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PhD Thesis

Precision livestock farming technologies addressing steroid biomarkers associated with animal welfare in modern livestock productive systems

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Index

List of figures

1. Animal welfare and assessment methods

2. Precision livestock farming (PLF) technologies towards the development of more efficient production systems of healthy livestock

4. Validation of a radioimmunoassay method for cortisol in buffalo milk whey. A preparatory step for future sensor technology

5. Cortisol concentrations in different matrices: predictive potential and relationship with productive level, lactation stage and parity in dairy buffaloes

6. Postnatal and postweaning endocrine setting in dairy calves through hair cortisol, dehydroepiandrosterone and dehydroepiandrosterone sulphate

7. Incorporation of Testicular Ultrasonography and Hair Steroid Concentrations in Bull Breeding Soundness Evaluation

8. Sow's pre- and post-delivery in different confinement systems evaluated by hair hormones concentrations

10. On the use of 3D camera to accurately measure volume and weight of dairy cow feed

11. Use of an automated walk-over-weighing system to monitor and forecast sheep liveweight in grazing sheep.

List of tables

1. Animal welfare and assessment methods

2. Precision livestock farming (PLF) technologies towards the development of more efficient production systems of healthy livestock

5. Cortisol concentrations in different matrices: predictive potential and relationship with productive level, lactation stage and parity in dairy buffaloes

6. Postnatal and postweaning endocrine setting in dairy calves through hair cortisol, dehydroepiandrosterone and dehydroepiandrosterone sulphate

7. Incorporation of Testicular Ultrasonography and Hair Steroid Concentrations in Bull Breeding Soundness Evaluation

8. Sow's pre- and post-delivery in different confinement systems evaluated by hair hormones concentrations

9. Association between a single measurement of progesterone and cortisol blood concentrations at two to one week before parturition, and number of fetuses in the Teramana goat

10. On the use of 3D camera to accurately measure volume and weight of dairy cow feed

12. Use of an automated walk-over-weighing system to monitor and forecast sheep liveweight in grazing sheep.

ABSTRACT

Animal welfare is a debated topic in modern society. Its meaning is a very extensive concept since several definitions have been proposed. A theoretical approach to animal welfare takes into consideration not only the physical and health conditions of animals, but also the psychological well-being and the ability to express species-specific behaviours. Therefore, the welfare status can be assessed and measured through indicators that must consider both the environmental conditions of the farm (management, facilities and climatic parameters) and the adaptive efforts of the animal itself. In recent years, animal care professionals working in managed settings have focused on identifying effective measures for systematically monitoring and assessing welfare. Different indicators can be considered: direct (or animal based), such as behavioural, physiological, pathological and productive parameters, and indirect (or environmental factors), as farming structures and systems, management and human-animal relationships.

Among the animal-based measures, several studies have been carried out on non-invasive physiological biomarkers to gain insight into an animal's physical condition, psychological health and overall welfare status. For this reason, glucocorticoids (especially cortisol), are frequently used to evaluate the physiological response to stress, since they provide information about the activity of the hypothalamic–pituitary–adrenal (HPA) axis. Recent research has incorporated other biomarkers of HPA activity, namely dehydroepiandrosterone (DHEA) and its sulphate ester (DHEA-S). Despite the extensive literature concerning steroid biomarkers, conflicting results have been reported about the HPA axis functioning at different phases of productive career of farm animals, depending on factors such as inflammatory and reproductive status, chronicity of exposure to stressors, and so on.

However, as stated above, a correct evaluation of welfare cannot leave an accurate monitoring of the animal environment out of consideration. In this view, recent studies focused on precision livestock farming (PLF), that can be defined as "*the management of livestock by continuous automated real-time monitoring of production/reproduction, health and welfare of livestock, and environmental impact*". PLF technologies consist of sensors at animal level (accelerometers, Radio-Frequency Identification, (RFID), rumen boluses, temperature and pH sensors, etc.) and in the environment (cameras, temperature loggers, gas sensors, microphones, etc.). The specific applications of these tools can help the decision-making processes by providing early detection of health or welfare problems in individual animals and the

application of targeted corrective practices. Therefore, the potential of PLF technologies to address livestock welfare is promising.

The aim of this thesis was to study cortisol and DHEA(S) as biomarkers of the HPA functioning and provide essential evidence for the practical purpose of targeting PLF technologies towards an animal welfare improvement within the modern livestock productive systems. The integrative approach between PLF and endocrinological measurements has been used in different species and across some sensitive phases of their productive career.

In the ***first experiment***, a radioimmunoassay (RIA) method for cortisol in buffalo milk was validated. Three formulations of milk and three solvents were tested: whey cortisol concentrations showed a significant correlation with whole extracted (methanol) milk and were not affected by fat content variation during the milking session. It was concluded that the RIA suited the cortisol measurement in buffalo milk and the ranges could be employed in the calibration of a biosensing method for non-invasive assessment of cortisol directly integrated in milking parlour systems.

The ***second experiment*** aimed at studying the relationship of cortisol in blood, milk, whey and hair with parity, lactation stage and productive level in dairy buffaloes and to study their predictive potential. Multiparous (n = 30) and primiparous (n = 38) buffaloes were assigned to 4 productive classes and 3 lactation stages and cortisol concentrations were measured using an in-house RIA method. Parity did not show a significant effect on cortisol concentrations of the four media, conversely the stage of lactation largely influenced cortisol concentrations in all matrices. Moreover, hair cortisol concentrations were negatively correlated to mature equivalent milk yield, mature equivalent protein content and mature equivalent corrected milk. Finally, milk had the predictive potential to estimate cortisol levels in other matrices.

Through the ***third experiment*** the hair concentrations of cortisol, DHEA, DHEA-S and their ratios in dairy calves during postnatal and postweaning periods were investigated. Hair sampling was conducted on healthy dairy calves at the ages of 64.8±0.65 days (postnatal) and at 155.3±0.85 (postweaning) days. Hair cortisol concentrations were higher during the postnatal compared to the postweaning period. Similarly, the cortisol:DHEA and cortisol:DHEA-S ratios were higher in the first period, showing a higher animal allostatic load at birth. A reduction of the allostatic load of the calves was demonstrated by the reduction in hair cortisol concentrations at 5 months compared to those measured at 60 days, as well as by the significant reduction in the cortisol:DHEA and cortisol:DHEA-S ratios.

The ***fourth experiment*** investigated testicular ultrasonography and steroid concentrations (cortisol, DHEA-S, cortisol/DHEA-S ratio, testosterone) in hair for their utility in the bull breeding soundness evaluation (BBSE). Beef and dairy bulls underwent routine semen collection twice weekly for 12 weeks. Ultrasonography through a B-mode ultrasound scanner equipped with a linear array probe and hair sampling for steroid RIA were performed at the last semen collection. Semen was analysed immediately after collection and post-thawing. Bulls with homogeneous parenchyma had a higher ($p < 0.05$) percentage of motile sperm post-thawing and hair DHEA-S was positively related to motile sperm, progressively motile sperm and motility yield. It was concluded that the inclusion of testicular ultrasonography and hair DHEA-S in the standard BBSE could provide a more integrated and comprehensive assessment of fertility in bulls.

Cortisol, DHEA and their ratio in hair were also evaluated as biomarkers of allostatic load and resilience in 296 pregnant sows around farrowing in the ***fifth experiment***. Four different models of farrowing crates and four different batches were considered. Each sow was sampled for the first time (ST1) 2.6 ± 1.6 (mean ± SE) days before parturition and for a second time (re-growth hair, ST2) at 88.9 ± 3.3 days after parturition. Thus, the sows were allocated to the February, March, April, May, or June batch. No differences in terms of hair steroids concentration were found between the four models of farrowing crates and between the sampling times ($P > 0.05$). A significant interaction emerged between the batch and the sampling time for all the biomarkers considered. In particular, ST1 showed always higher HC/HDHEA ratios than ST2. Thus, the pregnancy period in collective pens was more challenging than the early postpartum and lactation in individual crates.

The ***sixth experiment*** aimed at assessing the possible association between the maternal concentrations of plasma progesterone (P_4) and cortisol and the number of fetuses in Teramana goats. Twenty- four pregnant does were enrolled in the study. Two to one week before the expected date of parturition, each doe was submitted to blood sampling. Plasma cortisol and P_4 concentrations were determined by RIA. At birth, the number of kids for each doe was recorded and does were retrospectively grouped based on the number of fetuses (single, twin or triplet). Three (13%) does delivered single kids, 16 (69.6%) twins, and 4 (17.4%) triplets. Does bearing triple fetuses showed significant higher concentration of cortisol in comparison to does with single pregnancy. On the contrary, P_4 concentrations did not differ between does bearing different number of fetuses. Significant positive correlations emerged between plasma P_4 and cortisol concentrations and the number of fetuses, and between the two hormones. It was

concluded that the single measurement of cortisol one week before the expected parturition might be useful to distinguish between does bearing singleton and triplet pregnancies.

The ***seventh experiment*** focused on the management of weaning at individual level by investigating how PLF technologies can be used to monitor individual dry matter intake. A method was proposed using a 3D depth camera and a proper algorithm to measure the volume and weight of eaten feed. To preliminarily assess the feasibility of the proposed method, a suitable measurement setup was implemented in laboratory conditions, using a 3D Depth camera (Realsense™ D455, Intel©). A dedicated MATLAB™ code was implemented to control the camera and retrieve the distances measured in the framed scene. The MATLAB function *ginput()* was exploited to graphically determine the extent of the considered regions according to shown distance image. The 3D camera was positioned at 65 cm from the reference plane whose distance could be modified and controlled thanks to a hand crank. The volume of feed was associated with MATLAB reconstructions, showing differences lower than 2%.

The ***eighth experiment*** studied the use of an RFID-linked walk-over-weighing (WoW) system in a pastoral sheep production system to predict future liveweight (LW) of sheep with different lead times. The experiment lasted 94 days, the flock consisted of 144 Merino and crossbred lambs (White Suffolk × Merino) of both genders. Each animal was tagged with an electronic RFID ear tag and the remote walk-over-weighing station was installed at the entrance of a yard enclosing the single water source to record the liveweight of animals each time they came across. The station was configured to allow free flow of animals. Growth rate was calculated as the first derivative throughout the predicted LW curve. The future predicted LW (PW) was calculated daily on the 20, 30, 40, 50, and 60 days ahead of any actual day throughout the trial by multiplying each animal's actual growth rate by the target days and adding the actual observed LW (OW). The accuracy of the weight predictions was assessed using a linear mixed-effects and Lin's concordance correlation coefficient (CCC). As expected, the accuracy and the precision of the PWs calculated showed a decreasing trend. The concordance correlations coefficients showed an overall agreement between the PWs and the OWs, with CCCs ranging from 0.692 and 0.967. The same trend emerged by studying the PWs and the OWs through multiple linear regressions. It was concluded that the WoW system allowed to record LW of individual sheep daily.

Introduction

Animal welfare is a debated topic in modern society. Consumer's concern for the welfare of animals in livestock production is increasing. The consumer is interested in clear, detailed and reliable information on farming techniques and compliance with animal welfare parameters (Sanco, 2015). Furthermore, it should be considered that animal derived foods potentially acquire an additional intrinsic value, represented by the market demand exerted by consumers who are increasingly sensitive to these issues. Finally, the farmer can also benefit from the use of good farming practices, as improved animal welfare conditions lead to promote the immune system, reduce the use of antimicrobials and produce higher quality products.

The specific attention to animal welfare led the European Community itself and the political bodies towards considering animal welfare as a driving factor for the livestock sector to change along with environmental sustainability. Animal welfare is currently one of the pillars of European economic policy, its compliance is a necessary condition (conditionality) to gain access to dedicated Community funds, prior structural and managerial changes aimed at improving welfare conditions. However, guaranteeing conditions of welfare above the minimum and compulsory levels to farmed animals implies higher production costs which could hamper the process set up and the economic sustainability of the farm. It's necessary that the communication between breeders, veterinary technicians, animal scientists and consumers be transparent regarding the characteristics of final products, instructing on animal welfare respect and environmental sustainability, thus matching supply with demand. A complicated but achievable solution would be setting up an equation that combines the economic sustainability, achieved through the production of healthy livestock, and healthy animal derived food products, in respect of the environment and the animal welfare.

1. Animal welfare and assessment methods

Animal welfare science has expanded rapidly during the last 30 years (Broom, 2023). Animal welfare is a multifaceted concept and even among the experts it is still difficult to interpret. However, a distinction can be made between “objective” measures of welfare, which include all the scientifically measurable parameters, reflecting the real status of the animal, and "subjective" measures of welfare that evaluate the emotional state of the animal in the environment and are not scientifically definable (Bracke et al., 1999). These latter include philosophical concepts inspired to the ethics of farming, which are highly argued by the scientific community. Therefore, a theoretical approach to animal welfare takes into consideration not only the physical and health conditions of animals, but also the psychological well-being and the ability to express species-specific behaviors (Fraser et al., 1997). Twenty years after the end of the Second World War (during the economic boom), starting from the Anglo-Saxon world a new and changed sensitivity of public opinion and scientific world rose towards the breeding conditions of livestock and broadly the animal welfare.

In London, Ruth Harrison published "Animal Machines" (1964), a book condemning the conditions of intensively raised animals. At that time, this work caused such a stir within the public opinion and the politicians, that the British government commissioned a committee of researchers, led by Professor Brambell, to draft a report on the welfare conditions of intensively reared animals. From this work arose the well-known "Brambell Report" (Brambell Report, December 1965) which defined the meaning of animal welfare (not only considering the state of health, understood as the absence of pathologies), introducing the concept of mental welfare of animals. Animals were thus recognized as sentient beings.

Starting from the "Brambell Report", in 1979 the Farm Animal Welfare Council defined the universally known "five freedoms" of animal welfare:

1) freedom from hunger and thirst;
2) freedom from discomfort;
3) freedom from pain, injury and disease;
4) freedom to express normal behaviour;
5) freedom from fear and distress.

They also usefully highlighted point-by-point animal management actions, known as the Five Provisions (Webster, 2008):

1) by ready access to water and a diet to maintain health and vigour;
2) by providing an appropriate environment;
3) by prevention or rapid diagnosis and treatment;
4) by providing sufficient space, proper facilities and appropriate company of the animal's own kind;
5) by ensuring conditions and treatment, which avoid mental suffering.

Later on, to deepen the welfare concept, the most common and recognized definitions were those of Hughes in 1976 "*welfare is a state of complete mental and physical health, where the animal is in complete harmony with its environment*" and of Broom (1986) "*The welfare of an individual is its state as regards its attempts to cope with its environment*". From a legal point of view, animal welfare reached an important goal in 1976 in Strasbourg, with the European Convention on the "Protection of Animals in Livestock Farms", which defined the principles of animal welfare applied to intensive farming (Strasbourg 1976).

Another important document was the "Universal Declaration of Animal Rights", signed in 1978 at the UNESCO headquarters in Paris, with the aim of providing an ethical code to establish animal rights. This act aimed at legally defining a path towards the recognition of real rights to the animal, perceived as being a "sentient" subject of its own life and bearer of interests. (Universal Declaration of Animal Rights - UNESCO, Paris, 15 October 1978).

Subsequently, in 1997 the Treaty of Amsterdam defined the "Protocol on the protection and welfare of animals" through which animals were recognized as "sentient beings" and, capable of experiencing pain. Therefore, both suffering and mistreatment must be avoided (Treaty of Amsterdam 2 October 1997). This concept was reiterated in the Article 13 of the Treaty of Lisbon (Treaty of Lisbon 13 December 2007): "*In the formulation and implementation of Union policies in the fields of agriculture, fisheries, transport, the internal market , research and technological development and space, the Union and the Member States shall take full account of the welfare requirements of animals as sentient beings, while respecting the legislative or administrative provisions and customs of the Member States regarding , in particular, religious rites, cultural traditions and regional heritage*". Furthermore, the "White Paper on Food Safety" in 2000 defined the proposed new legal framework, which will cover the various aspects of the food chain, including: animal health and welfare, strengthening the

fight against zoonoses and the integration of animal welfare issues into food policy. This concept was essential, as a strong link between animal welfare, animal health and food safety was officially recognized. Thus, all the components of livestock production chain (farmers, agronomists/animal scientists, veterinarians, and food sector operators) must follow these guidelines to protect animal welfare and to guarantee the product quality to the consumers. The topic of animal welfare proved to be fundamental in 2004 and subsequently in 2016, when the "Global conference on animal welfare" was organized by the OIE, in which the recommendations that should inspire the policy and international jurisdiction were defined (Bayvel, 2004).

Although several studies have been carried out, still there is not a definition of animal welfare that is universally recognized and unambiguous. This is due to the real nature of the welfare concept, that is not purely scientific: in addition to scientific assessments, it includes ethical considerations affected by the public opinion too. Several authors describe different definitions of "wellbeing" or "welfare", but it is a term with a broad meaning, including both physical and mental well-being; therefore, its evaluation must include both the biology and the ethology of the animal. Assuming that, it can be implied that the welfare of an animal cannot be separated from the interrelation with the environment and its ability to adapt. Therefore, animal welfare is a concept that refers to both physiological and psychological health and it defines how the individual animal is coping, both mentally and physically with its circumstances (EAZA, 2023). The scientific community agrees that animal welfare can be assessed and measured, hence it represents a scientific concept (Fraser, 2008). When the Five Freedoms were formulated, they aimed at detailing a broader dimension of animal welfare by incorporating subjective experiences, health status and behaviour (Webster, 1995).

Nevertheless, it is biologically obvious that even during short periods of its life, an animal is never completely free from the negative experiences or states of thirst, hunger, discomfort, pain, fear, distress, malnutrition, disease and injury (Broom, 2021). The definition of welfare, based exclusively on the assessment of the animal's physical and environmental health has a clear limitation. As a matter of fact, "*genetics and environment can produce desirable physical results, even if the animal's mental state is compromised*" (Hewson, 2003). Hence, the environment, the animal and their interaction represent a crucial point in the modern interpretation of animal welfare. The welfare turns out to be a variable that can be measurable through indicators of both the environmental conditions of the farm (management, structures

and climatic conditions) and the adaptive efforts of the animal itself. A system that responds to or prepares for challenges is a coping system and coping means having control of mental and bodily stability (Broom and Johnson 1993). Coping requires the functioning of the nervous system, including the brain, and the attempt to face challenges may be towards short- or long-term stimulations, or, sometimes, to both (Broom, 2023). The various types of responses are interdependent and most of them involve the brain. Hence, the research on welfare has involved measurements of brain function and its effects on behaviour and physiology. Each coping system is independent and some of them include feelings (i.e., pain, fear and pleasure) as a part of their functioning (Fraser 2008). Since animal feelings are currently impossible to measure directly, animal welfare assessment has become heavily reliant upon the indirect measurement of feeling-related factors (Veasey, 2017). Hence, physiological and health orientated measures have emerged as popular metrics for assessing welfare.

Animal welfare indicators provide key information about positive and negative feelings and other coping mechanisms such as those that affect health. In recent years, animal care professionals working in managed settings have focused on identifying effective measures for systematically monitoring and assessing welfare (Butterworth et al., 2018; Whitham et al., 2013). Indeed, while the animal welfare community has traditionally conducted audits by evaluating specific management practices and environmental conditions, researchers now emphasize the importance of regularly tracking multiple indicators of behavioural, psychological and physiological health (Butterworth et al., 2018; Whitham et al., 2013). Furthermore, a focus on identifying measures that do not require invasive sampling or handling on a regular basis is crucial (Whitham et al., 2013). Different types of indicators can be considered: direct (animal based) and indirect (environmental factors) (Macrì, 2012). The "animal based" or direct indicators aim to specifically record and measure the reactions of animals to the environment and include:

- Behavioral: species-specific ethogram analysis, behavioral tests, behavioral anomalies (Appleby et al., 1992);
- Physiological: neuro-endocrine, immune, metabolic, cardiac (Wiepkema and Koolhaas, 1993);
- Pathological: pathologies, lesions, metabolic disorders, mortality;

- Productive: fertility, growth, quantity of production, quality of production.

Indirect indicators or environmental factors detect the characteristics of the environment the animals live in. These include:

- Farming structures and systems (characteristics of functional areas, type of housing, quality of bedding, aeration, ventilation, feeding system);
- Management (feeding, milking, individual care, plant maintenance);
- Human-animal relations (quantity and quality).

The detection of both indirect and direct parameters can identify the causes of any potential welfare issue.

Currently, stress is defined as a state of threating to homeostasis (Lu et al., 2021). A wide range of biomarkers is available to measure the physiological response to a stressful situation (Botía et al., 2023). Moreover, welfare scientists have recently increased efforts to non-invasively measure physiological biomarkers to gain insight into an animal's physical condition, psychological health and overall welfare status (Whitham and Wielebnowski, 2013; Staley et al., 2018). Researchers are committed to incorporate biomarkers that reflect an animal's level of emotions and, if possible, also the valence (positive or negative) of that emotion (Whitham et al., 2020). These biomarkers include measurements of the heart rate, molecules such as alpha amylase linked to the sympathetic nervous system, proteins related to immune function, such as e.g., cytokines and immunoglobulin A (Staley et al., 2018; Hänsel et al., 2010; Nater and Rohleder, 2009; Pressman and Cohen, 2005). Most commonly, however, animal studies have focused on glucocorticoid hormones (particularly cortisol for the most of mammalian species) or their metabolites as a measure of both long-term and short-term stress (Dickens and Romero, 2013; Touma and Palme, 2005; Wielebnowski and Watters, 2007). The reason for which glucocorticoids are the group of biomarkers most frequently used to evaluate the physiological response to stress is because from a neuroendocrinological point of view, any stressful stimulus triggers the release of the adrenocorticotropic hormone (ACTH), which leads to the secretion of these molecules (Butterworth et al., 2018). Although glucocorticoids provide information about the activity of the hypothalamic–pituitary–adrenal (HPA) axis, there are some limitations to relying solely on these biomarkers (Miller et al., 2007; Wielebnowski, 2003).

Therefore, recent research has incorporated other biomarkers of HPA activity, known as "glucocorticoid antagonist", dehydroepiandrosterone (DHEA) and its sulphate ester (DHEA-S), to provide a more complete picture of how an individual's HPA axis is functioning (Guilliams and Edwards, 2010). In particular, DHEA(S) counteracts the effects of glucocorticoids by anti-aging, immune-enhancing and neuroprotective properties. Recent studies have also examined the ratio of glucocorticoids to DHEA(S) as a tool to better understand how the HPA axis is functioning. There is evidence that this ratio is a useful indicator of immune function, mental health, cognitive performance and overall welfare (Peric et al., 2017; Trevisan et al., 2017). A more detailed description of these biomarkers and of the HPA axis functioning is provided in the sections below.

1.1. Measurable markers of animal welfare

Humans and animals respond to environmental perturbations with a stress response that allows physiological adaptation to the stressor to maintain homeostasis. A major component of such a homeostatic response is the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis represents an intricate and robust neuroendocrine system that mediates the effects of stressors by regulating several physiological processes, such as metabolism, immune responses, and the autonomic nervous system (Sheng et al., 2021). In particular, the HPA axis consists of a cascade of endocrine pathways (hormonal response system) that respond to specific negative feedback loops, involving hypothalamus, anterior pituitary gland, and adrenal gland, and it is crucial for maintaining a basal homeostatic state (Miller et al., 2007; Wielebnowski, 2003). The hormonal cascade associated with this system can be activated by intrinsic or extrinsic factors (Guilliams and Edwards, 2010; Dantzer et al., 2014). Once the axis has been triggered by a stressor, the hypothalamus (specifically the neurosecretory parvocellular neurons in the paraventricular nucleus - PVN) produces the corticotropin-releasing hormone (CRH), which leads to the secretion of ACTH by the anterior pituitary gland (Sheng et al., 2021). The latter stimulates the conversion of cholesterol to pregnenolone, a precursor to steroid hormones including glucocorticoids, DHEA, progesterone, testosterone and estrogens (De Kloet et al., 2005; Leowattana, 2004; Raison and Miller, 2003). The ACTH regulates the zona reticularis and fasciculata of the adrenal cortex that represents the steroid-hormone producing part of the adrenal gland (Sun et al., 2018). Figure 1 has been adapted from Whitham et al. (2020) and summarizes these pathways.

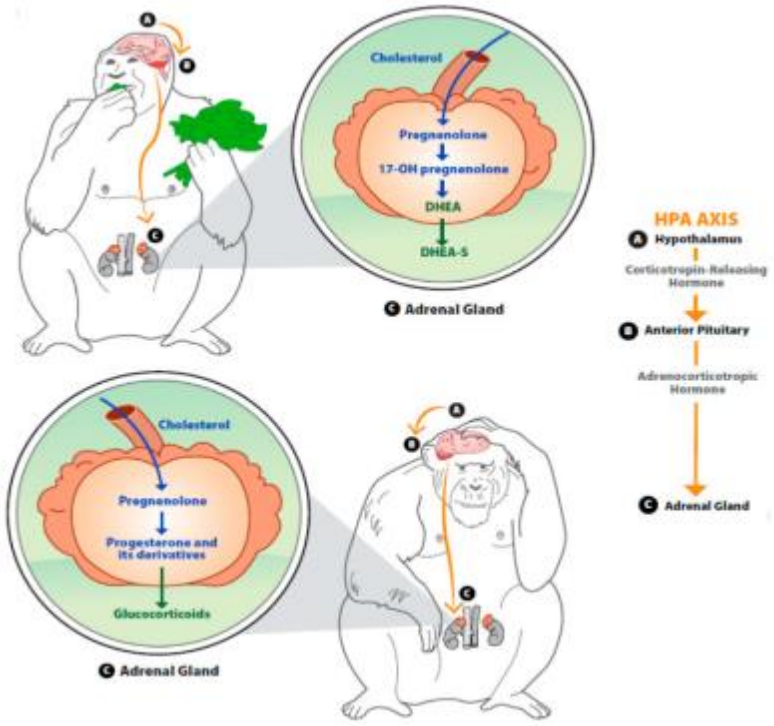

Figure 1. Schematic overview of the biosynthesis of glucocorticoid and dehydroepiandrosterone within the hypothalamus (A) - pituitary (B) – adrenal (C) axis (Whitham et al., 2020).

Both DHEA(S) and glucocorticoids are excreted mainly by the zona reticularis of the adrenal cortex (Leowattana, 2004) and impact on systems related to motivation, cognitive function, mood, immunologic status and sensory processing (Kamin and Kertes, 2017). If the HPA axis is functioning properly, when the stressor has diminished or passed, negative feedback inhibits the release of additional glucocorticoids (Sapolsky et al., 2000; Dickens and Romero, 2013). As a matter of fact, the HPA axis is controlled by a closed-loop glucocorticoids dependent negative feedback system, that is essential for the termination of the stress response. Hence, the normal HPA function is highly influenced by the dose and duration of glucocorticoids exposure (Abe and Critchlow, 1980; Sapolsky et al., 2000). On the other hand, DHEA(S) does not exert feedback on the HPA axis, as reported by Leowattana (2004) and Kamin and Kertes (2017).

The exposure to chronic stressors can cause the dysregulation of the HPA axis and may result in pathophysiological effects (Fries et al., 2005; Heim et al., 2000). Particularly, the dysfunction of the HPA axis occurs with an upregulation of the glucocorticoids and a reduced production of DHEA(S) (Guilliams and Edwards, 2010). This dysregulation may disrupt various homeostatic mechanisms, suppress the immune system, and inhibits the production of growth and reproductive hormones (Dickens ad Romero 2013; Heim et al., 2000).

Through the development of non-invasive monitoring techniques, glucocorticoids (and their metabolites) and DHEA(S) can be tracked in faeces (Wielebnowski and Watters, 2007), urine (De Clercq et al., 2015), saliva (Esposito et al., 2012), feathers (Frongia et al., 2020), hair (Peric et al., 2022), claws (Fusi et al., 2021) and wool (Zoratti et al., 2023). When measuring glucocorticoids and DHEA(S), the selection of the appropriate sample type requires researchers to consider both the aim and the duration of study (short- or long-term goals), the feasibility of collection, safety, and the physiology and behaviour of the animals involved (Whitham et al., 2020). But probably, the most important aspect is to evaluate whether a point-in-time hormone value or a cumulative concentration is preferred. Table 1 has been adapted from Whitham et al. (2020) and provides an overview of the characteristics of the biological matrices most widely used to measure glucocorticoids and DHEA(S). Glucocorticoids and DHEA(S) will be discussed more detailed in sections 1.2 and 1.3.

Table 1. Most common biological matrices for examining glucocorticoids and dehydroepiandrosterone.

Matrix	Description	Pros	Cons
Blood	Provide punctual hormone value, (subject's current state) (Sheriff et al., 2011).	Directly measures the hormone (Sheriff et al., 2011).	Invasive, cause stress (Kersey and Dehnhard, 2014). Need to distinguish into bound and free fractions (Bersano and Baumann, 1989).
Saliva	Offers punctual hormone concentration, similar to blood (Lutz et al., 2000).	Measures free fraction of the hormone (Teruhisa et al., 1981). Minimally invasive, little disruption to the routine (Lutz et al., 2000; Kobelt et al., 2003; Fels et al., 2019).	Training is necessary (Fels et al., 2019; Kersey and Dehnhard, 2014). Collection and sampling materials impact measurement (Gallagher et al., 2006). Must examine salivary flow rate and avoid contamination (Granger et al., 2007, 2012).
Urine	It's possible to detect the native hormone (Bahr et al., 2000; Behringer et al., 2012). Metabolites describe the cumulative concentration (Palme, 2019).	Non-to-minimally invasive. Little disruption to the subject's routine (Sheriff et al., 2011; Palme, 2019).	Requires designing holding areas for sample collection (Sheriff et al., 2011) and determining excretion rates (Teskey-Gerstl et al., 2000).
Faeces	Possible to detect native hormone (Price et al., 2019; Pauling et al., 2017). Metabolites describe cumulative concentration (Heistermann et al., 2006).	Non-invasive. No disruption to the routine (Millspaugh and Washburn, 2004).	Need to add a marker to distinguish the samples of socially-housed animals and to determine excretion rates (Palme, 2019).
Hair	Offers cumulative concentrations of hormone value providing retrospective concentrations over the preceding months (Wiechers et al., 2021)	Non-invasive. Little disruption to the subject's routine (Heimbürge et al., 2019)	Sampling protocols may affect measurement. Variations in hair growth parameters, various extraction protocols and quantification techniques (Greff et al., 2019; Heimbürge et al., 2019)

For a long time, blood has been the preferred sample type for measuring steroid hormones in many vertebrates (Sheriff et al., 2011). However, plasma and serum measurement of hormones has some disadvantages because blood collection is an invasive technique with handling and capture-induced stress. Therefore, the collection method itself causes an increase in glucocorticoids levels (Naidenko et al., 2007). On the other hand, blood offers a punctual evaluation of hormone concentration, providing insight into the individual's state at that moment (Sheriff et al., 2011). Moreover, when using blood to measure steroid hormones, it is important to distinguish between bound (hormones circulating with transport proteins in the bloodstream) and unbound (free hormones out of circulation) hormone (Henning, 1978). When assessing a stress response, researchers would focus their attention on unbound, free hormone concentrations, since they show stimulation and patterns of adrenal activity (Henning, 1978).

One less invasive technique to assess glucocorticoids and DHEA(S) levels is analysing saliva samples: it allows to measure the free, unbound concentration of these steroids and can be the sample type of choice in ex situ animal studies but is characterized by a difficult collection that affects its use in field (Teruhisa et al., 1981). So, saliva can be the matrix of choice to measure cortisol and DHEA(S) as long as optimal collection methods and materials are used for each hormone (Whitham et al., 2020).

Another non-to-minimally invasive approach for studying the HPA activity involves using urine samples (Palme, 2019): this matrix is most effective for trained animals, nevertheless urinary glucocorticoids and DHEA(S) are metabolized by the liver and kidneys, leaving only a small amount of native hormone, hence the measurement of metabolites is necessary (Bahr et al., 2000).

Faecal matrix has been widely used to measure steroids in animal welfare studies, because it can be collected without any disruption to the animal's daily routine and does not require handling, avoiding the capture-induced stress (Millspaugh and Washburn, 2004). It must be underlined that faeces yield by-products, or metabolites, of cortisol, corticosterone and DHEA(S), as for urine samples, (Bardi et al., 2010).

Recently, the possibility of measuring steroids in hair samples in a variety of species has been investigated. Measuring hair cortisol and DHEA concentrations, and examining the ratio of these two hormones, has already been used to assess stress in humans (Esposito et al., 2012), sows (Peric et al., 2023), cattle (Peric et al., 2022), sheep (Zoratti et al., 2023). In addition, the measurement of glucocorticoids and DHEA can be performed in coat and claws (Fusi et al.

(2021), and in fingernails (De Berker et al., 2007). Hair, fingernail, and claw samples offer a cumulative measure of the hormone over the period of interest (Whitham et al., 2020).

In veterinary sciences, immunoassays are the most common methods for measuring concentrations of glucocorticoids, DHEA(S) and their metabolites (Palme, 2019). A detailed description of those methods is beyond the scope of the present thesis, still a brief account of radio and enzyme-immunoassays is provided below.

Immunoassays analyses are techniques in which the hormones from a matrix compete with labelled hormone for limited antibody binding sites (Möstl et al., 2005). Radio-immunoassays (RIA) and enzyme-immunoassays (EIA) are the most used: the difference lies in the detection system involved into the quantification of the hormones. RIAs are heterogeneous assays that employs radiolabelled isotopes as detection label which generates a radioactive signal that can be measured by a gamma counter (Menargues et al., 2008). There are two different methods of RIA: the double-antibody RIA that adds a second antibody to facilitate the precipitation of the bound primary antibody; and the coated-tube RIA in which the primary antibody is coated on the inside of each tube. On the other hand, EIAs employs an enzymatic label, the signal produced is colorimetric and is measured by a spectrophotometer (Bardi et al., 2010). ELISA involves the use of an enzyme activity to detect the binding of an antibody–enzyme conjugate, the major disadvantage is the enzyme activity that highly rely on the physical and chemical environments (Pawliszyn, 2012).

1.2. Glucocorticoids

Glucocorticoids are endogenous adrenal hormones with a 21-carbon skeleton. As previously mentioned, they derive from cholesterol and are released in a stressful situation (Botía et al., 2023).

The most important glucocorticoids involved in the stress response are cortisol, cortisone, and corticosterone (Figure 2). Once released, they bind to the corticosteroid-binding globulin, and become available at systemic and tissue level (Perogamvros et al., 2011). They perform their function by binding intracellular receptors (De Guia, 2020).

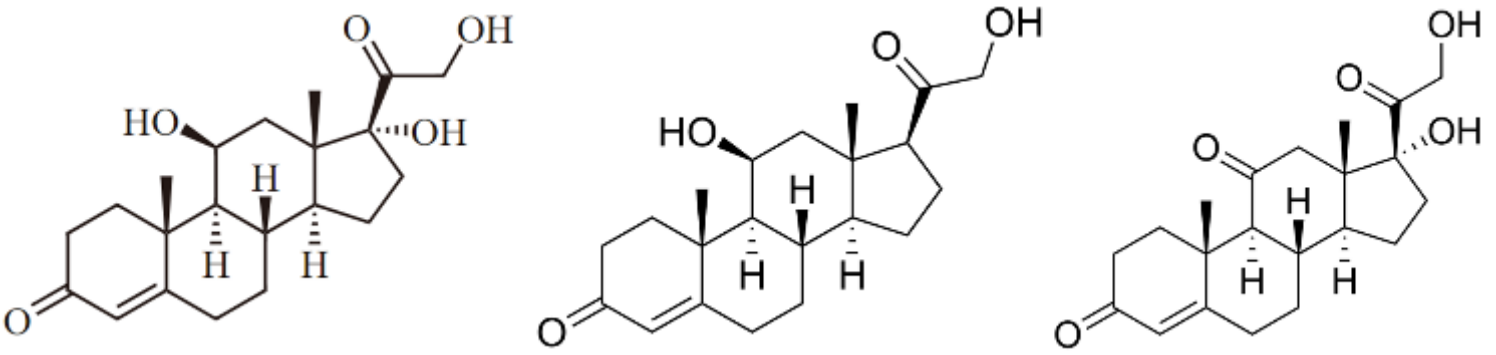

Figure 2. Skeletal formulas (left to right) of cortisol (11β,17α,21-trihydroxypregn-4-ene-3,20-dione), corticosterone (11β,21-dihydroxypregn-4-ene-3,20-dione) and cortisone (17-hydroxy-11-dehydrocorticosterone).

Glucocorticoids maintain the homeostasis by coordinating physiological responses to stress, exertion and arousal (Whitham et al., 2020). They are involved in catabolic metabolism, inflammatory and immune response, and other physiological functions (Nicolaides et al., 2014). In particular, an increase in glucocorticoid release has several effects, such as the secretion of glucose, that provides energy to overcome immediate challenges, the retrieval of homeostasis and the inhibition of non-essential functions (Gundlach et al., 2018).

The stress response is an adaptive/physiological response, therefore short-term stress responses are mostly positive. Conversely, a repeated or chronic stimulation of the stress response systems can lead to health-threatening consequences (Ralph and Tilbrook, 2016). While acute stress can be beneficial to an animal, the repeated exposure to stressors or the chronic activation of the HPA axis can lead to dysfunctional processes. In the initial phase of a chronic activation of the HPA axis, elevated levels of glucocorticoids are not effectively downregulated by the negative feedback loop, resulting in hypercortisolism (Guilliams and Edwards, 2010). Chronically elevated glucocorticoids lead to additional HPA axis dysfunction that are associated with pathologic symptoms:

1. Impaired neurological functions and cognitive performances (De Kloet, 2005);
2. Immune deficiency by reducing the production of lymphocytes, cytokines and antibodies (Bauer, 2008);
3. Reproductive problems, affecting the release of reproductive hormones, sexual receptivity and reproductive behaviour (Sapolsky et al., 2000);
4. Growth reduction (Wielebnowski, 2003).

After the initial over-response of the HPA axis, characterized by hypercortisolism, it may ultimately result in hypocortisolism or "*adrenal fatigue*" (Heim et al., 2000). Indeed, the chronic or repeated exposure to stressors leads to an adaptation within the HPA axis that aims to protect the organism from chronically elevated concentrations of glucocorticoids that would threaten long-term survival (Guilliams and Edwards, 2010). This adaptation alters the HPA axis at several levels: reduced glucocorticoid signalling, modified negative feedback loop, upstream changes in CRH and ACTH. These mechanisms result in reduced glucocorticoid production and contribute to hypocortisolism (Fries et al., 2005). Hypocortisolism can be just as damaging as hypercortisolism: in particular, the health issues associated with reduced glucocorticoid secretion include, but are not limited to, increased risk of developing

inflammatory disease, impaired cognitive function, and mental health or behavioral issues (Raison and Miller, 2003).

In conclusion, it is extensively recognized that the use of glucocorticoids as the sole measure of stress or welfare can provide only a partial picture (Dantzer et al., 2014), still the alteration of the baseline concentrations (both hypocortisolism and hypercortisolism) leads to detrimental effects on health status (Dickens and Romero, 2013; Miller et al., 2007). Hence, as discussed below, a comprehensive analysis of the HPA axis activity must integrate additional biomarkers.

