank (30º from the longer axis of the cow) at an approximate speed of 2 steps/s with her hands behind her back. The experimenter was not familiar to the cows. The animals were stationary and perceiving her from a distance of three to four meters before she started to walk towards them. The experimenter stopped walking when the cows started to move away from her. An assistant, watching from a distance, stood at the point where the animal started to move away, and the distance was measured using a laser measurer.

Other data such as number of lactations and previous production at 305 days of lactation was also collected. Animals that were visibly lame or sick were immediately excluded from the study.

Figure 6 - Straw yard housing and dry cows from one of the farms included in the study (original image).

On the second visit to the farms, the experimenter collected data about the occurrence of infectious and/or metabolic diseases of the dairy cows included in the study during the first 30 days of lactation. Information about their calves' health problems such as diarrhoea and respiratory diseases in the first month of life were also registered.

Nineteen diseased cows and eighteen controls were selected to be part of the study (for hair analysis), as the ELISA kit used in the analysis only allowed that number of animals to be tested. Diagnosis of disease was confirmed by the assisting farm veterinarian or a trained farm employee and registered in the farm records. The selection of the diseased cows was made based on reliability of data and if the animal met our criteria for a diseased animal.

From the recorded data, three types of metabolic disorders and three types of infectious diseases were identified and defined as follows:

1) Metritis was defined as an abnormal fetid discharge with systemic clinical signs such as inappetence and/or fever in the first 10 days post-partum;
2) Mastitis was defined as a clinical inflammation of the mammary gland;
3) Retained placenta was characterized as retention of foetal membranes for more than 12 hours;
4) Displaced abomasum was diagnosed by a veterinarian or farm employee by auscultation and clinical signs and confirmed through surgery;
5) Ketosis was diagnosed in animals that had more than 1.4 mmol/L of β-hydroxybutyrate in the blood;
6) Hypocalcaemia was defined as a decrease in the blood calcium below normal associated with clinical signs.

Animals that were classified as having metritis in the farm records but did not receive any antibiotic and/or anti-inflammatory treatment and had more than 10 days post-partum at the time of the diagnosis were excluded from the study since they did not meet the criteria of a sick animal nor a healthy one. Animals that died of unknown causes or that were culled before the first 30 days after parturition were also eliminated from the analysis.

Following this selection, diseased animals were paired with healthy cows (controls), based on farm, lactation, 305-day milk production in the previous lactation and body condition score within a range of 0.5 difference. A schematic of the study design can be found in figure 7.

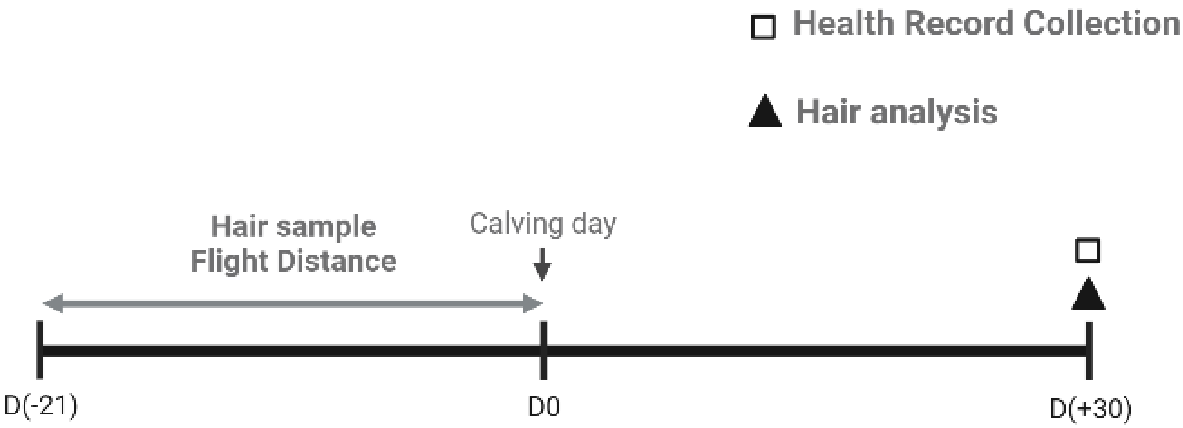


Figure 7 - Schematic of the experimental and sampling design of the study. Hair samples were collected once from each dry cow that had an expected calving date in the 21 days following the visit and the flight distance was measured on the same day. The health records were collected 30 days after calving and hair samples were subsequently analyzed (created in BioRender®).