1.3. DHEA(S)

Dehydroepiandrosterone (DHEA) and its sulphate-ester (DHEA-S) (Figure 3) have been described as glucocorticoid antagonists (Guilliams and Edwards, 2010), immunostimulants (Kamin and Kertes, 2017), biomarkers of aging (Muehlenbein et al., 2003) and of resilience (Bürgin et al., 2020), and neuroprotective hormones (Bauer, 2008). From now on, DHEA(S) will be used when referring to both DHEA and DHEA(S).

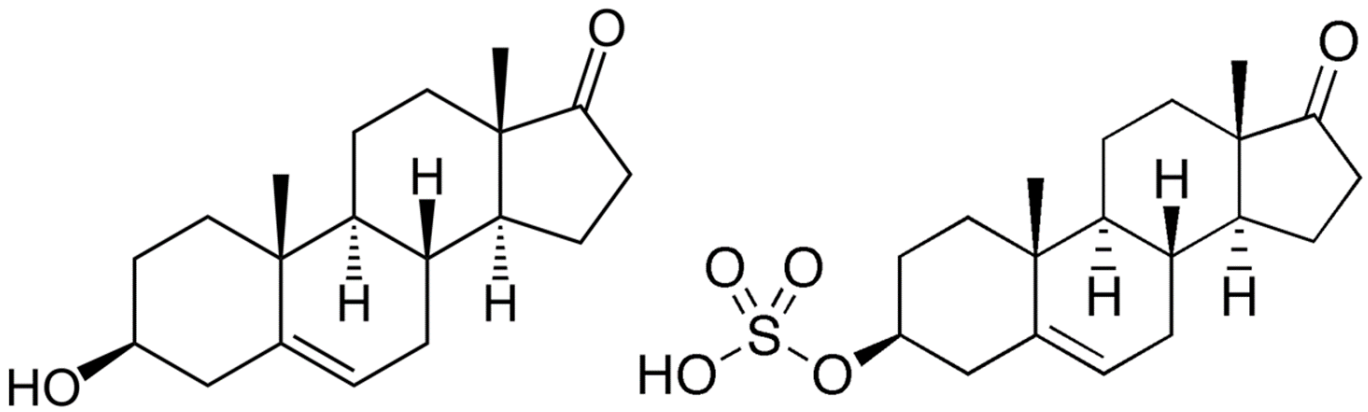

Figure 3. Chemical structures of dehydroepiandrosterone (left) and its sulphate ester, dehydroepiandrosterone sulphate (right).

DHEA(S) is precursor to both androgens in males and active estrogens in females (Leowattana, 2004; Muehlenbein et al., 2003). DHEA(S) is synthesized in steroidogenic tissues, as adrenals, gonads, and placenta, and in the nervous system from pregnenolone through the Δ^5 pathway (Gabai et al., 2020).

DHEA(S) has widespread physiological effects:

1. On cognition and mental health (Dong and Zheng, 2012), by memory improvements (Buvat, 2003);
2. On behaviours related to survival and reproduction (Kamin and Kertes, 2017), such as species-appropriate territorial behaviour and adaptive behaviours;
3. Immune-enhancing and anti-aging, by increasing the production of cytokines that promote white blood cell activity, inhibiting the production of cytokines responsible for inflammation (Cao et al., 2020), and contrasting immunosenescence (Bauer, 2008).

Whitin the scope of the present thesis, DHEA(S) has been thoroughly considered for his role in the short- and long-term stress response and his effects (Sahu et al., 2020). The antagonistic actions to glucocorticoids played by DHEA(S) have been previously described as "*the most intriguing aspect of DHEA and DHEAS*" (Gabai et al., 2020). At the same time, the need for a deeper understanding the co-actions of these hormones on physiological systems has been underlined (Gabai et al., 2020). An increase of DHEA(S) has been reported in response to acute stressors by Fustini et al. (2017). On the other hand, repeated or chronic stressors can lead to dysregulation of the HPA axis and a consequent reduction in DHEA(S) (Kamin and Kertes, 2017). Indeed, DHEA-S has been recommended to be rather incorporated into allostatic load indices (Edes et al., 2020). Similarly, suboptimal levels of DHEA(S) have been reported for humans suffering from chronic diseases (i.e. mood disorders, chronic pain disorders) and inflammatory diseases, such as inflammatory bowel disease (Guilliams and Edwards, 2010). Furthermore, it has been suggested that DHEA could better reflect the response to acute stress, with DHEAS being associated only to long-term perceived stress (Kamin and Kertes, 2017).

In conclusion, it is currently suggested that the phenotypic response to a stressor can be better described by expressing both cortisol and DHEA(S) simultaneously as glucocorticoid/DHEA ratio, which may be a more accurate indicator of the whole animal response (Gabai et al., 2020).

1.4. Glucocorticoid:DHEA Ratio

As mentioned before, in the last few years, there has been a growing interest in examining the glucocorticoids:DHEA(S) ratio rather than these two hormones alone, in order to discuss the HPA axis functioning in detail (Sollberger and Ehlert, 2016). Indeed, the antagonistic dynamic between cortisol and DHEA(S) has been described as "*cortisol and DHEA(S) mediate largely opposing biologic, neurologic, and immunologic functions […] measuring their levels simultaneously may be an important indicator of net glucocorticoid activity*" (Kamin and Kertes, 2017). If a disruption of the sensitive balance between glucocorticoids and DHEA(S) occurs because of a chronic stimulation of the HPA axis, the effects on physical, mental and emotional health may be detrimental (Kamin and Kertes, 2017). Kamin and Kertes continue arguing that cortisol and DHEA(S) do counterbalance one another and proper concentrations of each should be maintained, in particular: "*rather than assuming that a low level of cortisol and a high level DHEA(S) are optimal […] both hormones need to be maintained at certain levels depending on biological and psychological states*". In addition, the cortisol to DHEA(S) ratio combines the value of the two in one single piece of information.

The cortisol:DHEA(S) ratio has been extensively studied in humans, and it has been reported as an indicator of several conditions:

1. ***Immune functions***: high cortisol:DHEA(S) ratio has been used to predict the risk of infection or death (Beishuizen et al., 2002; Butcher et al., 2005);
2. ***Immunological effects***: immunological changes have been observed during aging (Buford and Willoughby, 2008);
3. ***Cognitive function and mental health***: high cortisol:DHEA(S) ratios have been associated to cognitive impairment (Kalmijn et al., 1998), treatment-resistant depression, anxiety, stressful life events, schizophrenia, angry temperament, hostility and symptoms of dissociation (Heaney et al., 2014; Markopoulou et al., 2009; Morgan et al., 2009 Ritsner et al., 2004).

In farm animal welfare research, several studies have referred to the cortisol:DHEA ratio as a potential biomarker of resilience and allostatic load (Peric et al., 2017; Trevisan et al., 2017; Bergamin et al., 2019), therefore ratio will be further analysed accordingly. Health status has been proved to influence the ratio in cattle, with lame cows showing 65 % higher cortisol:DHEA ratios compared to healthy subjects (Almeida et al., 2008). Other factors that have been proved to impact the cortisol:DHEA(S) ratio in farm animals are transport,

environmental conditions, housing and husbandry practices. Transport has been reported to increase cortisol to DHEA(S) ratio in pigs coping with a novel environment after being moved to a new facility (Trevisan et al., 2017) and in young bulls (Buckham Sporer et al., 2008). Similarly, Peric et al. (2017) described how deteriorating environmental conditions cause an increase in the cortisol:DHEA ratio in dairy cows. In piglets, welfare-enhancing housing conditions lowered the cortisol:DHEA ratios compared to more stressful housing systems (Fels et al., 2019). Finally, horses that experienced natural boarding practices had significantly lower cortisol:DHEA than subjects exposed to traditional stable management style (Placci et al., 2020).

Overall, the cortisol to DHEA(S) ratio should be interpreted with care in the context of animal welfare research, taking into account individual physiological factors such as age and sex (Goncharova et al., 2010; Rosado et al., 2010). Moreover, further research should focus on analysing hormone ratios with a proper statistical approach (Sollberger and Ehlert, 2016).

2. Precision livestock farming (PLF) technologies towards the development of more efficient production systems of healthy livestock

Terms such as precision livestock farming, precision agriculture (PA), smart farming and industry 4.0 are nowadays widespread (Morrone et al., 2022). The term Industry 4.0 refers to the 4th industrial revolution. It focuses on factory automation, incorporation of internet into industrial processes, and dissemination of information and communication technology (ICT) to create intelligent devices, machines, and systems (Lasi et al., 2014), leading to fully automated and interconnected industrial production (Maci, 2018). The PA has been described as an "*integrated information and production-based farming system designed to increase the efficiency, productivity and profitability of long-term, site-specific and entire farm production while minimizing impacts on wildlife and environment*" (Morrone et al., 2022). In the present thesis, a focus will be provided on the precision livestock farming (PLF), that is defined as "*management of livestock by continuous automated real-time monitoring of production/reproduction, health and welfare of livestock, and environmental impact*" (Berckmans, 2017).

In the next years, an increase of the global demand for animal derived food will be observed in response to the increasing world population and to meet consumers' requirements. At the same time, maintenance and improvement of animal welfare must be ensured (Tobin et al., 2022). In particular, a focus is provided about the ruminant sector, where according to FAOSTAT (2021), the total number of heads for both dairy and meat production in 2019 was 2.67 billion (table 2) out of a global ruminant population of 4.44 billion.

Table 2. Number of animals in the ruminant sector bred for dairy and meat production in 2019 (FAOSTAT, 2021).

Species	Attitude	Number of head
Buffalo	Dairy	75.743.127
Cattle		270.985.026
Sheep and goat		508.839.234
Buffalo	Meat	32.154.715
Cattle		365.076.041
Sheep and goat		1.422.142.701

However, increasing production intensity to meet this growing demand for animal-derived products leads to some resistance due to people's concerns about animal welfare and environmental limitations (Tobin et al., 2022). Therefore, the observational capacity and the practical experience which farmers relied on in the past is no longer sufficient for an efficient daily herd management (Parson et al., 2007) and the use of PLF tools can play a pivotal role towards the development of a more efficient, sustainable and healthy livestock production system (Hartung et al., 2017).

Several type of sensors can be used in livestock. From a classification point of view, 3 types of sensors can be distinguished (with reference to cattle, Knight 2020):

- At cow sensors: this large group includes all the sensors that are positioned on the cow, with different aims and roles. Among these, it is possible to mention the accelerometers, usually placed on the leg, the neck or the ear of the animals to evaluate activity or rumination; ruminal boluses, that can be useful i.e. to assess rumen pH and temperature; vaginal devices for temperature or pH detection;
- Near cow sensors: these are all the sensors positioned to monitor the environment in which the cow lives. Cameras, videocameras and depth cameras to assess weight or body condition score (BCS), sensors for GHG detection or climate parameters, microphones, drones, etc. are part of this group;
- From cow sensors: in this case sensors are utilized to monitor the characteristics of the animal derived products, such as milk, assessed through infrared spectroscopy, hair, faeces, etc.

Briefly, the technologies currently available include several types of sensors that can be used i.e. for automated milk yield and quality monitoring, automated estrus-detection, calving time forecast, detection of health issues such as lameness and mastitis and feeding-related metabolic problems (Abeni et al., 2019). The specific applications of these tools can help the decision-making processes by providing early detection of diseases in individuals and the application of targeted corrective practices (Caja et al., 2016). Nevertheless, some sensors are difficult to use in their management systems since they produce large and complex datasets that are difficult to interpret (Werkheiser, 2018). Thus, technologies that implement data output into precision livestock management (PLM) schemes have been commercially developed for large- and small-scale livestock managers (Bailey et al., 2021). Research focusing on PLM could improve our comprehension of animal behaviour and facilitate management action before performance

and welfare of the animal are compromised (Ikurior et al., 2020). Particularly, the aim of the PLM in intensive livestock systems is to build "*a management system based on continuous automatic real-time monitoring and control of production/reproduction, animal health and welfare, and the environmental impact of livestock production*" (Berckmans, 2014). Such approach can provide opportunities to improve data driven and proactive decisions, rather than reactionary actions that are typical of traditional management (Tobin et al., 2022). The motivation behind PLM is to observe the individual animal (fine-grained) who has different nutritional, health and physiological needs compared to the herd average (Werkheiser, 2018).

As described above, the precision livestock farming is a multidisciplinary paradigm, and the decompartmentalization into scientific disciplines has been extensively discussed (Terrasson et al., 2017).

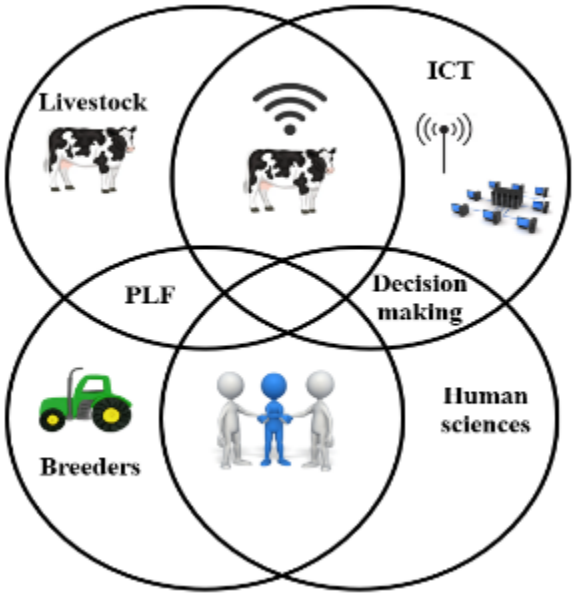

Figure 4. Schematic representation of the smart interfaces involved in the transdisciplinary paradigm of the precision livestock farming (adapted from Terrasson et al., 2017).

Among the decompartmentalization approaches, PLF has been used to study the five freedoms and demonstrate its contribution to livestock welfare improvement (Tobin et al., 2022). A detailed description concerning this conceptual framework is provided in the next sections.

2.1. Potential of PLF technologies to address animal welfare

Thanks to technological development and progress, nowadays, accurate, powerful and affordable tools are commercially available (Berckmans, 2014). Modern sensors, such as 3D cameras, microphones, accelerometers, temperature sensors (i.e. thermography), skin conductivity sensors and glucose sensors, systems for automated milk quantity and quality monitoring, at-line milk progesterone determination, are only some of the sensors that were developed and that replace the farmer's eyes and ears, aimed to improve health, welfare, yields and environmental impact (Berckmans, 2014; Abeni et al., 2019; Antanaitis et al., 2020).

The evaluation of the use of on-animal sensors to monitor welfare using the five domains model was provided by Fogarty et al. in 2019. In particular, they retrospectively classified the application of sensors using the five domains model as a framework for animal welfare assessment and concluded that PLM can be used to improve the welfare of livestock grazing on extensive rangeland systems. Conversely, Mellor in 2017 argued that the advancements in our comprehension of the biological processes are not included in the five freedoms concepts, nevertheless they continue to have value, because they can simply and clearly outline appropriate management actions and help the decision-making processes (Webster, 2016). To help elucidate the five freedoms conceptual framework, table 3 was adapted from Tobin et al. (2022) to include intensive system applications and updated for newly available technologies; it lists the precision livestock management technologies, the metric provided by each device, and examples of which could help issues related to each of the five freedoms.

Table 3. Examples of the main precision livestock farming technologies with the measurements provided by each device that could help identifying issues related to each of the five freedoms.

Sensor technologies	**Accelerometer**	**Cameras (3D, thermography), GPS Tracking**	**Unmanned Aerial Vehicles (mainly grazing systems)**	**Stationary Sensors, near infrared spectroscopy**
Measurements	Movement, intensity, energy, entropy	Distance travelled, interactions with other animals, BCS, body temperature	Forage quantity and quality, real time location	Consumption quantity and quality, frequency and obstruction
Freedom from thirst, hunger, and malnutrition	Increasing movement intensity due to water deprivation, feed consumption	Increasing distance from peers throughout grazing season, reduced BCS	Limited forage availability, potential decreased intake	Decreased water consumption and quality of TMR/forage, and frequency of water visits
Freedom from discomfort	Detection of panting (heat stress?)	Increased time spent near or within repairs, water source or shade (heat stress?), reduced body temperature	Increased concentration of animals near water or shade because of heat stress	Increased water intake and frequency of visits (heat stress?)
Freedom from pain, injury, and disease	Reduced movement intensity from illness (lameness?)	Reduced velocity and limited travel range (lameness?), body lesions, fever	Increased time standing in/near water because of injury/injury	Reduced water intake (injury, disease?)
Freedom to express normal behaviour	Detection of rumination and estrus behaviour	Changes in feeding pattern or reduced travel, absence of estrus behaviour	Typical forage defoliation rates, consumption of palatable forages	Typical water intake
Freedom from fear and distress	Increased nighttime movement intensity	High density of animals or changes in diurnal pattern	Limited grazing sites, overutilization	Reduced intake of water

Currently, the most common devices used in ruminants are accelerometers to detect calving, estrus and lameness (based on activity data); cameras to determine standing heat (combined with machine learning), BCS, and estimate weight; reticulum boluses to monitor estrus, calving and physiological factors (i.e., body temperature or pH); and ear sensors to monitor the temperature (Schillings et al., 2021).

A detailed description of the contributions provided by the PLF technologies to each of the freedoms of animal welfare will be provided in the sections below, with a focus on those technologies applied to ruminants.

2.1.1. Freedom from thirst, hunger, and malnutrition

The monitoring of drinking and feeding behaviours (including grazing, ruminating and jaw movements, chewing and feed intake), and gastrointestinal health are the main applications related to the nutrition domain (Schillings et al., 2021). The daily water requirement ranges from 3 to 6 liters for sheep and goats, 20 liters for horses, above 100 liters per day for cattle (Tobin et al., 2022). Water deprivation is rarely fatal within the first 24 h, it implies a loss of appetite and mobilization of body fat reserves (Marques et al., 2012); but it becomes more critical after 24 h, with cows losing 20% body weight within 3 days and leading to death within 5 days (Siebert and Macfarlane, 1975). An increment in sweating and panting occurs during periods of dehydration and heat stress. In particular, panting allows the blood passing through the nasal area to cool, lowering the brain temperature in comparison to the body (Robertshaw and Damiel, 1983; Baker, 1989). Panting, decreased movement, heavy breathing, and lack of coordinated movement could be symptoms of heat stress and can potentially be identified by accelerometers and GPS-tracking tools in extensive systems (Tobin et al., 2022). On the other hand, in intensive systems stationary sensors, such as radio frequency identification (RFID) readers and cameras can provide valuable behavioural data including drinker and feeder visit duration, intake rates, and drinking rates (Chapinal et al., 2007; Allwardt et al., 2017).

The utilization of near infrared spectroscopy (NIRS) has been highly widespread in the last few years. It uses the infrared region of the electromagnetic spectrum (from 800 to 2,500 nm) to evaluate the characteristics of different samples (Givens et al., 1997). The main advantages of this technique are that it is rapid, cheap, not disruptive and is able to supply information on the physiochemical features of several matrices with accurate predictive ability. NIRS is currently utilized to achieve information on the chemical-physical composition of raw materials, total mixed ration (TMR), faeces and digestibility, chemical and technological analysis of milk (Evangelista et al., 2021). This technology is largely used in dairy cattle farms to monitor all the critical points linked to the feeding and encounter the requirements of the animals.

2.1.2. Freedom from discomfort

Monitoring environmental parameters plays a pivotal role in helping address negative experiences by minimizing thermal, physical, respiratory and olfactory discomfort due to inappropriate temperatures or inappropriate levels of ammonia (Schilling et al., 2021). Ensuring optimal environmental conditions benefits animal welfare by minimizing risks of infectious and respiratory diseases and heat stress, and promoting feelings of comfort (Tobin et al., 2022). Furthermore, the identification of discomfort and, consequently, the provision of supplementary feed and water, or shelter or ventilation could be vital to guarantee survival and welfare of livestock (Tobin et al., 2022). Unlike the freedom from pain, injury and disease, within the discomfort domain, the potential impact of experiences on affective experiences remains within the negative-to-neutral valence range (Schilling et al., 2021). Body temperature is the most important factor for assessing heat stress in domestic livestock and is closely associated with health, welfare, and reproductive success (Lewis Baida et al., 2021). In cattle, rumen boluses can be useful for monitoring body core temperature, whereas ear sensors can be used to monitor body temperature or pH. In any case, both tools are useful for detecting heat stress (Lewis Baida et al., 2021). Cold stress is also a concern for animal discomfort, especially in extensive systems (Tobin et al., 2022) and in species of tropical origin, like buffalo (Matera et al., 2022). In extensive rangeland systems, livestock typically seek shelter or protected terrain during cold and windy weather (Black Rubio et al., 2008). In addition, they tend to decrease feed consumption during cold stress (Gregory, 1995). Therefore, real-time GPS and accelerometers could potentially be able to detect when livestock are not coping to cold stress.

During extreme heat and cold conditions, grazing livestock must find shelter to prevent hyper/hypo-thermia (Bailey, 2016). Livestock responses to heat stress could potentially be determined by real-time GPS too, because animals may move to areas with water and shade (DelCurto et al., 2005; Cheleuitte-Nieves et al., 2020). Moreover, real-time accelerometers and remote sensing have the potential to identify heat stress-associated symptoms and inform livestock managers for instantaneous decisions (Tobin et al., 2022).

In conclusion, the potential for real-time GPS tracking and accelerometer monitoring to detect cold and heat stress needs to be studied in detail. Tracking and physiologic status of the animals would be examined in different weather conditions, in landscapes with variable topographic

features and in both extensive and intensive systems, in order to improve livestock’s freedom from discomfort under different conditions.

2.1.3. Freedom from pain, injury, and disease

A variety of technologies are commercially available to monitor parameters related to the freedom from pain, injury and disease, from specific diseases to foot health and stress, to physiological parameters such as heart rate, respiratory rate or temperature. The effects of a disease cause a wide range of behaviour changes in livestock. In this context, e.g., parturition represents a crucial event and requires increased observation and livestock management to reduce offspring susceptibility to disease, decrease offspring mortality, and improve animal welfare (Cornou and Kristensen, 2014; Chang et al., 2020). Accelerometers and GPS technologies have the potential to identify common behaviours associated with parturition including reduction in drinking and feeding, seeking sheltered areas, increasing standing, and reduction in rumination (Chang et al., 2020). The use of sensors, such as accelerometers and GPS, could also detect subclinical issues related to diseases before production and welfare are affected (Ikurior et al., 2020; Tobin et al., 2020). Commercially available technologies appear to apply mostly to cattle, but they can also be found for pigs, poultry, sheep and fish (Schillings et al., 2021).

In cattle, body-mounted accelerometers can be used to detect calving, estrus and lameness, while cameras combined with machine learning can help identifying standing heat, BCS, lameness, and estimate weight (Williams et al., 2016). Estrus can also be monitored by boluses placed in the reticulum, alongside calving and physiological factors such as body temperature or pH, and ear sensors can monitor temperature (Röttgen et al., 2020). Mastitis can be monitored using sensor, sound- and image-based technologies (Steensels et al., 2016; Vandermeulen et al., 2016; Yazdanbakhsh et al., 2017; Zaninelli et al., 2018). Finally, pphysiological parameters such as respiration rate (Strutzke et al., 2019), temperature (Nogami et al., 2013) or heart rate can be monitored using image or sensor-based technologies (Stewart et al., 2017).

Other technologies apply to the pig sector: camera-based systems can determine BCS, estrus and weight; microphones placed in barns can detect coughing sounds and monitor respiratory health; sensor-based systems can detect pig farrowing (Manteuffel et al., 2015; Pastell et al., 2016; Liu et al., 2018); lameness can be detected using images and sensors (Pluym et al., 2013; Stavrakakis et al., 2015).

In sheep, tri-axial accelerometers could detect abnormal gait patterns due to lameness (Barwick et al., 2018a) and could help determine the onset of diseases (Ikurior et al., 2020) or heat stress (Soriani et al., 2013); and parameters such as respiration rate, temperature or heart rate can be monitored through physiological sensors (Fuchs et al., 2019).

2.1.4. Freedom to express normal behaviour

Cattle are social animals and associate with other individuals, possibly creating a synchronization of behaviours (Tobin et al., 2022). Nevertheless, management practices could hinder the expression of normal behaviour, affecting the social interactions among individuals (Stephenson et al., 2017; Sprinkle et al., 2021). The use of PLF technologies improves our ability to identify and categorize normal behaviours in several situations and determine whether environment or management are adversely impacting animals' behaviour (Tobin et al., 2022). The behaviours that can be evaluated to monitor animal activity patterns include lying, walking/swimming, standing and ruminating. Commercially available systems to monitor activity have been developed for most farmed species and rely on image- and sensor-based technologies, other technologies detect agonistic behaviours, as well as social interactions and maternal behaviours in pigs, cattle and poultry (Schillings et al. 2021).

In grazing systems, behaviours can be predicted from traveling velocities calculated from GPS tracking (Augustine and Derner, 2013; Nyamuryekung'e et al., 2020). Through GPS tracking or accelerometers daily activity budgets can be calculated from predicted behaviours, and they can be affected by season (Cheleuitte-Nieves et al., 2020), quality and quantity of forage (Tobin et al., 2021), and physiological status (Werkheiser, 2018). Accelerometers are mostly available for ruminants and are usually attached to the animals' bodies (neck or ear). They allow to monitor behaviour, location, or postures of individual animals such as lying, standing or walking. Particularly, rumination is part of a ruminant's physiological behaviour, and the measurement of rumination activity can be used to identify metabolic disorders and physiological patterns, such as reproduction (Stangaferro et al., 2016). Accelerometers can remotely monitor rumination patterns and may be particularly useful in monitoring grazing livestock (Chang et al., 2022). Abnormal rumination patterns may be symptom of several factors, unusual rumination patterns may be useful for determining conditions when the diet does not allow livestock to express normal behaviour, for example abnormal patterns in dairy cattle may also indicate an excessive or insufficient level of fiber in the diet that livestock are consuming (White et al., 2017).

In sheep, pedigree matchmakers based on RFID tags can be used to identify the maternal pedigree of lambs and to monitor behaviour traits in extensive systems, providing information on potential changes in relationships (Brown et al., 2011; Morris et al., 2012).

Other recently developed technologies can help monitor activity, such as drones in goats (Vayssade et al., 2019) and pigs (Mainau et al., 2009; Thompson et al., 2016). In pigs, depth sensors, 3D cameras and computer vision can monitor tail biting and fighting behaviours (Lee et al., 2016; Chen et al., 2019; Viazzi et al., 2014; D'eath et al., 2018); image analysis can detect excessive mounting (Nasirahmadi et al., 2016); accelerometer data control nest building (Oczak et al., 2015); and video analysis can monitor nursing behaviour (Yang et al., 2019).

In cattle, sensors have been developed to monitor feeding- and drinking-related agonistic behaviour and dominance (Foris et al., 2019), while mounting behaviours and social interactions can be monitored through image-based technologies, such as side-view cameras (Chung et al., 2015; Guo et al., 2019; Guzhva et al., 2016), and accelerometers that can estimate locomotor play in dairy calves (Luu et al., 2013). Local positioning sensor network can also monitor the proximity interactions of individual dairy cows within large herds (Chopra et al., 2020). Moreover, RFID can explore social behaviour such as cow-calf affiliations (Swain and Bishop-Hurley, 2007; Boyland et al., 2013).

2.1.5. Freedom from fear and distress

The management of fear and distress in livestock farming systems is mainly connected to the management of human-animal relationships (Tobin et al., 2022). Reducing the frequency of visual and physical intervention that can impact stockpeople's attitudes and behaviour toward their animals is crucial for an appropriate human animal relationship and to prevent fear and distress of livestock (Schilling et al., 2021). This could be problematic on systems with larger numbers of animals (e.g., intensive livestock farming, poultry, aquaculture or extensive systems). In extensive systems the animals are allowed to roam free and are generally fear- and stress-free (Tobin et al., 2022). However, the interaction with livestock is needed during certain periods, such as pregnancy detection, calving, branding, and weaning (Bailey, 2016). Due to varying management styles and skills, these interactions could cause distress and fear (Grandin, 1997). Interactions between livestock and livestock managers that increase stress and discomfort are detrimental to an animal's welfare and livestock managers' attitudes toward their animals (Tobin et al., 2022). PLF technologies can allow an easier monitoring of larger numbers of animals, also in extensive systems (Rutter, 2014), and can reduce the stress resulting from repeated handling and moving of livestock (Kashiha et al., 2014). Real-time accelerometers may indicate higher activity levels caused by increased anxiety and stress upon the animal (Hibbard, 2012). Moreover, the human animal relationship is an important aspect which can also influences productivity. For example, reduced milk yields can be found in dairy farms where farmers exhibit negative attitudes toward cows during milking routine (Waiblinger et al., 2002). This is one of the reasons for which several automated milking systems (AMS) have been adopted in the last years. Aversive handling also impacts the growth performance of pigs and negative relationships has been described between level of fearfulness toward humans and egg production (Hemsworth and Barnett, 1991; Cransberg et al., 2000). In some cases, the automatic estrus detection can reduce the need for stressful handling (e.g., in pigs) too, potentially addressing negative states such as anxiety or fearfulness. Monitoring changes in heart rate can help livestock managers determine the causes of fearful and distressful situations. In extensive systems, another form of stress experienced by livestock is predation, which almost certainly impacts animal welfare and increases fear and distress of livestock. When experiencing predation, ungulates usually aggregate into larger groups reducing the probability of being preyed upon (Hebblewhite and Pletscher, 2002). The application of sensors such as real-time GPS tracking and accelerometers can help identifying the presence of predators. So, congregation of livestock and reduction in the distances travelled can alert managers and lead

to implement strategies to minimize the impacts of predation (Breck et al., 2011, 2012). Finally, in intensive dairy farms, the use of estrus detection systems and automated milking systems have the potential to help improving the human animal interactions (Hostiou et al., 2017). In particular, the latter reduces the handling of dairy ruminants connected to the milking routine and monitor the performances by daily recording of quantity and quality parameters of milk (Hostiou et al., 2017).

3. Problem statement and thesis objectives

The contribution of PLF to address animal welfare has been questioned in the past by proposing possible threats that PLF itself may pose to farm animal welfare (Tuyttens et al., 2022). Others wondered whether farm animals will be the victim or the beneficiary of the digital revolution (Dawkins, 2021). These authors argue that the true PLF breakthrough regards production efficiency and labor quality of life rather than animal welfare. They concluded that it is still unclear whether PLF technologies that focus on animal welfare will ever be widely adopted commercially or will have the hoped-for beneficial effect for the animals. However, the potential of the PLF technologies to address animal welfare is still promising, since technologies can detect health issues at an early stage and help ensure optimal environmental conditions (Schillings et al., 2021).

The hypothalamus pituitary adrenal axis dysregulation may disrupt various homeostatic mechanisms, suppress the immune system, and inhibit the production of growth and reproductive hormones (Dickens ad Romero 2013; Heim et al., 2000). Despite the extensive literature concerning steroid biomarkers, conflicting results have been reported about the HPA axis functioning at different phases of productive career of farm animals, depending on a number of factors, such as inflammatory and reproductive status, the chronicity of exposure to stressors, and so on. The biological properties observed in one species cannot be straightforward translated to another. Overall, it is believed that the phenotypic response to a stressor can be better described by expressing both steroids simultaneously as glucocorticoid/DHEA ratio, which may be an important indicator of the whole animal response, but a systematic comparative investigation on the biology of these steroids is lacking (Gabai et al., 2020). Furthermore, a more detailed definition of the stress phenotype could be achieved by studying the downstream responses of both glucocorticoids and DHEA (De Almeida et al., 2019). Hence, the present thesis focused on two biomarkers of the HPA functioning, namely cortisol and DHEA(S), during sensitive phases of the productive career of farm animal.

Therefore, the aim of this thesis was to study cortisol and DHEA(S) as biomarkers of the HPA functioning and provide essential evidence for the practical purpose of targeting PLF technologies towards an animal welfare improvement within the modern livestock productive systems. Since the adaptive physiology of animals related to several PLF technologies still need to be elucidated in different phases of their productive career, the rationale behind the

present thesis was deepening the adaptation processes of farm animals in relation to the sensors used in the livestock sector and contribute to fill the existing literature gap regarding the link between PLF and biomarker analysis. The integrative approach between precision livestock farming and endocrinological measurement has been studied in different species of farm animals and across some sensitive phases of their productive career.

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4. Validation of a radioimmunoassay method for cortisol in buffalo milk whey. A preparatory step for future sensor technology

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ABSTRACT

One animal-based method to evaluate welfare is the presence of stress. In dairy ruminants, the responses to stressors include the activation of both neuroendocrine and autonomous nervous system that can be evaluated through an endocrine assessment. The present study aimed to validate a radioimmunoassay method for cortisol in buffalo milk. Three formulations (whole and skimmed milk and whey) and three solvents (methanol diethyl ether and dichloromethane) were tested: methanol was characterised by the best extraction efficiency (69.88%), whey cortisol concentrations showed a significant correlation with whole extracted milk and were not affected by fat content variation during the milking session. The RIA used in the present study showed good precision, sensitivity and specificity: the dilutions test indicated the high reproducibility of the results, overlapping of the dilution curve and standard curve highlighted high specificity and the lack of interfering factors by buffalo whey matrix. It is concluded that the present assay suits the cortisol measurement in buffalo milk and the ranges described could be employed in the calibration of a biosensing technologies directly integrated in milking parlour systems.

HIGHLIGHTS

- Buffalo milk whey revealed to be a matrix of great interest because of its high stability in terms of storage, transportation and processing.
- RIA method suits the cortisol measurement in buffalo milk.
- Ranges described can be employed in the calibration of biosensors for non-invasive assessment of cortisol.

4.1. Introduction

Nowadays, ensuring animal welfare is of utmost importance in the production of animal-derived foods and in particular in dairy industry (Hötzel et al. 2014). Welfare must be assessed considering the animals and their attempt to cope with the environment (Broom 1991). A frequent but accurate welfare assessment requires reliable protocols that can be carried out in a couple of hours (van Eerdenburg et al. 2021), although a 'gold standard' for welfare assessment is still lacking. Many methods were proposed for assessing animal welfare at farm level called as farm based. Among them, the Animal Needs Index score (ANI 35 L) (Bartussek 1999) is focussed on environmental conditions and attributes high and positive scores to pasture. This index has a high repeatability among evaluators and is objective (Amon et al. 2001). Other methods described by Capdeville and Veissier (2001) and Whay et al. (2003) involve the direct observations of animals (animal-based). Thus, environmental and animal-based criteria should be included together in an appropriate index for welfare assessment, as proposed by the Welfare Quality® Consortium (Welfare Quality® 2009).

One of the main parameters that can be recorded to evaluate welfare in animal-based methods is the presence of stress (Broom 2001). Several factors are responsible of stress in dairy ruminants including climatic conditions (De Rensis et al. 2015), management techniques (Canaes et al. 2009; Marsico et al. 2009; Olmos et al. 2009) and animal-related factors such as mammary gland health status (Decarvalho et al. 2009), lactation stage (Trevisi et al. 2009), breed (Negrão 2008), parity number (Van Reenen et al. 2002) and milk yield (Sevi et al. 2001). Responses include behavioural changes, changes to the immune system and activation of both neuroendocrine and autonomous nervous system (Moberg 2000); the latter can be evaluated also through endocrine assessment.

Endocrinological analysis can provide a picture of such responses; in particular, cortisol is considered a useful tool to monitor the response of the hypothalamo-pituitary-adrenocortical axis (HPA) to stress (Sevi 2009; Kirovski et al. 2014). Furthermore, its release over time reflects also the 'allostatic load' of the animal, that is the cumulative effect of experiences in daily life, that involve ordinary events (subtle and long-standing life situations) as well as major challenges (McEwen 2007). Therefore, the evaluation of the allostatic load of animals could be used as a complement to other welfare assessment methods (McEwen 2003).

The radioimmunoassay for steroids used in this study belonged to an in-house method already validated in plasma (Neglia et al. 2012), feathers (Frongia et al. 2020), hair (Peric et al. 2013; Prandi et al. 2018), wool (Peric et al. 2020) and bovine whey (Comin et al. 2005). So far, cortisol in buffalo milk has never been assayed with immunomethods. The RIA assay is the traditional gold standard method for immunoassays (Reimers et al. 1981) as it is sensitive, specific and reproducible.

The present study aimed to validate a reliable radioimmunoassay method to assess cortisol concentration in buffalo milk in order to provide a preliminary data for the calibration of future biosensing technologies for non-invasive assessment of cortisol to be integrated in milking parlour systems.

4.2. Materials and methods

Milk sampling represents a non-invasive procedure and within standard farm practices. All experimental procedures and the care of the animals complied to the Italian legislation on animal care (DL n.116, 27/1/1992) and were approved by the Ethical Committee of the University of Naples 'Federico II' (Protocol number: 25539-2022).

Animals and milk sampling

The study was carried out at a commercial buffalo dairy farm in southern Italy (Campania region, 41°03'40.6''N - 14°02'16.5''E), where a total of 950 buffaloes were bred.

The trial involved 71 randomly selected Italian Mediterranean dairy buffaloes (Bubalus bubalis) with an average weight of 423.02 ± 3.05 kg and an average age of 4.40 ± 0.29 years, with different parity (33 multiparous and 38 primiparous) and days in milk (DIM = 121.52 ± 8.46). They were maintained in pens with a concrete floor and were milked twice daily in the morning and afternoon; the animals were clinically mastitis-free.

In 63 out of the 71 buffaloes, individual milk samples were collected by the mean of sterile falcon tubes (Falcon® 50 mL, Corning Science, Mexico) from the tank of an automatic sampler (MM15 DeLaval) calibrated by breeders' association (Campania region Breeders Association, ARAC) and placed in the milking parlour. Thanks to the automated sampler each individual specimen was representative of the animal's whole milking. After collection, samples were immediately placed into dry ice (-78 °C) and transported to the laboratory where they were stored at -20 °C until lab processing.

An integrative individual milk sampling was performed in the milking parlour on 8 out of the 71 buffaloes. In this case milking was carried out manually by trained professionals and the sampling was performed in two different timeframes: at the beginning and at the end of milking. The samples were collected in sterile falcon tubes (Falcon® 50 mL, Corning Science, Mexico), immediately placed into dry ice (-78 °C) and transported to the laboratory where they were stored at -20 °C until lab processing.

Milk sample preparation for first testing

After thawing, 5 randomly selected milk samples (out of the 63 automatically gathered) were divided into three 5 mL aliquots to be used in analysis as whole milk (first aliquot), skimmed milk (from the second aliquot) and whey (from the third aliquot). The second aliquot of whole

milk was skimmed in tubes centrifugated at 2000 × g for 10 min at 4 °C and the fatty supernatant was removed through a pipette obtaining skimmed milk. The third aliquot was used to obtain whey from whole milk that undergone coagulation procedure. Briefly, 400 mL of rennet (60 g of a commercial compound in 250 mL of ultrapure water) were added to 5mL of whole milk, gently mixed for 3 min at room temperature and incubated at 37 °C for 30 min to complete the clotting process. After incubation, samples were centrifuged at 3500 × g for 10 min at 4 °C and separated from the fat and curd phases supernatant twice.

Whole milk, skimmed milk and whey extraction for first testing

A solvent evaluation was carried out to identify the most efficient one also by the mean of the test on cortisol recovery. Three different solvents were chosen for the extractions: methanol (Sigma-Aldrich, St. Louis, MO, 99.8%), diethyl ether (Sigma-Aldrich, St. Louis, MO, 99.8%) and dichloromethane (Fluka Honeywell, 99.5%). Briefly, to 400 mL of each sample (whole milk, skimmed milk, or whey) obtained as described previously were added 20 mL of antigen tracer (hydrocortisone {cortisol [1,2,6,7-3H (N)]-}, PerkinElmer Life Sciences Inc., Waltham, MA, USA) and kept at 4 °C. After 20min, 5mL of one of the three solvents were added to each tube, mixed for 5 min at room temperature and centrifuged for 15 min at 3500 × g and 4 °C. The vials were then kept at -20 °C, and once frozen the solvent was moved to a tube to dry at 37 °C under an airstream suction hood. The remaining residue was dissolved in 0.5 mL of RIA buffer (0.05M phosphate-buffered saline, pH 7.5, 0.1% BSA).

Milk sample preparation and extraction for second testing

For the second testing the rest of the 63 milk samples collected automatically were divided after thawing in two aliquots to obtain whole milk and whey as described previously. Then, 400 mL of the whole milk and 400 mL of the whey has been extracted adding 5 mL of methanol (Sigma-Aldrich, St. Louis, MO, 99.8%), mixed for 5 min at room temperature and centrifuged for 15 min at 3500 × g and 4 °C. Once having frozen the content of the vials (-20 °C), the solvent was moved to a tube to dry it at 37 °C under an airstream suction hood. The remaining residue was dissolved in 0.5 mL of RIA buffer (0.05M phosphate buffered saline, pH 7.5, 0.1% BSA).

Milk sample preparation for third testing

The milk samples collected manually from the 8 buffaloes at the beginning and the end of milking were curdled after thawing as described previously to obtain whey.

Cortisol analysis by RIA

The concentration of cortisol was measured using a solid-phase microtiter radioimmunoassay (RIA). In brief, a 96-well microtiter plate (OptiPlate; PerkinElmer Life Science, Boston, MA, USA) was coated with goat antirabbit γ-globulin serum diluted 1:1000 in 0.15 mM sodium acetate buffer (pH 9) and incubated overnight at 4 °C. The plate was then washed twice with RIA buffer (pH 7.5) and incubated overnight at 4 °C with 200 mL of the antibody serum diluted at ratios of 1:20,000 for cortisol (Analytical Antibodies, Bologna, Italy). The cross-reactivities of the anti-cortisol antibody with other steroids were as follows: cortisol, 100%; corticosterone, 1.8%; aldosterone, <0.02%. After washing the plate with RIA buffer, the standards (5–200 pg/well), the quality-control extract, the test sample or extract and the tracer (hydrocortisone {cortisol [1,2,6,7-3H (N)]-}, PerkinElmer Life Science, Boston, MA, USA) were added, and the plate was incubated overnight at 4 °C. The bound hormones were separated from the free hormones by decanting and washing the wells in RIA buffer. After the addition of 200 mL of scintillation cocktail (MicroScint-20, PerkinElmer Life Science, Boston, MA, USA), the plate was counted on a β-counter (Top-Count, PerkinElmer Life Science, Boston, MA, USA). Moreover, a 50 mL sample spiked with the tracer and 200 mL of scintillation cocktail were placed in the plate and counted. The extraction recovery value for each unknown well was determined by expressing the count rate for that well as a percentage of the counts added before extraction. The cortisol content of the unknown well was then corrected by the extraction recovery percentage for that well and the cortisol content of the unknown sample expressed in pg/mL.

Cortisol RIA validation tests

Each validation tests involved different pools constituted by five whey samples and were analysed by quintuple. The parallelism test consisted of determining the deviation from the standard curve of a series of whey samples containing known amounts of cortisol, they were prepared by serial dilution of whey samples from animals that showed high concentrations of cortisol. Linear regression was used to determine if whey samples and the standard cortisol curve deviated from parallelism.

The recovery test was conducted to evaluate the system response to an increasing amount of cortisol standard added to a whey sample with low concentration. The percentage of recovery was determined as follows:

[(measured cortisol in spiked sample) / (measured cortisol in non-spiked sample + added cortisol) x 100].

The sensitivity of the curve was as the hormone concentration resulting in a displacement of the labelled hormone at least two standard deviations from maximal binding.

Precision was estimated by repeatedly assaying samples in the inter- and intra-assay and was expressed as the coefficients of variation (CV%).

Statistical analysis

Statistical analyses were carried out using SPSS (28.0) for Windows 10 (SPSS Inc., Chicago, IL). To compare cortisol concentrations between different milk formulations and sampling timeframes Student's t-test was used. Pearson correlation test was performed to assess possible correlations between different milk formulations. Results are expressed as mean ± standard error mean (SEM). A statistically significant difference was accepted at $p < .05$.

4.3. Results

First testing – solvent efficiency

The test on cortisol recovery to evaluate the solvents extraction efficiency gave the following rates when used dichloromethane: 52.03 ± 3.42% on whole milk, 60.71 ± 2.17% on skimmed milk, 60.00±1.75% on whey. Diethyl ether showed a recovery of 54.76 ± 5.69%, 63.03 ± 1.87% and 68.67 ± 1.64% respectively for whole, skimmed and whey. Finally, methanol reported recoveries of 58.89 ± 3.47%, 59.09 ± 4.03% and 69.88 ± 1.43% for whole, skimmed and whey, respectively.

The cortisol concentrations recorded in the three types of samples (whole milk, skimmed milk and whey) with the three solvents tested for hormone extraction are described in Figure 1.

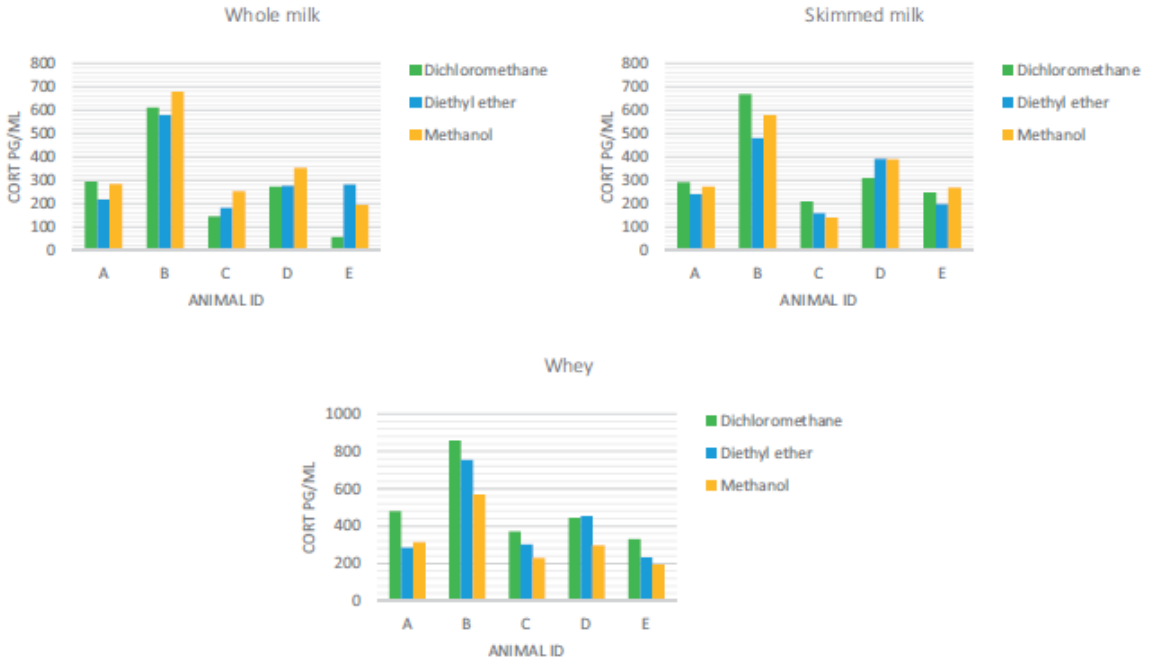

Figure 1. Concentration of cortisol (pg/mL) in five samples (A–E) tested as skimmed milk, whole milk and whey extracted with dichloromethane, diethyl ether and methanol.

Second testing

Based on the first testing results only methanol was chosen as solvent for the subsequent second testing. The cortisol concentrations (pg/mL) of the 63 milk samples that were assayed as whole extracted milk, extracted whey and whey are displayed in Figure 2.

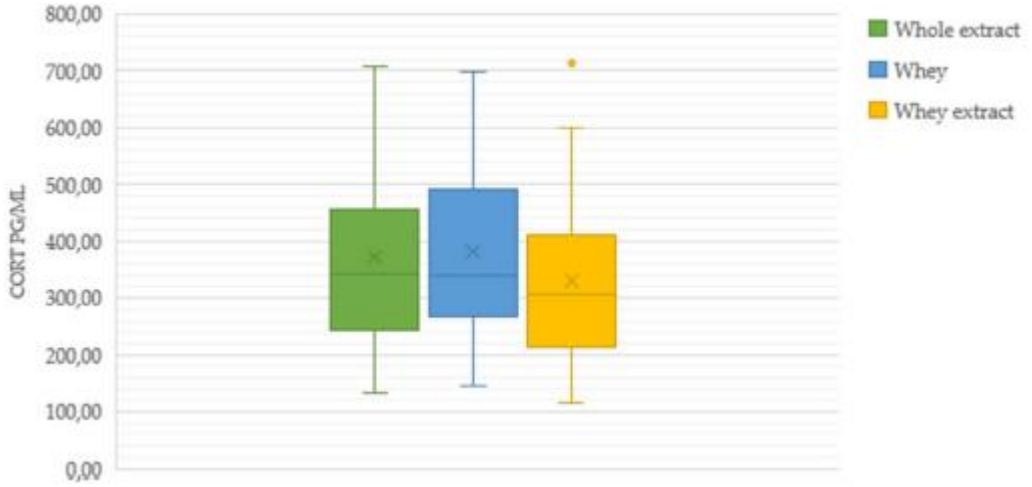

Figure 2. Cortisol concentrations (pg/mL) in 63 milk samples assessed as whole extracted milk, extracted whey and whey. When relevant the extraction was made with methanol.

No significant differences were recorded between the three formulations.

The cortisol concentrations (pg/mL) detected in whey and whole extracted milk showed a significant correlation (p <.001, correlation coefficient + 0.51) (Figure 3).

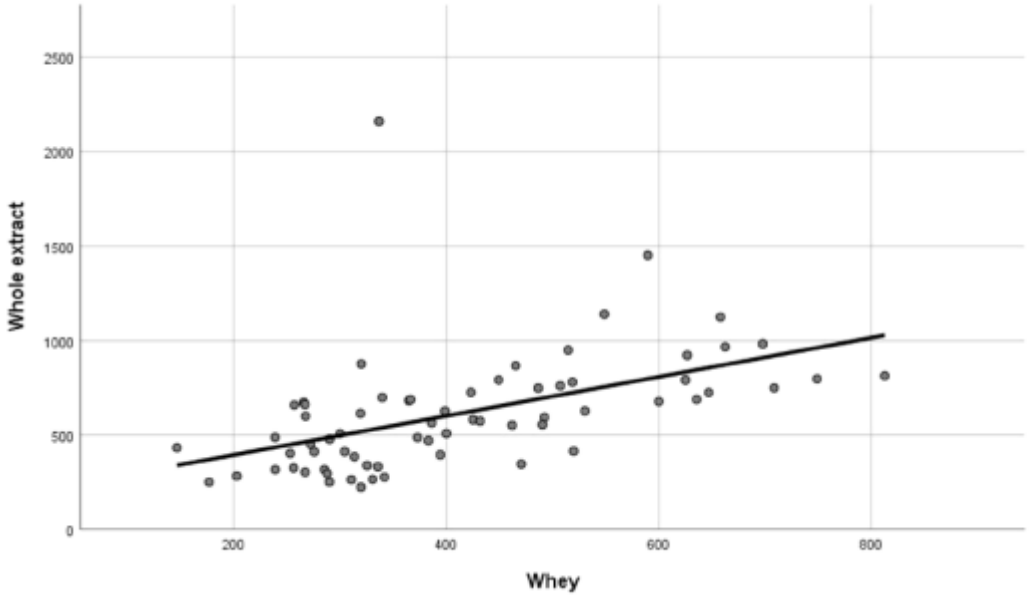

Figure 3. Pearson correlation between cortisol concentrations (pg/mL) of whole extracted milk and whey.

Third testing

The whey cortisol concentrations of milk sampled in the two different timeframes of the milking session are displayed in Figure 4 along with the percentage of cortisol's variation and the difference in whey cortisol concentrations between the beginning and the end of milking.

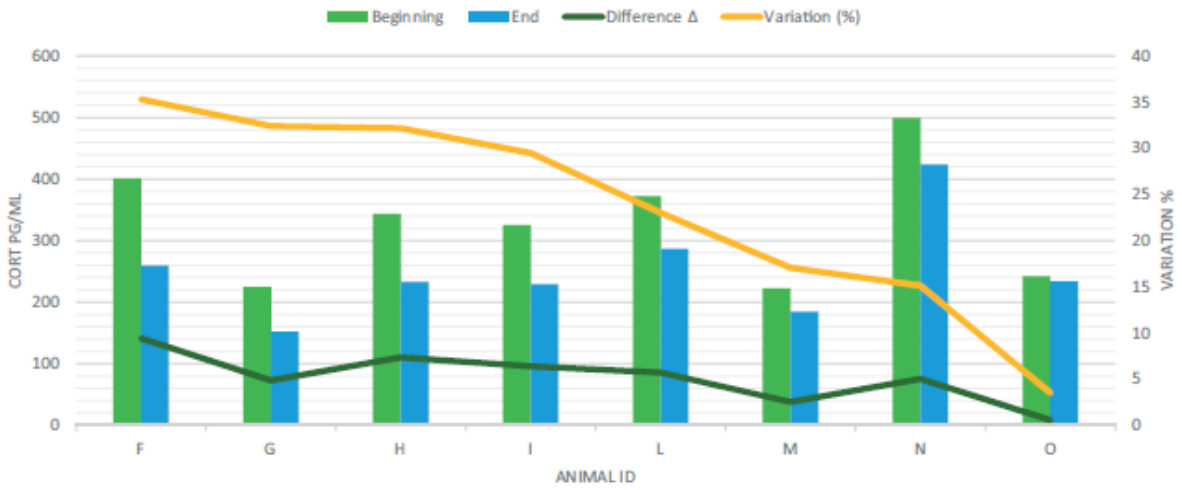

Figure 4. Cortisol concentrations (pg/mL) of whey from milk sampled at the beginning and at the end of milking session. Difference (Δ) between the two sampling times was calculated as Cb - Ce; percentage of cortisol's variation (variation %) was calculated as follows: [(C_b - C_e)/ C_b] * 100. C_b is the whey cortisol concentration at the beginning of the milking and C_e the cortisol at the end of the milking session.

Average values were 328.84 and 250.29 pg/mL, respectively, for beginning and end of milking. Among the two timeframes sampled for milk, whey cortisol concentrations ranged between a minimum of 152.10 (pg/mL, end of milking) and a maximum of 499.20 (pg/mL, beginning of milking). No significant differences were highlighted by comparing the two concentrations (p >.05).

RIA validation

The parallelism between the dilution curves and the standard one indicated that milk cortisol and standard cortisol reacted identically to the antibodies because of the high correlation (r = 0.99) observed between the concentrations obtained and those expected (Figure 5).

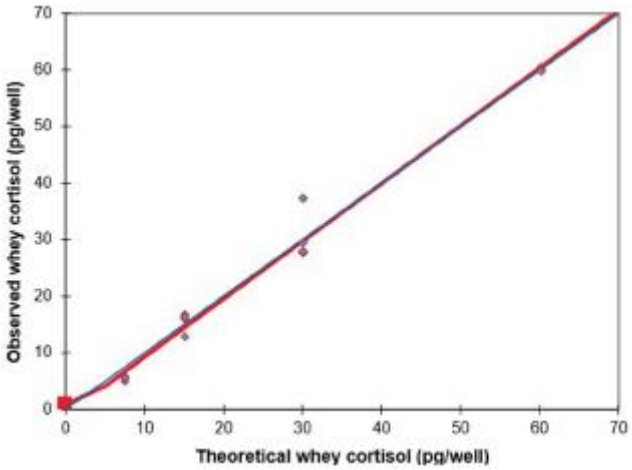

Figure 5. Relationship between theoretical whey cortisol concentration and the observed whey cortisol.

The relationship between whey cortisol concentrations and the standard cortisol curve was given by the equation y = 1.02x - 0.97 (Figure 5).

The recovery test aimed to evaluate the reaction of the system to an increasing amount of cortisol standards and revealed a recovery rate of 104.4 ± 6.1% (mean ± SD).

The assay sensitivity was 16.8 pg/mL. Whey sample, in repeated determinations, showed intra- and interassay coefficients of variation of 7.6 % and 12.7 %, respectively.

4.4. Discussion

The assessment of cortisol concentration in biological samples is one of the main tools to evaluate the stress in animals. E.g. cortisol has been used to study automatic milking systems (Gygax et al. 2006) versus conventional milking parlours, or to investigate the association with animal behaviour (Fukasawa and Tsukada 2010). It is known that steroid hormones can permeate cell membranes and may cross the epithelial blood-milk barrier (Rushen et al. 2008). A high correlation between milk and plasma cortisol concentrations after ACTH administration has been found (Thinh et al. 2011). Considering that milking for dairy cows is a routinary process, milk might represent a non-invasive alternative to blood in HPA axis evaluation (Diaz et al. 2013): it can be sampled without any extra animal manipulation and offers the advantage to monitor hormones concentration directly in milking parlour in line or at the official milk recording.

The purposes of the present study were to validate a radioimmunoassay to assess for the first-time cortisol concentration in buffalo milk. The RIA assay is the traditional gold standard method for immunoassays (Reimers et al. 1981) as it is sensitive, specific and reproducible. It would provide essential evidence, as the physiological cortisol concentrations range of buffalo species is, for the practical purpose of calibrating a sensor able of automatically measuring cortisol in buffalo milk. Moreover, in this study we considered two variables that could interfere with sensor's activity on a farm basis: the milk formulation and the sampling timeframe. Differently from Claycomb and Delwiche (1998) who integrated a rapid enzyme immunoassay (EIA) for progesterone in an on-line sensor, the RIA technology won't be part of the device developed.

According to previous studies performed on bovine milk, three formulations were chosen: whole milk (Butler and Des Bordes 1980), skimmed milk (Gellrich et al. 2015) and whey (Comin et al. 2005).

Two out of three milk formulations showed some criticalities concerning their versatility in sample preparation/using. Great attention had to be paid when pipetting the whole milk samples because of the lipidic film that adhered on the surface of the pipette tips. In the literature it has been reported that milk lipids can be detrimental to give an accurate measurement of cortisol (Butler and Des Bordes 1980). Therefore, it has been hypothesised that the removal of fat would make the pipetting easier without affecting the concentrations of cortisol in milk.

Nevertheless, milk from Italian Mediterranean water buffaloes is characterised by high fat content (approximately 8.00%) and favourable coagulation characteristics (Costa et al. 2020). These features conditioned the skimming procedure, that turn out to be troublesome and time-consuming. Conversely, the whey proved to be a matrix of great interest: coagulation process was effective and timesaving in buffalo milk too and allowed to remove both peptide and lipidic interference. In addition, whey has already been described as a trustworthy milk formulation to measure a steroid such as progesterone in cow milk (Comin et al. 2005). As usual for steroid hormones, to exclude any possible interference due to the matrix components, the analytical measurement can be preceded by an extraction step. The extraction solvent must disrupt the binding of the steroid to protein and must extract the steroid of interest quantitatively and leave behind in the aqueous medium non-specific interfering substances (Makin et al. 2010).

Several extraction protocols have been described in ruminants reporting different procedures. We choose to refer to Hagen et al. (2004) who performed extraction of bovine milk with diethyl ether and Castro-Gómez et al. (2014) who extracted whole milk with a dichloromethane-methanol solution. According to our data, methanol was characterised by the best results based on cortisol recovery to evaluate the solvents extraction efficiency compared to diethyl ether and dichloromethane. Also, visually the samples extracted with methanol formed a homogenous solution after the solvent addition and a clear separation between the two phases after centrifugation. Furthermore, methanol has been already used for steroid extraction in several studies on different matrices (Gleixner and Meyer 1997; Ashley et al. 2011; Palme et al. 2013) providing convenient and efficient extraction. This interesting finding leads to point at methanol as the most reliable solvent for steroid analysis in buffalo milk.

Subsequently, the evaluation of the biological matrix to be used in milk cortisol evaluation has been extended to a larger sample size. We reported the absence of significant differences ($p > .05$) in cortisol concentrations among the formulations, suggesting that all of them can be used in cortisol measurement, but a significant correlation was found between whey and whole extracted milk. In any case, considering that the whole milk was hard to store before analysis and to handle throughout sample preparation, milk whey was chosen as the best matrix to be used because of its high stability in terms of storage, transportation and processing.

Some modifications have been reported in bovine milk composition during milking session (Nielsen et al. 2005), with a marked increment in milk fat concentration at the beginning of milking and a small decrement in non-fat milk solids at the end of the session. The exact reason

for such variation remains unclear, but several theories have been proposed: the progressive filtration of milk fat globule clusters through the mammary ducts, the mechanical shearing of alveolar contractions and decreasing adsorption of fat globule to the alveolar wall during milking (Rico et al. 2014). Moreover, a circadian rhythm of milk fatty acids concentration has been reported and it may be linked to a stratification of the fat content and fatty acids profile of the cistern and alveolar milk (Daly et al. 1993; Rottman et al. 2014). Given that buffalo milk has higher fat concentration than bovine milk and the present study aimed to provide preliminary data for sensor-based technologies, an integrative analysis was performed to study if the high fat percentage of buffalo milk and sampling timeframe could affect measurements (Pope et al. 1976) and to properly describe what should be the best timeframe to be sampled throughout milk ejection.

Our findings revealed that whey cortisol concentrations were not affected by fat content variation during the milking session, showing no significant difference between the concentrations of the two timeframes sampled (p >.05). Moreover, endpoint cortisol concentrations were always lower than the beginning with a variation always less than 0.1 ng/mL. Such evidence seems to suggest that cortisol concentration in whey doesn't significantly change with the increasing amount of fat in buffalo milk.

The RIA analysis method used in the present study showed good precision, sensitivity and specificity also for determining cortisol in buffalo whey. The analysis of cortisol concentrations in buffalo whey resulted to be feasible and reproducible; the dilutions tested indicated the high reproducibility of the results. Overlapping of the dilution curve and standard curve indicates the method's high specificity and the lack of interfering factors by buffalo whey matrix.

4.5. Conclusions

To our knowledge, this is the first study aimed to measure cortisol in buffalo milk matrix.

The measurement of cortisol in buffalo milk has been evaluated step-by-step, approaching the potential interferences and variables given by the peculiarity of buffalo milk itself. This aimed to provide reliable and solid data for the calibration of future biosensing technologies for non-invasive assessment of cortisol to be integrated in milking parlour systems. In case of extraction, a more time-consuming procedure, it has been demonstrated the necessity to work with a suitable solvent for buffalo milk, but the results have also shown that it is possible to opt for a more versatile matrix such as whey without losing in quality of the data obtained in assay. The RIA method revealed good sensitivity and specificity for measuring cortisol in buffalo milk whey, the present assay suits the cortisol measurement in buffalo milk and the ranges described can be employed in the calibration of a biosensing method for non-invasive assessment of cortisol directly integrated in milking parlour systems.

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Ethical approval

All experimental procedures and the care of the animals complied to the Italian legislation on animal care (DL n.116, 27/1/1992) and were approved by the Ethical Committee of the University of Naples ‘Federico II’ (Protocol number: 25539-2022).

Disclosure statement

No potential conflict of interest was reported by the author(s).

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(Innovative technologies for smart farming in the buffalo sector). Sportello Agrifood F/200090/01-03/X45, CUP B61B20000570005.

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5. Cortisol concentrations in different matrices: predictive potential and relationship with productive level, lactation stage and parity in dairy buffaloes

ABSTRACT

Cortisol is the primary biomarker associated with activation of the hypothalamic-pituitary adrenal (HPA) axis. Plasma and milk cortisol concentrations provide short term evaluations about the HPA axis activity (Sgorlon et al., 2015). Hair is used for the assessment of retrospective levels of cortisol (Sharma et al., 2019). This research aimed at studying the relationship of cortisol in blood, milk, whey and hair with parity, lactation stage and productive classes in four media and to study their predictive potential. Multiparous (n = 30) and primiparous (n = 38) buffaloes were used and assigned to 4 productive classes, and cortisol concentrations were measured using a in house radioimmunoassay method. Parity did not show a significant effect on cortisol concentrations of the four media. The catabolic stage of lactation (up to 90 DIM (days in milk)) was characterized by higher cortisol concentrations compared to the second anabolic (beyond 150 DIM) in both milk formulations. The plasmatic concentrations of cortisol were higher at the catabolic and the first anabolic stage (91 to 150 DIM) compared to the second anabolic ($P = 0.022$ and $P = 0.009$ respectively). Buffaloes beyond 150 DIM differed from those below 90 DIM ($p < 0.001$) and between 91 and 150 DIM ($p < 0.05$) in hair. Finally, hair cortisol concentrations were negatively correlated to mature equivalent milk yield (EMY), mature equivalent protein content (EPC) ($p < 0.01$) and mature equivalent ECM (eECM) ($p < 0.05$). The whey showed a potential to predict the concentrations of cortisol in whole extracted milk ($R^2 = 0.31$).

HIGHLIGHTS

- The lactation stage significantly influences plasma, hair and milk cortisol concentrations.
- Hair cortisol concentrations are negatively correlated to milk quality parameters.
- The whey is a potential predictor of the cortisol concentrations in whole extracted milk.

KEYWORDS: Dairy buffaloes; lactation stage; cortisol concentration; parity; Milk yield

5.1. Introduction

Nowadays, animal welfare in dairy farms remains a major concern, in order to protect livestock from poor conditions and improve the quality of animal derived food. Therefore, it is imperative to interpret their behavior, as well as their cognitive needs and capacities (Nawroth et al., 2019) to both encounter their requirements and reduce stressful situations. Stress is an animal condition resulting from the action of one or more stressors of either external or internal origin (Bova et al., 2014). Several physiological and pathological conditions can be responsible for an alteration of the homeostasis in the animals. Parity and stage of lactation are physiological factors, as such they can influence metabolic profile (Kuczyńska et al., 2021), milk yield and somatic cell count (Sabek et al., 2021), and acid base balance (Walter at al., 2022) of dairy cattle.

The lactation stage and the period around calving are characterized by physiological processes that cows enact to adapt to the new lactation, such as reduction of immune competence, negative energy balance, hypocalcemia, inflammatory responses, and oxidative stress (Trevisi and Minuti, 2018).

Similarly, milk yield may represent a physiological burden in dairy ruminants and may be associated with the hypothalamic-pituitary-adrenal (HPA) axis (Otten et al., 2023). As a matter of fact, milk production challenges the metabolism and health of dairy cows (Gross and Bruckmaier, 2019) since complex adaptation processes take place to enable the maintenance of the animals' energy and nutrient homeostasis to meet the requirements for the metabolically prioritized mammary gland in early lactation (Drackley, 1999; Ingvartsen, 2006; van Knegsel et al., 2014). Consequently, metabolic stress arises with various effects on the immune system, reproductive performance, milk yield, product quality, and the overall well-being of the dairy cow (Drackley et al., 2005; Bradford et al., 2015; Bruckmaier and Gross, 2017).

The glucocorticoid hormone cortisol, due to its multifaceted role in the physiological stress response, is the primary physiological biomarker for many phenotypic changes in animals associated with activation of the HPA axis (Hellhammer et al., 2009). A typical stress response pattern begins with a pulse of ACTH in response to the stimulus, hereafter it accesses the adrenal cortex and promote glucocorticoid synthesis and release. The timing of both ACTH and corticosterone responses are dependent on stressor modality and intensity (Herman et al., 2016).

In dairy ruminants, conflicting results have been reported about the influence of parity and lactation stage on cortisol concentrations. Some authors (Wu et al., 2019) reported that days in milk and parity do not affect serum cortisol concentration in Holstein cows, whereas Saqib et al. (2022) concluded that lactation numbers and days in milk have an impact on blood cortisol in buffaloes. Other studies carried out in dairy cattle (Fukasawa et al., 2008; Gellrich et al., 2015) and goats (Diaz et al., 2013) demonstrated that both parity and days in milk affect milk cortisol levels, but no clear effects are recorded on hair, since contrasting results have been reported (Burnett et al., 2014; 2015). In Italian Mediterranean buffalo parity and stage of lactation strongly affect milk yield and quality (Costa et al., 2020), response to THI (Matera et al., 2022) and milk electrical conductivity (Matera et al., 2022).

Plasma cortisol concentrations have been widely used to evaluate acute responses to stressful stimuli (Kovács et al., 2021). However, the process of blood sampling is accompanied by additional stress, which can affect the test results due to the stress response subsequent to immobilization and handling. Therefore, milk has been suggested as an alternative to blood for dairy ruminants (Pošćić et al., 2017) and the protocol for assessing cortisol has been recently validated also in buffalo (Cotticelli et al., 2022). Both these biological fluids provide short term evaluation about the HPA axis activity (with a lag time) (Sgorlon et al., 2015). On the other hand, hair analysis can be used for the assessment of long-term retrospective levels of cortisol, since it is non-invasive and has a long-time lag for changes (Sharma et al., 2019).

The aim of this study was to evaluate the influence of parity, stage of lactation and productive levels on cortisol concentrations in several matrices sampled in Italian Mediterranean buffaloes and to study which one could show a promising predictive potential.

5.2. Materials and methods

All the experimental procedures and the care of the animals complied to the Italian legislation on animal care (DL n.116, 27/1/1992) and received the approval of the Ethical Committee of the University of Naples "Federico II" (Protocol number: 25539-2022).

Animals and sampling procedures

The Italian Mediterranean dairy buffaloes (*Bubalus bubalis*) used in the present study were located in a commercial dairy farm in southern Italy (Campania region, 41°03'40.6"N - 14°02'16.5"E). Multiparous (n = 30) and primiparous (n = 38) buffaloes (4.34 ± 0.26 years old, mean ± standard error) were kept in pens with concrete floor and were routinely milked twice daily (morning and afternoon). At beginning of the trial an area of 10 cm^2 in the prescapular region was shaved in all the animals. After 50 days, three substrates (blood, milk and regrown hair) were collected concurrently on each animal, at different lactation stage and productive level (see statistical analysis section),. Milk quality traits included milk yield (as daily yield (kg)) fat, protein, lactose content of milk (expressed as daily percentage), somatic cells count (SCC), mature equivalent milk yield (EMY), mature equivalent fat content (EFC) and mature equivalent protein content (EPC) (expressed as kg/lactation, Tris and Buttazioni, 1990).
SCC was log-transformed into somatic cell score (SCS) using the following formula (Ali and Shook 1980):

$$SCS = \log_2\left(\frac{\mathrm{SCC}}{100}\right) + 3$$

Energy corrected milk (ECM = 740 kcal) was calculated according to the formula from Campanile et al. (1998):

$$ECM = milk\ yield\ \times \{[fat\ (g/kg) - 40 + protein\ (g/kg) - 31] \times 0.01155\}\ +\ 1$$

The same formula was adapted for the calculation of mature equivalent energy corrected milk (eECM):

$$eECM = dEMY\ \times \{[EFC\ (g/kg) - 40 + EPC\ (g/kg) - 31] \times 0.01155\}\ +\ 1$$

Where:
EFC and EPC are percentages, calculated by dividing mature equivalent fat and protein contents (kg/lactation) by mature equivalent milk yield (kg/lactation);

dEMY is the daily mature equivalent milk yield, calculated by dividing mature equivalent milk yield (kg/lactation) by 270 (standard lactation length (days) of buffalo cows).

Biological samples collection

Sterile falcon tubes (Falcon® 50 ml, Corning Science, Mexico) were used to collect individual milk samples from the at-line sampler (MM15 DeLaval). It automatically collects a representative sample of milk from an individual buffalo during milking. So, each individual specimen was representative of the whole milking as previously described (Cotticelli et al., 2022). After collection, samples were immediately placed into dry ice (-78 °C) and transported to the laboratory where they were stored at -20°C until lab processing.
Blood (10 mL) was collected from the mammary vein into vacutainer tubes (lithium heparin anticoagulant) puncture. Samples were centrifuged at 1500 x g for 15 minutes and the plasma was aliquoted into Eppendorf tubes (1 mL) and stored at -20°C.

Hair was obtained from the scapular region of the buffalo using a razorblade. The concentration of steroids in hair provides an integrated history of secretion during the preceding 3 months approximately (Meyer and Novak, 2012; Russel et al., 2012; Caslini et al., 2016). The animals were shaved 50 days prior the substrates collection and reshaved to collect regrown hair (Meyer and Novak 2012). Therefore, the samples represented the integrated steroids concentrations for December 2020 – January 2021.

Samples processing and analysis

Milk processing

After thawing, two aliquots of whole milk were constituted for each specimen. The first was extracted as per Cotticelli et al. (2022). In brief, 5 mL of methanol (Sigma-Aldrich, St. Louis, MO, 99.8 %) were added to 400 µl of sample, mixed for 5 min at room temperature and centrifuged for 15 min at 3500 RPM and 4 °C. The vials were frozen, and the solvent was moved to a tube to dry at 37 °C under an airstream suction hood. The residue was dissolved in 0.5 ml of RIA buffer (0.05 M phosphate-buffered saline, pH 7.5, 0.1% BSA).
The second aliquot was processed to obtain the whey. The coagulation procedure was exactly as per Cotticelli et al. (2022). Briefly, rennet (400 µl) was added to whole milk (5 mL), mixed, and incubated at 37 °C for 30 min. Samples were then centrifuged at 3500 x g for 10 min at 4 °C and fat and curd phases were separated twice.

Plasma processing

Plasma samples were aliquoted (0.25 mL) in a glass vial, extracted with 5 mL of ≥ 99.8% diethyl ether (Sigma-Aldrich St. Louis, MO), centrifuged at 1500 x g for 5 minutes and incubated at -20 °C for 18 h. Next, the liquid in the vial was dried at 37 °C under an airstream suction hood. The remaining residue was dissolved in 0.5 mL of phosphate-buffered saline (PBS), 0.05 M, pH 7.5.

Hair processing

Hair samples were prepared for cortisol assay as per Peric et al. (2022). In brief, approximately 60 mg of trimmed hair were washed twice in 3 mL isopropanol (Sigma -Aldrich, St. Louis, MO) and extracted in a glass vial with 3 mL of methanol (Sigma-Aldrich, St. Louis, MO). The vials were incubated at 37 °C for 18 h and then evaporated to dryness at 37 °C under an airstream suction hood. The remaining residue was dissolved in 0.60 mL of phosphate-buffered saline (PBS), 0.05 M, pH 7.5.

Cortisol radioimmunoassay

The cortisol concentrations were measured in whole extracted milk, whey, hair and plasma using the in-house radioimmunoassay (RIA) method (Cotticelli et al., 2022; Peric et al., 2022). The cross-reactivities of the anti-cortisol antibody with other steroids were as follows: cortisol, 100%; corticosterone, 1.8%; aldosterone, <0.02%. After washing the plate with RIA buffer, the standards (5–200 pg/well), the quality-control extract, the test sample or extract and the tracer (hydrocortisone {cortisol [1,2,6,7-3H (N)]-}, Perkin-Elmer Life Science, Boston, MA, USA) were added, and the plate was incubated overnight at 4 °C. The bound hormones were separated from the free hormones by decanting and washing the wells in RIA buffer. After the addition of 200 µl of scintillation cocktail (MicroScint-20, Perkin-Elmer Life Science, Boston, MA, USA, the plate was counted on a β-counter (Top-Count, Perkin-Elmer Life Science, Boston, MA, USA).

Statistical analysis

Statistical analyses were carried out using SPSS (29.0.1.0) for Windows 10 (SPSS Inc., Chicago, IL). According to the lactation stage, buffaloes were assigned to three classes (Matera et al., 2021): class C (catabolic phase of lactation, between 60 and 90 DIM, n = 23), class A1 (first anabolic phase, from 91 to 150 DIM, n = 19), and class A2 (second anabolic phase,

beyond 150 DIM; n = 26). Furthermore, animals were divided according to their productive levels (4 classes), based on percentiles of EMY (kg) and eECM (Table 1).

Table 1. Descriptive statistic of the 4 classes of productive levels according to percentiles of EMY (mature equivalent milk yield) and eECM (mature equivalent ECM).

Class	EMY (kg/die)	eECM
1	8,944	15,851
2	10,015	17,569
3	11,089	19,739
4	14,185	24,345

The normal distribution of data was verified using the Shapiro-Wilk test. Possible correlations between cortisol concentrations of the four matrices and mature equivalent milk parameters were studied by using Spearman's correlation. Cortisol concentrations between the two milk formulations were compared using Wilcoxon signed-ranks test for related samples The Kruskal–Wallis test was used to compare the cortisol concentrations of the four matrices between lactation stages, productive levels and parities. The predictive potential of the matrices was tested by including whey, whole extracted milk and plasmatic cortisol concentrations in a linear regression model (prior log-transformation). A statistically significant difference was accepted at $P < 0.05$, and tendency was discussed at $p < 0.10$. Unless otherwise stated data are medians ± standard deviations.

5.3. Results

The days in milk and the age of the dairy buffaloes used in the present study alongside the quality and quantity parameters of milk recorded are showed in Table 1.

Table 2. Descriptive statistic of the buffaloes enrolled in the trial, and quality and quantity parameters of milk recorded throughout the trial. Data are means ± SE.

	Mean	**Standard error**
Parity	1.971	0.183
DIM (days)	119.574	8.580
Milk yield (l/day)	8.513	0.399
Fat content (%)	9.683	0.479
Protein content (%)	3.960	0.156
ECM	14.855	1.062
Lactose (%)	3.933	0.174
SCS	2.949	0.176
Mature equivalent milk yield (kg/lactation)	2,732.436	60.603
Mature equivalent fat content (kg/lactation)	253.436	5.616
Mature equivalent protein content (kg/lactation)	119.909	2.541
Mature equivalent ECM	17.792	0.368

The distribution of the concentrations of cortisol in the four matrices analyzed in the present study is presented in table 2.

Table 3. Descriptive statistic of cortisol concentrations of the four matrices.