2.2. Measurement of cortisol concentration

The hair samples were taken out the freezer (temperature of -18ºC) on the day before the analysis and kept at room temperature for about 12 hours.

Subsequently, hair samples were washed three times with 5 mL of isopropanol and left to dry in a petri dish protected from light for the next 24 hours.

The hair was minced with a surgical scissors to a size of approximately 2 mm (Figure 8) and about 50 mg of hair mass was weighted out and transferred to a test-tube.

Figure 8 - Hair samples minced with a surgical scissors (original image).

Cortisol was extracted by adding 1.5 mL of methanol to the test-tubes and shaking in a bain-marie at a temperature of 50ºC for exactly 16 hours.

About 0.75 mL of methanol was evaporated to dryness with nitrogen and each sample was reconstituted with 0.25 mL of PBS H8. Quantification of cortisol in the hair was determined using a salivary cortisol ELISA kit (DRG Instruments GmbH, Marburg, Germany). The intra-assay variation was analysed, and the coefficient of variance (CV) was 4.55%.

2.3. Statistical analysis

The collected data was organized and recorded in Microsoft® Excel® version 2209. Descriptive statistics and statistical analysis were performed using the software program IBM® SPSS® Statistics for Windows version 28.0.1.0.

A logistic regression was used to analyse the impact of variables "hair cortisol concentration", "farm", "number of lactations", "body condition score", "production at 305 days" with developing a disease as the dependent variable. Three more logistic regressions were used with the same predictor variables to study their impact on the development of an infectious disease, the development of a metabolic disease and the development of both infectious and metabolic diseases in the dairy cows.

A multiple linear regression was used to test if "disease in dairy cow", "farm", "number of lactations", "BCS", "flight distance", "production at 305 days", and "disease in the calf" significantly predicted hair cortisol concentrations (dependent variable).

The backward elimination method for variable selection was used in every regression (logistic and multiple linear). It consisted of removing insignificant predictors from the model one by one until all variables remaining in the model had a p-value of less or equal to 0.157 (Heinze and Dunkler 2017).

P-values were considered significant when $p<0.05$. Statistical tendencies to differences were accepted if p-values ranged from 0.05 to 0.10.

3. Results

In the period between December 2021 and February 2022, a total of 37 multiparous cows from five different farms in the Lisbon and Tagus valley area were selected to be part of the study. Nineteen diseased cows were paired with eighteen healthy cows (controls). Table 3 illustrates the number and percentages of animals selected from each farm.

Table 3. Number of animals selected from each farm.

Farm	Healthy (control)	Diseased	N	%N
Farm A	8	9	17	45.9
Farm B	2	2	4	10.8
Farm C	2	2	4	10.8
Farm D	3	3	6	16.2
Farm E	3	3	6	16.2
Total	18	19	37	100.0

The mean, standard deviation, and minimum and maximum values of parity, flight distance and body condition score from all animals included in the study are visible in Table 4.

Table 4. Descriptive statistics of BCS, production at 305 days, flight distance and number of lactations of 37 dairy cows included in the study.

	Body Condition Score	Production at 305 days (Kg)	Flight Distance (meters)	Number of lactations
Mean	3.50	12155	1.596	2.84
SD	0.34	2015	0.799	1.72
Minimum	3.00	8157	0.331	1.00
Maximum	4.50	17850	3.458	8.00

Body condition score mean of animals with a metabolic disease was 3.45 ± 0.45 points. Animals with an infectious disease had a BCS mean of 3.55 ± 0.40 points, whereas animals with both a metabolic and infectious disease scored 3.38 ± 0.32 points. Healthy animals had a BCS mean of 3.51 ± 0.29 points (Table 5).

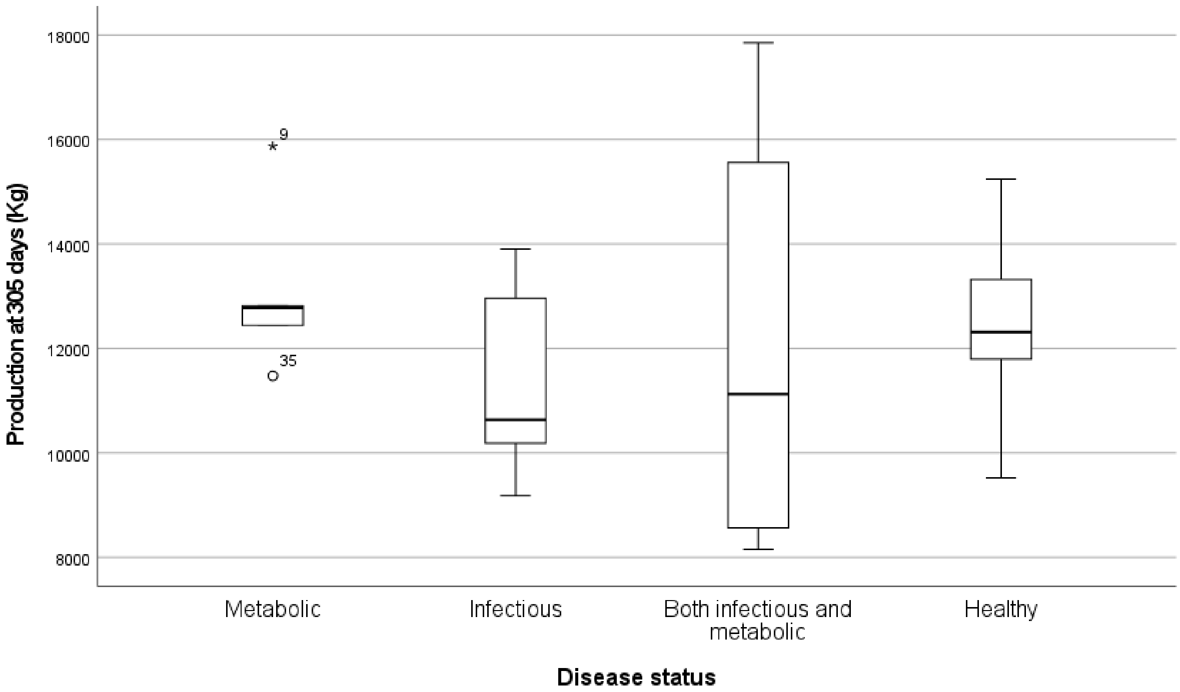
Table 5. Body condition score mean in animals with metabolic disease (n=5), infectious disease (n=10), both infectious and metabolic disease (n= 4) and healthy controls (n=18).

	Body Condition Score		
Disease status	Mean	N	Std. Deviation
Metabolic	3.45	5	0.45
Infectious	3.55	10	0.40
Both infectious and metabolic	3.38	4	0.32
Healthy	3.51	18	0.29

The mean production at 305 days in the previous lactation was 12155 ± 2015 kg.

Animals with a metabolic disease had an average production at 305 days in the previous lactation of 13079 ± 1654 kg, cows with an infectious disease had a mean production of 11177 ± 1611 kg and animals with both metabolic and infectious diseases had a mean production of 12065 ± 4463 kg. Healthy controls had an average milk yield at 305 days of 12461 ± 1465 kg (Graph 1).

Graph 1. Boxplot representation of the distribution of 305-day milk production (kg) in the previous lactation in animals with a metabolic, infectious or both diseases and healthy controls in the post-partum period. The median is indicated by the line across each box. The central box represents the interquartile range from the first to the third quartile.



The flight distance mean discriminated for animals with diseases and healthy controls is illustrated in Table 6. The average number of lactations of the dairy cows in the study with a metabolic, infectious or both diseases and healthy controls is depicted in Table 7.

Table 6. Flight distance mean according to disease status.

	Flight Distance (meters)		
Disease status	Mean	N	Std. Deviation
Metabolic	1.783	5	0.459
Infectious	1.282	10	0.667
Both infectious and metabolic	1.300	4	0.588
Healthy	1.785	18	0.937

Table 7. Number of lactations mean according to disease status

	Number of lactations		
Disease status	Mean	N	Std. Deviation
Metabolic	3.20	5	2.78
Infectious	2.70	10	1.82
Both infectious and metabolic	3.75	4	1.71
Healthy	2.61	18	1.38

The mean hair cortisol concentration in this study was 48.137 ± 42.777 pg/mg for all cows (n=37). The minimum value for hair cortisol concentration was 6.250 pg/mg and the maximum value was 160.550 pg/mg. The mean hair cortisol concentration for healthy dairy cows was 44.695 ± 35.674 pg/mg, whereas the mean hair cortisol concentration for diseased cows was 51.397 ± 49.344 pg/mg (Table 8).