Matrix	**Median**	**Standard error**
Whole extracted milk (pg/ml)	338.53	22.18
Whey (pg/ml)	340.65	19.74
Plasma (ng/ml)	4.06	0.50
Hair (pg/mg)	2.05	0.09

Parity did not show any significant effect on the cortisol concentrations of the four matrices, considering all animals, with similar values in whey, whole extracted milk, hair and plasma ($p > 0.10$). Similarly, no differences were recorded in each lactation phase.

The analysis of the stage of lactation (Figure 1) on cortisol concentrations (pg/ml) in whole extracted milk revealed that significantly ($p < 0.05$) higher levels were recorded during the catabolic compared to second anabolic phase in whole extracted milk. A similar trend was also observed in whey, although in this case the values did not reach the statistical significance ($p < 0.10$).

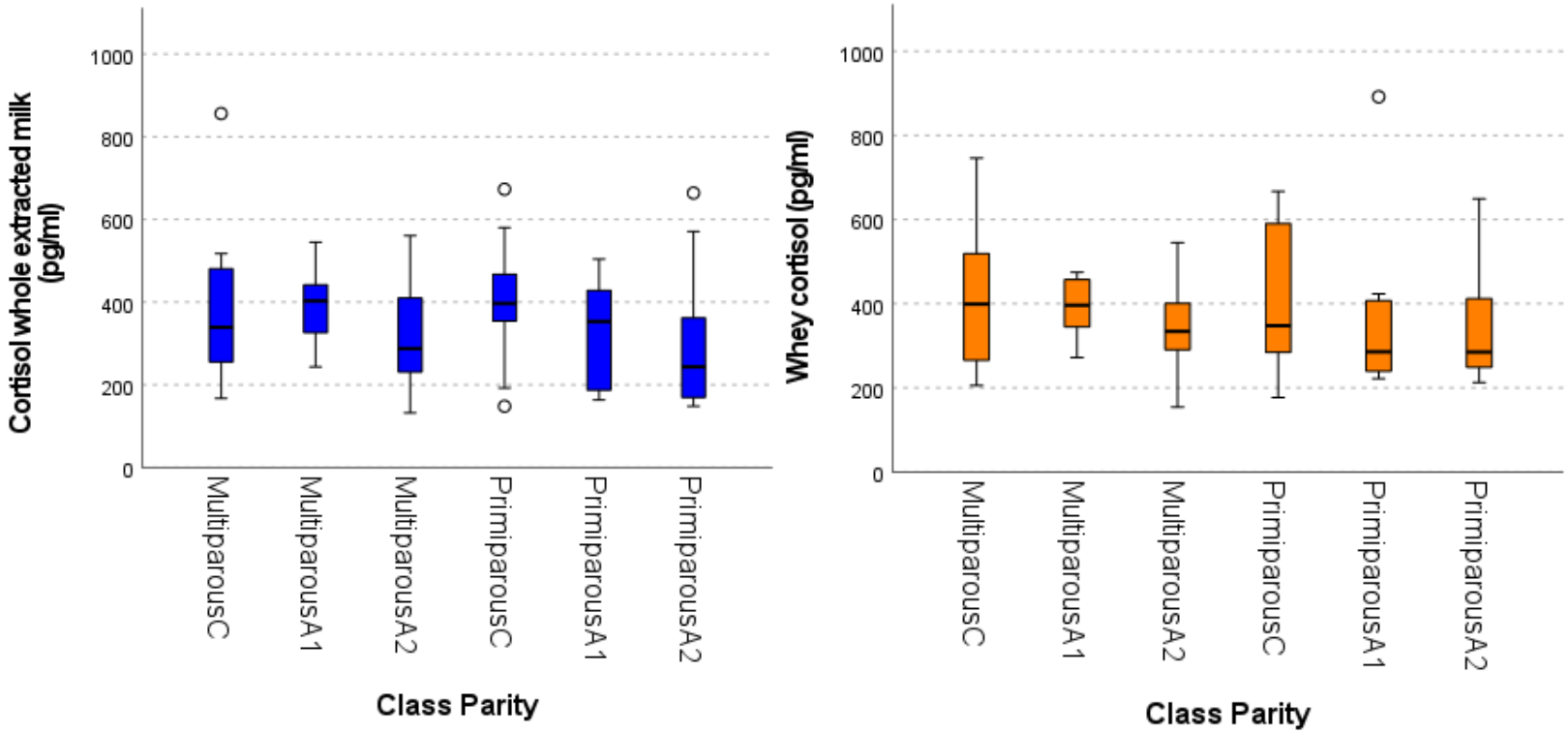

Figure 1. Cortisol concentrations (pg/ml) of 68 milk samples according to parity and stage of lactation.

In general, cortisol concentrations (pg/ml) did not differ ($p > 0.10$) between whey and whole extracted milk (Figure 2) with median values of 340.65 and 338.53 pg/ml for whey and whole extracted milk, respectively.

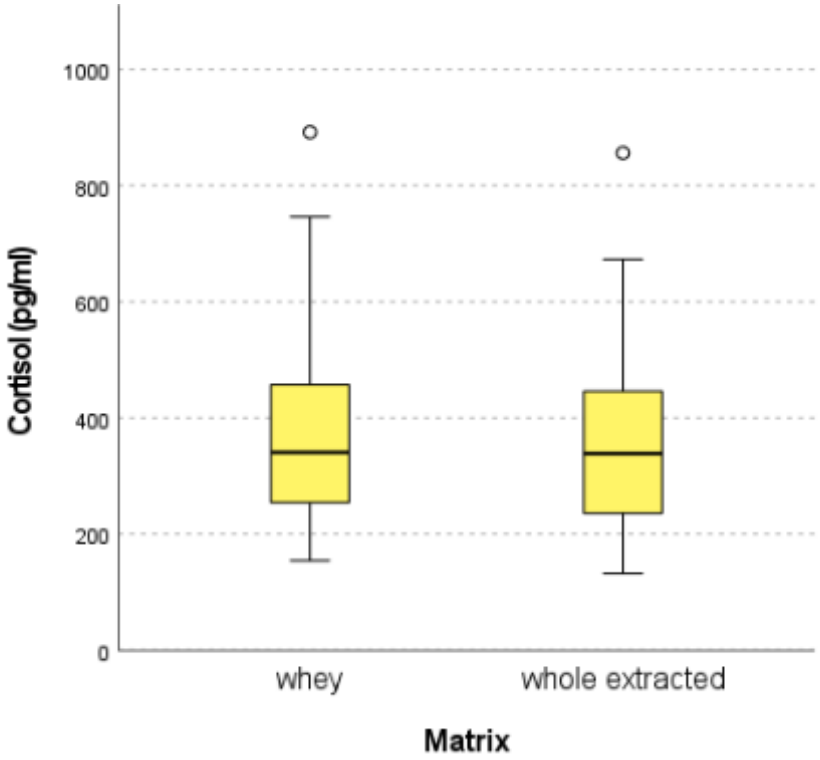

Figure 2. Comparison between whey and whole extracted milk cortisol concentrations (pg/ml)

Plasma cortisol concentrations were higher during the catabolic and the first anabolic stage compared to the second anabolic ($P = 0.022$ and $P = 0.009$, respectively). The lactation stage had a significant effect on plasma and hair cortisol concentrations (Figure 3). The three lactation phases differed significantly in hair, with the second anabolic stage that differed both from the catabolic ($p < 0.001$) and the first anabolic stage ($p < 0.05$), similarly these last two tended to differ ($p = 0.051$).

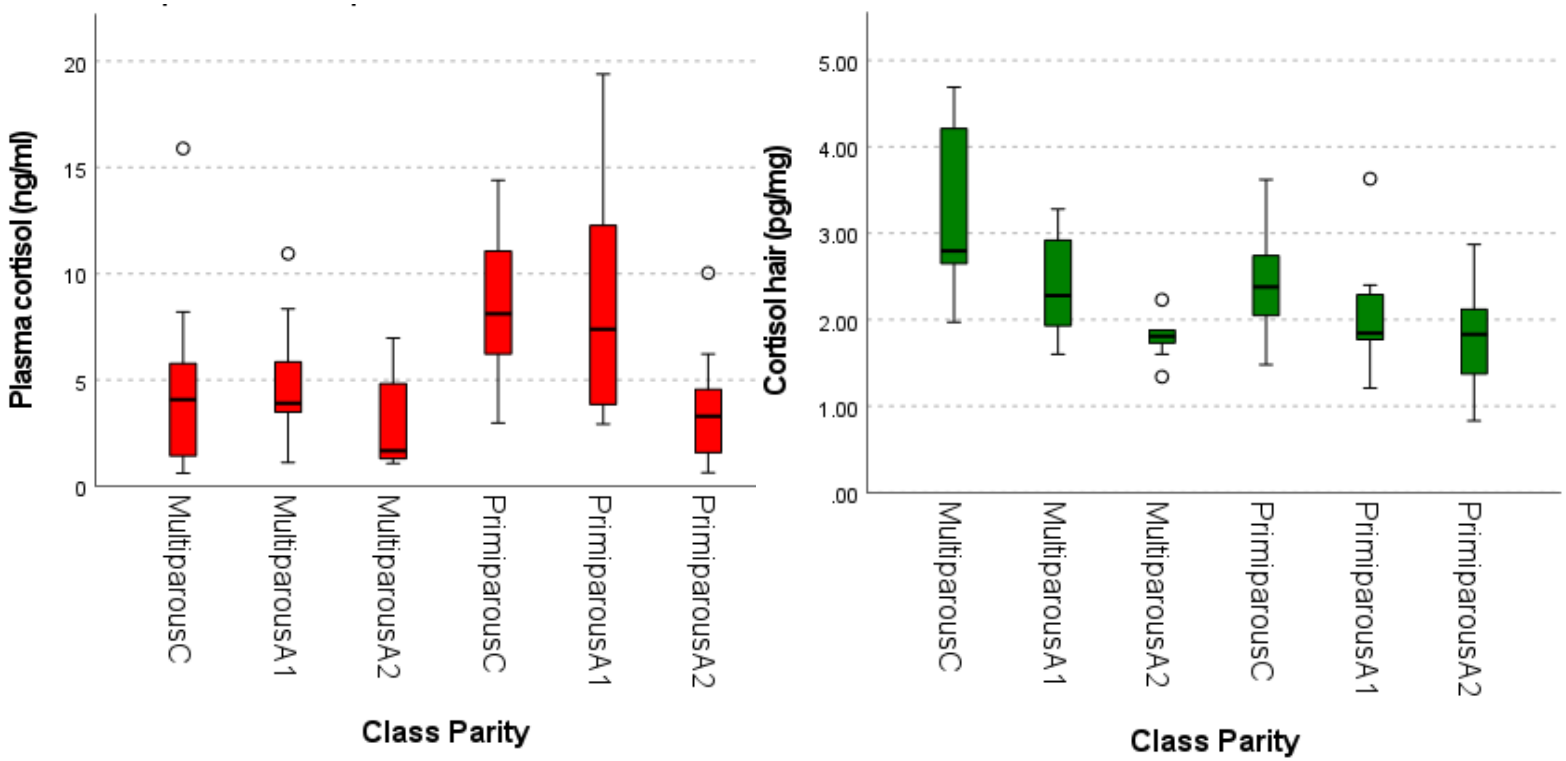

Figure 3. Cortisol concentrations in hair (pg/mg) and plasma (ng/ml) according to parity and stage of lactation.

The concentrations of cortisol of the four matrices were analyzed according to productive levels of buffaloes and are presented in Figure 4. The medians showed a repeatable pattern among the four matrices, although the comparison tended to be significant only in whole extracted milk between the first two productive classes (294.18 and 455.14 pg/ml, for classes 1 and 2 respectively, $p < 0.10$).

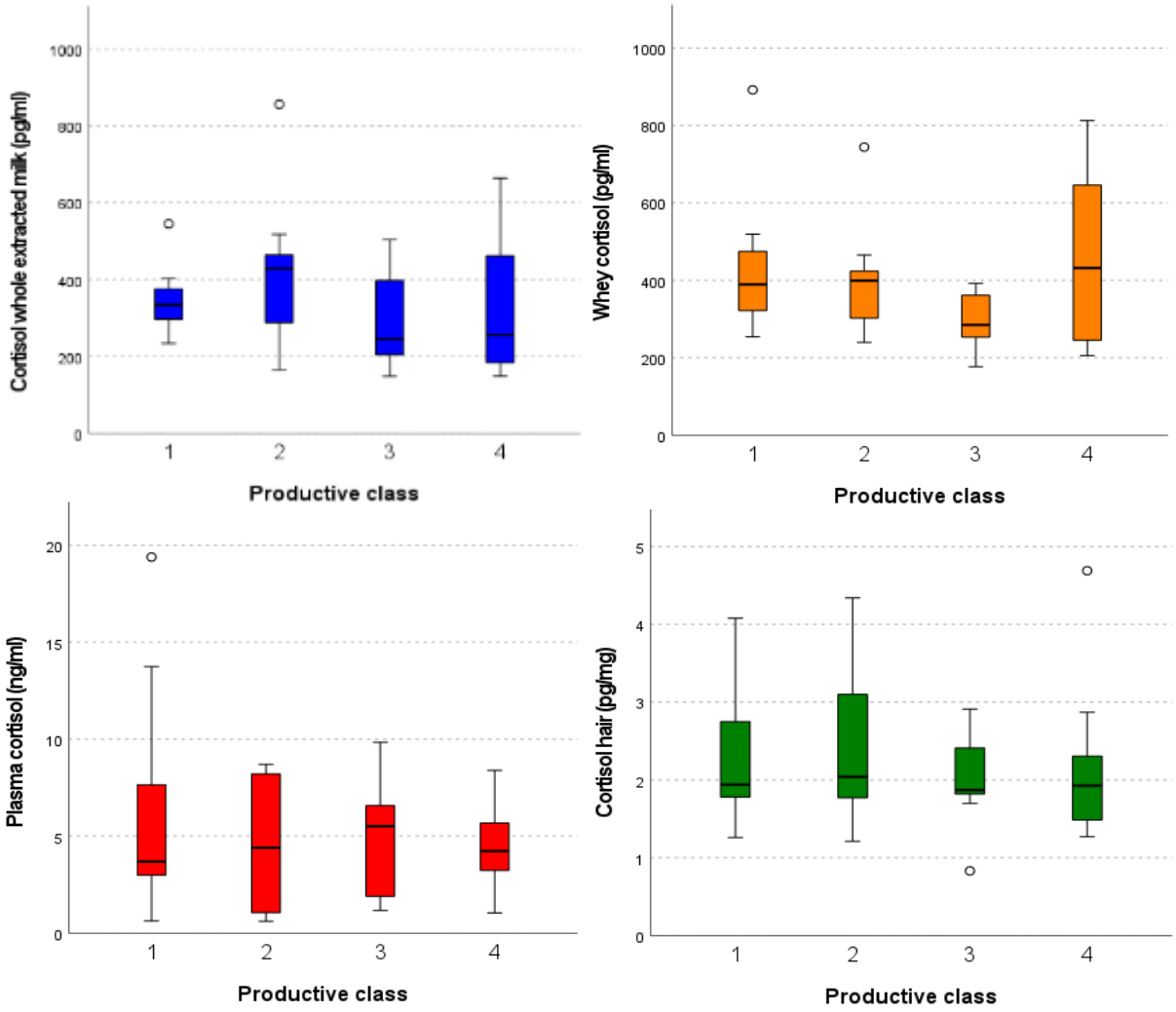

Figure 4. Cortisol concentrations of whole extracted milk (pg/ml), whey (pg/ml), plasma (ng/ml), and hair (pg/mg) sorted by productive classes (1 – 4).

The Spearman's correlation showed that hair cortisol concentrations were negatively correlated with EMY (ρ = - 0.358; $p < 0.01$), EPC (ρ = - 0.347; $p < 0.01$) and eECM (ρ = - 0.288; $p < 0.05$), and a tendency was observed with EFC (ρ = - 0.247; $p = 0.069$). Similarly, a negative correlation tended to be significant between whey cortisol concentrations and EFC (ρ = - 0.229; $p < 0.10$).

Table 4. Correlations between the cortisol concentrations of the 4 matrices and the mature equivalent milk parameters (EMY, EFC, EPC, eECM).

Milk parameter	Spearman	Cortisol whole extracted (pg/ml)	Cortisol whey (pg/ml)	Cort plasma (ng/ml)	Cort hair (pg/mg)
EMY (kg/lactation)	rho	-0,093	-0,097	-0,115	-0,358
	p	0,500	0,483	0,417	0,007
EFC (kg/lactation)	rho	-0,182	-0,229	-0,064	-0,247
	p	0,183	0,096	0,653	0,069
EPC (kg/lactation)	rho	-0,091	-0,152	-0,118	-0,347
	p	0,507	0,273	0,406	0,009
eECM	rho	-0,142	-0,187	-0,088	-0,288
	p	0,301	0,176	0,534	0,033

Furthermore, significant positive correlations were recorded between whey cortisol concentrations and both plasma cortisol ($\rho = 0.229$, $p < 0.10$) and whole extracted milk ($\rho = 0.631$, $p < 0.001$), and between these last two ($\rho = 0.298$, $p < 0.05$), suggesting the potential of the two milk formulations to predict plasmatic concentrations of cortisol in buffalo. Therefore, milk concentrations were tested in a linear regression model (Figure 5).

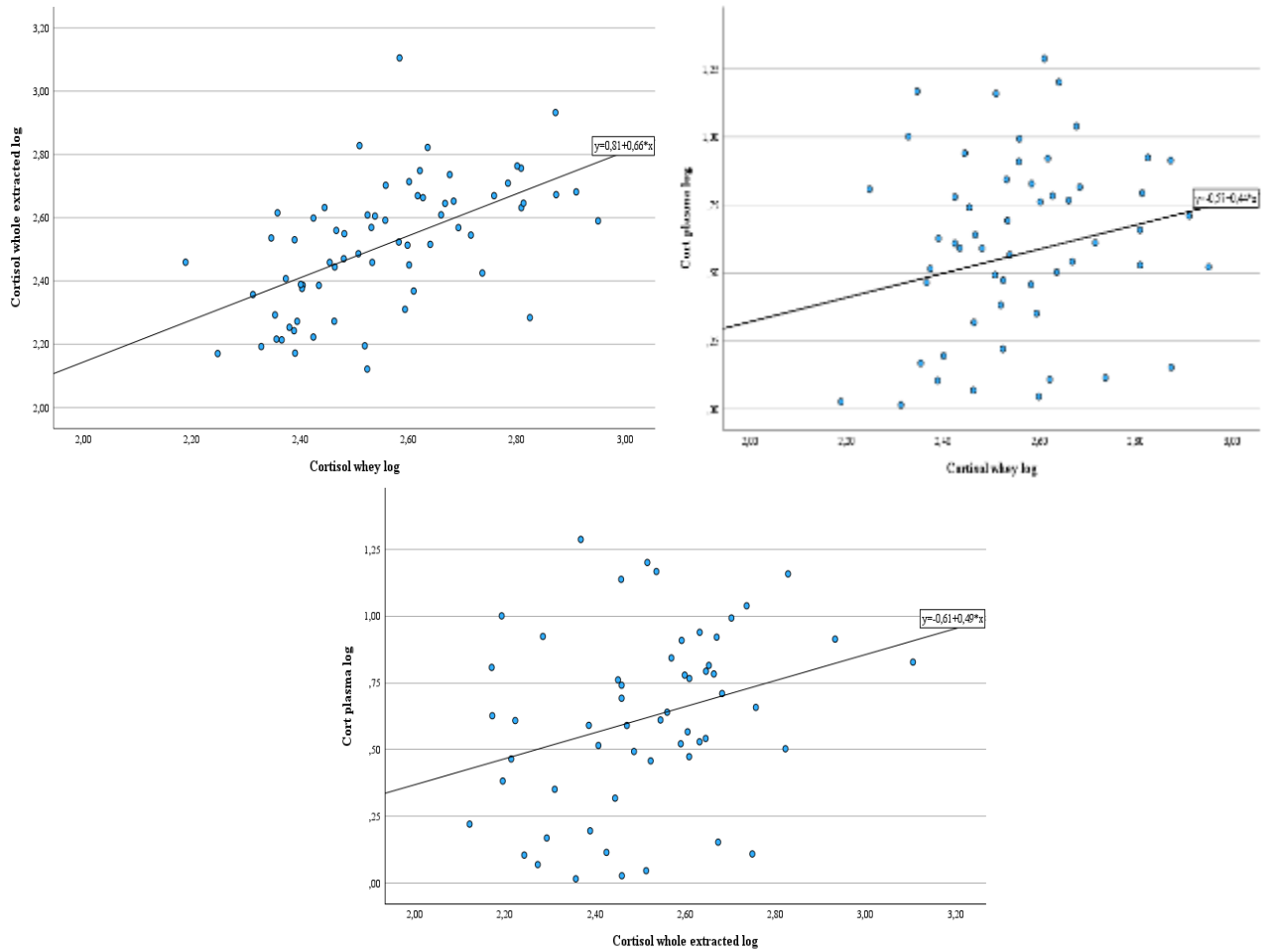

Figure 5. Cortisol concentrations of whole extracted milk (pg/ml) (left panel) and plasma (ng/ml) (right panel) regressed on whey (pg/ml) and of plasma (ng/ml) regressed on whole extracted milk (below panel) (pg/ml) in a linear model. The whole extracted milk, whey and plasma cortisol concentrations were log-transformed and included as independent and dependent variables, respectively.

5.4. Discussion

The objective assessment of animal welfare is one of the challenges that livestock systems are actually obliged to face with. Cortisol evaluation may represent a simple, reliable and cheap parameter that could be considered in this sense, since this hormone may be assessed in several matrices with different significance: in fact, the measurement of cortisol concentration in milk and blood is an indicator of responses to acute stress, while its concentration in hair is used to evaluate the retrospective condition of the animals. In the present research, we aimed at studying both the relationship of concentrations of cortisol with parity, lactation stage and productive levels in Italian Mediterranean buffaloes and to evaluate physiological levels in different matrices.

Few studies are reported in literature on cortisol levels in buffalo species. In particular, cortisol has been investigated in milk (Cotticelli et al., 2022), whereas the impact of parity and lactation stage on its concentrations was studied on plasma in Pakistani buffaloes (Saqib et al., 2022). However, to our knowledge, no information is present by using different matrices from the same animals. We used two formulations of buffalo milk that proved to be highly correlated in a previous study (Cotticelli et al., 2022). A significant positive correlation was observed also in the present study, and cortisol concentrations didn't differ ($p > 0.05$) between the two formulations. It confirms the stability, the reliability and the reproducibility of whey and whole extracted milk for the measurement of cortisol by radioimmunoassay.

In contrast, as specified above, cortisol in hair samples reflects the hormone concentration over a prolonged period of time (Heimbürge et al., 2018). Dairy cattle hair grows approximately 0.6 to 1 cm per month, with a complete molt every three months (Schwertl et al., 2003). So, hair specimen reflects cortisol levels from a period of approximately three months.

Therefore, the parity was expected to exert an effect on cortisol concentrations depending on the matrix. As reported by Siewert et al. (2019), primiparous animals must deal with the milking management practices for the first time and are less used to milking practices. In addition, it is largely known that primiparous cows may be more affected from stress than multiparous cows, especially during the transition period (Burnett et al., 2014). Nevertheless, previous studies reported conflicting results even within the same species, arguing that more physiological stress parameters are needed to evaluate the parity effect (Ferreira et al., 2021).

In the present study, whole extracted and whey cortisol levels were similar between primiparous and multiparous buffaloes. Contrasting results are present in literature. In dairy cattle, some authors (Fukasawa et al., 2008) reported no differences according to parity, whereas others (Gellrich et al., 2015) highlighted that milk cortisol tends ($p = 0.07$) to be lower in second parity animals in comparison with those with higher parity. The concentrations of cortisol described in the present study were higher than those reported for dairy cattle and no differences were observed regarding the parity. Therefore, it's possible to suggest that the thresholds considered for the stress-response of the bovine species should not be directly transferred to buffaloes, and the analysis of the effect of parity should be adapted accordingly. It is worth pointing out that a higher variability was observed in the stress-response of primiparous buffaloes compared to multiparous, since they highlighted a more asymmetrical distribution of whey cortisol concentrations.

. Our results are consistent with a recent study, that reported no significant effect of parity on plasma concentrations of cortisol in Nellore cows (Ferreira et al., 2021); on the contrary Saqib et al. (2022) compared dairy buffaloes of first, second and third parity and reported a declining trend of serum cortisol concentrations with the increasing lactation number.

The parity effect was proved to be not significant in hair of Holstein dairy cows by some authors (Burnett et al., 2014), although the same authors in a subsequent study (Burnett et al., 2015) reported a more pronounced effect of parity by including only animals with absence of clinical diseases. In this case, multiparous cows showed greater concentrations of cortisol than primiparous counterparts. As mentioned above, hair cortisol concentration represents a long-term window (till 3 months) for retrospectively analyzing animal stress: it is likely that other effects can influence this aspect and further studies are needed to better understand the significance of this matrix.

The stage of lactation largely influenced cortisol concentrations in all matrices. It is known that after calving buffaloes undergo several physiological changes, including, but not limiting, negative energy balance, uterine involution and ovarian cyclic activity resumption, (Campanile et al. 2006; Campanile et al., 2010). All these conditions represent stressful events for the animals that may respond to increasing cortisol levels. As expected, our results showed an increase of cortisol concentration in animals in the first stages of lactation in almost all matrices considered. Buffaloes were characterized by higher cortisol concentrations during the catabolic phase of lactation (within 90 DIM) compared to the second anabolic phase (beyond 150 DIM)

in both whey ($p < 0.10$) and whole extracted milk ($p < 0.05$). Our results agree with both Fukasawa et al. (2008) and with Diaz et al. (2013), who reported higher values of milk cortisol in early lactation than in mid and late lactation in Holstein cows and in Murciano-Granadina goats, respectively.

Plasma cortisol concentration was higher in buffaloes between 50 and 90 DIM compared to other groups, as reported by Saqib et al. (2022) in dairy buffaloes. Also in this case, different plasma cortisol levels were recorded during post-partum period, although this study was focused on the transition period and only animals within 56 days from calving were recruited.

Similarly, the lactation stage significantly affected hair cortisol concentrations. Our results are consistent with those reported by Burnett et al. (2015) for Holstein dairy cows, even if their experimental design compared hair cortisol within 126 days from calving and with the trend described by Endo et al. (2017) who reported highest levels of hair cortisol at 60 – 90 days after parturition and a subsequent decrement in Brown Swiss cross-bred and Holstein cows.

The interrelationships between productive levels and cortisol concentrations have been investigated in dairy cattle with contrasting results (Otten et al., 2023; Tallo-Parra et al., 2018). In our study, the relationship between cortisol levels and productive parameters were further examined by correlating the values of the four matrices to the mature equivalent milk parameters. Mature equivalent parameters are lactation records that have been adjusted for age at freshening, frequency of milking and season of the year at calving. They estimate how much a cow would have produced if she were of a mature age, calved during an average month, and were milked twice a day.

The negative correlations between the hair cortisol, EMY, EPC and eECM and between whey cortisol and EFC seem to reveal a detrimental effect of the cortisol on both quality and quantity parameters of buffalo milk. Our results are consistent with the higher hair cortisol observed in cows with lower milk yield reported by Burnett et al. (2015) and Tallo-Parra et al. (2018). On the contrary a positive correlation between hair cortisol and milk yield was reported by other authors (Braun et al., 2022). Hence, it is suggested that more research is needed to deepen the effect of productive level on cortisol concentrations of dairy buffaloes.

To pursue the final aim of the study, the cortisol concentrations of the two milk formulations were included in a regression model, since they showed high correlation coefficients with plasma. Our results suggest that the whey and the whole extracted milk could be further investigated as potential predictor of the concentrations of cortisol of other matrices. If the

predictive potential of the whey on the whole extracted milk would be confirmed, it could serve the purpose of avoiding the extraction process that is necessary for measuring cortisol concentrations in whole milk and is both expensive and time-consuming. Also, predicting the plasma cortisol concentrations by milk could help sparing the animals the blood sampling, that is always invasive and stressful.

5.5. Conclusions

This study aimed to analyze for the first time the effect of parity, lactation stage and productive level on cortisol concentrations of dairy buffaloes in four matrices. In fact, these factors are major concerns in animal welfare and occasionally can represent prolonged stressors throughout the productive career of dairy animals.

According to our results, it can be concluded that the lactation stage showed a comparable influence on cortisol concentrations across the four media, and it was also consistent with the results previously reported in other ruminants. On the contrary, further studies are needed to get conclusions about the effects of parity and of productive level on cortisol concentrations in dairy buffaloes. Finally, milk seems to have predictive potential to estimate cortisol levels in buffalo and may be used as parameter for stress determination.

Ethical approval

All experimental procedures and the care of the animals complied to the Italian legislation on animal care (DL n.116, 27/1/1992) and were approved by the Ethical Committee of the University of Naples "Federico II" (Protocol number: 25539-2022).

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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6. Postnatal and postweaning endocrine setting in dairy calves through hair cortisol, dehydroepiandrosterone and dehydroepiandrosterone sulphate

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Importance of the work: The care of calves on dairy farms between birth and weaning can improve their long-term development and growth. In fact, a poor newborn health status and a high allostatic load may adversely affect development in dairy cows. To determine cortisol, dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulphate (DHEA-S) individually is useful for an understanding of the individual state, being biomarkers of hypothalamic-pituitary-adrenal (HPA) axis activity.

Objectives: As a preliminary study, to investigate the hair concentrations of cortisol, DHEA, DHEA-S and their ratios in dairy calves in two key periods of their growth characterized by considerable environmental changes.

Materials & Methods: Hair sampling was conducted on clinically healthy dairy calves during the postnatal period at age 64.8 ± 0.65 d (POP; mean ± standard error; n = 73) and during the postweaning period at age 155.3 ± 0.85 d (PWP, n = 62). The hair hormone concentrations were measured using a radioimmunoassay.

Results: Hair cortisol concentrations were higher in the POP than in the PWP. Furthermore, the cortisol:DHEA and cortisol:DHEA-S ratios were higher in the first period of evaluation, showing a higher animal allostatic load at birth.

Main finding: Identification was achieved non-invasively of calves with a high allostatic load through biomarkers of HPA axis activity. The evaluation of this activity is very important given

its influence on many biological processes, such as energy balance, development of the reproductive system and immune response.

6.1. Introduction

The care of newborn animals on dairy farms in the time between birth and weaning can improve the long-term development and growth of calves. In fact, a poor newborn health status, together with other factors, such as improper handling or inadequate facilities and feeding, may adversely affect the development of dairy cows (Bazeley et al., 2016). In high-production dairy farms, the calves are separated from their mothers within 24 h of birth (Broom and Leaver, 1978). The allostatic load related to the separation procedure from the mother and the perinatal period can expose the newborn calf to environmental and social stressors that act simultaneously and, at times, in a sequential way (Enriquez et al., 2011). These can alter homeostasis, thus compromising the immune defenses and, potentially, lead to a poor health status, which can negatively influence the animal's future productive life (Weary et al., 2008; Van de Stroet et al., 2016). These environmental changes and stressors in their various forms, durations and intensities during the postnatal period can induce dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and epigenetic alterations (Lee and Sawa, 2014; Burns et al., 2018). This can lead to a consequent impairment of the biorhythms and a hormetic response of steroid hormones (Chung et al., 2011), resulting in pathological conditions (Nicolaides et al., 2014). The endpoint of the HPA axis is cortisol, which regulates many physiological processes, such as energy balance, the development of the reproductive system and immune and stress responses (Hill and McEwen, 2010).

Cortisol has long been considered a marker of the allostatic load, which is correlated with the body's attempt to adapt to environmental changes (Mormède et al., 2007; Burnett et al., 2014). Cortisol is usually determined in blood samples (Cook et al., 2000; Negrao et al., 2004), saliva (Negrao et al., 2004), urine (Hay and Mormède, 1998), faeces (Möstl and Palme, 2002) and milk (Verkerk et al., 1998). In these matrices, the concentrations of this steroid hormone reflect punctual changes in the circadian rhythm, the diet or stress before sampling. The advantage of using hair is that it provides an integrated measure of hormone concentrations over medium and long periods (Meyer and Novak, 2012), it can be simply and non-invasively collected and it does not require any special storage (Wright et al., 2018).

Dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulphate (DHEA-S) are hormones related to resilience and the allostatic load (Charney, 2004; Whitham et al., 2020). They are primarily secreted by the zona reticularis of the adrenal glands and act as prohormones for sexual steroids in both males and females. They also have anti-glucocorticoid qualities,

presumably as competitive inhibitors of cortisol (Hazeldine et al., 2010). These steroid hormones act on multiple levels, playing a role in immune system activation and they have anti-inflammatory effects, antioxidant properties and are involved in lipid metabolism. In humans, Charney (2004) reported positive associations between plasma DHEA concentrations and adaptation to extreme stress. Russo et al. (2012) indicated a possible relationship between a high concentration of DHEA and positive coping. Because of the opposing effects of DHEA and cortisol, a common measure applied to test the impact of both hormones simultaneously is the ratio between them (Hechter et al., 1997; Goodyer et al., 1998; Qiao et al., 2017). Studies in lame cows demonstrated a decrease in serum DHEA and a higher cortisol:DHEA ratio compared to clinically healthy cows (Almeida et al., 2008). In humans (Shen et al., 2009a; Chen et al., 2013; Gao et al., 2013), cows (Peric et al., 2017), horses (Placci et al., 2020), pigs (Trevisan et al., 2017; Bergamin et al., 2019) and guinea pigs (Shen et al., 2009b), the DHEA hair sample assay has been investigated, but, to date, no study has been published on hair DHEA concentrations in calves. In fact, DHEA-S in calf hair was first described by Probo et al. (2021).

Currently, the emphasis on efficiency, sustainability, welfare and production quality in livestock breeding is leading to new approaches in animal management and monitoring. Among the latter, sensor-based technology is expected to play a pivotal role. Nevertheless, in spite of the latest scientific advancements, there remains a gap between the technological measurement of signals, behaviors and the processes of physiological adaptation of farm animals (Neethirajan, 2020). Hence, “traditional” determination of hormone concentrations remains a valid manner for measuring the physiological status of farm animals; additionally, the new approach relies on the use of specimens non-invasively collected.

Thus, the current study aimed to test the hypothesis that cortisol, DHEA and DHEA-S hair concentrations are different in the perinatal period and in the postweaning period at age 5 mth, when weaning and adaptation to extrauterine life must be already accomplished. Finding differences between them would be important to evaluate variations in the allostatic load and resilience in this species using a non-punctual and non-invasively collected specimen, such as hair, which is not influenced by acute HPA axis stimulations.

6.2. Materials and Methods

Ethics statements

Although hair sampling is a non-invasive and unproblematic procedure, the study was carried out in accordance with EU Directive 2010/63/EU (2010) on the protection of animals used for scientific purposes and Italian legislation on animal care (DL n. 26, 04/03/2014) (2014).

Animals

The trial was conducted with 142 Italian Friesian female calves reared on one farm with an intensive dairy production system. Within 3 h of birth, the colostrum was administered and calves were transferred to individual pens where they remained until aged 7 d; they were fed with bulk milk. After this period, the animals were moved to a first multiple box (with an area of 800 m2) in which they were kept until weaning. The nutrition planning included the controlled administration of bulk milk (through automatic suckles that have the ability to recognize individual animals) supplemented by commercial, pre-weaning, concentrated pellets offered ad libitum. The weaning of the calves took place when they were aged between 63 d and 80 d. Then, the weaned calves were transferred to a second multiple box (with an area of 800 m2) in which they were fed with hay ad libitum and with a concentrate composed of 0.6 kg of corn and 0.4 kg of soybean meal. The diets were formulated to meet the nutritional requirements of animals in agreement with the standards stipulated by Institut National de la Recherche Agronomique (2010). All animals were in good condition as verified by the official veterinarian, except for seven traumatized calves (TRA) that, remained blocked in the feed fence for a few hours due to a mechanical problem following the move from the first to the second box.

Hair sampling and animal weighing

White hair samples were collected from the scapular region of 73 calves randomly selected during the postnatal period at weaning (POP; aged 64.8 ± 0.65 d; mean ± SE) and during the postweaning period, both from healthy calves (PWP; aged 155.3 ± 0.85 d, n = 62) and from TRA (aged 158.9 ± 2.58 d, n = 7). The hair segments in which cortisol is incorporated require time to emerge because the hair cells that take up the hormones are part of the hair follicles beneath the skin. On the other hand, this kind of cells start to capture systemic hormonal concentrations soon after the hair follicle has been developed in the fetus (Kapoor et al., 2016). Considering both the lag time in hair availability above the skin level and the intrauterine

development of hair, the first hair sample collected in this study represented the cortisol concentrations characterizing the fetal period and approximately the first 45–50 d after birth.

The second hair sample was taken at about age 155 d, which corresponded to the hormone concentrations characterizing the postweaning period because approximately 4 mth are required for a complete change of hair; during shedding, almost all mature hairs are lost from the skin follicles (Hayman and Nay, 1961).

The hair samples were collected from the withers by shaving close to the skin using an electric razor. This area was chosen as the cleanest and most easily accessible. The hair samples were stored in paper envelopes in the dark at room temperature until the end of the study. Each calf was weighed at birth and before hair collection.

Hair hormonal assay

The hair strands were washed and extracted, as described in Peric et al. (2013). The concentrations of hair cortisol (Peric et al., 2013), DHEA and DHEA-S (Probo et al., 2021) were measured using a solid-phase microtiter radioimmunoassay (RIA). For DHEA, a 96-well microtiter plate (Optiplate; Perkin-Elmer Life Science; Boston, MA, USA) was coated with goat anti-rabbit γ-globulin serum diluted to 1:1,000 in 0.15 mM sodium acetate buffer, pH 9 and the plate was incubated overnight at 4 °C. Then, the plate was washed twice with RIA buffer, pH 7.5 and incubated overnight at 4 °C with 200 μL of the anti-hormone serum diluted to 1:2,000 for DHEA. The rabbit anti-DHEA antibody used was obtained from Sigma-Aldrich (St. Louis, Missouri, USA) and showed the following cross-reactions: DHEA, 100%; pregnenolone, 0.1%; androstenediol, 0.08%; dihydrotestosterone, 0.05%; sulphate DHEA, 0.02%; testosterone, < 0.01%; 5α-androstane-diol-3β,3 α, < 0.01%; 5β-androstane-3α, < 0.01%; estradiol, < 0.01%; progesterone, < 0.01%; estrone, < 0.01%; and cholesterol, < 0.01%. After washing the plate with RIA buffer, standards (5–200 pg/well), a quality control extract, the test extracts and tracer (DHEA; Perkin-Elmer Life Science; specific activity: 70.5 Ci/mmol, 15 pg/well) were added in duplicate and the plate was incubated overnight at 4 °C. The bound hormone was separated from the free hormone by decanting and washing the wells in RIA buffer. After the addition of 200 μL of scintillation cocktail, the plate was counted on a β-counter (Top-Count; Perkin-Elmer Life Science; Boston, MA, USA).