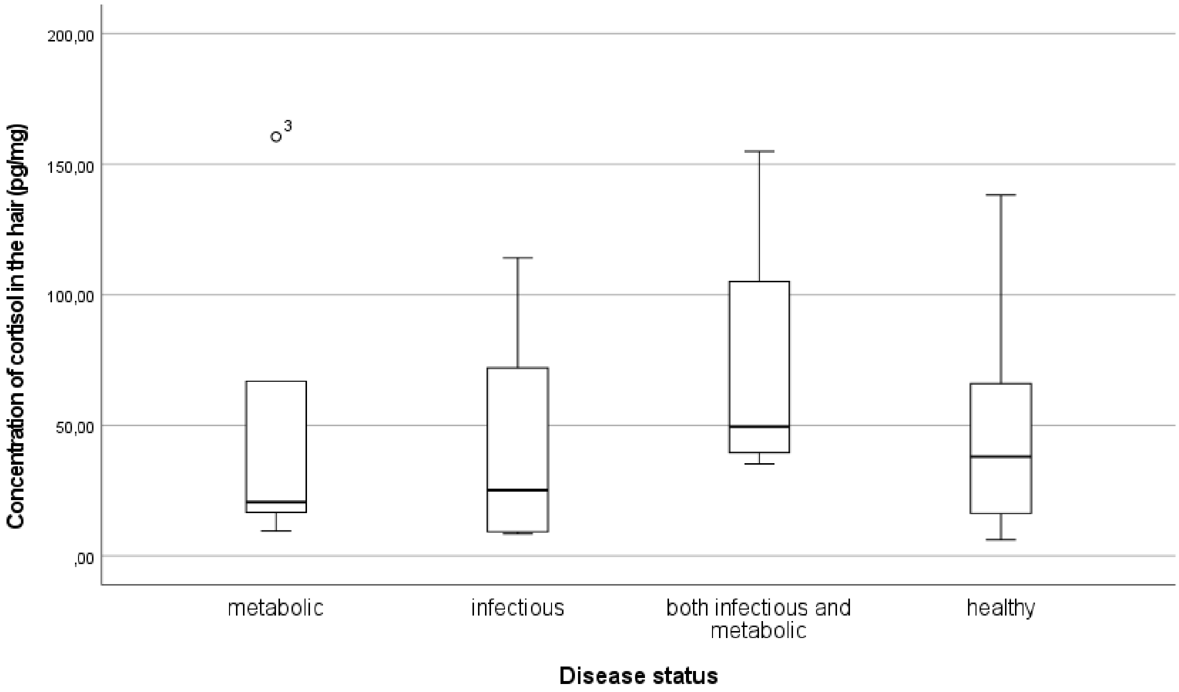
Table 8. Hair cortisol concentrations (pg/mg) in healthy (n=18) and diseased (n=19) dairy cows.

Hair Cortisol (pg/mg)	Mean	N	SD	Min	Max
Healthy	44.695	18	35.674	6.250	138.180
Diseased	51.397	19	49.344	8.450	160.550
Total	48.137	37	42.777	6.250	160.550

Within the nineteen diseased animals that were part of the experiment, 13.5% were diagnosed with at least one metabolic disease, 27% were diagnosed with at least one infectious disease, 10.8% were diagnosed with both an infectious and metabolic disease. The mean hair cortisol concentration for dairy cows diagnosed with a metabolic disease was 54.908 ± 63.212 pg/mg. The mean hair cortisol concentration for cows with an infectious disease was 41.274 ± 41.762 pg/mg. Animals that were diagnosed with both an infectious and metabolic disease had a hair cortisol concentration of 72.315 ± 55.707 pg/mg.

Graph 2 demonstrates a boxplot with the cortisol concentration in the hair (pg/mg) and the different categories (Metabolic disease/ Infectious Disease/ Both infectious and metabolic disease/ Healthy).

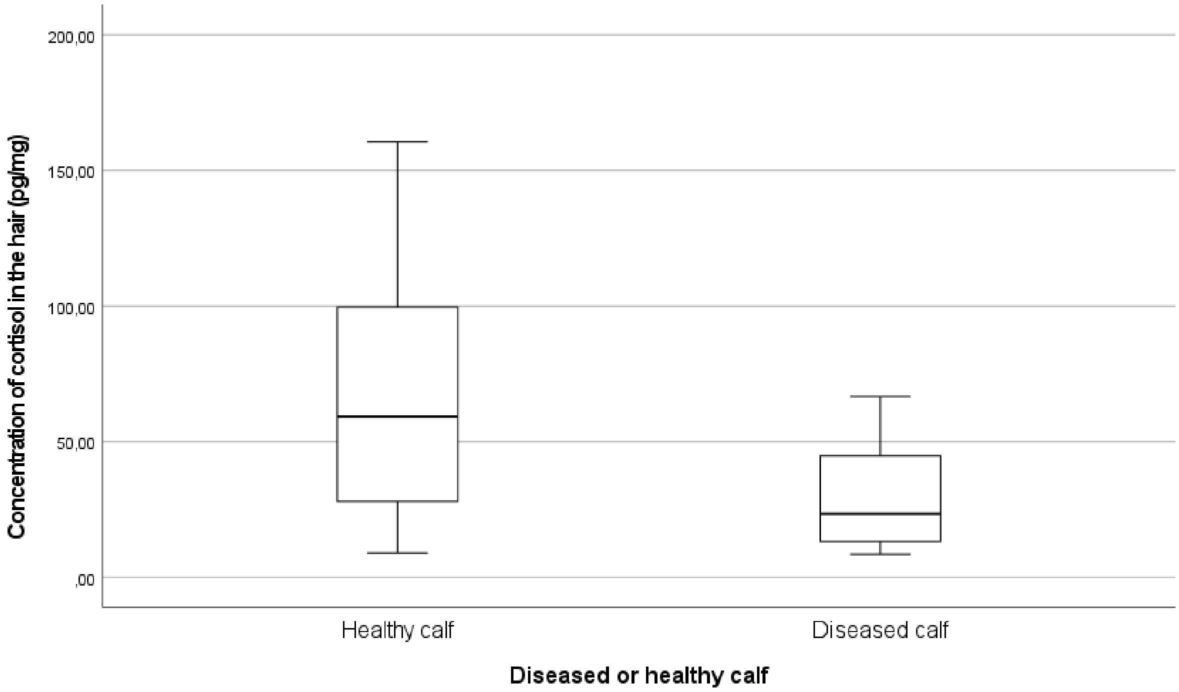
Graph 2. Hair cortisol concentrations (pg/mg) in cows with metabolic disease (n=5), infectious disease (n=10), both infectious and metabolic disease (n=4) and healthy controls (n=18). The median is indicated by the line across each box. The central box represents the interquartile range from the first to third quartile.



Within the 37 calves that were born only 28 were eligible to enter the study. Eight males and one stillborn, were eliminated because they did not meet the criteria of "staying in the farm during the first month of life". Within the 28 calves, 20 stayed healthy (71.4%) and 8 were diagnosed with a disease (28.6%) throughout the first 30 days of life. The calves' diseases were recorded by a veterinarian or a farm employee, seven calves were diagnosed with diarrhoea, and one calf was diagnosed with respiratory disease.

Graph 3 demonstrates a boxplot with the cortisol concentration in the hair (pg/mg) of dams and disease in their respective offspring.

Graph 3. Boxplot representation of hair cortisol concentrations (pg/mg) in the dams and disease (n=8) or healthy (n=20) calves. The central box represents the interquartile range from the first to third quartile.



A logistic regression was used to assess the impact of “hair cortisol concentration”, “farm”, “number of lactations”, “BCS”, “production at 305 days” on developing a disease. The backward elimination method was used, and no variable was significant.

A second logistic regression was built to evaluate the impact of “hair cortisol concentration”, “farm”, “number of lactations”, “BCS”, “production at 305 days” on developing a metabolic disease. The backward elimination method was used, and no variable was significant.

A third logistic regression was built to estimate the impact of “hair cortisol concentration”, “farm”, “number of lactations”, “body condition score”, “production at 305 days” on the development of both infectious and metabolic disease. The backward elimination method was used, and no variable was significant.

A fourth logistic regression was used to evaluate the impact of “hair cortisol concentration”, “farm”, “number of lactations”, “body condition score”, “production at 305 days” on developing an infectious disease. The backward elimination method was used and only one variable stayed in the model with a p-value of 0.097. Table 9 shows the output of the logistic regression model.