For DHEA, the intra- and inter-assay coefficient of variation (CV) were 4.3 and 10.1%, respectively. The detection limit of the assay, as calculated using the software Riasmart (Perkin- Elmer Life Science; Boston, MA, USA), was 8.2 pg/mL. To determine parallelism

between DHEA standards and endogenous DHEA in bovine, hair samples containing high concentrations of endogenous DHEA were serially diluted in 0.05 M phosphate-buffered saline (PBS), pH 7.5. There was a linear relationship between the hair DHEA concentrations and the standard DHEA curve, determined through linear regression, with a correlation coefficient (r) of 0.99 and the model was described by the equation $y = 0.838x + 1.812$. The recovery test was conducted to evaluate the system response to an increasing amount of DHEA standard added to a hair extract with low DHEA. The percentage of recovery was determined as: [(measured DHEA in spiked sample) / (measured DHEA in non-spiked sample + DHEA added) × 100]. The recovery test revealed a mean (± SD) recovery rate of

97.3 ± 4.8%.

Statistical analysis

The statistical analysis was carried out using R software version 3.4.1, (R Core Team, 2017). Data were analyzed using a boxplot and their normal distribution was evaluated using the Shapiro-Wilk test. The probability density functions to assess the hormone distribution were obtained with the package fitdistrplus (Delignette-Muller and Dutang, 2015). The density functions of normal, log-normal, logistic, exponential and gamma were tested. The most appropriate density functions were selected based on the Akaike Information Criterion (AIC; Burnham and Anderson, 2010).

The differences in hormone concentrations between groups of animals were analyzed using the Kruskal-Wallis test; for the multicomparisons, the Mann-Whitney test was applied. This procedure is the nonparametric analog to one-way ANOVA followed by Fisher's LSD as a post-hoc test (Lin and Haseman, 1977).

6.3. Results

Boxplots of DHEA, cortisol and DHEA-S are shown in Figs. 1–3, respectively.

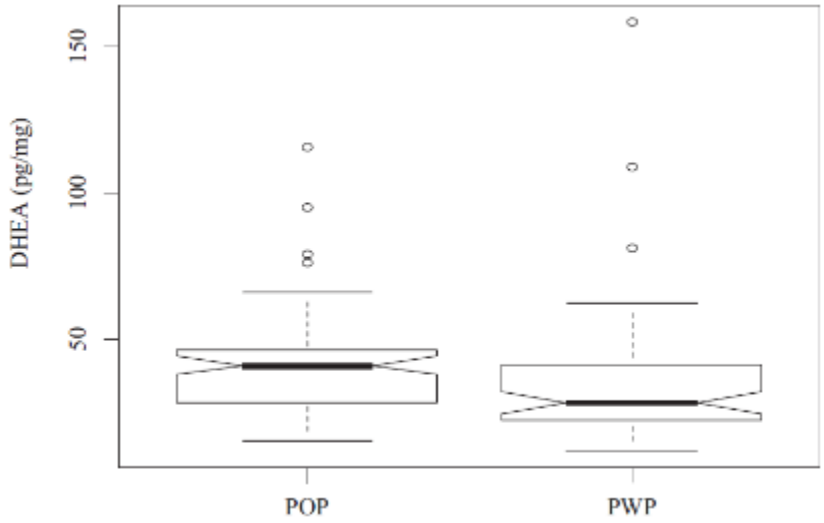

Fig. 1 Hair DHEA concentrations in postnatal (POP) and postweaning (PWP) periods of calves

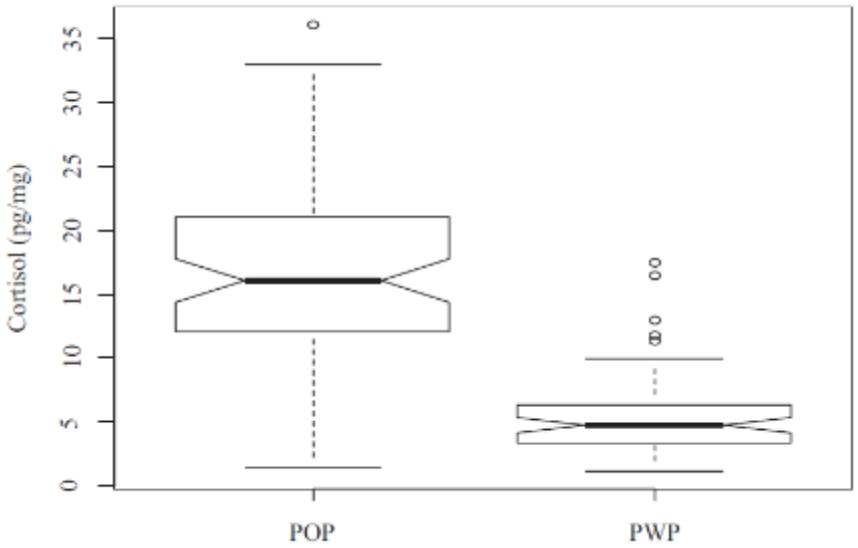

Fig. 2 Hair cortisol concentrations in postnatal (POP) and postweaning (PWP) periods of calves

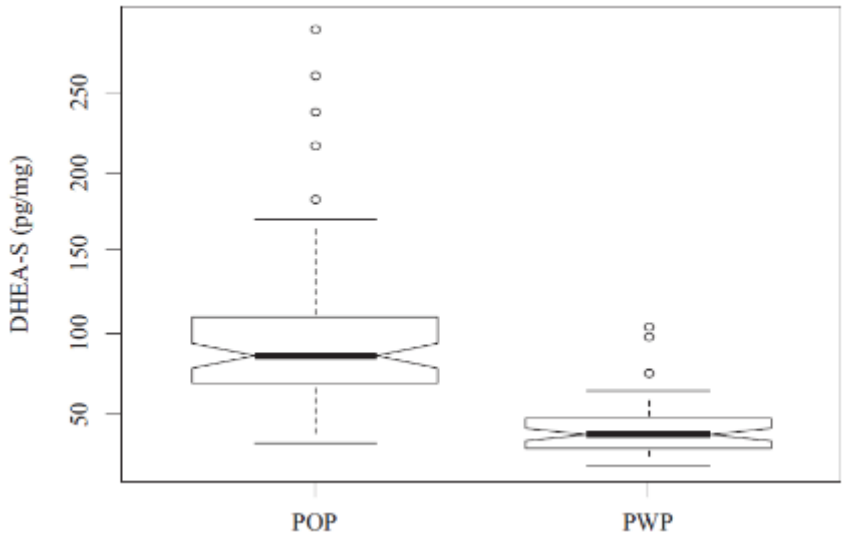

Fig. 3 Hair DHEA-S concentrations in postnatal (POP) and postweaning (PWP) periods of calves

The hair cortisol concentrations recorded in the POP had a normal distribution; however, the logistic distribution can be considered to have substantial support since the ΔAIC was lower than 2 (ΔAIC = 1.02; Burnham and Anderson, 2004). Conversely, hair cortisol concentrations recorded in the PWP had a log-normal distribution. Values regarding both distributions are described in Table 1.

Hair DHEA and DHEA-S concentrations followed log- normal distributions both in the POP and the PWP. Values regarding DHEA and DHEA-S distributions are described in Table 2 and Table 3, respectively.

The average weights of the animals at birth, POP, PWP and TRA hair sampling were 37.6 ± 0.36 kg (n = 142), 89.1 ± 1.04 kg (n = 73) and 176.7 ± 1.52 kg (n = 62) and 179.4 ± 3.50 kg (n = 7), respectively (data not reported in Tables). The average daily gains from birth to sample collection were 0.797 ± 0.011 kg/d, 0.895 ± 0.005 kg/d and 0.881 ± 0.007 kg/d for the POP, PWP and TRA, respectively (data not reported in Tables). Interestingly, the average daily gain of the PWP was similar to that of the TRA ($p > 0.05$; data not reported in Tables). The comparisons between hair hormones in POP, PWP and TRA are reported in Table 4.

Table 1 Best fitting probability density functions to hair cortisol distribution in postnatal and postweaning periods of calves

Function	Parameter 1 (± SE)	Parameter 2 (± SE)	AIC
Postnatal period			
Normal	Mean: 16.89 (0.85)	SD: 7.26 (0.60)	500.57
Logistic	Location: 16.62 (0.84)	Scale: 4.14 (0.40)	501.99
Gamma	Shape: 4.36 (0.70)	Rate: 0.26 (0.04)	504.53
Log-normal	Mean: 2.71 (0.06)	SD: 0.55 (0.05)	518.02
Exponential	Rate: 0.0592 (0.0069)	-	560.66
Postweaning period			
Log-normal	Mean: 1.58 (0.06)	SD: 0.50 (0.05)	290.31
Gamma	Shape: 3.98 (0.69)	Rate: 0.72 (0.13)	295.35
Logistic	Location: 5.06 (0.34)	Scale: 1.53 (0.17)	310.34
Normal	Mean: 5.53 (0.40)	SD: 3.15 (0.28)	322.22
Exponential	Rate: 0.1808 (0.0230)	-	338.08

AIC = Akaike Information Criterion

Table 2 Best fitting probability density functions to hair dehydroepiandrosterone distribution in postnatal and postweaning periods of calves

Function	Parameter 1 (± SE)	Parameter 2 (± SE)	AIC
Postnatal period			
Log-normal	Mean: 3.63 (0.05)	SD: 0.40 (0.03)	607.35
Gamma	Shape: 6.25 (1.01)	Rate: 0.15 (0.03)	610.34
Logistic	Location: 38.88 (1.76)	Scale: 8.85 (0.88)	618.52
Normal	Mean: 40.68 (2.06)	SD: 17.59 (1.46)	629.79
Exponential	Rate: 0.0246 (0.0029)	-	689.02
Postweaning period			
Log-normal	Mean: 3.45 (0.06)	SD: 0.47 (0.04)	513.98
Gamma	Shape: 4.03 (0.70)	Rate: 0.11 (0.02)	526.11
Logistic	Location: 32.06 (2.10)	Scale: 9.86 (1.07)	543.37
Normal	Mean: 35.74 (2.90)	SD: 22.86 (2.05)	568.01
Exponential	Rate: 0.0280 (0.0035)	-	569.47

AIC = Akaike Information Criterion

Table 3 Best fitting probability density functions to hair dehydroepiandrosterone sulphate distribution in postnatal and postweaning periods of calves

Function	Parameter 1 (± SE)	Parameter 2 (± SE)	AIC
Postnatal period			
Log-normal	Mean: 4.47 (0.05)	SD: 0.43 (0.04)	739.52
Gamma	Shape: 5.25 (0.84)	Rate: 0.05 (0.01)	747.45
Logistic	Location: 88.79 (4.54)	Scale: 23.11 (2.34)	762.19
Normal	Mean: 96.40 (5.67)	SD: 48.46 (4.01)	777.77
Exponential	Rate: 0.0104 (0.0012)	-	815.01
Postweaning period			
Log-normal	Mean: 3.62 (0.05)	SD: 0.39 (0.04)	512.79
Gamma	Shape: 6.43 (1.13)	Rate: 0.16 (0.03)	516.04
Logistic	Location: 38.35 (1.94)	Scale: 8.90 (0.95)	525.26
Normal	Mean: 40.24 (2.18)	SD: 17.14 (1.54)	532.32
Exponential	Rate: 0.0249 (0.0032)	-	584.16

AIC: Akaike Information Criterion

Table 4 Median [minimum, maximum] values of hair hormones in healthy and traumatized animals

Hair hormone	Clinically healthy calves		Traumatized calves	*p* value
	Postnatal period	Postweaning period		
Cortisol (pg/mg)	16.06 [1.38, 36.10] ^{B}	4.74 [1.15, 17.50] ^{A}	28.34 [21.87, 60.83] ^{C}	< 0.001
DHEA (pg/mg)	40.96 [15.04, 115.56] ^{a}	28.06 [12.02, 158.11] ^{b}	35.13 [19.89, 57.91] ^{ab}	0.026
DHEA-S (pg/mg)	86.06 [32.05,289.05] ^{A}	37.02 [18.02, 104.27] ^{B}	54.22 [25.91, 72.41] ^{B}	< 0.001
Cortisol:DHEA *100	40.68 [3.27, 229.85] ^{B}	17.11 [2.36, 57.39] ^{A}	83.72 [51.82, 281.05] ^{C}	< 0.001
Cortisol:DHEA-S *100	16.84 [1.58, 44.96] ^{B}	12.25 [4.40, 40.02] ^{A}	80.21 [36.39, 234.77] ^{C}	< 0.001
DHEA:DHEA-S	0.43 [0.08, 1.58] ^{A}	0.80 [0.42, 3.00] ^{B}	0.71 [0.52, 1.07] ^{B}	< 0.001

DHEA = dehydroepiandrosterone; DHEA-S = dehydroepiandrosterone sulphate

Values in the same row superscripted with different uppercase or lowercase letters are highly significantly ($p < 0.01$) or significantly ($p < 0.05$) different, respectively.

The highest ($p < 0.01$) hair cortisol concentration, cortisol:DHEA ratio and cortisol:DHEA-S ratio were in the TRA, while the lowest ($p < 0.01$) were in the PWP. The DHEA concentrations were higher in the POP than in the PWP ($p < 0.05$) and the TRA group showed similar DHEA concentrations compared to clinically healthy calves both in the POP and PWP ($p > 0.05$). The highest DHEA-S concentrations were in the POP ($p < 0.01$), with the TRA showing similar concentrations compared to the PWP group ($p > 0.05$). Conversely, the lowest DHEA:DHEA-S ratio was in the POP ($p < 0.01$), with the TRA showing similar concentrations in comparison to the PWP group ($p > 0.05$).

6.4. Discussion

The neonatal period is characterized by several environmental changes faced by newborn calves, with an endocrine setting that allows them to cope with the allostatic load of this particular period (Hammon et al., 2012). As described by Weary et al. (2008), several postnatal stressors (early separation from the mother, dietary change from the mother's milk to the bulk tank milk, introduction into new living area and modifications of social groups) can affect HPA-axis activity and consequently affect the development of the animal. It is also known that a calf that adapts badly to this period has a high chance of presenting lower productivity throughout its life; for example, the heifer stage is the weak link in many dairy farms, and if not well managed, it can cause serious losses, both in economic and animal welfare terms (Fantini, 2009; Bazeley et al., 2016). Despite a number of commercially available biosensors that quantify stress responses through parameters such as heart rate variability, rectal temperature and respiration rate, resting, laying and ruminating (Riaboff, 2020) the fruitfulness of such technological approaches has yet to be evaluated.

The "traditional" endocrine approach combined to a newer specimen as hair might be an interesting way for individual evaluations in calves and also on a large scale.

To date, several studies have been carried out on calves to investigate hair cortisol concentrations. At around age 60 d, Probo et al. (2021) found beef calves had lower hair cortisol concentrations than those in the current study on dairy calves, while at 150 days the cortisol values were common in both studies; Braun et al. (2019) at this time-point measured lower cortisol concentrations for non-regrowth samples taken at the slaughterhouse. The difference at age 60 d could be surely explained by the non-regrowth sample analyzed in the current study that also included the higher cortisol concentrations characteristic for the newborn calf (Maiero et al., 2005; Comin et al., 2008; González-de-la-Vara et al., 2011). This study was the first published report on the measurement of hair DHEA concentrations in calves, while the only study reporting hair DHEA-S concentrations in calves (Probo et al., 2021) was in accord with the concentrations observed in the current trial.

The analysis of the hair cortisol, DHEA, and DHEA-S concentrations from the hair samples collected at around age 60 d (at the end of the weaning) showed that all the animals had significantly higher hormone concentrations than those found at age 5 mth, excepting the hair cortisol concentrations measured in the seven calves that were stuck for a certain time in the feed fence (the TRA group). The first hair sample not only includes information linked to the

extrauterine life, but also events that occurred during pregnancy and birth. In fact, this matrix is capable of cumulatively recording hormonal variations. Therefore, hair hormone concentration is assumed to be a retrospective marker of integrated hormone secretion over longer periods (Dettenborn et al., 2010; D'Anna-Hernandez et al., 2011; Pereg et al., 2011; Stalder and Kirschbaum, 2012). Several studies have shown that pregnancy plays a very important role in the future of the unborn animal (Funston et al., 2010; González-Recio et al., 2012; Noya et al., 2019) and that high cortisol levels have an important effect on the activity of many systems (Uetake et al., 2014; Strong et al., 2015) and fetal programming (Holt, 2002; Xiong and Zhang, 2013).

The reduction in hair cortisol concentrations at about age 5 mth compared to those measured at age 60 d demonstrated a reduction in the animal's allostatic load. This was not surprising, since in the postweaning rearing period, the number of environmental changes greatly reduced. Furthermore, it is conceivable that an increase in the calves' resilience did not result in an increase in DHEA and DHEA-S concentrations by itself but rather in a significant reduction in the cortisol:DHEA and cortisol:DHEA-S ratios, as already observed in piglets (Fels et al., 2019) and dairy cows (Peric et al., 2017).

The TRA group, which were stuck in the feeding fence for several hours at weaning time and that probably re-experienced this trauma every time they entered the feeding rack to feed, showed significantly higher cortisol concentrations compared to the animals sampled at age 5 mth and even to those sampled earlier. The cortisol:DHEA and cortisol:DHEA-S ratios of the TRA group were also significantly higher than those of the POP and PWP groups. This suggested that an acute stressor, such as trauma, can transform into a chronically repeated feeling that triggers a recurrent activation of the HPA axis. On the other hand, the TRA group demonstrated a similar average daily weight gain to the PWP group having, thus overcome the possible influence of the dysregulated HPA axis on the metabolic rate of the animals.

In conclusion, the simultaneous evaluation of cortisol, DHEA and DHEA-S in hair samples and their ratios may provide more information than an assessment based on each steroid alone, as it provides a more complete picture of the hypothalamic-pituitary-adrenal axis activity and functionality. Such assessments appear to be crucial to capitalizing on the genetic potential of livestock, to safeguard both animal welfare and their productive life. In addition, this may represent a preparatory phase in the development of predictive clustering trees (Nikoloski et al., 2019), which will aid machine learning tools and decision-making processes. Indeed, cortisol, DHEA and DHEA-S are steroid hormones related to the organism's ability to cope

with environmental changes. Consequently, they affect the development of an animal. Evaluation of the hair cortisol, DHEA and DHEA-S can provide useful information about the ability of a calf to react to environmental changes; thus, facilitating the identification of calves with a higher allostatic load that may interfere with their ability to be resilient.

6.5. References

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7. Incorporation of Testicular Ultrasonography and Hair Steroid Concentrations in Bull Breeding Soundness Evaluation

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Simple Summary: Bulls' subfertility has a major impact on the efficiency of production and profitability of cattle enterprises. Bulls typically undergo a bull breeding soundness evaluation (BBSE) to predict potential fertility. The present study investigated if a more comprehensive index of indicative fertility could be developed in bulls by including testicular ultrasonography and hormonal status in the BBSE. Bulls with homogeneous testicular parenchyma showed a higher percentage of motile sperm post-thawing compared with bulls with heterogeneous parenchyma. In bulls with homogenous parenchyma, the percentage of motile sperm, progressively motile sperm, and motility yield were positively correlated with hair DHEA-S concentration. The use of testicular ultrasonography and DHEA-S status in the BBSE would provide a more comprehensive assessment of potential fertility in bulls. In addition, ultrasonography can be used in the BBSE when the evaluation of semen parameters is not available.

Abstract: Testicular ultrasonography and steroid concentrations (cortisol, dehydroepiandrosterone sulfate (DHEA-S), cortisol/DHEA-S ratio, testosterone) in hair were examined for their utility in the bull breeding soundness evaluation (BBSE). Beef and dairy bulls ($n = 16$; 2.7 ± 0.4 years old; body condition score 3.2 ± 0.1) of five breeds were maintained under the same conditions at an accredited semen collection center. Bulls underwent routine semen collection twice weekly for 12 weeks and semen was processed and cryopreserved. Ultrasonography and hair sampling were undertaken at the last semen collection. Bulls with homogeneous testicular parenchyma ($n = 8$) had a higher ($p < 0.05$) percentage of motile sperm post-thawing compared with bulls with heterogeneous parenchyma ($n = 8$). There were no differences ($p > 0.05$) in the hair concentrations of cortisol, DHEA- S, and testosterone between bulls with homogeneous and heterogeneous parenchyma. In bulls with homogeneous

parenchyma, hair DHEA-S concentration was positively correlated with percentage motile sperm (R2 = 0.76), progressively motile sperm (R2 = 0.70), and motility yield (R2 = 0.71). The findings indicate that the integration of testicular ultrasonography and hair DHEA-S status in the BBSE could provide a more comprehensive assessment of indicative fertility in bulls. Additionally, ultrasonography can be used in the BBSE when the evaluation of semen parameters is not available.

Keywords: testicular ultrasonography; hair steroids; semen; bull

7.1. Introduction

Bulls with low fertility have a major negative impact on the efficiency of production and profitability of cattle enterprises [1,2]. Hence, bulls routinely undergo a bull breeding soundness evaluation (BBSE) before they are used for natural or assisted breeding. BBSE involves an assessment of overall structural soundness, integrity of the reproductive organs, and semen quality [1]. Noninvasive testicular ultrasonography has undergone preliminary investigation as an additional parameter for inclusion in the BBSE. Ultrasonography provides information on the integrity of testicular parenchyma, and the relationship to spermatogenesis [3,4]. The homogeneity of testicular parenchyma, as judged by ultrasonography, was reported to have an important bearing on spermatogenesis and fertility in males. In men, testicular inhomogeneity, characterized by the presence of fibrotic tissue on ultrasound, was associated with impaired sperm quality and azoospermia [5]. The relationship between testicular homogeneity and sperm production and fertility is less clear for bulls. In an early study, there were no differences in sperm abnormalities between bulls with fibrotic foci in testicular parenchyma and bulls without fibrotic foci [6]. Subsequent studies also reported no clear association between the integrity of testicular parenchyma and semen quality in bulls [7–9]. However, in a study with a small number of bulls, testicular lesions were associated with a low BBSE score and poor semen quality [10].

Spermatogenesis is influenced by many factors including metabolic and endocrine status [11]. The brain–adrenal axis is involved in metabolic homeostasis [12] and it also influences the brain–gonadal axis [13,14]. Adrenal glucocorticoids, including cortisol, typically have a negative impact on testicular function including spermatogenesis [15,16]. Glucocorticoids are elevated during stress, and chronic stress can be associated with impaired sperm production [17]. The adrenals also secrete the androgens dehydroepiandrosterone (DHEA) and DHEA sulphate (DHEA-S) [18,19]. In cattle, DHEA and DHEA-S are suppressed when cortisol is elevated during chronic stress [20]. The inverse relationship between cortisol and DHEA/DHEA-S led to the proposal that DHEA and DHEA-S could be antagonistic to cortisol [20].

Allostasis is a term used to describe mechanisms whereby the body adapts to stressors to maintain healthy homeostasis. Allostatic load is the build-up of stressors over time and the impact on the brain and somatic tissues. In cattle and other species, the amount of cortisol present in hair is reflective of the short- to medium-term activity of the brain–adrenal axis and

provides an index of allostatic load [21,22]. Hair and blood concentrations of DHEA and DHEA-S are also reflective of allostatic load in cattle [22]. The effect of cortisol and DHEA-S on semen parameters and fertility has not been thoroughly investigated in bulls. The present study investigated the effects of testicular ultrasonography and hair steroids (cortisol, DHEA-S, cortisol/DHEA-S ratio, testosterone) on semen parameters in bulls. The aim was to determine the association between testicular ultrasonography and hair steroids with semen parameters in bulls. If ultrasonography and hair steroids were shown to be related to semen parameters, they could be used in BBSE if semen assessment was not available. The hypothesis tested was that the integrity of testicular parenchyma is related to semen parameters in bulls. The accurate selection of bulls for fertility is particularly important when bulls of high commercial value are used extensively in assisted breeding programs.

7.2. Materials and Methods

All experimental procedures complied with the Italian legislation on animal care (Legislative Decree n. 116, 27/1/1992). The study had approval from the Ethical Animal Care and Use Committee of the University of Naples Federico II (Protocol PG72021/0130477).

7.2.1. Animals

The study involved sixteen bulls (2.7 ± 0.4 years old, body condition score 3.2 ± 0.1) of five breeds: Pezzata rossa italiana (n = 7), Holstein Friesian (n = 5), Limousine (n = 2), Charolaise (n = 1), and Chianina (n = 1). Animals were maintained under the same management at an accredited National Semen Collection Center. The study lasted 12 weeks and testicular ultrasound examination and hair sampling were undertaken at the same time as the last semen collection, according to the retrospective value provided by the hair matrix.

7.2.2. Testicular Ultrasonography

For testicular ultrasound examination, bulls were restrained in a bovine steel stanchion. The scrotal skin was cleaned and ultrasonographic gel was applied to increase the quality of the ultrasound image. A B-mode ultrasound scanner (MyLab™AlphaVET- Esaote S.p.a, Genova, Italy) equipped with a 13–3 MHz linear array probe was used to image the testes of each bull; the same settings were used for focus, gain, brightness, and contrast, standardized at the machine median settings. The ultrasound transducer was held vertically (parallel to the long axis of the testes) on the caudal surface of the scrotum. The image was aligned until the mediastinum of the testes was clear and apparent [7]. The image was then frozen and saved. This process was repeated with the ultrasound transducer in the horizontal plane (at the widest part of the testis) and both views were repeated for the other testis. A validated scoring system was used to identify bulls with a homogeneous testicular parenchyma and bulls with a heterogeneous parenchyma [23,24]. In brief, the scoring system adopts a six-point scale with scores of 0–5 encompassing normal homogenous patterns of echotexture to very severe fibrosis throughout the testis [8]. All images were obtained by the same operator.

7.2.3. Sample Collection and Processing

7.2.3.1. Hair

The hair in cattle grows at approximately 0.6–1.0 cm per month and animals show a full molt approximately every 3 months [25]. The concentration of steroids in hair therefore provides an

integrated measure of secretion during the preceding 2 to 3 months [25–27]. The integrated value avoids the short-term and diurnal variations in steroid secretion and is a more accurate indicator of the prevailing steroidal status of animals. Hair samples can be readily obtained and processed compared with blood samples. Hence, hair steroid concentrations were used in the present study. Hair was obtained from the scapular region of bulls using a razorblade and cut close to the skin at the same time as the last semen collection. Samples represented the integrated steroid concentration over the 12-week duration of the study. Samples were stored in dry tubes at room temperature and in the dark until analysis.

7.2.3.2. Semen

Bulls underwent semen collection twice weekly for 12 weeks as part of the routine commercial activity of the authorized National Semen Collection Center. Bulls were trained to serve an artificial vagina (IMV, L'Aigle, France). A total of 384 ejaculates were collected during the study.

7.2.4. Semen Evaluation

Semen was assessed and processed immediately after collection. The volume of semen was estimated by weighing the ejaculates within 5 min of collection and incubation at 35 °C. Initial assessments on fresh semen included the following: ejaculate volume (mL), motility (% motile spermatozoa), and total concentration (10^6 sperm/mL) [28,29]. Semen concentration and motility were determined by the computer-assisted semen analysis system (CASA, Sperm Vision, Minitube GmbH, Tiefenbach, Germany). The technical settings used in the present study were the following: depth of sample chamber 20 µm, light adjustment 100–155, temperature of analysis 37 °C, dimension of sperm heads 22–99 μm^2, frame rate 60 s−1, immotile AOC < 3.5 μms^{-1}, lateral motile DSL < 15 μms^{-1}, VSL < 20 μms^{-1} and VAP < 24.9 μms^{-1}, progressively motile STR > 0.5 and LIN > 0.35, non-linear STR < 0.5 and LIN < 0.35, curvilinear DAP/Radius = 3 and LIN < 0.5, round pat area average < 40 μm^2, BCF = 0, and AOC > 8 [30]. Semen samples were diluted to a final concentration of 40 to 80 × 10^6 spermatozoa/mL with egg yolk-based freezing extenders (Tryladil, Minitube GmbH, Tiefenbach, Germany) before packaging into 0.25 mL straws. Straws containing extended semen were then incubated at 5 °C overnight. Freezing was initiated by transferring the straws into a fixed temperature freezing chamber (Minitube GmbH, Tiefenbach, Germany) at −140 °C for 15 min, and subsequently plunging them into liquid nitrogen. Assessments on frozen-thawed semen were performed within 5 min after thawing and included the percentage of

motile sperm (% motile), percentage of progressively motile sperm (% sperm PM), percentage of viable sperm (% live sperms), and motility yield (proportion of sperm migrating into the medium).

7.2.5. Hair Steroid Assays

Hair samples were prepared for the steroid assay as previously described [25]. Briefly, the hair samples were washed in isopropanol (Sigma-Aldrich, St. Louis, MO, USA), and approximately 60 mg of trimmed hair was extracted with methanol (Sigma- Aldrich, St. Louis, MO) for 16 h. Vials were then evaporated to dryness at 37 °C under an airstream suction hood and the remaining residue was dissolved in 0.35 mL of phosphate- buffered saline (PBS), 0.05 M, pH 7.5.

Cortisol [25,26], DHEA-S [25], and testosterone [27] concentrations were measured using solid-phase microtiter radioimmunoassay assays. Cortisol was measured using a commercial rabbit anti-cortisol antibody (Analitical Antibodies-Bologna, Italy) with cross- reactivities: cortisol 100%; cortisone 4.3%; corticosterone 2.8%; 11-deoxycorticosterone 0.7%; 17-hydroxyprogesterone 0.6%; dexamethasone 0.1%; progesterone < 0.01%; 17-hydroxypregnenolone < 0.01%; DHEA-S < 0.01%; androsterone sulfate < 0.01%; pregne- nolone < 0.01%. DHEA-S was measured using a commercial rabbit anti-dehydroepi- androsterone sulfate-7ß-CM-BSA antibody (SpiBio, Montigny le Bretonneux, France) with cross-reactivities: DHEA-S 100%; 4-Androsten-3,17-dione (4-androstenedione) 0.2%; 4-Androsten-17-ol-3-one (testosterone) < 0.01%; 5-Androsten-3-ol-17-one (dehydroepi-androsterone, DHEA) < 0.01%; 5-Androstan-3-ol-17-one (androsterone) < 0.01%. Testosterone was measured using a commercial anti-testosterone-3-carboxymethyloxime-BSA antibody (Analytical Antibodies-Bologna, Italy) with cross-reactivities: testosterone 100%; 5α-dihydrotestosterone 43.2%; 5α-androstanedione 33.1%; 5β-androstanedione 11.4%; 5α-androstan-3a,17b-diol 9.4%; androstenedione 0.4%; progesterone, DHEA, oestradiol 0.01%; cortisol < 0.001%. For cortisol, intra- and inter-assay coefficients of variation (CV) were 3.6 and 9.8%, respectively, and the sensitivity was 24.6 pg/mL (Riasmart software, Perkin-Elmer Life Sciences, Boston, MA, USA). For DHEA-S, the intra- and inter-assay coefficients of variation were 3.6% and 12.7%, respectively, and the sensitivity was 15.8 pg/mL. For testosterone, the intra- and inter-assay CV were 3.5% and 12.8%, respectively, and the sensitivity was 17.6 pg/mL.

7.2.6. Statistical Analyses

Statistical analyses were carried out using SPSS (28.0) for Windows 10 (SPSS Inc., Chicago, IL, USA). The initial dataset was edited, discriminating both for missing information and outliers (values lying 3 standard deviations below/above the mean). The number of samples excluded was the same between the homogenous and heterogenous bulls, which were characterized by similar coefficients of dispersion. The final dataset consisted of 236 ejaculates (14.7 ± 1.4/bull). The normal distribution of all data was confirmed using the Shapiro–Wilk test. Bulls were used as the experimental units. Multivariate analysis of variance (general linear model) was used to compare hair steroids of bulls (dependent variables); testicular parenchyma and breed were the fixed factors, and their interaction was also considered. Data on semen characteristics were analyzed by ANOVA for re- peated measures with testicular parenchyma (homogenous/heterogenous) as the main factor, and breed and cortisol as random. The day of collection was the repeated measure. Multiple linear regression was performed (forward stepwise procedures) with steroid concentrations as independent variables, and fertility parameters (mean values) as dependents. Potential independent and dependent variables were first tested for potential correlations using Pearson correlations and only significant correlations ($p < 0.05$) were included in the regression model. Pearson correlation was also used to exclude possible intercorrelations between the independent variables. Unless otherwise stated, the results are presented as mean ± standard error and significance was set at $p < 0.05$.

7.3. Results

7.3.1. Testicular Ultrasonography

Eight bulls had homogeneous testicular parenchyma (Holstein Friesian, 4; Pezzata rossa italiana, 2; Limousine, 1; Charolais, 1) (fibrosis score 0–2) (Figure 1). For the other eight bulls, four bulls had slight to mild generalized heterogeneous parenchyma (Pezzata rossa italiana, 2; Holstein Friesian, 1; Limousine, 1), three bulls had clearly noticeable hy- perechoic foci of calcification (Pezzata rossa italiana, 2; Chianina, 1), and one bull had se- vere hydrocele (Pezzata rossa italiana) (score 3–5) (Figure 1).

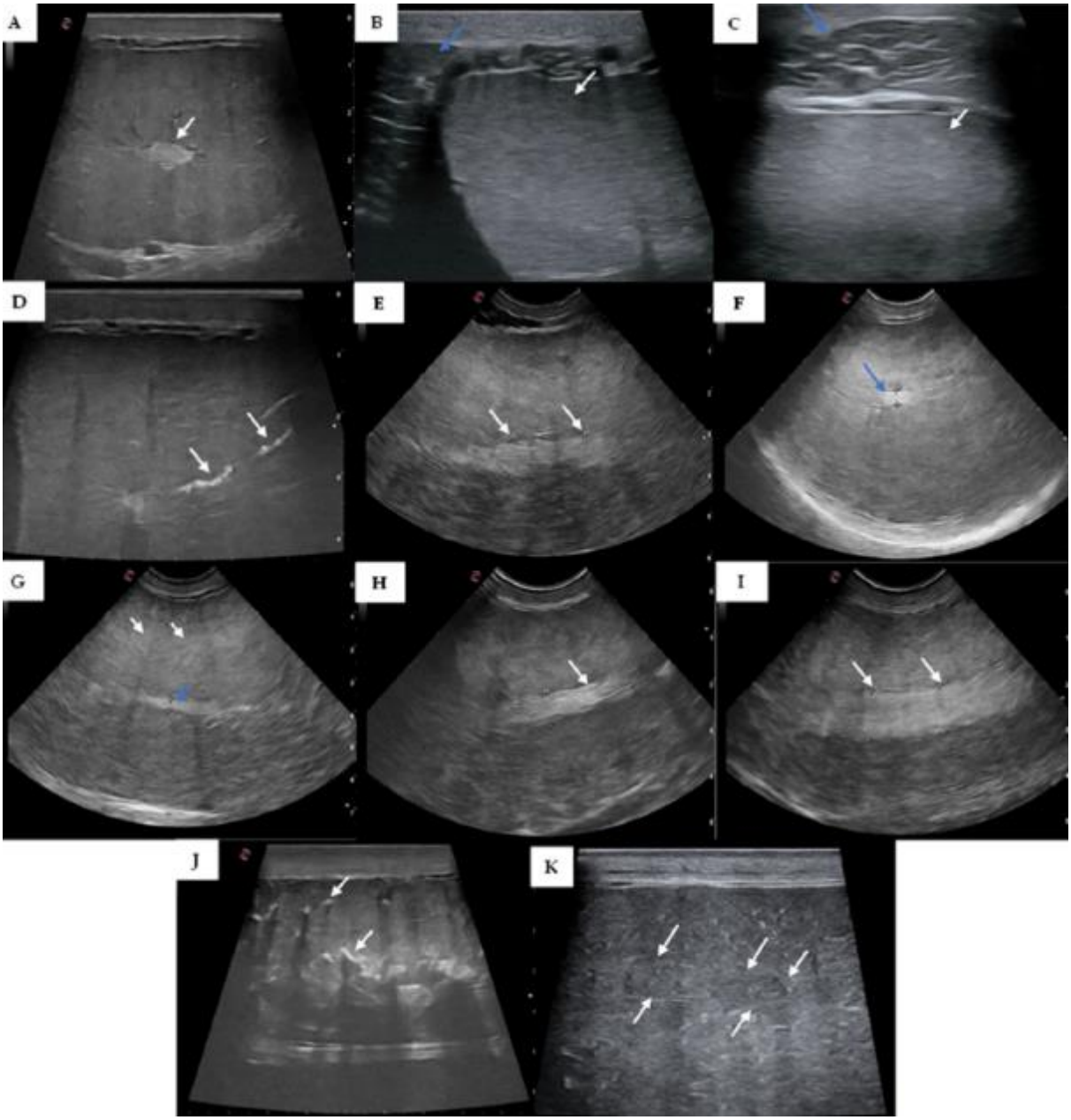

Figure 1. Representative ultrasound images for bulls with homogeneous testicular parenchyma (A– C): (A) medium echogenicity with the mediastinum testis as a central or slightly eccentric hyperechoic focus (white arrow); (B) homogenous testicular parenchyma (white arrow) and head of the epididymis (blue arrow); (C) homogenous testicular parenchyma (white arrow) and body of the epididymis (blue arrow); and bulls with heterogeneous testicular parenchyma (D–K): (D) linear hyperechoic, non-shadowing foci (white arrows) filling the rete tubules indicative of spermiostasis; (E) slight heterogeneous testicular parenchyma and distension of mediastinum testis (8.7 and 8.8 mm left and white arrow respectively); (F) slight heterogeneous testicular parenchyma and mediastinum testis of 4.2 mm (blue arrow); (G) slight heterogeneous testicular parenchyma with small hyperechoic foci (white arrows) and mediastinum testis of 3.6 mm (blue arrow); (H) heterogeneous testicular parenchyma, distension, and thickening of mediastinum testis (6.8 mm, white arrows); (I) bilateral testicular hypotropia and severe mediastinum distension (10.4 and 10.8 mm left and white arrow respectively); (J) coarse echotexture with multiple hyperechoic foci (white arrows) with distal acoustic enhancement scattered throughout the parenchyma; (K) inhomogeneous echotexture with hypoechoic areas scattered throughout the parenchyma.

7.3.2. Testicular parenchyma and spermatozoan parameters

Fertility parameters of fresh semen did not differ between homogenous and heterogenous bulls (Table 1).

Table 1. Fertility parameters for fresh semen in bulls with homogeneous (n = 8) and heterogeneous (n = 8) testicular parenchyma. Results are mean ± sem.

	Testicular parenchyma		
Fertility parameters (fresh semen)	**Homogenous**	**Heterogenous**	***p***
Ejaculate volume	6.7 ± 0.5	6.6 ± 0.5	0.86
Sperm concentration (× 10^6/mL)	710 ± 133	940 ± 186	0.32
motile sperm (%)	54.5 ± 8.2	60.5 ± 9.6	0.64

Bulls with homogeneous parenchyma had a higher ($p < 0.05$) percentage of motile sperm post-thawing (Table 2).