Table 9. Logistic regression with dependent variable "infectious disease" and predictor variable as production at 305 days.

	B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B) Lower	95% C.I.for EXP(B) Upper
Production at 305 days	-0.000	0.000	2.757	1	0.097	1.000	0.999	1.000
Constant	3.512	2.412	2.121	1	0.145	33.527		

A multiple linear regression was used to test if "disease in dairy cow", "farm", "number of lactations", "BCS", "flight distance", "production at 305 days", and "disease in the calf" significantly predicted hair cortisol concentrations. The backward elimination method was used and the variable "Disease in the calf" was the only predictor that stayed in the model with a p-value of 0.039. Table 10 shows the output of the linear regression model.

Table 10. Linear regression model with independent variable disease or healthy calf and dependent variable "Concentration of cortisol in the hair".

Model		Unstandardized Coefficients B	Unstandardized Coefficients Std. Error	Standardized Coefficients Beta	t	Sig.	95% Confidence Interval for B Lower Bound	95% Confidence Interval for B Upper Bound
1	(Constant)	67.813	9.382		7.228	<0.001	48.529	87.098
	Disease or healthy calf	-38.061	17.552	-0.391	-2.168	0.039	-74.139	-1.983

a. Dependent Variable: Concentration of cortisol in the hair (pg/mg)

4. Discussion

4.1. Hair cortisol concentration and diseases

In this study, cortisol concentration in the hair of dairy cows was not a predictor for the occurrence of a metabolic or infectious disease during the first month post-partum.

The mean hair cortisol concentration in this study was 48.137 ± 42.777 pg/mg, a result that is not within the range reported for lactating dairy cows by other similar studies (Burnett et al. 2014; Fischer-Tenhagen et al. 2018). There is a high variability of results in different studies that may be related to the method used for measuring the cortisol concentration. In this study the intraassay variability was 4.55%, which was higher compared with 3.51% in the study of Burnett et al. (2015) and lower compared with 5.45% in the study of Comin et al. (2013).

It seems to be difficult to define a baseline hair cortisol concentration for a non-stressed dairy cow. Braun et al. (2017) found an increase in levels of hair cortisol during pregnancy, reaching maximum concentrations in the month of calving. Because hair samples in our study were collected during the dry period, a physiological increase in cortisol associated with pregnancy status would be expected. We cannot sustain the idea that dairy cows in our study with higher levels of hair cortisol were chronically stressed or were within the normal physiologic range, since it is a comparative study between only 37 animals (small sample size) and to this date there is no cortisol threshold indicative of chronic stress in dairy cows' hair.

Furthermore, hair samples were taken between December 2021 and February 2022, corresponding to a retrospective interval of two months before (the winter period), so we can affirm that heat was not a present stressor for the cows in this study.

There are studies in dairy cows that prove a direct relationship between diseased animals and increased hair cortisol concentrations (Comin et al. 2013; Burnett et al. 2015). However, a study by Fischer-Tenhagen et al. (2018) found no significant differences between hair cortisol levels of lame and non-lame dairy cows.

We could not find any association between hair cortisol levels and the occurrence of metabolic or infectious disease. The fact that our study was performed in five different farms and with a sample of only 37 animals is a limitation. Moreover, not every disease was diagnosed by a veterinarian, with a trained farm employee being responsible for the diagnosis and reporting of some diseases, which can bias our data.

4.2. Body condition score and diseases

Body condition score (BCS) in the dry period did not have an impact in the cows' postpartum health. Cows with an excessive BCS in the dry period are at increased risk for left-side displacement of the abomasum (Constable et al. 2016). However, in our data, BCS values did not diverge much.

In our study, the mean BCS was 3.50 ± 0.34, the minimum value for BCS was 3.00 and there was only one animal with a BCS of 4.50, which means that our study population was within the desired values of BCS for a dairy cow in the dry period (Garnsworthy and Wiseman 2006). Therefore, it was expected that BCS would not impact greatly the cows' postpartum health.

4.3. Body condition score and hair cortisol concentrations

Body condition score was also not associated with hair cortisol levels in this study. These results disagree with Burnett et al. (2015), where they found a significant effect of body condition score on hair cortisol concentrations in multiparous cows at 30 DIM. Burnett et al. (2015) found that animals with a BCS > 2.75 had higher cortisol levels than animals with BCS of ≤ 2.75.

In our study, none of the animals had a BCS of less or equal to 2.75 points. Additionally, as mentioned previously, our study population was within the desired values of BCS for a cow in the dry period. Due to this, it was expected for BCS not to influence hair cortisol concentrations.

4.4. Previous milk yield and diseases

The 305-day milk yield in the previous lactation was obtained for each dry cow in our study. The logistic regression model showed that there is a tendency (p=0.097) for cows with lower milk production in the previous lactation to develop an infectious disease during the first month after parturition. This could be explained by a possible subclinical mastitis in the previous lactation that decreased the 305-d yield (Gonçalves et al. 2018) and could possibly have an impact in the development of a clinical mastitis in the current lactation.

Conversely, Gröhn et al. (1995) and Fleischer et al. (2001) found an increase in clinical mastitis in animals with a higher milk yield in the previous lactation, explained by genetic factors associated with the animal and higher incidence of teat lesions, predisposing for mastitis in the following lactation. Dohoo and Martin (1984) explained that cows with a subclinical mastitis that flared up to a clinical mastitis are more likely to get treatment and consequently eliminate the subclinical condition and increase the milk production.

4.5. Flight distance and hair cortisol concentrations

The flight distance measurement is a recognized method for the evaluation of an animal’s reaction to humans (Waiblinger et al. 2003). In our study, we failed to prove an association between the flight distance and hair cortisol levels. While it is true that intense fear can cause chronic stress and consequently have an impact in the flight distance, the flight distance can also be influenced by many different factors difficult to control such as lameness, past experiences of the animal, genetics (Grandin and Deesing 2014), the observer to be known/unknown to the animal, etc.

In the methodology used, we tentatively addressed these different factors by excluding all animals that were apparently sick or visibly lame. The same experimenter measured the flight distance of every animal and was unfamiliar to all animals in the study.

All animals could move freely in the straw yard. Dairy cows perceive wavelengths of light up to a maximum of approximately 620 nm (Philips and Lomas 2001). For this reason, in the methodology, a yellow shirt was used since it has a wavelength of 550-590 nm (Choudhury 2014).

The fact that the flight distance was measured only once decreases invariably the sensitivity of the results, however, it was clear for the experimenters that the time spent in the dry pen was an agitating factor for the animals and therefore could have an impact on the flight distance measures taken a second time. For this reason, the authors decided to only measure the flight distance once for each cow.

4.6. Number of lactations and hair cortisol concentrations

The number of lactations did not significantly affect hair cortisol levels in the animals from our study. Burnett et al. (2014) found a tendency for multiparous cows to have higher hair cortisol levels than primiparous cows.

In our study, the variable “parity” was controlled as a possible confounder, and only multiparous cows were selected for hair analysis.

4.7. Hair cortisol concentrations and diseases in the offspring

In human studies, there is growing empirical evidence that intrauterine environment affects fetal development, possibly having long-term effects in the offspring development.

During pregnancy, placenta and fetal membranes produce a large amount of peripheral CHR. Placental CHR is stimulated by glucocorticoids in a positive feedback loop resulting in a gradually increase in CRH during pregnancy (Zijlmans et al. 2015; Chavatte-Palmer and Tarrade 2016). As a consequence, maternal cortisol increases two to four times throughout a normal gestation in women (Davis and Sandman 2010).

It is well known that glucocorticoids, including cortisol, are indispensable for the normal development and maturation of fetal organs before birth. In many animal species, a rise in cortisol levels during late pregnancy parallels with increased maturity of fetal organs. Glucocorticoids are responsible for fetal lung development and promote the maturation of other organs such as the thymus, gastrointestinal tract, liver, and kidney (Murphy et al. 2006).

However, according to a review by Zijlmans et al. (2015), a prolonged exposure to elevated maternal cortisol concentrations in pregnant woman can have harmful and long-term effects on fetal growth and organ development.

A few studies have demonstrated that maternal stress attributable to extreme stressors (e.g., environmental disasters) is related to worse performance on cognition, behaviour, motor development and health outcomes in children. Milder forms of stress experienced by pregnant women were also associated to altered infant outcomes (Zijlmans et al. 2015).

Several studies have shown that the human fetus is more susceptible to maternal cortisol concentrations in certain gestational periods. Class et al. (2011) found that the risk for adverse birth outcomes is higher in pregnant women during gestational months five and six.