Table 2. Fertility parameters post-thawing in bulls with homogeneous (n = 8) and heterogeneous (n = 8) testicular parenchyma. Results are mean ± sem. a,b $p < 0.05$

	Testicular parenchyma		
Fertility parameters (post-thawing)	**Homogenous**	**Heterogenous**	***p***
Motile sperm (%)	65.7 ± 4.7 [a]	51.6 ± 3.8 [b]	0.02
Progressively motile sperm (%)	41.3 ± 4.5	33.8 ± 3.9	0.21
Viable sperm (%)	49.1 ± 4.5	44.2 ± 4.2	0.44
Motility yield (%)	41.9 ± 2.4	41.8 ± 2.5	0.97

7.3.3. Steroid concentrations

Concentrations in hair of cortisol, DHEA-S, and testosterone, and the cortisol/DHEA-S ratio, are shown in table 3. For all three steroids, there were no significant differences between bulls with homogeneous or heterogeneous testicular parenchyma.

Table 3. Hair steroid concentrations (pg/mg) for bulls with homogenous testicular parenchyma and bulls with heterogenous testicular parenchyma. HCC, hair cortisol concentration; HTC, hair testosterone concentration; HDC, hair DHEA-S concentration; C/DHEA

	Testicular parenchyma		
Hair steroid concentrations	**Homogenous**	**Heterogenous**	***p***
HCC	1.1 ± 0.1	1.3 ± 0.1	0.21
HDC	6.7 ± 0.7	7.8 ± 0.6	0.54
HTC	4.8 ± 0.6	4.2 ± 0.6	0.53
C/DHEA-S ratio	17.6 ± 1.8	16.6 ± 1.9	0.70

7.3.4. Steroid Concentrations and Semen Parameters

The relationships between hair DHEA-S concentration and motile sperm (%), progressively motile sperm (%), and motility yield for bulls with homogeneous testicular parenchyma are shown in Table 4. Hair DHEA-S was positively related to motile sperm (R^2 = 0.76), progressively motile sperm (R^2 = 0.70), and motility yield (R^2 = 0.71).

Table 4. Relationship between hair dehydroepiandrosterone sulphate (DHEA-S) and fertility parameters in bulls with homogeneous testicular parenchyma.

Fertility parameter		*p*
Motile sperm (%)	22.88 + 2.88 (DHEA-S); R^2 = 0.76	0.003
Progressive motile sperm	8.53 + 2.66 (DHEA-S); R^2 = 0.70	0.006
Motility yield	17.19 + 2.86 (DHEA-S); R^2 = 0.71	0.006

7.4. Discussion

The present study examined whether the incorporation of testicular ultrasonography and hair steroid concentrations in the bull breeding soundness evaluation (BBSE) would provide a broader and more comprehensive index of indicative fertility. Another objective was to determine whether testicular ultrasonography could be used in the BBSE when semen evaluation is not available. Bulls with homogeneous testicular parenchyma had a higher percentage of motile sperm post-thawing compared with bulls with heterogeneous parenchyma. This finding could be interpreted to suggest that the sperm of bulls with homogenous parenchyma has a higher tolerance to cryopreservation and thawing com- pared with the sperm of bulls with heterogeneous parenchyma. This was an important observation as sperm motility is related to fertility in bulls [28,29]. Ultrasonography rep- resents a practical, non-invasive procedure and adds important information to the BBSE. In an earlier study, the condition of the parenchyma was reported to be predictive of se- men quality 2 to 4 weeks after ultrasound examination in bulls [7].

There were no differences in hair concentrations of testosterone, cortisol, and DHEA- S between bulls with homogeneous or heterogeneous testicular parenchyma. Previous studies in cattle and other species have reported an inverse relationship between cortisol and DHEA-S, and it was suggested that DHEA-S may partly counterbalance the negative impact of elevated cortisol on physiological and endocrine functions [20,22,31,32]. The cortisol/DHEA-S ratio was also considered an index of allostatic load [22]. The finding on the cortisol/DHEA-S ratio in the present study was interpreted to indicate that bulls with homogeneous and heterogeneous testicular parenchyma experienced the same allostatic load and did not have compromised endocrine function. This could be expected as all bulls experienced the same handling and management at an accredited National Semen Collection Center. Therefore, factors other than cortisol, and the cortisol/DHEA-S ratio, contributed to differences in testicular parenchyma condition in the present study. In this regard, testicular status was reported to have a genetic component [33].

Percentage motile sperm, progressively motile sperm, and motility yield were positively correlated with hair DHEA-S concentration in bulls with homogeneous parenchyma. This relationship may have been partly due to the prohormonal role of DHEA-S and its conversion to androgens and/or estrogens in peripheral target tissues [34]. As noted above, sperm motility and morphology are closely correlated with fertility [29]. Hair DHEA-S concentrations reflect

adrenal secretion and assimilation in hair during the preceding weeks, and give a longer-term integration of DHEA-S status. Ultrasonography is now used routinely for monitoring reproductive function in females, and hair sampling is used for genomic testing in males and females. Hair sampling is more practical than blood for hormonal and genetic evaluation.

As noted, there are conflicting reports on relationships between testicular parenchyma and testis hormonal and spermatogenic function in bulls. The present study has provided strong evidence that the condition of the parenchyma is reflective of spermatogenesis. Given the practical implementation of ultrasonography and hair sampling, the case can be made for inclusion in the BBSE, or ultrasonography can be used when semen evaluation is not available.

7.5. Conclusions

The present study has shown that bulls with homogeneous testicular parenchyma have sperm with a greater resilience to cryopreservation than the sperm of bulls with heterogeneous testicular parenchyma. This is an important finding as bulls of high commercial value are used extensively in artificial insemination. The study also highlighted a positive relationship between hair DHEA-S and important sperm fertility parameters. A limitation of the present study was the relatively small number of bulls tested and the absence of the BBSE. Notwithstanding, it could be concluded that the inclusion of testicular ultra- sonography and hair DHEA-S in the standard BBSE is practical and would provide a more integrated and comprehensive assessment of fertility in bulls. Finally, ultrasonography can be used when the evaluation of semen parameters is not available.

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8. Sow's pre- and post-delivery in different confinement systems evaluated by hair hormones concentrations

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H I G H L I G H T S

- Some biomarkers of allostatic load and resilience have been evaluated in sows around farrowing via non-invasive sampling.
- No differences were found in terms of hair hormones between the four models of farrowing crate and the sampling times.
- The interaction between batch and sampling time has been significant for all the biomarkers considered.
- During the pregnancy period in collective pens (ST1) HC/HDHEA ratios were almost never lower than those in the following characterized by the postpartum in individual crates (ST2).
- The influence of the social interaction in collective pens that cyclically re-occurs in the standard swine production system should be further investigated.

A B S T R A C T

Several matrices are available to detect the concentrations of cortisol and dehydroepiandrosterone (DHEA) as markers of allostatic load and resilience but most of them provide a measurement of the concentration at a single- time point or within a 12– 24 h period and are subjected to daily fluctuations; conversely, keratinized derivatives, as hair is, provide a measure of the hormonal concentrations over medium- and long-term periods, appearing unaffected by circadian changes or by factors inducing short-term variations. Aim of this study was to evaluate hair cortisol (HC), hair DHEA (HDHEA) and their ratio (HC/HDHEA ratio) as biomarkers of allostatic load and resilience in sows around farrowing, in relation to four different models of farrowing crates, and in relation to different batches by a non-invasive sampling. The study has been conducted on 296 pregnant sows managed in a batch farrowing system from February to June. Sows were kept in multiple pens of 10–15 sows until 5 days before farrowing, then were randomly divided in 4 different models of farrowing crates. The hair samples were taken by shaving for the first time (ST1) 2.6 ± 1.6 days before the expected delivery date and for the second time (ST2) 88.9 ± 3.3 days after parturition. The environmental temperature and the relative humidity data were recorded and used to calculate the Temperature Humidity Index (THI). HC and HDHEA were determined using a solid-phase microtiter radioimmunoassay (RIA) while HC/HDHEA ratio has been calculated. No differences in terms of hair steroids concentration were found between the four models of farrowing crates and the sampling times ($P > 0.05$). The interaction between batch and sampling time has been significant for all the biomarkers considered (HC ($P < 0.01$), HDHEA ($P < 0.05$) and their ratio ($P < 0.01$)). During the pregnancy period in collective pens (ST1) HC/HDHEA ratios were never lower (with an exclusion of February) than those in the following characterized by the early postpartum and lactation in individual crates (ST2). It seems that an aggregate effect of different stressors acts on the sows exhausting their ability to cope with adverse events; it should be further investigated the influence of the social interaction in collective pens that cyclically re-occurs in the standard swine production system.

Keywords: Swine, Cortisol, DHEA, Non-invasive, Pregnancy, THI

8.1. Introduction

The swine production in Italy is mainly linked to production of Protected Designation of Origin (PDO) hams (ISMEA, 2021). Along with the growing interest to certain products there is also a growing concern of consumers about how products are obtained and the animal welfare in intensive farming systems (Schröder and McEachern, 2004; Velarde et al., 2015).

Nowadays, the management of farrowing period that usually involve housing sows in restrictive systems such as pregnancy stalls and farrowing crates for parturition and lactation (Baxter et al., 2018) and the heat stress (Muns et al., 2016) are two of the main welfare concerns in the Italian pig industry.

The farrowing crates are widely use in modern pig farms, but they could inhibit the sow's innate behavior to build a nest before parturition, generating a decrease in maternal endogenous hormones that could in turn lead to detrimental effects on farrowing and lactating performances (Jarvis et al., 2001). Nevertheless, the farrowing systems aim to avoid crushing risk due to piglets attempts to reach the teats, and thus reduce piglets' mortality (van Nieuwamerongen et al., 2014). In time the genetic selection brought to remarkable improvements in reproductive efficiency of sows, though leading to a rise in metabolic heat production of the animals (Ross et al., 2015). Consequently, sows are more sensitive to heat stress effects even under conditions of a temperate climate (Bloemhof et al., 2013). The negative impact of heat stress around farrowing might result in prolonged farrowing and reduced feed intake during lactation and might also have a negative impact on piglet performance (Muns et al., 2016).

A major component of the stress response of mammals is activation of the hypothalamus-pituitary-adrenal (HPA) axis to restore homeostatic conditions; this increase in circulating glucocorticoid levels with a rising energetic demand is termed allostatic load (Wingfield, 2005). Stress is also strictly linked to the concept of resilience, which is the ability of an individual to be minimally affected by a disorder or to quickly return to the state present before exposure to the stressor (Colditz and Hine, 2016). The allostatic load and resilience can be investigated through biological markers, of which cortisol, dehydroepiandrosterone (DHEA) (Mc Ewen, 2003; Charney, 2004; Peric et al., 2017)) and their ratios (Logan et al., 2008; Maninger et al., 2009; Saczawa et al., 2013) are probably the most studied. Because of the opposite effects of cortisol and DHEA, a common measure employed to test the impact of both hormones simultaneously is the ratio between DHEA and cortisol (Qiao et al., 2017). The cortisol/DHEA ratio has been proposed as an appropriate method of assessing the degree of

functional hypercortisolemia (Gallagher and Young, 2002) and also in animals is of great importance (Whitham et al., 2020). Several matrices are available to detect the concentrations of these markers. Most of them provide a measurement of the concentration at a single-time point or within a 12–24 h period and are subjected to daily fluctuations (Meyer and Novak, 2012; Davenport et al., 2006); conversely, keratinized derivatives, as hair is, provide the measure of hormone concentrations over medium- and long-term periods (Meyer and Novak, 2012), appearing unaffected by circadian variations in the hormone or by factors inducing short-term variations (Russell et al., 2012). In the past, some studies have been already carried out on sow restraints (Bacci et al., 2014, López-Arjona et al., 2020; Morgan et al., 2021) but only hair cortisol (and cortisone) has been taken in account as biomarkers of the HPA axis activity.

Given that some studies reported that the complete confinement of sows in farrowing crates throughout the lactation period can be detrimental to animal welfare (Baxter et al., 2012), the hypothesis was that different models of farrowing crate could differently affect the allostatic load and the resilience of the sows.

Hence, the present study aimed at evaluating HC, HDHEA and HC/ HDHEA ratio as biomarkers of allostatic load and resilience in sows around farrowing, in relation to four different models of farrowing crates, to the period spent in multiple pens and to different batches by a non-invasive sampling.

8.2. Materials and methods

8.2.1. Ethics

Although hair sampling is non-invasive and it is not a troublesome procedure, the study was carried out in accordance with Directive 2010/ 63/EU on the protection of animals used for scientific purposes.

8.2.2. Animals and housing conditions

The study was conducted in the province of Brescia (Po Valley, Italy), at a pig farm of about 2000 Danish sows (Dan Bred International®, Denmark) specialized in production for Parma ham according to Pro- tected Designation of Origin (PDO). Animals were reared according to the current Italian legislation which implements the Council Directive 2008/120/EC 2008 concerning the laying down minimum standards for the protection of pigs.

The study involved 296 pregnant sows with a mean parity of 4.40 ± 0.15 (mean ± SE). Gilts were excluded from the study because their behavior in the delivery room is less predictable than the multiparous sows (Roelofs et al., 2019). After mating, sows remained in the gestation crate for 28 days. Here they received ultrasound pregnancy confirmation and were transferred to multiple pens of 10–15 individuals where they remained until 5 days before farrowing. The multiple pens were rectangular with a slatted floor. During gestation the animals had a space allocation of 2.25 m^2 per sow in accordance with the welfare European legislation. The feeding was liquid and supplied through an automatic system. Nipple drinkers were available for make water available ad libitum to animals. In reaching the farrowing rooms the sows were randomly housed in 4 different models of crates: Conventional, Evoslide (Pig Evoslide VR®; Evoteck, Manerbio, BS, Italy), Elevoteck (Elevoteck VR®; Evoteck, Manerbio, BS, Italy) and Liberty (Liberty VR®; Evoteck, Manerbio, BS, Italy). Thus, the sows were divided as follows: 66 animals in the Conventional farrowing crate, 66 in the Evoslide, 48 in the Ele- voteck and 116 in the Liberty. Sows remained in the farrowing crates for 26 days postpartum. The management was organized in a batch farrowing system, resulting in 14.80 ± 2.93 (mean ± SE) sows per batch per farrowing model.

In the Conventional farrowing crate, the movements of the sow are limited both during delivery and during lactation. The Conventional crate (2700 × 1700 mm) contains a sow area (2100 × 600 mm) surrounded by solid metal dividers. The movements of the sow are limited by lateral anticrushing bars intended to prevent the sow rolling onto her side so that the risk of crushing

piglets is reduced (Wischner et al., 2010; Nicolaisen et al., 2019) and operators safety increased (Baxter et al., 2012; Glencorse et al., 2019).

The Evoslide crate (2600 × 1800 mm) provides a sow area (1810 × 585 mm) equipped with a nest (steel box of 400 × 500 mm) at a lower level and located behind the sow. In this crate, after delivery, the piglets are allowed to slide on an inclined plane, dropping in the nest. The in- clined plane let the vital piglets climb up to the upper side immediately after farrowing, whereas the less viable piglets are left to lie under the red lamp to warm themselves, avoiding crushing by the mother.

The Elevotek crate (2700 × 1700 mm) is composed of a central area (2100 × 630 × 500 mm) for the sow and two lateral sides for the piglets, with anticrushing bars. A mechanic sensor is activated by the sow changing position from laying down to standing, and the central sow area rises from the floor to a 20 cm higher plane using a motorized technology. It allows piglets to stay on a lower level, forbidding them to reach the mother. Conversely, when the sow from a standing position tries to lay down, reaching a safe position for the piglets, the mechanic sensor lowers the sow platform, letting the piglets reach the teats.

The Liberty crate is a flexible structure, in which the sides are closed for the first 72 h post-delivery in order to allow time for the litter to establish their hierarchy (De Passillé et al., 1988) reducing the risk of crushing and then they open letting the sow move freely.

In all the create models the sows were housed on paper strips bedding during farrowing. All the farrowing crates were located in the same plant without any environmental control system.

For all the sows included in the study only a spontaneous farrowing took place. During lactation, the animals were fed with a liquid feeding through a semiautomatic system and nipple drinkers were available for make water available ad libitum to animals.

The management followed the standard operating procedures (SOPs) regarding vaccinations, placement, cleaning, waste management and biosecurity measures. The health status of the animals included in the study was considered "conventional": Mycoplasma hyopneumoniae posi- tive, Circovirus positive, PRRS (Porcine reproductive and respiratory syndrome) positive but stable-inactive at time of trial (Holtkamp et al., 2011).

8.2.3. Environmental temperature, relative humidity and THI

The environmental temperature and the relative humidity were recorded in collaboration with ARPA Lombardia (Regional Agency for Environmental Protection, Italy). A meteorological

station recognized by the World Meteorological Organization regulations and located 15 km away from the farm was used for the data collection throughout the study (daily average for each factor). The Temperature Humidity Index (THI) was calculated according to Mader's formula (Mader et al., 2006):

$$THI = (0,8 \times T) + (RH / 100) \times (T - 14,4) + 46,4 \quad (1)$$

where T is the environmental temperature in °C and RH the relative humidity in %.

8.2.4. Hair sampling

Hair samples were obtained from all the sows included in the trial. According to the expected delivery date, each sow was sampled for the first time (ST1) 2.6 ± 1.6 (mean ± SE) days before parturition. Thus, the sows were allocated to the February, March, April, May, or June batch.

Then, each sow was sampled for a second time (re-growth hair, ST2) at 88.9 ± 3.3 days after parturition.

The collection of the hair was carried out on the back at the level of the last rib and for about 10 cm at the side of the vertebral column; this area was chosen because of its cleanliness. The animal was shaved as close as possible to the skin with a Heiniger cordless electric razor (Ukal) for large animals. Samples were then stored in paper envelops, in the dark at room temperature until analysis.

8.2.5. Hair washing procedure, extraction and hormone analyses

Hair strands were washed, extracted and analysed for both HC and HDHEA concentrations as described by Bergamin et al. (2019). The washing with isopropanol is essential to minimize the risk of extracting steroids from the surface of the hair, which have been deposited by sweat and sebum.

8.2.6. Statistical analysis

The statistical analysis was performed with the R vers. 3.4.0 and the packages nlme (Pinheiro et al., 2022), car (Fox and Weisberg, 2019), lmerTest (Kuznetsova et al., 2017) and emmeans (Lenth, 2022) were considered. The normality of the data distribution was tested with the Shapiro-Wilk test. In case of non-normal distribution, the data has been transformed. The model adopted was a mixed model for repeated measures where the batch and the farrowing crate were considered between subject factors, whereas the day of the sampling was considered as a repeated measure for each animal, all the possible interactions were taken into account. The covariance structure was chosen in according to the Akaike Information Criterion as

suggested by Wang and Goonewardene (2004). The post hoc test was conducted according to Holm-Sidak. Significant statistical differences were declared at $P \leq 0.05$.

8.3. Results

The temperature and the relative humidity are shown in Table 1. THI ranged between a minimum of 41.67 in the March batch and a maximum of 75.53 in the July batch (Table 1).

Table 1. Temperature, Relative humidity, and THI mean values from the beginning of February to the end of September. THI has been calculated according to Mader et al. (2006) as abovementioned.

Month	Temperature (° C)	Relative humidity (%)	THI
February	5.47 ± 0.29	92.46 ± 1.85	42.53 ± 0.52
March	4.68 ± 1.03	87.22 ± 3.17	41.67 ± 1.63
April	14.00 ± 0.54	82.35 ± 2.33	57.27 ± 0.86
May	20.75 ± 0.68	76.74 ± 1.41	67.88 ± 1.03
June	25.03 ± 0.38	66.17 ± 1.07	73.46 ± 0.59
July	25.84 ± 0.35	73.85 ± 1.27	75.53 ± 0.43
August	25.08 ± 0.48	73.32 ± 1.20	74.30 ± 0.69
September	21.01 ± 0.54	75.14 ± 0.54	68.17 ± 0.86

Values are expressed as monthly means (± standard error), temperature is expressed in Celsius degrees (°C) and relative humidity in percentage (%). THI: Temperature Humidity Index.

As reported in Table 2 the April, May and June batches showed hair cortisol (HC) significatively higher than the February and March batches ($P < 0.01$). In addition, the February batch had the lowest HDHEA (16.80 pg/mg, $P < 0.01$). No differences were found in terms of hair hormones concentration between the four differently designed farrowing crates and the sampling times (Table 2).

Table 2. Estimated marginal means of hair hormones concentrations (pg/mg) and HC/HDHEA ratio recorded in sow (n=296).

Main effects	**HC**	**HDHEA**	**HC/HDHEA*100**
Cage type			
Conventional	21.82	39.85	85.94
Elevoteck	18.65	28.67	80.92
Evoslide	17.17	27.64	66.67
Liberty	18.05	28.06	78.75
Sampling time			
ST1	21.08	28.33	84.82
ST2	16.76	33.78	71.34
Batch			
February	12.67^{a}	16.80^{A}	76.86^{bc}
March	9.31^{a}	29.20^{B}	47.02^{a}
April	16.25^{c}	50.56^{B}	55.00^{ab}
May	27.83^{c}	31.22^{B}	100.06^{c}
June	28.55^{c}	27.50^{B}	111.41^{c}
SEM	1.393	2.681	5.378
P-value			
Batch	<0.001	<0.001	<0.001
Cage type	0.316	0.817	0.620
Sampling time	0.272	0.476	0.084
Batch × Cage type	0.524	0.056	0.263
Batch × Sampling time	<0.001	0.013	<0.001
Cage type × Sampling time	0.642	0.382	0.410
Batch × Cage type × Sampling time	0.274	0.253	0.112

^{a,b,c,d} Means within a column and within mean effect not sharing the same superscript differ at $P < 0.05$;

^{A,B} Means within a column and within mean effect not sharing the same superscript differ at $P < 0.01$.

SEM: standard error of the mean.

As shown in Table 2, the only significant interaction emerged between the batch and the sampling time. So, in Fig. 1 are showed the hair hormones concentrations of each sampling time throughout the batches. In the February batch HC was higher at ST2 than ST1 ($P < 0.01$), while in both the April and June batches the HC at ST2 was lower than ST1 ($P < 0.05$). Similarly, in the March batch HDHEA concentrations differed between the sampling times ($P < 0.01$). The HC/HDHEA ratio showed differences at three points: in the February batch was higher at ST2 than ST1 ($P < 0.01$), while in both the April and June batches the ratio recorded at ST2 was lower than ST1 ($P < 0.01$ and $P < 0.05$, respectively).

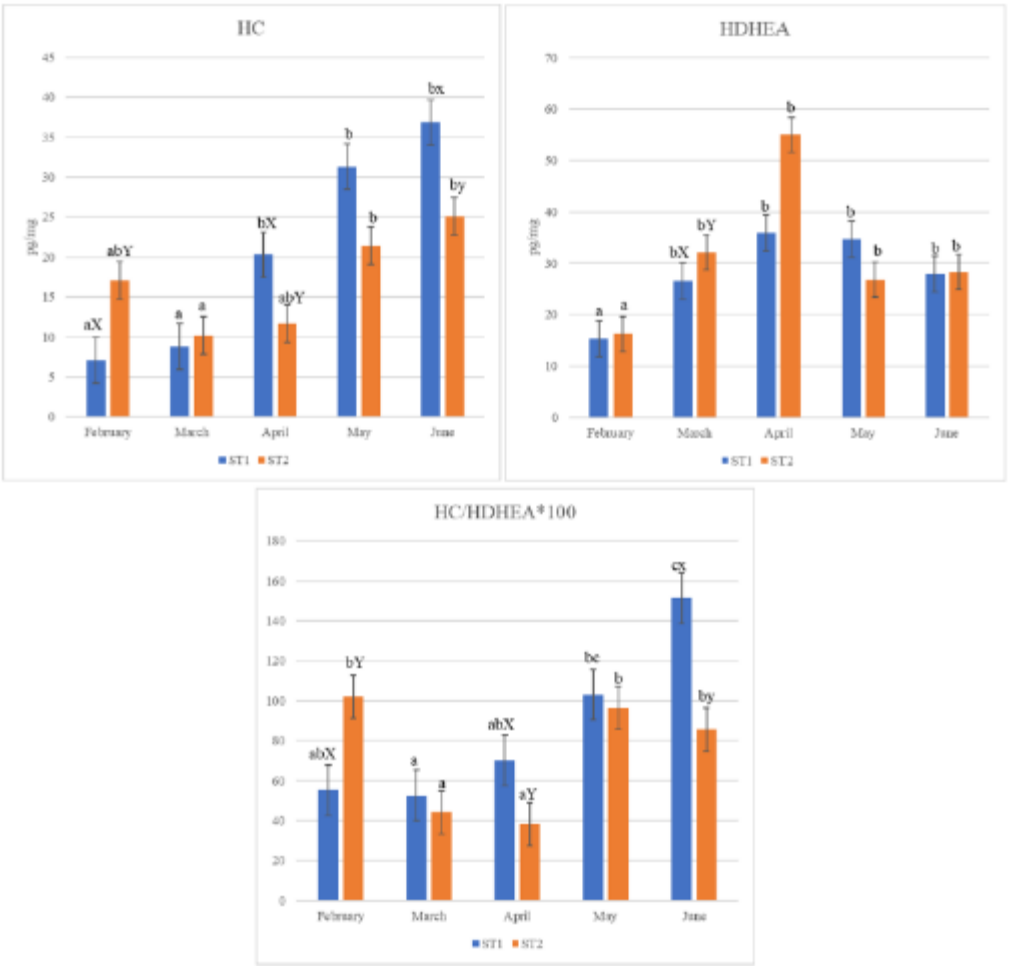

Fig. 1. Estimated marginal means of hair hormone concentrations (hair cortisol (HC), hair DHEA (HDHEA), pg/mg) and their ratio (HC/HDHEA ratio) recorded in sows (n = 296, 59.20 ± 3.18 (mean ± SE per batch) in each sampling time throughout the batches. The hair was sampled for the first time (ST1) 2.6 ± 1.6 (mean ± SE) days before parturition and for a second time (re-growth hair, ST2) at 88.9 ± 3.3 days after parturition.

[a,b,c] Indicate differences between batches at P < 0.05; [x,y] Indicate differences between sampling times at P < 0.05; [X,Y] Indicate differences between sampling times at P < 0.01

8.4. Discussion

In this study we obtained a first sample (ST1) few days before delivery, considering the retrospective information provided by the hair. The HC and HDHEA concentrations represent the hormonal status prior delivery, with the exclusion of the last 15 days of the pregnancy (time requested by a hair section to reach the skin surface (Russell et al., 2012). As a matter of fact, the section of hair located beneath the skin of the animals is never collected as hair is always shaved close to the skin and not plucked. Then, a second sample was collected by re-shaving the same area to evaluate the influence of the different farrowing crates on biomarkers of allostatic load and resilience in sows. As abovementioned sows spend 26 days in farrowing crate, therefore the second regrowth sample represents the delivery and post-delivery with its lactation, weaning of piglets and the subsequent period until ultrasound pregnancy confirmation. For each marker investigated (HC, HDHEA and HC/HDHEA ratio) no statistical differences were observed between the four different models of farrowing crates. The same was observed in a recent study in which Wiechers et al. (2021) compared conventional farrowing crates with single loose-housing systems in which animals were free to move. In spite of the lack of difference between the four models of crates in terms of the biomarkers investigated, it is worth to underline that different designed farrowing systems remain an improvement in piglet crushing reduction (Mazzoni et al., 2018). In fact, with conventional cages, Jarvis et al. (2005) report that 50 - 80% of total piglet mortality happens in the farrowing room even if they guarantee the sow and the operators' safety (Baxter et al., 2012; Glencorse et al., 2019).

The sampling operations started in February and lasted till the end of the summer period to evaluate the biomarkers of allostatic load and resilience in relation to different batches. The THI has been used as a reliable index to assess heat stress in pigs (Lallo et al., 2018). The THI remained in the comfortable range (below 74) described for swine species during the whole duration of the trial, reaching the threshold value of mild stress (75–78) described by He et al. (2019) and Wegner et al. (2016) only in July (75.53 ± 0.43).

Because the interaction was significant, the main effect of batch and sampling time could not be discussed separately. The interaction has been significant for all the biomarkers considered (HC ($P < 0.01$), HDHEA ($P < 0.05$) and their ratio ($P < 0.01$)). The HC/HDHEA ratio in the May and June batches were similar to the February batch when observing ST2 while the June batch in ST1 showed the highest HC/ HDHEA ratio and significantly differed from the ratio of

the first three batches. Moreover, ST1 (with an exclusion of February) showed HC/ HDHEA ratios that were never lower than the ST2 ratios. Similar results were observed for HC.

Thus, it seems that the pregnancy period in collective pens was more challenging than the following characterized by the early postpartum and lactation in individual crates. In collective pens animals are subjected to the determined hierarchy and the social structure of the group (Couret et al., 2009; Ringgenberg et al., 2012; Marchant Forde, 2009) that in individual crate are lacking. In such scenario, it seems likely that the aggregate effect of different stressors acts on the sows exhausting their ability to cope with adverse events, as described also in other species (Barton and Iwama 1991).

The farrowing system and the early postpartum management play a pivotal role in preventing piglet mortality and in sows' welfare (Wiechers et al., 2021; Morgan et al., 2021; Nicolaisen et al., 2019), and the modern swine industry is paying much effort implementing such sensitive phases. Though, our findings suggest that particular attention should be paid to all environmental parameters rather than confinement systems alone.

8.5. Conclusions

Our study aimed at assessing HC, HDHEA and their ratio as bio- markers of allostatic load and resilience within a multifactorial experimental design. The hypothesis that different models of farrowing crates could differently affect the allostatic load and the resilience of the sows was disproved by the lack of a significant effect on the markers used. Still, it is worth to underline that they represent an improvement in piglet crushing reduction. Our experimental design showed that during the pregnancy period in collective pens HC/HDHEA ratios were never lower than those in the following characterized by the early postpartum and lactation in individual crates, because of a cumulative effect of different stressors. Though, our findings suggest that particular attention should be paid to all environmental parameters rather than confinement systems alone; moreover, it should be further investigated the influence of the social interaction in collective pens that cyclically re-occurs in the standard swine production system.

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CRediT authorship contribution statement

Tanja Peric: Conceptualization, Methodology, Validation, Formal analysis, Visualization, Writing – review & editing, Supervision. Claudio Mazzoni: Conceptualization, Methodology, Visualization, Writing – review & editing, Supervision. Francesca Quai: Investigation, Writing – original draft, Visualization. Alessio Cotticelli: Investigation, Writing – original draft, Visualization. Isabella Pividori: Formal analysis. Mirco Corazzin:. Antonella Comin: Methodology, Supervision, Writing – review & editing. Carla Bresciani: Conceptualization, Resources, Supervision. Alberto Prandi: Conceptualization, Resources, Supervision.

Declaration of Competing Interest

None.

8.6. References

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9. Association between a single measurement of progesterone and cortisol blood concentrations at two to one week before parturition, and number of fetuses in the Teramana goat

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Summary

The Teramana goat is an at-risk breed, needing population protection and programs to increase their numbers. The first step for a population increase is the best management of reproduction, leading to an as high as possible number of healthy and viable kids born. To this purpose, beside the optimization of mating, the best possible management of pregnancy and parturition is mandatory. The goat is a prolific farm animal in which single, double, or triple ovulations can occur, leading to singleton, twin or triple pregnancies, and the birth of multiple kids. Twins and triplets are associated to increased risk for perinatal mortality and need a special surveillance and possible assistance at birth. Knowledge of the number of fetuses that have to be delivered from each goat could be a practical tool for a better management of parturition. Among the methods to define the number of fetuses in the goat, the measurement of blood progesterone (P4) concentrations has provided inconsistent results. Therefore, the present study was aimed to assess the possible association between the maternal concentrations of plasma P4 and cortisol (C), two hormones possibly associated to the number of fetuses, measured only once at about two to one week before parturition in Teramana goats, and the number of fetuses. The results, obtained from 23 does, showed that both plasma P4 and C are higher in does bearing multiple fetuses than does with singleton pregnancies. However, the single measurement of plasma C, but not P4, two to one week before the expected parturition in the Teramana goat is useful to distinguish between does bearing singleton and triplet pregnancies for a better surveillance and assistance at delivery. Therefore, it could represent a tool for the best management of reproduction in a breed population at risk for extinction.

Keywords: goat, late pregnancy, cortisol, progesterone, number of fetuses

9.1. Introduction

The Teramana goat breed is an ancient Italian breed typical of the Abruzzo region, and especially of the Appennini mountains region around the city of Teramo, obtained by crossbreeding with the Garganica breed, typical of the Gargano area in the near Puglia region. The Teramana breed is fully adapted to hostile environments and rugged or bushy pastures. Since 2002, an official breed registry defined the typical breed characteristics: a medium body-size breed, its height is 60-85 cm, and body weight ranges between 45 and 80 kg. Although, in origin this breed was a double- attitude breed for meat and milk production, at present its main attitude is the production of milk, with and average production of 250-400 L of milk in 180-240 days of lactation. Although in the past the Teramana breed had over 500 animals, since 2014 the national pastoral association (ASSONAPA) reported a total of 56 recorded does, reared in only two flocks in the L'Aquila area. The Teramana goat breed was considered among the endangered breeds, which needed population protection and programs to increase its numbers. The first step for a population increase is the best management of reproduction, leading to an as high as possible number of healthy and viable kids born. For this purpose, beside the optimization of mating the best management of pregnancy and parturition is mandatory.

The goat is a prolific farm animal in which single, double, or triple ovulations can occur, leading to singleton, twin or triple pregnancies, and the birth of multiple kids. The number of ovulations and, in turn, the number of fetuses is influenced by multiple factors such as breed, age, parity, nutrition, season and bodyweight (16). Twin or triplets' pregnancies in goats were associated with increased risk of perinatal death (16). It was reported that in milk producing goats, perinatal mortality can amount to 0-17% in singleton (9, 18), 13-18% in twin, and 18-83% in ≥ triplet (1, 12) pregnancies. Therefore, for a better management of parturition and to improve kids' survival the knowledge of the number of fetuses carried by each doe could be useful in a practical setting.

From a physiological perspective, pregnancy in does is maintained by the action of progesterone (P4) entirely produced by the corpora lutea, developed after the single or multiple ovulations, and possibly associated to a similar number of developing embryos. This aspect led to a scientific investigation on the possible relationship between the number of corpora lutea, circulating P4 concentrations and number of fetuses. However, the studies on the association between blood P4 concentrations and number of fetuses provides inconsistent results. In 1991,

Jarrel and Dziuk (8) reported that blood P4 was higher in goats with multiple corpora lutea from 7 to 30 days of pregnancy, while the number of corpora lutea or of fetuses did not show an effect on plasma P4 concentrations after 30 days of pregnancy. A recent study (17) reported that plasma P4 does not correlate with the number of fetuses during early pregnancy in goats with a relation between fetuses' number and P4 at 51 days of pregnancy. In mid pregnancy, the plasma P4 was greater in does with twins than those bearing singleton pregnancies (18.91 ± 0.67 vs 14.51 ± 0.47 ng/mL) (17).

Beside P4, in late pregnancy also cortisol (C) plays an important role, mainly for final fetal maturation processes and for triggering the parturition in several species. Studies in sheep have shown that cortisol produced by the fetal adrenal glands is pivotal to initiate the hormonal cascade leading to the parturition onset (10), and, similarly, increased C plasma concentrations were reported in goats during labor (7). The maternal circulating C concentrations could therefore sum up C produced by the fetus(es) and by the mother.

According to this, the measurement of maternal blood C concentrations could vary depending on the number of fetus(es) carried by the mothers. However, the body homeostasis could balance the overall maternal blood C concentrations, independently to the number of fetus(es).

Therefore, the aim of the present study was to assess the possible association between maternal P4 and C plasma concentrations and number of fetuses in the Teramana goat. The possible effect played by maternal age and parity on number of fetuses was also assessed, as well as the correlation among all the studied factors.

9.2. Material and methods

Ethics. The study was approved by the Comitato per il Benessere Animale Università di Bologna (Prot N 62126- 2018).

Animals. The study was performed on a group of goats reared in the Abruzzo region under a program for population increase in the Teramana breed. All the Teramana goats were kept under natural photoperiod at a latitude of 42°43'34.351"N and a longitude of 13°46'21.539"E, at about 270 m above sea level.

The flock consisted of 44 does and 2 bucks kept in open paddocks and fed hay *ad libitum* and 400 g/d of a commercially prepared food. Water was available *ad libitum*. At spontaneous estrus occurring during the breeding season, 30 does were submitted to natural mating. Pregnancy was detected by trans-rectal ultrasonography using a portable ultrasound machine (®Ge Logiq Book Xp, Best Medical srl, Santarcangelo di Romagna, RN, Italy) equipped with a linear 7.5 MHz probe during the 4th week after mating and confirmed by trans-abdominal ultrasonography at 35-40 days of pregnancy, using a convex 3.5 MHz probe. Twenty- four does, 1-6 years old, 1-4 parity (4 primiparous and 20 multiparous) were found pregnant and enrolled in the study. After a normal pregnancy course, at impending parturition the does were allowed to deliver spontaneously, but under kidding surveillance for a prompt obstetrical intervention in case of dystocia occurrence. At birth, the number of kids for each doe was recorded. The timing of placenta shedding was also considered normal when it was < 12 hours from birth (13). In the first week postpartum, clinical daily monitoring was also performed to assess the normal postpartum period in does and to assess the survival of kids.

Blood sampling. At about two to one week before the expected date of parturition, each doe was submitted to a single blood sampling. Blood samples were collected into heparinized vacuum tubes from the jugular vein. Samples were centrifuged (20 min at 1000 × g) within 30 min of collection, and plasma was stored at –20°C until analysis.

Hormonal analyses

Progesterone. Plasma progesterone concentrations were determined by radioimmunoassay (RIA) as described and validated in goats by Bono et al. (2). The antiserum, raised in a rabbit to 11α-hydroxyprogesterone-hemisuccinate- BSA, was used at a dilution at 1 : 12000. The cross-reactions of other steroids were: 11α-hydroxyprogesterone 83.3%; 11β-

hydroxyprogesterone 15.7%; 21-hydroxy- progesterone 4.0%; 17α-hydroxyprogesterone 1.7%; 20α-dihydroprogesterone < 0.1%. The assay sensitivity was 21.9 ± 0.74 pg/tube.

Cortisol. Plasma cortisol concentrations were determined as reported by Tamanini et al. (19) using a RIA method. The sensitivity (90% B/B0) of the cortisol antibody was 4.9 ng/ml, Intra-assay and the cross-reactivities were as follows: 20.4% with cortisone, 74.6% with deoxycortisol- 11a, 1.1% with corticosterone and 0% with progesterone and oestrogens.

Grouping of animals. Does were retrospectively grouped on the basis of number of fetuses: i.e. single, twin or triplet pregnancy.

Statistical analysis. Data about the possible association between the number of fetus(es) and: 1) the maternal blood P4 and C concentrations one week before parturition; 2) the maternal age and parity were assessed through ANOVA with the number of fetus(es) as fixed factor, and plasma P4, C, maternal age and parity as random factors, followed by a post-hoc test. The Pearson's correlation test was used to assess possible correlations among all the studied parameters, while the Spearman's correlation test was used to assess possible correlations between the number of fetus(es) and the plasma P4 and C, and maternal age and parity (Jamovi ver. 1.6.23 for Windows operating system). Significance was set with $p < 0.05$.

9.3. Results and discussion

Clinical findings. Out of the 30 mated does, 24 (80%) resulted in pregnancies. However, one primiparous doe was found dead before parturition and its samplings were excluded from further analysis. In the other 23 does, kidding occurred at term, with pregnancy lasting 149 ± 5 (143-157) days from mating. In twenty-one (91.3%) does, kidding occurred spontaneously without the need for obstetrical assistance, while 2 goats (8.7%) needed manual obstetrical assistance. Three (13%) does delivered single kids, 16 (69.6%) twins, and 4 (17.4%) triplets. Therefore, a total of 47 kids were born, with the mean of 2.0 kids/goat. One kid was stillborn (2.1%), while all the other kids were healthy, viable and survived the first postpartum week. The one stillbirth was delivered by a twin pregnant doe. Two (8.7%) of the does showed placental retention with spontaneous expulsion between 12 and 24 hours after parturition but did not develop any postpartum disturbance.

Results from ANOVA test. The ANOVA showed a significant ($p < 0.05$) association between the number of fetuses and plasma C concentrations, with higher C concentrations in does bearing triple fetuses, and with a trend ($p = 0.057$) for higher plasma C concentrations in does with two fetuses in comparison to does bearing only one kid. No significant associations were found between maternal age and parity on number of fetus(es). On the contrary, P4 concentrations did not show statistically significant association with the number of fetus(es).

Data (mean ± SD) about the maternal plasma P4 and C concentrations in does grouped according to the number of fetus(es) are reported in Table 1.

Tab. 1. Data about the maternal plasma P4 and C concentrations in the 23 does grouped according to the number of fetus(es) (mean ± SD)

Hormonal concentrations	**Number of fetus(es)**		
	1 (n = 3)	**2 (n = 16)**	**3 (n = 4)**
P4 (ng/mL)	8.9 ± 3.98 [a]	15.5 ± 5.26 [a]	19.1 ± 9.95 [a]
C (ng/mL)	15.7 ± 2.91 [a]	23.0 ± 10.42 [ab]	29.1 ± 9.66 [b]
Explanation: a, b – superscripts in rows indicate significance with $p < 0.05$			

Results from Pearson's correlation test. The Pearson's correlation test showed a significant positive correlation between maternal age and parity ($r = 0.97$, $p < 0.001$); between maternal plasma P4 concentrations and the number of fetus(es) ($r = 0.39$, $p < 0.05$); between maternal

plasma C concentrations and the number of fetus(es) ($r = 0.36$, $p < 0.05$); and between maternal plasma P4 and C concentrations ($r = 0.40$, $p < 0.05$).

Results from Spearman's correlation test. When the correlation among all the other parameters (maternal age and parity, plasma P4 and C concentrations) was assessed in relation to the number of fetuses, age and parity were significantly positively correlated within pregnancies with one ($r = 1.0$, $p < 0.001$), two ($r = 0.99$, $p < 0.001$) or three ($r = 1.0$, $p < 0.001$) fetuses.

In order to increase the knowledge about reproduction in the at risk for extinction of Teramana the goat breed, the aim of the present study was to assess the possible relationship between the number of fetuses and maternal plasma P4 and C concentrations measured one week before the expected date of parturition, and maternal age and parity.

From a clinical standpoint, the 80% pregnancy rate is very similar to the 83-85% reported by Jarrell and Dzink (8) and by Singh et al. (17). However, a recent review by Robertson et al. (16), reported that conception rates in goats depends on multiple factors and could range widely between 60 and > 93%. Pregnancy length was 149 ± 5 days, which is in agreement with the 146-151 day range reported by Hydbring et al. (7), and with the 150 (145-155 days) reported by Probo et al. (14) for other goat breeds.

The does gave birth to 47 kids, with only one kid loss (stillborn) (2.1%) from a twin pregnant doe. This stillborn rate is very low when compared to the 4.2% reported by Mellado et al. (11) for dairy goats. Similar to other reports (16), this stillborn kid in our study was not caused by dystocia. At parturition, the 8.7% of dystocia was also the 8.7% placental retention (however, not occurring in the same does with dystocia), which is a bit higher than the 3-5% rate reported by Braun (3).

According to the number of fetuses, the distribution found in the present study agrees with data previously reported for other breeds, in which most goats give birth to twins (8, 14, 16, 17). This result was also confirmed by the 2.0 mean number of kids/goat, in agreement with previous reports in other goat breeds (14, 16). One week before the expected date of parturition, the mean maternal plasma P4 concentrations were lower in does bearing singleton pregnancies than those bearing twins, while does bearing triple pregnancies showed the highest concentrations (8.9; 15.5 and 19.1 ng/mL, respectively). Although there was an apparent difference among the mean plasma concentrations in relation to the number of fetuses, the

absence of statistically significant differences prevents the use of plasma P4 concentrations measurement to predict the number of fetuses bearing by the goat.

This result agrees with a previous study (8) in which plasma P4 concentrations were correlated to number of fetuses only in early pregnancy and converged toward the concentration of about 5 ng/ml by 35 days of pregnancy, irrespective by the number of fetuses. A more recent study showed that plasma P4 concentrations in does during mid-pregnancy was affected by fetal number, with higher concentrations in does carrying twins than singleton fetuses (18.91 + 0.67 vs 14.51 + 0.47 ng/ml). A study from Haldar et al. (6) found a significant association between the number of fetuses and plasma P4 concentrations with higher concentrations in does bearing triplets than twins or singleton pregnancy, between 84 and 21 days before parturition. It should be noted that the plasma P4 concentrations observed in the present study were higher than the study from Jarrell and Dziuk (5-10 ng/ml at 35 days of pregnancy) (8), but comparable to those reported by Probo et al. (12.9 ng/ml) (14) at a similar timing of sampling. In contrast, the plasma P4 concentrations found in the present study were lower than those reported by Singh et al. (17) in mid-pregnancy does with singleton fetuses (14.5 ng/ml), but very similar when twin pregnancies were concerned (18.9 ng/ml). In the present study plasma P4 concentrations were also lower than those reported by Gaafar et al. (24.5 ng/ml) (5). However, as stated by Singh et al. (17), the different values reported by the different studies could be attributable to multiple factors, such as the breed, age, breeding system and especially by the method of P4 analysis.

The mean plasma C concentrations, ranging between 15.7 to 29.1 ng/ml, were markedly higher than the concentrations reported by Probo et al. (5 ng/ml) (14). The mean plasma C concentrations showed a similar trend of increase in dependence of the number of fetuses, but, differently to plasma P4 concentrations, the plasma concentrations of C were significantly higher in goats bearing triplets than singleton pregnancies. This result suggests that the measurement of plasma C on a single blood sample collected one week before the expected day of parturition could be a useful tool to estimate in advance the number of fetuses that each goat will deliver. From a practical standpoint, this is important for parturition surveillance and assistance, allowing a more focused attention to the goats at the time of delivery and a better assistance to the newborn kids.

The increasing plasma C concentrations according to the number of fetuses found one week before parturition could be reasonably addressed to the cumulative secretion of cortisol by

multiple fetuses at the end of pregnancy. In goats, in fact, the direct effect of the fetus in the process of triggering parturition through the activation of placental C21-steroid 17alphahydroxylase was demonstrated (4).

The association between increasing plasma P4 and C concentrations and increasing number of fetuses were also confirmed by the significant positive correlation found for both hormones.

The similar trend of higher plasma P4 and C concentrations with increasing number of fetuses were also corroborated by the significant correlation found between the two hormones. If higher plasma C concentrations could be attributable to the increased number of fetuses, the higher plasma P4 concentrations found in does bearing multiple fetuses could be the result of the cumulative activity of multiple corpora lutea, even if to the authors knowledge this aspect at the end of pregnancy was not investigated in the goats.

Although the significant positive correlation between maternal age and parity is not surprising, age and parity resulted positively correlated to the number of fetuses bearing by the does. It was reported that the ovulation rate in goats is influenced by many factors, among them age and parity have a significant effect, with a lower rate in primiparous goats (16).

In conclusion, although both plasma P4 and C were higher in does bearing multiple fetuses than does with singleton pregnancies, the results from the present study showed that the single measurement of plasma C, but not of P4, one week before the expected parturition in the Teramana goat might be useful to distinguish between does bearing singleton and triplet pregnancies and for a better surveillance and assistance at delivery. Therefore, it could represent a tool for the best management of reproduction in an at risk for extinction breed population.

9.4. References

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10. On the use of 3D camera to accurately measure volume and weight of dairy cow feed

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Abstract - The paper discusses the challenges facing the dairy industry due to increased farm sizes and reduced staff-to-animal ratios, which are impacting animal welfare. The development of precision livestock farming (PLF) technologies has gained momentum to address these challenges. PLF technologies can assess animal welfare and health status by monitoring animal behavior and biological changes, and alerting farmers of any issues. However, the applicability of PLF tools in other productive phases of the dairy cattle is still limited. The article focuses on the challenges of managing unweaned dairy calves, particularly the variability in relation to when calves start consuming solid feed, and how PLF technologies can be used to monitor individual calf intake and manage weaning at the individual level. The attention is mainly focused on the advantages of using automated feeders for unweaned dairy calves, including labor savings, greater precision in measurement and control of individual intake of liquid and solid feed, and higher preweaning growth rates. In particular, a method is proposed, involving a 3D depth camera and a proper algorithm to measure the volume and weight of eaten feed. The method is preliminarily assessed in tests conducted in laboratory, which highlight a remarkable concurrence (differences as low as 2 %) with respect to nominal values.

10.1. Introduction

Nowadays, the dairy industry is facing an accelerated trend towards larger farm sizes and higher-yielding animals [1]. Consequently, the husbandry staff to animal ratio is decreasing [2] and the implications of mass-production systems on the welfare of dairy animals are troubling the consumers [3]. In response to these challenges, the development of new technologies has gained momentum. Precision livestock farming technologies have been developed to assess the welfare and health status of dairy animals by reducing labour demands [4]. A variety of systems using technologies (i.e., sensors, cameras, or microphones) are currently available and several countries are already investing in their development to be part of strategies to move toward sustainable agriculture [5].

Through this system, the farmer can monitor the animals' everyday lives irrespective of the size of the herd [6]. Particularly, they monitor the animal behaviours, the behavioural and biological changes that influence the animals' health and welfare status [7]. The detection of such behavioural changes triggers a warning signal, suggesting an immediate action, and leading to an early problem diagnosis or an immediate housing practices assessment [8].

Currently, the most common devices used in cattle are accelerometers to detect calving, estrus and lameness (based on activity data); cameras to determine standing heat (combined with machine learning), body condition scores (BCS), and estimate weight; reticulum boluses to monitor estrus, calving and physiological factors (i.e., body temperature or pH); and ear sensors to monitor the temperature [5].

However, there is still a lack of knowledge on the applicability of precision livestock farming tools in other productive phases of the dairy cattle.

10.2. Problem statement

The current calf management practices need profound changes to improve dairy calf health and survival, enhance the long-time performance of dairy heifers and satisfy consumer interests in farm animal welfare [9]. The development of calves depends on prenatal and postnatal conditions. At birth, calves are defined as functional monogastric, relying on nutrients from milk or milk replacer [10].

Therefore, the pre-weaning stage represents a biological critical window that may affect the performance and overall well- being of calves for their entire life [11]. Early weaning is adopted to accelerate the early intake of solid feed and the development of the forestomach system [12]. Nevertheless, a later weaning regimen allows a smooth transition of physiological functions from the pseudomonogastric status to full ruminant status avoiding potential consequences for later health and metabolic performance [12]. In later weaning method, body maturation of calves is supported not only by milk/milk replacer but also by solid feed (concentrate and hay).

Nowadays, dairy calves are typically weaned from milk to solids according to a gradual weaning method (step-down technique) based on age [13]. It consists of gradually reducing the milk allowance, from four weeks of age until weaning. Still, even with the use of a step-down weaning program, a variability is observed in starter intakes of intensively managed calves, suggesting that the calves begin to consume starter based on their individual ability cope with early weaning [14].

Heinrichs and Heinrichs [15] reported a 27 % coefficient of variance in the age to consume 0.91 kg of starter; de Passillé and Rushen [16] reported ranges of 59 days of age for calves to first consume 0.2 kg/day of starter and of 36 days to first consume 1.4 kg/day of starter throughout the milk-feeding period (12 L/day). Similar results were reported for different levels of milk allowances ([17]-[18]).

This variability in relation to when calves begin to consume starter suggests that individuals will vary in how well they cope with early weaning. Hence, moving toward individual-based and data-driven farm management, there is growing interest in monitoring the solid intake of unweaned dairy calves and managing weaning at the individual level [16].

10.3. Related works

A In the last few years, there has been a growing interest in the use of automated feeders for unweaned dairy calves. The advantages of automated feeders include labor savings [19]; greater precision in the measurement and in the control of individual intakes of liquid and solid feed [16], [17]; simpler feeding of unweaned dairy calves more milk/milk replacer; and higher preweaning growth rates [10], [20].

There is mounting evidence that high preweaning growth is associated, in some way, with increased first-lactation milk yield [21], [22]. Previous research demonstrated that the same automated feeder used for milk can be used to supply starter and record both milk and starter intake independently [16], [23], [24]. They gave calves access to automated feeders supplying milk and starter, controlled by a single computer (CF 1000 CS Combi, DeLaval Inc., Tumba, Sweden). This computer recognized individual calves from their radio frequency ID (RFID) tags and independently controlled and recorded milk and starter intake for each calf. Hay and water were available ad libitum from automated feeders that weighed the intake of each calf at each meal (RIC, Insentec B.V., Marknesse, the Netherlands).

Rosenberger et al. (2017) [24] tested a step-down procedure, where milk allowance was initially reduced at 42 days of life and then again at weaning (50–54 days), providing social housing and access to forage, and observed a lack of difference in total starter intake throughout the experimental period. Still, in their conclusions, they stated that further research was needed to compare weaning protocols and identify which features are required to set up weaning protocols tailored to meet the needs of different individuals [16].

In the present study, we used a 3D camera to accurately measure the volume and weight of dairy calves during the pre- weaning period.

10.4. Proposed method

The key idea underlying the proposed method is the exploitation of a 3D depth camera in order to digitize the distance with respect to the surface of the solid feed thus making it possible to estimate its volume and, knowing the density, the associated weight ([25]-[29]). In particular, the 3D camera is capable of reconstructing a suitable map of the distance of each pixel in its frame and, by means of straightforward calculations, the volume of the regions of interest. For the sake of clarity, the operating steps of the proposed method will be discussed in detail in the following with respect to an application example.

1. The first step accounts for the digitization of the framed scene. To this aim, the camera subdivides its field of view into a defined number of pixels (let us suppose, for example, M × N pixels in the whole image) ([30]-[33]). The measured values are arranged according to an M × N matrix, whose entries are the distance of the specified pixel with respect to the camera. It is possible to provide an image in fake colours, where each colour corresponds to a specific distance with respect to the camera (Figure 1).

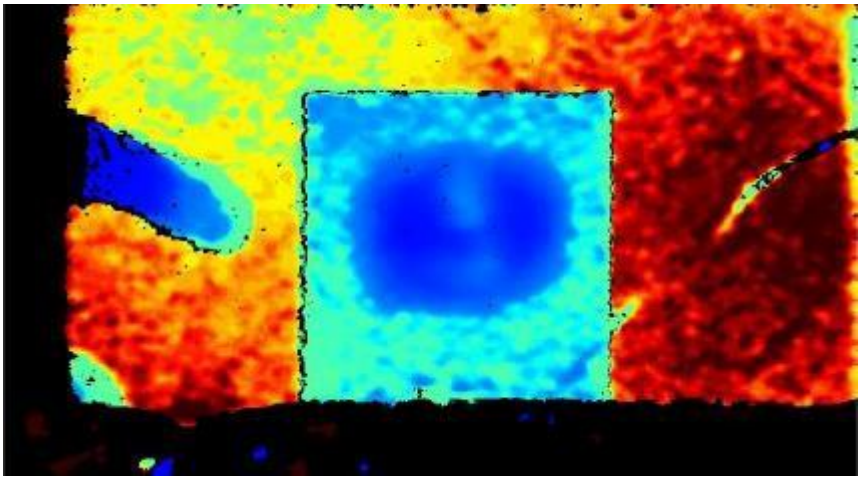

Figure 1. Example of 3D distance map represented in false colours.

2. The region of interest (i.e., the one whose volume we are interested in) is then determined by selecting the coordinates (i.e., row and column indexes) of two points in the acquired 3D image (as an example, the points A and B in Figure 2).

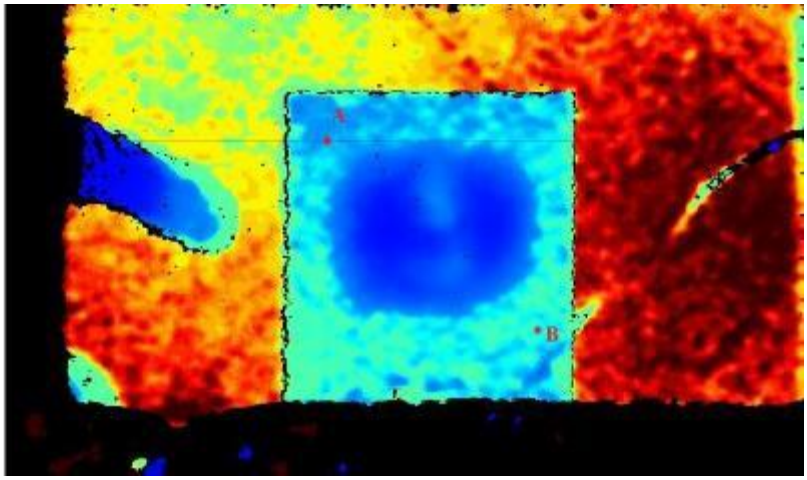

Figure 2. Determination of the region of interest by selecting two opposite points A and B.

3. The coordinates of two further points are singled out (as an example, the points C and D in Fig. 3) in order to define the distance of each point of the manger with respect to the camera. To this aim, the distance *d* of a generic point of the bottom of the trough (whose coordinates are referred to as *x* and *y*) is obtained according to a bi-linear approximated model:

$$db\,(x,y) = \frac{d1(xD - x)(yD - y)}{(xD - xC)(yD - yC)} + \tag{1}$$

$$+\frac{d2(x - xC)(yD - y)}{(xD - xC)(yD - yC)} +$$

$$+\frac{d3(xD - x)(y - yC) + d4(x - xC)\,(y - yC\,)}{(xD - xC)(yD - yC)}$$

where $(xC\ ,\ yC)$, $(xD,\ yD)$, $d1$ and $d4$ are the coordinates of the pixel C and D, respectively; while, $d2$ and $d3$ represent the distances of the points whose coordinates are equal to $(xC\ ,\ yD)$ and $(xD,\ yC)$, respectively.

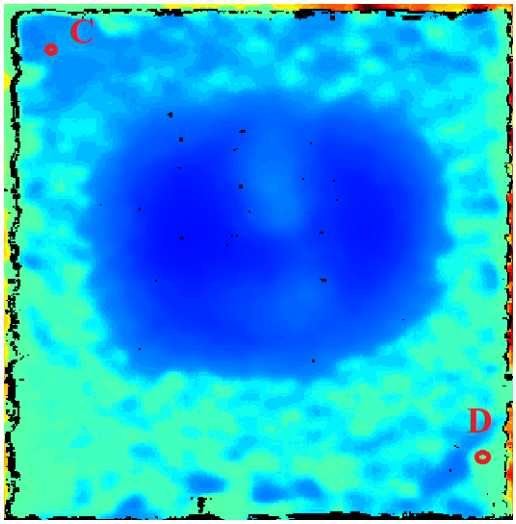

Figure 3. Determination of the trough base by selecting two opposite points C and D.

4. The volume associated with the generic pixel (x, y) can thus be evaluated as the parallelepipedon whose height is given by the difference between the distance of the pixel from the camera $d(x, y)$ and the estimated distance of the trough at the same pixel $d_b(x, y)$. As for the area associated with the pixel, it strictly depends on the distance with respect to the camera; in particular, said Δx and Δy the base and height of the pixel and α and β the angle defining the depth field of view, the area of the pixel (i,j) can be expressed as

$$A(x,y) = \Delta x \cdot \Delta y = (d(x,y) \cdot tan(\alpha)) \cdot (d(x,y) \cdot tan(\beta)) \quad (2)$$

5. The volume of interest can be finally achieved by adding all the volumes of the pixels involved in the region of interest selected in step #2.

10.5. Measurement setup

To preliminarily assess the feasibility of the proposed method, a suitable measurement setup was implemented in laboratory conditions, using a 3D Depth camera, namely Realsense™ D455 by Intel© (Figure 4).

Figure 4. The 3D depth camera adopted for the preliminary method assessment.

The RealSense D455 is a high-resolution depth camera developed by Intel that utilizes Time-of-Flight (ToF) technology to provide accurate and detailed depth data [34]. It is a compact, lightweight device, measuring 101 mm × 24 mm × 9.5 mm and weighing only 45 g, making it easy to integrate into a wide range of applications.

One of the key features of the RealSense D455 is its depth range, which extends from 10 cm to 10 m. This makes it well-suited for a variety of applications, including robotics, drones, augmented reality, virtual reality, and more. The camera is capable of capturing depth data with a resolution of up to 1024 × 768 pixels, as well as colour data with a resolution of up to 1920 × 1080 pixels. The RealSense D455 can capture data at frame rates of up to 90 frames per second, depending on the resolution and mode selected. It has a wide field of view, with a horizontal field of view of 91.2°, a vertical field of view of 65.5°, and a diagonal field of view of 100.6°. This allows the camera to capture a large area of the scene, making it easier to track objects and navigate through the environment. The main specifications are summarized in Table 1.

Table 1. Main specifications of the 3D camera Realsense D455 exploited for method feasibility assessment.

Specifications	**Value**
Depth Technology	Time of Flight
Depth Range	10 cm to 10 m
Depth Resolution	Up to 1024 × 768 pixels
RGB Resolution	Up to 1920 × 1080 pixels
Frame Rate	Up to 90 fps (@640 × 480)
Field of View	87° × 58°
Interface	USB 3.1 Gen-1 Type-C
Dimensions	101 mm × 24 mm × 9.5 mm
Weight	45 g

The RealSense D455 uses a USB 3.1 Gen 1 Type-C interface for data transfer and power. It is compatible with a wide range of operating systems and development environments, and Intel provides an SDK (Software Development Kit) that allows developers to create their own applications and interfaces for the camera.

In particular, the authors implemented a dedicated MATLABTM code to control the camera and retrieve the distances measured in the framed scene. The code involves the steps of the method described in Section 4. With specific regard to the identification of both region of interest and trough base, the MATLAB function *ginput()* was exploited to allow the user to graphically determine the extent of the considered regions according to shown distance image.

10.6. Preliminary results

The 3D camera was installed on a camera holder whose distance from a reference plane could be modified and controlled thanks to a hand crank mechanism. The reference plane acted as the trough, and rabbit feed was exploited to assess the method's feasibility (Figure 5).

Figure 5. Realized setup for volume measurements of rabbit feed.

The 3D camera was positioned at a distance of 65 cm from the reference plane. A starting volume of 1500 cm^3 of feed was initially placed on the reference plane and the proposed method was applied to the acquired image. Figure 6 shows a picture *(top image)* along with the MATLAB reconstruction *(bottom image)* obtained by means of the proposed method; as for the measured volume, the value of 1490 cm^3 was obtained.

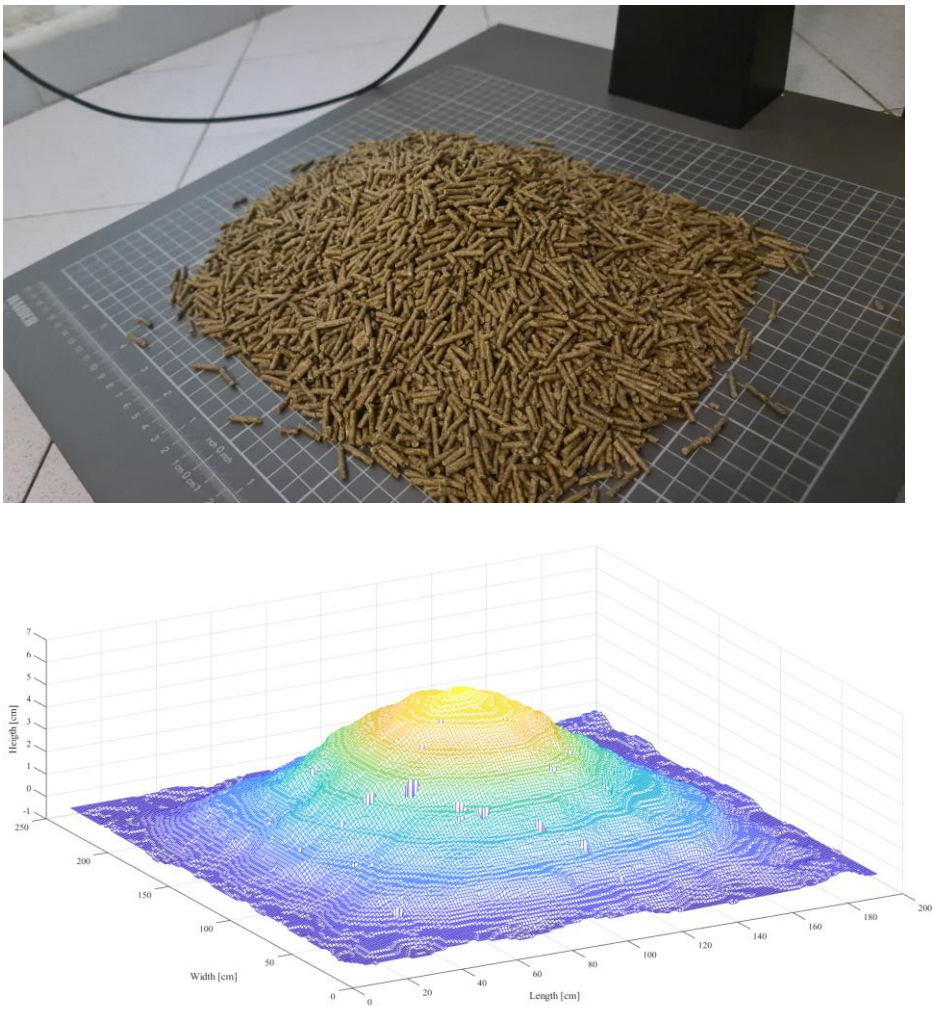

Figure 6. A picture (top) along with the reconstructed surface in the case of 1500 cm3 of rabbit feed (bottom).

The volume of feed was then reduced in two successive steps, whose value was 300 cm^3 and 200 cm^3, respectively. The associated MATLAB reconstructions are shown in Figure 7 and Figure 8, while the corresponding volume measures were equal to 1220 cm^3 and 1007 cm^3. As it can be appreciated, differences with respect to the nominal values always lower than 2 % were observed ([35]-[46]).

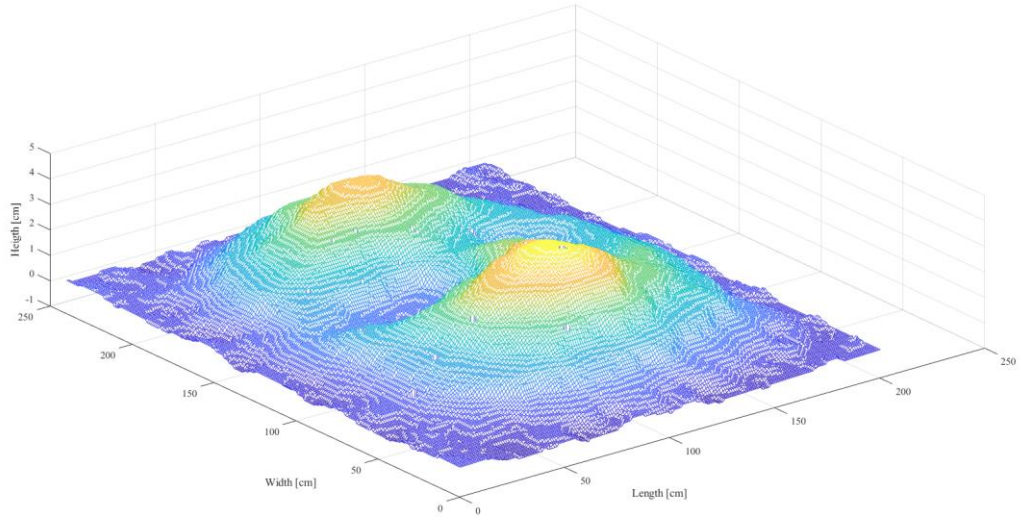

Figure 7. Reconstructed surface in the presence of nominal feed volume equal to 1200 cm3.

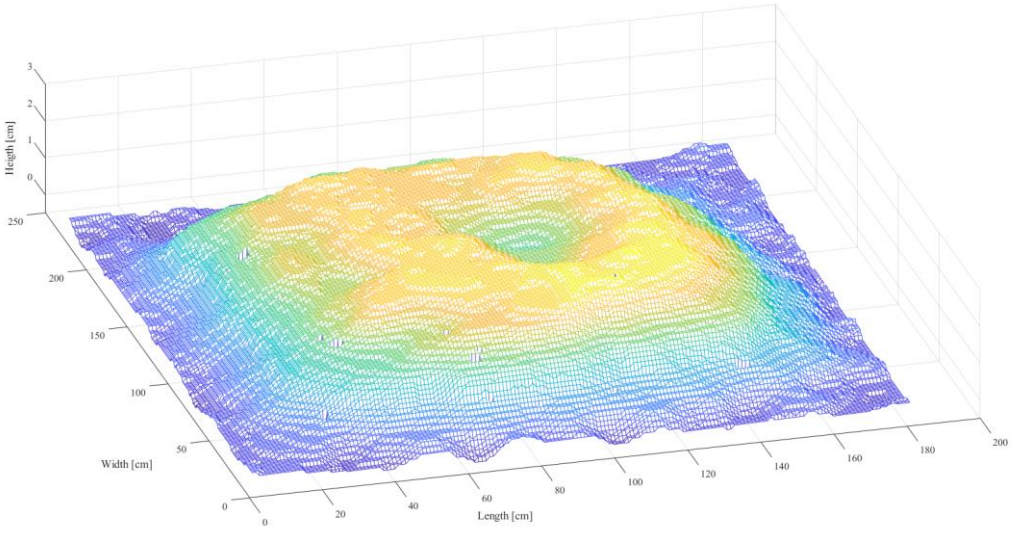

Figure 8. Reconstructed surface in the presence of nominal feed volume equal to 1000 cm3.

10.7. Conclusions

The paper presented a method based on both a 3D depth camera and a suitable digital signal processing algorithm for the measurement of feed volume for precision livestock farm applications.

In particular, the 3D camera provides a distance map of the framed scene, and the algorithm allows extracting measures of both the feed surface and the trough base. Geometrical considerations allow measuring the volume of feed as the sum of the volumes of all the parallelepipedon whose bases are associated with the pixel dimension at the measured distance and whose height is evaluated as the difference between the pixel measured and base-estimated distances.

Preliminary tests to assess the feasibility of the proposed method were carried out in the laboratory, employing a 3D depth camera by Intel. The volume of rabbit feed (nominally decreasing from 1500 cm^3 to 1000 cm^3) was then measured with differences expressed in relative percentage values as low as 2 %.

Ongoing activities are mainly focused on the metrological characterization of the proposed method with respect to possible parameters of the measurement setup, such as the distance and alignment of the camera, scene illumination and geometrical artifacts.

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11. Use of an automated walk-over-weighing system to monitor and forecast sheep liveweight in grazing sheep.

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Keywords

Grazing ruminants, Liveweight, Growth rate, Remote monitoring

11.1. Introduction

Sheep production remains a cornerstone of Australia's agricultural industry and it is mostly managed under extensive grazing conditions. Interest in intensive lamb finishing, defined as any system that aims to optimise lamb growth, has increased across Australia in recent years. A recently published guide for producers identifies "running a viable finishing system" as a key factor influencing cost efficiency (MLA, 2020). Besides finishing, young animals should be weighed, and fat scored regularly to guarantee adequate growth rates, respect welfare, recognize problems and poor performances, to adjust management accordingly and determining the time of sale (Leroux et al., 2023). In particular, sheep liveweight (LW) recording can be used to aid in decision making around weight gain, body condition, animals' health and nutritional status, responses to feeding programs, scheduling pasture rotation, developing precision nutrition strategies, or setting finishing programs, slaughtering schedules and market benchmarking (Brown et al., 2014; Wishart et al., 2017). However, frequent monitoring of LW in commercial sheep farms is still rare because it is time-consuming and stressful for both animals and farmers and it is even more challenging in pasture systems (Brown et al., 2014). As a matter of fact, there is still a perception that weighing causes stress and negatively impacts performance. A survey conducted by Jones et al. (2011) on a sample of sheep producers highlighted that only 17% of producers 'usually' weigh their ewes. When it comes to lambs, it has been reported that weighing every three to four weeks can help ensuring satisfactory lamb performances (MLA, 2020). Ideally, days to market and average daily gain (ADG) should be calculated for each animal to obtain a much more accurate picture of the performance of animals in the flock, because individual animal performances and management

can have a big impact on profitability, moreover considering sheep as groups rather than individuals conceals many poor performing individuals by the averages.

Animal liveweight is also an informative indicator of growth rate and body composition (McAuliffe et al. 2018). The ability to predict these attributes early in the production cycle could be of substantial value to sheep producers to enable tailored flock management strategies for different groups of animals (Jones et al., 2021). Growth rate is affected by the animals' own ability to grow (genetics), gender and several environmental factors. Biological growth can be defined as the weight gain of an animal until it reaches adulthood. In the early stages of the animal life, weight gain is greater than the adulthood and follows a sigmoid curve (Lupi et al., 2015). As the animal matures, growth rate increases until it reaches the highest growth rate (inflection point), and gradually decreases afterwards (Fitzhugh, 1976; Gómez et al., 2008). Conversely, commercial growth is the weight gain of the animals solely during the period comprised between birth and slaughter (determined by cultural aspects such as carcass weight demanded by consumers and technological criteria such carcass fat).

Walk-over weighing (WoW) is an emerging alternative to the conventional weighing in handling facilities (Morris et al., 2012). This is a method of automated liveweight data collection whereby sheep are encouraged to traverse a strategically placed weighing platform to access attractants such as feed or water as part of their daily routine. Mob-based walk-over weighing is a method of automated liveweight data collection, providing flock average liveweight estimates to make nutritional management decisions on a whole flock basis (Brown et al., 2014). A walk-over-weighing system (WoW) prototype was developed combining radio frequency identification (RFID) and load cells, resulting in an RFID-linked body weight of individual sheep with controlled sheep flow (González-García et al., 2018a). Such prototype could be less stressful compared with human handling and capable of collecting a much higher frequency of LW records per unit of time compared to the standard static weighing system (Brown et al., 2014; González-García et al., 2018a, 2018b, and 2021). The same system was validated for the first time under Mediterranean grazing conditions, with post-weaned ewe lambs (Leroux et al., 2023).

However, most of the reports using this technology have been tested only with adult females (Brown et al., 2012; González-García et al., 2021 and 2018b; Morris et al., 2012; Polat et al., 2013). Also, the repeatability and frequency of raw data collected by RFID-linked WOW have been questioned, since the timeframe required to collect a suitable number of observations per

sheep for on-farm decision making was large and variable within and across flocks (Brown et al., 2013).

Another important application of remotely collected LW data from sheep is on the prediction or forecasting of future LW to allow more precise animal marketing that meet market specifications (LW or carcass weight) and taking advantage of the market opportunities. However, no studies have assessed the potential of WOW technologies to predict LW of sheep with different lead time.

Hence, the aim of the present study was to evaluate the use of an RFID-linked walk-over-weighing system in a pastoral sheep production system to predict future LW with different lead times. This information could help with decision making around marketing of the animals to meet market specifications, optimise income, transport, and profitability. The hypothesis was that the WoW system could effectively track LW and growth patterns of animals and provide reliable data to accurately forecast LW of sheep in a grazing system.

11.2. Materials and methods

Animals and flock management

All experimental procedures were undertaken in accordance with the guidelines of the Australian code for the care and use of animals for scientific purposes (2013) and were approved by the Research Integrity and Ethics Administration of The University of Sydney (Approval # 983).

The research took place on a commercial property near Wallendbeen (Wallendbeen, NSW). The region receives an average annual rainfall of 612 mm predominantly through winter and spring. The experiment was carried out from mid-March to mid-June for a total of 94 days. The trial flock of lambs undergone a 21-day of adaptation period to the experimental setup similar to that described by Gonzalez et al. (2014). The flock consisted of 144 lambs grazing freely on approximately 22 acres of annual grass and subterranean clover. This pasture is typical of the southwest slopes region of NSW and included mainly of *Lolium rigidum*, *Hordeum glaucum* and *Trifolium subterraneum* good soil fertility accommodated by bi-decade fertiliser application to provide a nutritious forage diet for grazing sheep. The flock consisted of *Merino* and crossbred lambs (*White Suffolk* x *Merino*) of both genders as follows: 58 merino ewes, 47 merino wethers, 18 crossbred ewes, and 21 crossbred wethers.

These breeds are targeted for their quick growth and production of a large, lean carcase. The lambs were marked in November and weaned in December the year prior to the start of the trial. All animals received clostridial vaccine (GLANVAC ®6, Zoetis Inc., Australia) and an external antiparasitic application of a water-soluble suspension containing the active principle cyromazine (iO Venus Liquid, 500g/L, Aussie Vet Products, Gumdale, Australia). The central yard facilities included a walk-through foot bath treatment in zinc sulphate to water solution aimed at preventing ovine foot rot (*Dichelobacter nodosus*).

Instrumentation and paddock set-up

Each animal was manually tagged with an electronic RFID ear tag on the right ear, composed of low frequency (134.2 kHz), high-performance, non-reusable HDX High Performance Ultra EID Tag ISO Compliant ear tags (Allflex, Rahway, NJ, USA).

The remote walk-over-weighing station was installed at the entrance of a yard enclosing the single water source to record the liveweight (LW) of animals each time they came across, as showed in figure 1.