In our study, hair cortisol concentrations in dairy cows affected significantly (p=0.039) the incidence of disease in their offspring. We found that higher cortisol concentrations in the hair of the dam were associated with healthier calves.

A study by Davis and Sandman (2010) observed that higher concentrations of salivary cortisol in early gestation of women was associated with slower rate of development and lower scores on mental development of the offspring over the first year, whereas higher levels of maternal salivary cortisol in late gestation were associated with better child outcomes.

Maternal cortisol can cross the placenta of humans and ruminants (Dixon et al. 1970; Gitau et al. 200). In early gestation, to protect the fetus from physiologic increasing levels of maternal cortisol, the enzyme 11β-HSD2 is secreted by the placenta, converting cortisol into its inactive form, cortisone (Murphy et al. 2006; Davis and Sandman 2010). Excessive levels of maternal cortisol during early gestation will result in an overexposure to cortisol that the placental enzyme is unable to oxidize, leading to deleterious effects for the fetus (Davis and Sandman 2010). Conversely, with the progression of the gestation towards parturition, exposure to high levels of cortisol is necessary and beneficial for maturation of fetal organs (Murphy et al. 2006).

In our study, higher levels of hair cortisol in the mothers were associated with calves without disease (diarrhea and respiratory disease). To this date, it was not possible to find a baseline for hair cortisol levels that predicts stress in dairy cows. Hence, elevated levels of hair cortisol from the animals in our study could be associated with the pregnancy status and not with chronic stress conditions/factors, even though all animals sampled being within 21 days of the expected calving date.

Hair samples were taken during the last month of the dry period, which gives a retrospective view of circulating cortisol concentrations in the last pregnancy trimester (7th and 8th months). In this phase, as previously explained, cortisol is essential for lung maturation and the development of the fetus' vital organs. It is possible that elevated levels of cortisol in dairy cows in the last trimester of gestation were beneficial for the development and maturation of the fetus' vital organs and therefore associated with healthier calves.

Even though the study of Davis and Sandman (2010) established a relation between higher levels of maternal salivary cortisol in late gestation and better child outcomes, we should acknowledge that there are vast and notorious differences in the reproductive system and physiology of women compared to dairy cows, thus the ability to generalize from human models/research to dairy cows is limited. Furthermore, there are significant differences in the type of prenatal stress experienced with different species that should also be taken into account.

Considering that this study was conducted in five distinct farms, the calves' husbandry and environment was inevitably different. The interpretation of these results should be cautious since many factors can affect disease in a new-born calf such as quantity and quality of colostrum, composition of the diet fed to calves, ventilation and density, vaccination status, and others (Roy 1980).

5. Conclusions

We could not find any association between hair cortisol concentrations in dairy cows in the dry period and the development of a metabolic and/or infectious disease in the first 30 days in milk.

In this study, a tendency ($p = 0.097$) was demonstrated for cows with a lower milk yield in the previous lactation to develop an infectious disease during the first month after parturition. A subclinical mastitis in the previous lactation could influence the lower milk production, predisposing for the development of a clinical mastitis in the following lactation.

Hair cortisol concentrations in dairy cows affected significantly ($p=0.039$) disease prevalence in their offspring. We found that higher cortisol levels in the hair of the dam were associated with healthier calves.

Cortisol naturally increases during the course of a pregnancy. To this date, there is no baseline hair cortisol concentration for a non-stressed dairy cow. Due to this, higher hair cortisol levels from cows in our study could be related to the pregnancy status and not with stressful situations. Higher concentrations of hair cortisol in late gestation are beneficial for the development and maturation of the fetus vital organs, and therefore, higher levels of hair cortisol in the dam could be associated with greater vitality and health in their calves. Nonetheless, it is prudent to acknowledge that other factors contribute for the development of a disease in the calf during the neonatal period.

The number of cows from which hair was collected was too small to establish a reliable correlation between hair cortisol concentration in the dry period and disease in the postpartum period. More research should be done with a larger sample size. In addition, further research is needed to determine the mechanisms of cortisol deposition in the hair shaft and establish the extent of impacts affecting hair cortisol concentrations in dairy cows. A standardized protocol for sampling and hair cortisol concentration measurement and defining a baseline hair cortisol level for chronic stress should be taken in place to minimize the differences in hair cortisol concentrations reported in the literature.

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