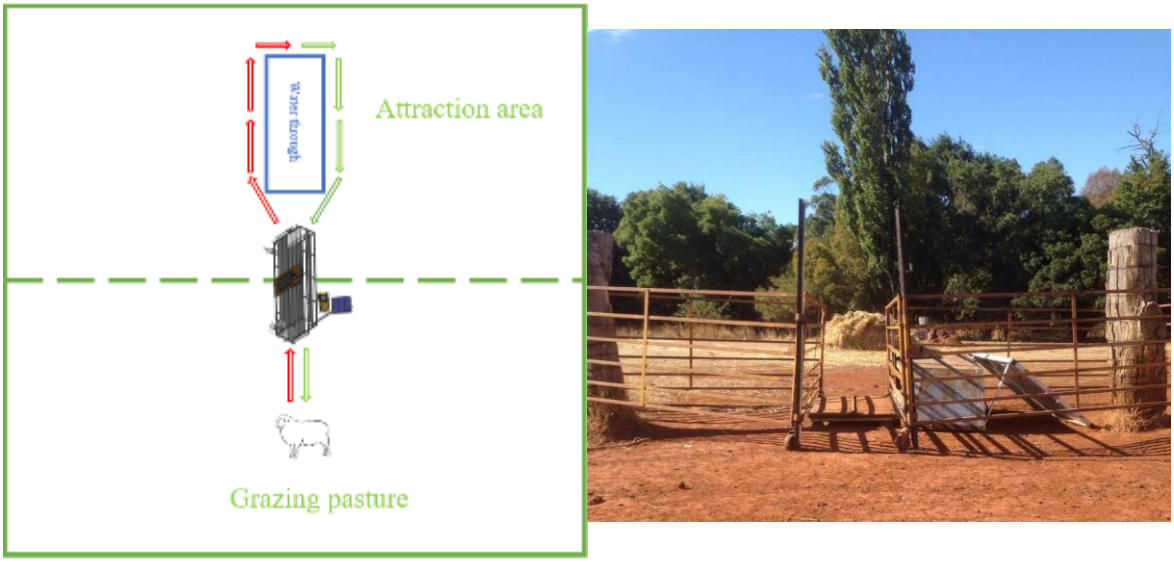

Fig. 1. Schematic representation of the paddock plan and the set-up of the walk-over-weighing (WoW) system to allow two-way flow of sheep to the water trough.

The station was configured to allow free flow of animals either way going in or out of the yard enclosing the water trough. Using the existing fencing infrastructure, a permanent gateway was further narrowed with portable yarding equipment to funnel the sheep into a 30m x 30m yard containing a fixed water trough. The weighing system consisted of load bars attached to a reinforced steel platform of dimensions 72 x 130 cm that acted as the weighing platform, a single RFID panel reader attached to the side panel on the right-hand side of the scale (as animal enters watering area). The single-entry point also acted as the exit point for all animals. One 12V AGM Deep Cycle battery was used to supply power to the unit with a portable 12V solar panel connected to increase battery longevity. A second battery was kept on site and interchanged when necessary. A schematic representation of the system has been previously reported by González-García et al., 2018a. The system recorded animal individual electronic identification (EID), date and time of visit and liveweight (kg). The flock was placed in the paddock with no WoW infrastructure for an adaptation period of three weeks before the trial commencement. This aimed to help the animals familiarizing with the permanent gateway leading to the watering location (attractant). One week prior to commencement the portable yarding panels were installed to narrow the gateway gap, still without the presence of a weigh platform. Two 3-yr old merino ewes were run with the flock for the initial stages of

infrastructure to facilitate adaptation of the lambs. This was done on recommendation of the owner to provide 'flock leaders' that would teach the younger, less experienced lambs as routinely performed by the company. On the day of trial commencement, the weighing platform and RFID panel reader were installed, and the flock was mustered from the external grazing area and encouraged across the platform. Animals were then left to return freely to the grazing area before the process was repeated. This mustering was conducted again the following day. On the third day the animals were observed from distance to voluntarily cross the platform and access watering facilities, no further mustering was deemed necessary.

The LW data were recorded as each animal entered voluntarily and walked through the WoW platform. Once inside, the antenna read the EID while the animal was traversing the platform and sent it to the XRP2 panel reader (Tru-Test). The animal identification was then sent to the weighing scale indicator (WoW-enabled XR3000, Tru-Test) which recorded the corresponding LW, date and time data. Thereafter, the operator downloaded the stored files into a personal computer for further processing and interpretation. The WoW scale system was calibrated by the manufacturer (Tru-Test) to have a resolution of minimum ± 1 kg.

Data analysis

Data from the weighing station was processed by first filtering for erroneous and biologically implausible data and then deleting outliers as per González et al. (2014), All data lines containing missing EID records or containing EID having LW lower than 12 kg or greater than 65 kg were deleted because all animals involved in the present study were within these ranges. The data was then fitted according to Eilers and Marx (1996). Data points below or above 1.5 times the residuals for each animal were deleted and then fitted again to calculate the predicted LW. Growth rate was then calculated as the first derivative throughout the predicted LW curve. The time between two successive LW observations for each animal was also calculated.

All statistical analyses were performed using R software (R Core Team, 2021). The future predicted LW (PW) of all animals was calculated daily on the next 20, 30, 40, 50, and 60 days ahead of any actual day throughout the trial. This PW was calculated by multiplying each animal's actual growth rate multiplied by the target days and adding the actual observed LW (OW). Daily growth rate can change dramatically over time which could affect the accuracy of PW, and more data used to calculate growth could yield more accurate predictions. Therefore, we estimated the average growth rate for the last 7 and 14 days calculating daily rolling average growth rates with the daily growth rates of the 7 and 14 previous days (7DGR and 14DGR,

respectively) for each animal and experimental day. This growth rate was then used to forecast the LW with different lead (target) days in the future (20, 30, 40, 50, 60 days) according to the following equation:

$$Predicted\ weight\ (PW) = (growth\ rate\ \times target\ days\ + actual\ observed\ weight\ (OW))$$

Where PW is the predicted weight at 20, 30, 40, 50, and 60 days from the actual date (PW20, PW30, PW40, PW50, and PW60), growth rate is either 7DGR or 14DGR, target day is 20 to 60 days ahead of the actual date, and OW is the actual observed LW on the day when the calculations are being made. Therefore, PW was calculated for the combination of 7DGR or 14DGR, and PW20 to PW60 (e.g., 7DGR-PW20, 7DGR-PW60, 14DGR-PW20, 14DGR-PW60).

The accuracy of the weight predictions was assessed using a linear mixed-effects in the lme4 package (Bates et al., 2015) with PW as dependent variable and the OW as independent for each 20 to 60 target days, whereas both animal ID and date were random effects. The hypothesis tested if the slope was different than 1 and the intercept different from 0. Lin's concordance correlation coefficient (CCC) was computed using the epi.R package to evaluate the prediction accuracy and precision of future PWs against the OWs on the lead day (Lin, 1989). The CCC combines the measurements of accuracy and precision to determine the deviation of the predictions from a perfect concordance (i.e., CCC = 1.0). The correction bias factor (C_b) was also reported to estimate how far the method resulted from the perfect correlation (deviation from a line at 45 degrees, no deviation from the 45-degree line occurs when $C_b = 1$) (Lin, 1989). In addition to analyzing the data for the entire trial, we also assessed the effect of predicting the weights both early and late in the trial, since growth rate may slow down as the animal matures. To achieve this, the analysis was split into two time periods (early or late) according to the mean date of the trial.

11.3. Results

The raw data contained liveweights of extreme values not likely to occur in these animals which were deleted before statistical analysis (Table 1). There was a total of 20,556 records throughout the 93 days of the trial, from which 5,117 (24.89 % of the original dataset) were implausible values ($12 < LW > 65$ kg) and were considered outliers. Out of the 144 total animals included in the trial, 7 were removed due to outlier values, and 5 had less than 89 days of observations. The final number of animals was 132 and the final predicted weights were calculated by interpolating two consecutive predicted weights (n = 7869) over a specific period to generate an average weight and fill the days with missing information. The final dataset included 12,360 final predicted weights with an average of 57.23 ± 14.66 days with at least one observation per animal.

Table 1 Descriptive statistics of liveweight data remotely collected in grazing sheep.

	n	Minimum	Mean	Maximum	Standard deviation
All data					
No. observations/an	144	5	142.6	295	43.23
Live weight (kg)	20,556	0	24.75	78.00	16.53
Live, weight, kg (12 kg < LW < 65 kg)	15,439	12.00	32.12	65.00	9.57
No. of animals after outlier removal					
No. days with observations/animal	137	4	56.57	81	15.31
Length of data (days)	137	37	91.42	94	6.94
Days with data (% of all days)	137	29.0	64.2	86.2	16.3
Predicted weight (kg)	8,006	14.93	32.07	65.12	8.36
Final predicted weight (kg/day)	12,641	14.93	33.21	65.12	8.72
Growth rate (kg/day)	12,641	-0.09	0.25	0.98	0.11
Number of animals with more than 89 days of observations					
No. days with obs	132	26	57.23	81	14.66
Length of data (days)	132	59	91.85	94	5.18
Predicted weight (kg)	7,869	14.93	31.96	65.12	8.24
Final predicted weight (kg)	12,360	14.93	33.00	65.12	8.45
Growth rate (kg/day)	12,360	-0.07	0.25	0.96	0.11

In figure 2 is presented the example of one sheep to show the raw data and the predictions (PWs) obtained with the aforementioned methodology (Gonzalez et al., 2014). An 8-days gap occurs between the predicted weights (green circles) due to battery dysfunction issue.

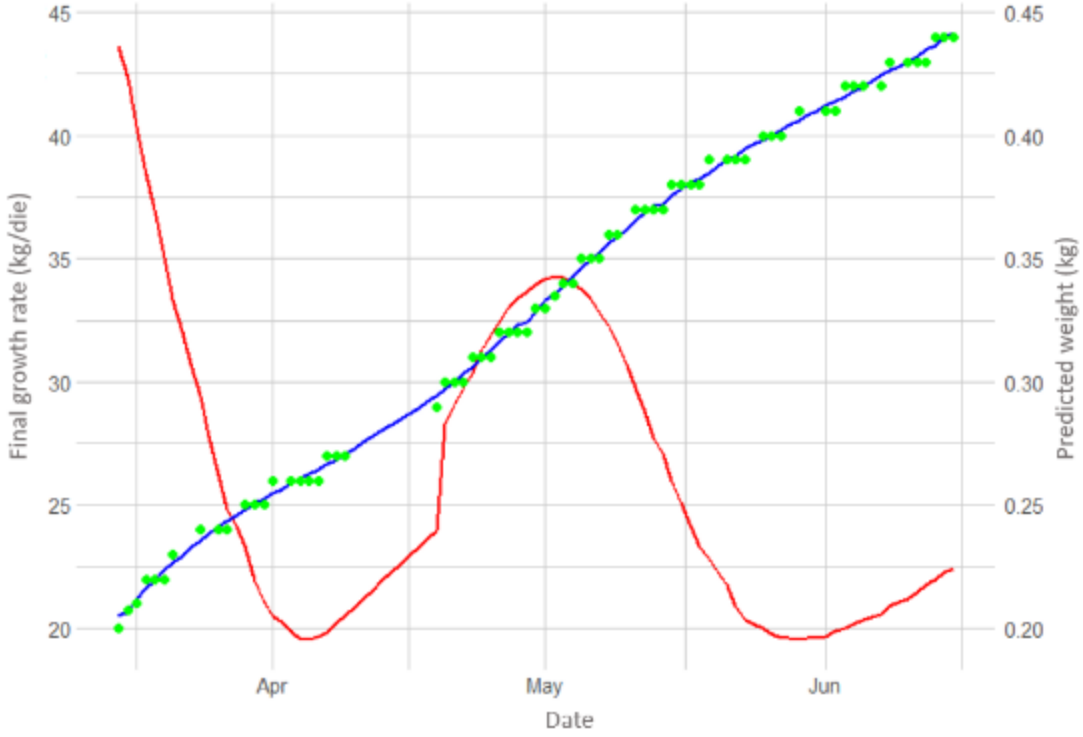

Fig. 2 Example of data from the weighing station, recorded LWs (predicted weights, kg) are presented as green circles, final predicted weights (kg) as solid continuous blue line, and growth rate (kg/die) solid continuous red line.

The 132 sheep included in the final dataset showed a growth rate of 0.25 ± 0.11 kg/die (mean ± standard deviation) throughout the 93 days of the trial (figure 3).

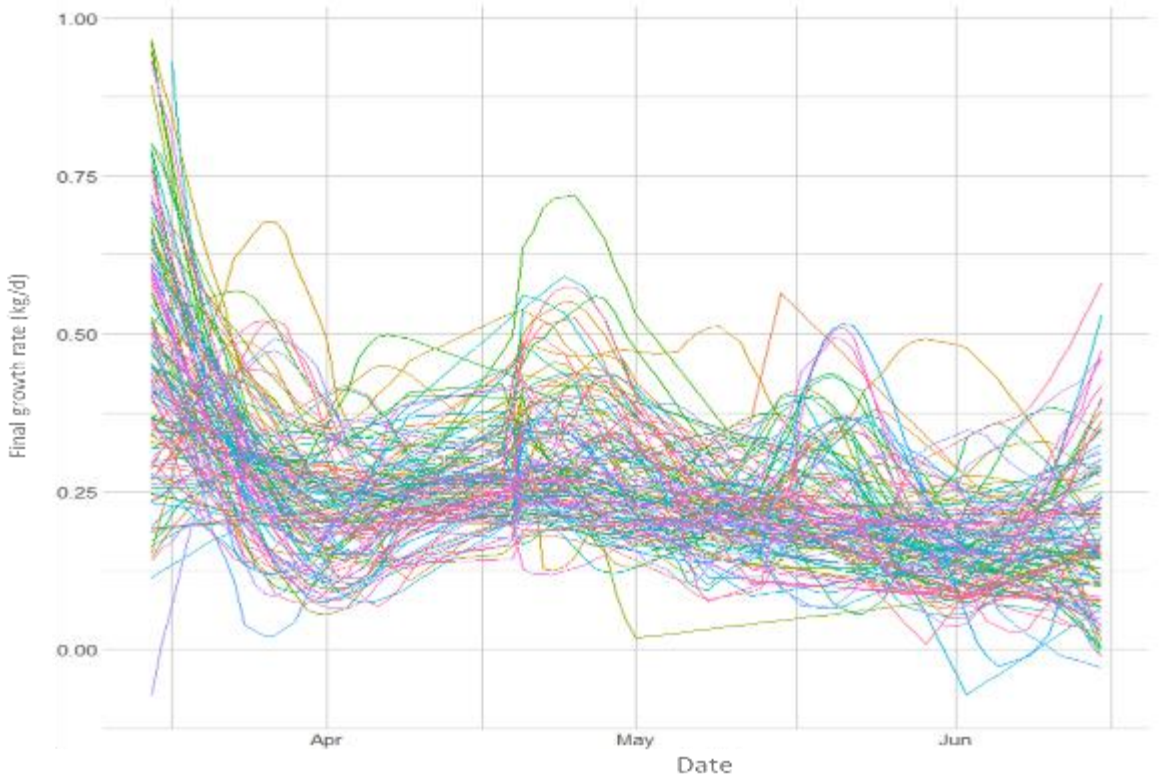

Fig. 3 Growth rate per individual sheep throughout the entire duration of the trial. Each line represents a growth curve of an individual animal (kg/day).

An average final predicted weight of 33.00 ± 8.45 kg (mean ± standard deviation) was recorded throughout the trial and is presented as solid continuous blue line in figure 4 alongside final growth rate (kg/die). The gaps between the predicted weights have been filled as described above.

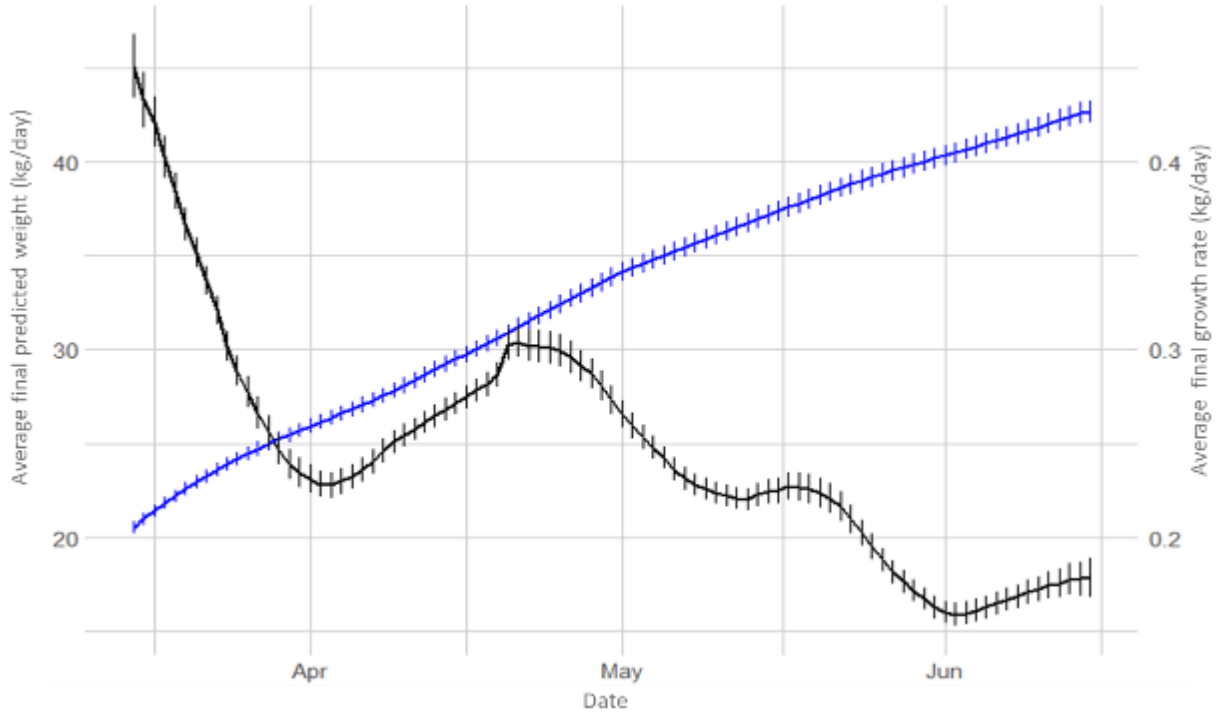

Fig. 4 OW (kg) and growth rate (kg/day) of the flock sorted by day are presented as blue line and black solid continuous lines, respectively. Data are means ± (standard error, SE).

As expected, the accuracy and the precision of the PWs calculated showed a decreasing trend: the further the target day, the lower the CCC. Still the concordance correlations coefficients showed an overall agreement between the PWs and the OWs, with CCCs ranging from 0.692 and 0.967 and C_b always higher than 0.90 (table 2).

Table 2 Descriptive statistics and Lin's concordance correlation coefficient between PWs (calculated as 7 and 14 DGR) and actual OWs at different lead days (from 20 to 60 days).

			Independent variable (OW, kg)				Dependent variable (PW, kg)				Concordance correlation coefficient	
Lead day (days)	**DGR**	**n**	**Mean**	**SD**	**Min**	**Max**	**Mean**	**SD**	**Min**	**Max**	**Lin's CCC**	**C_b**
20	7DGR	8901	36.268	7.232	20.443	65.117	36.873	7.707	19.947	67.756	0.964	0.99
	14DGR	7969	37.125	6.968	22.127	65.117	37.482	7.572	20.661	67.322	0.967	0.99
30	7DGR	7571	37.481	6.870	22.830	65.117	38.522	7.831	21.295	70.568	0.918	0.98
	14DGR	6639	38.275	6.681	24.689	65.117	39.362	7.806	22.420	70.797	0.923	0.98
40	7DGR	6241	38.591	6.617	25.380	65.117	40.285	8.205	22.605	75.895	0.851	0.95
	14DGR	5309	39.296	6.496	26.986	65.117	40.972	8.099	24.008	75.721	0.867	0.95
50	7DGR	4911	39.586	6.454	27.629	65.117	41.698	8.304	23.308	81.316	0.782	0.93
	14DGR	3979	40.248	6.376	29.015	65.117	42.103	8.016	25.597	79.048	0.824	0.94
60	7DGR	3581	40.518	6.356	29.555	65.117	43.006	8.584	23.706	77.914	0.692	0.91
	14DGR	2649	41.115	6.342	30.464	65.117	43.354	8.313	27.161	75.405	0.757	0.92

The same trend emerged by studying the PWs and the OWs through multiple linear regressions. The conditional R^2 was always higher than marginal R^2, suggesting that the inclusion of the random effects of the day and the animal in the model improve the comparison between predicted and observed weights (table 3). Similar to Lin's CCC, the lowest agreement between PWs and OWs emerged at 60 target day (7 DGR, $R^2 = 0.769$).

Table 3 Multiple linear regressions between PWs (calculated as 7 and 14 DGR) and OWs at different lead days (from 20 to 60 days).

		Intercept			Slope					
Lead day (days)	**DGR**	**Coefficient**	**SE**	**P-value**	**Coefficient**	**SE**	**P-value**	**R^2 (cond.)**	**R^2 (marg.)**	**RMSE**
20	7DGR	0.222	0.265	0.402	1.011	0.007	0.092	0.953	0.935	1.623
	14DGR	1.371	0.346	0.000	0.973	0.009	0.002	0.952	0.927	1.518
30	7DGR	3.274	0.581	0.000	0.941	0.014	0.000	0.898	0.837	2.261
	14DGR	15.284	1.010	0.000	0.629	0.024	0.000	0.882	0.514	1.986
40	7DGR	4.084	0.897	0.000	0.939	0.022	0.005	0.841	0.718	2.878
	14DGR	8.415	1.145	0.000	0.830	0.028	0.000	0.865	0.655	2.409
50	7DGR	6.880	1.353	0.000	0.880	0.032	0.000	0.801	0.588	3.251
	14DGR	13.805	1.692	0.000	0.705	0.041	0.000	0.851	0.481	2.450
60	7DGR	10.125	2.013	0.000	0.813	0.048	0.000	0.769	0.442	3.734
	14DGR	14.060	2.297	0.000	0.715	0.055	0.000	0.870	0.420	2.452

Multiple linear regressions and Lin's concordance correlations were repeated early and late in the trial after splitting the dataset in the two timespans (below/above the mean date of the predictions, respectively). As reported in table 4, early in the trial the concordance correlation coefficients showed a slightly wider range compared to the entire dataset (0.712 – 0.960), also the bias correction factors deviated further from the perfect correlation on the 60 target day (0.81 and 0.86 for 7DGR and 14DGR respectively).

Table 4 Descriptive statistic and Lin's concordance correlation coefficient early in the trial.

		Independent variable (OW, kg)					Dependent variable (PW, kg)				Concordance correlation coefficient	
Lead day (days)	**DGR**	**n**	**Mean**	**SD**	**Min**	**Max**	**Mean**	**SD**	**Min**	**Max**	**Lin's CCC**	**C_b**
20	7DGR	4391	32.555	6.042	20.443	58.892	32.726	6.076	19.947	62.280	0.947	0.99
	14DGR	3991	34.004	6.071	22.127	59.734	33.760	6.232	20.661	63.422	0.960	0.99
30	7DGR	3726	34.522	6.062	22.830	59.944	34.812	6.385	21.295	64.024	0.892	0.99
	14DGR	3326	35.854	6.093	24.689	60.713	35.945	6.513	22.420	66.214	0.929	0.99
40	7DGR	3061	36.305	6.090	25.380	60.899	37.065	7.007	22.605	64.474	0.822	0.98
	14DGR	2661	37.472	6.114	26.986	61.644	37.979	6.966	24.009	66.482	0.880	0.98
50	7DGR	2396	37.881	6.110	27.629	61.831	39.776	7.962	23.308	70.032	0.721	0.93
	14DGR	1996	38.961	6.120	29.015	62.576	40.379	7.690	25.597	67.021	0.807	0.95
60	7DGR	1731	39.331	6.119	29.555	62.762	43.386	9.031	23.706	77.550	0.604	0.81
	14DGR	1331	40.265	6.169	30.464	63.524	43.534	8.535	27.161	72.780	0.712	0.86

The predicted and the observed weights were compared early in the trial and the regressions equations are presented in table 5. The conditional R^2 were consistent throughout the target days, moreover the predictions calculated on 14 days growth rate were highly accurate, showing R^2 between 0.923 and 0.954 (50 and 20 target days, respectively).

Table 5 Multiple linear regressions between PWs (calculated as 7 and 14 DGR) and OWs at different targets (from 20 to 60 days) early in the trial.

		Intercept			Slope					
Lead day (days)	**DGR**	**Coefficient**	**SE**	**P-value**	**Coefficient**	**SE**	**P-value**	**R^2 (cond.)**	**R^2 (marg.)**	**RMSE**
20	7DGR	2.347	0.451	0.000	0.934	0.013	0.000	0.942	0.893	1.414
	14DGR	3.610	0.541	0.000	0.887	0.015	0.000	0.954	0.896	1.197
30	7DGR	3.661	0.825	0.000	0.904	0.022	0.000	0.898	0.781	1.938
	14DGR	3.381	0.871	0.000	0.910	0.023	0.000	0.940	0.835	1.451
40	7DGR	1.118	1.230	0.364	0.993	0.032	0.834	0.879	0.706	2.438
	14DGR	3.603	1.277	0.005	0.920	0.033	0.016	0.930	0.747	1.727
50	7DGR	3.610	1.796	0.046	0.959	0.044	0.353	0.841	0.565	3.012
	14DGR	5.164	1.872	0.006	0.908	0.046	0.049	0.923	0.627	1.872
60	7DGR	2.097	2.465	0.396	1.054	0.059	0.365	0.841	0.521	3.566
	14DGR	-1.036	2.403	0.667	1.113	0.057	0.050	0.946	0.657	1.857

The Lin's concordance correlation coefficients between the PWs and the OWs showed high accuracy and precision late in the trial (table 6). The furthest prediction (60 target day) was still characterized by CCCs of 0.801 and 0.810 (7 and 14 daily growth rates, respectively). Likewise, the bias correction factors didn't deviate much from the perfect correlation, showing the lowest value (0.90) on the 40 target day (14 DGR).

Table 6 Descriptive statistic and Lin's concordance correlation coefficient late in the trial.

		Independent variable (OW, kg)					Dependent variable (PW, kg)				Concordance correlation coefficient	
Lead day (days)	**DGR**	**n**	**Mean**	**SD**	**Min**	**Max**	**Mean**	**SD**	**Min**	**Max**	**Lin's CCC**	**C_b**
20	7DGR	4510	39.882	6.419	28.258	65.117	40.911	6.946	28.499	67.756	0.953	0.98
	14DGR	3978	40.257	6.380	29.015	65.117	41.217	6.930	29.196	67.322	0.955	0.98
30	7DGR	3845	40.349	6.373	29.193	65.117	42.118	7.415	28.041	70.568	0.903	0.96
	14DGR	3313	40.706	6.354	29.909	65.117	42.793	7.485	30.191	70.797	0.893	0.94
40	7DGR	3180	40.792	6.352	30.083	65.117	43.384	8.079	26.667	75.895	0.835	0.91
	14DGR	2648	41.128	6.351	30.464	65.117	43.980	8.044	27.771	75.722	0.831	0.90
50	7DGR	2515	41.211	6.353	30.553	65.117	43.529	8.208	25.423	81.316	0.811	0.92
	14DGR	1983	41.543	6.367	30.908	65.117	43.838	7.963	26.966	79.048	0.825	0.93
60	7DGR	1850	41.628	6.372	30.997	65.117	42.650	8.129	26.223	77.914	0.801	0.96
	14DGR	1318	41.973	6.398	31.612	65.117	43.171	8.078	27.594	75.405	0.810	0.96

Finally, the agreement between the OWs and the PWs was tested late in the trial and the regressions equations are summarized in table 7. The lowest conditional R^2 was 0.920 (7 DGR, 40 target day) and the range (0.967 was the highest at 60 target days, 14 DGR) was the narrowest recorded in the present trial, highlighting the best agreement between the predictions and the actual weights.

Table 7 Multiple linear regressions between PWs (calculated as 7 and 14 DGR) and OWs at different targets (from 20 to 60 days) late in the trial.

		Intercept			Slope					
Lead day (days)	**DGR**	**Coefficient**	**SE**	**P-value**	**Coefficient**	**SE**	**P-value**	**R^2 (cond.)**	**R^2 (marg.)**	**RMSE**
20	7DGR	3.697	0.491	0.000	0.933	0.012	0.000	0.944	0.907	1.461
	14DGR	5.290	0.473	0.000	0.892	0.011	0.000	0.954	0.889	1.273
30	7DGR	5.898	1.016	0.000	0.898	0.024	0.000	0.929	0.808	1.661
	14DGR	5.792	1.086	0.000	0.910	0.026	0.000	0.955	0.814	1.323
40	7DGR	-1.197	1.252	0.340	1.093	0.030	0.002	0.920	0.813	2.120
	14DGR	-1.788	1.343	0.185	1.113	0.032	0.000	0.948	0.831	1.714
50	7DGR	-1.379	1.603	0.391	1.090	0.037	0.017	0.924	0.755	2.125
	14DGR	0.423	1.701	0.804	1.044	0.040	0.260	0.958	0.764	1.508
60	7DGR	6.586	2.248	0.004	0.866	0.053	0.012	0.925	0.579	1.904
	14DGR	7.636	2.333	0.001	0.846	0.055	0.006	0.967	0.582	1.244

11.4. Discussion

The modern management of grazing livestock production systems must be supported by technologies collecting information with high temporal and spatial detail (Gonzalez et al., 2014). In sheep production systems, few studies have been conducted on the accuracy of walk-over weighing technology (Brown et al., 2013). However, the focus has been the mob-based walk-over-weighing due to elimination of the need to identify each animal (Brown et al., 2012). It has also been argued that single individual weights (RFID-linked WoW) are inaccurate unless repeated weights over a specific period are combined to generate an average weight (Richards et al. 2006). Hence, the aims of the present study were to validate the use of a RFID-linked walk-over-weighing system in a pastoral based sheep production system to predict future LW with different lead times. This information could serve as preparatory step to the development of prediction tools that would help the operators' decision making.

Previous studies described features such as acclimatisation with the system (González-García et al., 2018), incentive provision (Brown et al., 2012) and behaviours (Brown et al., 2014, 2014b) as key factors for the data collected by the WoW system. Consequently, data collection relies on animals using the WoW platform voluntarily. In the present experimental design, we pre-exposed the sheep to RFID-linked-WoW equipment for an adaptation period of 3 weeks, and we provided a single water source as attractant. Considering that we had an 8-day window of time with missing data due to battery malfunction issues, we achieve a satisfactory efficiency of the WoW station, since we obtained an average of 57.23 days with at least one observation per animal and a 63.66 % of the animal that visited the system (7869 predicted weight / 12360 final predicted weight). Also, 91.67 % of the animals had more than 89 observations throughout the trial (132 out of the initial 144).

The next step of data processing considered the frequency which the sheep attended the water through to reduce the noise of the dataset: 5 animals with less than 89 days of observations were removed and the final dataset included 132 sheep. The final frequency of data available in the final dataset from the WoW platform ranged between a minimum of 26 and a maximum of 81 visits/day (57.23 ± 14.66, mean ± SD). This could be considered an acceptable frequency of data collection for decision making around animal nutrition and for marketing purposes.

Previous research concluded that the repeatability and the frequency of data recorded by RFID-linked walk-over-weighing systems was not suitable for decision making in sheep grazing

systems (Brown et al. 2013). It was suggested that sheep behaviour contributes to the low repeatability of RFID-linked WoW data, because they don't usually pass over the platform in a slow, repeatable fashion. However, the same authors stated that factors such as access to the attractant, the proximity of the weighing platform to the attractant, the type of attractant, whether the weigh platform is single or multi-directional, the number of animals in the mob, and physiological status of the sheep could contribute to congestion in proximity of the platform.

The efficiency of the RFID-linked walk-over-weighing station in the present trial allowed the estimation of individual LW in a flock-based system without any extra handling that can cause stress to the animals. The data also allowed to record and visualise the growth curve thanks to the amount and frequency of data recorded. This could be considered a solution on a major concern of monitoring animals in sheep grazing where the visual observation is impracticable and the WoW is a fully automated system and provides a remote regular assessment of sheep live weight (Brown et al., 2014). The WoW system used in the present study captured the temporal trends in LW and DGR, which could be used for timely management decisions and warning notifications could be sent to operators when certain thresholds are reached or animals do not attend the water trough (Gonzalez et al., 2014). Moreover, behavioural monitoring has significant potential to improve animal welfare and production efficiency, managing health issues and marketing processes based on growth rates (Richards et al. 2005). Throughout the 93 days of the trial, the LW ranged between a minimum weight of 14.93 kg and a maximum of 65.12 kg with an average daily weight increment of 250 g/ d. Hence, our results look promising because of both the frequency of visits per day and the total number of days during which the sheep voluntarily walk across the platform.

The accuracy of the PWs in the next 20 to 60 days was studied through a comparison of the OWs on those days against the LW predicted by multiplying the 7/14 DGR by the target day, and adding the actual observed LW on the day when the calculations were being made. We found substantial agreement between PWs and OWs studied on the entire dataset, with R^2 ranging between of 0.953 and 0.769 (7DGR, 20 and 60 target days respectively). We expected decreasing precision and accuracy the more distant the predictions were set, and with PWs calculated from the 14DGR compared to 7DGR. On the entire dataset, this was confirmed for all predictions except those at 20 and 30 days ahead of any current day which registered a higher R^2 when the calculation based on the 7DGR. A possible explanation could be the higher

sample size involved in the predictions calculated by 7DGR or the lower overestimation (lower intercept coefficients).

The coefficients of under/overestimation ranged between 0.22 and 15.28 kg (20 target days, 7DGR and 30 target days with 14DGR, respectively). The overestimation showed by the PW at 30 days calculated with the 14DGR was unexpected. It seems likely that the inflection point described in that phase of the growth curve of the flock conditioned the agreement between this prediction and the OW leading to a high overestimation.

The Lin's concordance correlation coefficients on the entire dataset reflected the trend described by the linear regressions. In particular, the most accurate PW was at 20 days and calculated by 14 DGR (0.967), whereas the lowest CCC and C_b were recorded for the prediction at 60 days (7DGR). The 7DGR with PW at 50 days can be suggested as the prediction that balanced accuracy (R-square of 0.801 and 0.782 CCC) and a limited overestimation (6.880 kg), therefore it could possibly provide useful information for the operator decision making.

Afterwards, a second analysis was performed by splitting the dataset in two timespans according to the mean date of the predictions and aimed at studying which stage of the growth curve of the flock would best fit the predictions and provide the most accurate agreement between OWs and PWs. Mathematically, the growth curve is a function of age and live weight covering all or part of the animal's lifespan (Echeverri et al., 2013). These growth curves show initially a self-accelerating stage (slope increase) followed by a deceleration stage (slope decline) (Brody, 1945). Among the many mathematical functions used to represent growth curves in sheep, the most accurate models are the non-linear Brody, von Bertalanffy, Verhulst, logistic and Gompertz models (Topal et al., 2004; Hamouda and Atti, 2011; Tariq et al., 2013). During the first stage of the present trial the growth curve showed a slope decline until an inflection point around 0.225 kg/d, which remained stable ranging between 0.15 and 0.30 kg/d for the rest of the trial. Early in the trial, the weight of the flock ranged between a minimum of 20.443 kg and a maximum of 63.524 kg; whereas late in the trial, the minimum and maximum weights were 28.258 kg and 65.117 kg respectively.

Seven out of 10 predictions showed a better agreement with the OWs late in the trial. All predictions calculated on the 7DGR had higher CCCs late in the trial. It seems likely that predicting LW based on a shorter daily growth rate period (7DGR) affects the agreement with the OWs mainly early in the trial because of the higher variance of the growth curve. For instance, the flock showed more variable growth curves during the first period as shown by the

slope of the curve. The PWs calculated according to the 14DGR agreed more to the OWs late in the trial only at the 50- and 60-days targets. It could be suggested that LW predictions using longer time window to calculate dynamic growth rate (14DGR) improves the accuracy of predictions when the target day is not too distant, even in a stage of high variability of the growth curve. The R^2 values ranged between 0.841 and 0.954 early in the trial (PW at 60 days, 7DGR and PW at 20 days, 14DGR, respectively), and between 0.920 and 0.967 (PW at 40 days, 7DGR AND PW at 60 days 14DGR, respectively) late in the trial. The regressions equations showed an overall agreement between the predicted and the observed weights, like CCC, both on the entire dataset and below/above the mean date of the predictions. Accounting for the random effects of the animal and the date improved the agreement between the prediction and the actual weight since the conditional R^2 were always higher than the marginal R^2. The highest R^2 values emerged late in the trial for most of the predictions late in the trial. These findings suggest the prediction of LW are accurate for calculating reliable LW over weeks or months, especially during less variable stage of the growth curve of the flock.

11.5. Conclusions

The WoW system allowed to record LW of individual sheep daily. After outlier removal, these data were frequent and reliable in a pastoral based lamb production system. The predicted LW were in substantial concordance to the observed LW recorded by the system and suggest the WoW technology and data processing methods used in the present study are suitable for applications in commercial farms. The WoW system was validated in a pastoral based sheep production system and can be recommended to help with on-farm decision making of individual sheep.

CRediT authorship contribution statement

Luciano Gonzàlez: Conceptualization, Project administration, Resources, Supervision, Validation, Visualization, Funding acquisition. Augustus Jacobs: Formal analysis, Investigation, Methodology; Alessio Cotticelli: Roles/Writing - original draft, Writing - review & editing, Data curation, Software.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on reasonable request to Professor Luciano Gonzalez.

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12. Conclusions

Animal welfare in livestock is nowadays mandatory for several reasons, from the concern of the consumers about farming conditions, to the increasingly conscious relationship between animal welfare, health and product quality, which is crucial for the preservation of human health and the environment in a One-Health vision. These aspects encourage political decisions towards the promulgation of new rules for animal protection and the development of several methods to assess animal welfare. However, based on farming practices adopted in the last years, welfare cannot disregard a proper environment monitoring and the ability of the animal to adapt and cope with daily events. Indeed, a comprehensive approach to welfare status in modern livestock productive systems must take into account an accurate monitoring of the environment and the assessment of new biomarkers for stress evaluation. The utilization of checklists such as ClassyFarm, is not always correct to evaluate livestock welfare, thus new approaches are pursued. The Precision Livestock Farming (PLF) is recognized as the most accurate method to obtain a real time monitoring of animals and environment. At the same time, the evaluation of biomarkers of the hypothalamus pituitary axis (HPA) is an animal-based measure that defines the stress phenotype under different conditions.

The present thesis contributed to deepen the knowledge about the co-actions of cortisol, dehydroepiandrosterone (DHEA) and its sulphate ester (DHEA-S) on physiological systems, confirming (or in some cases identifying for the first time as in buffalo) the pivotal role played by these steroids during stressful events in different species. Furthermore, as demonstrated in buffalo, the endocrinological measurements of these hormones may be used as preparatory step to develop and calibrate a sensor for the automatic measuring of cortisol in milking parlour systems. This technology could be used in the entire dairy sector to target new PLF technologies towards the animal welfare improvement and could help addressing the identification of stress related to the milking routine.

Another important result obtained through this project of thesis was the stress evaluation through non-invasive sampling and procedures, such as the assessment of steroid hormones in hair in several species exposed to different environmental conditions. The reliability of the analysis was verified in dairy calves, bulls, buffaloes, and sows. In dairy calves a protocol was developed to investigate the hair concentrations of cortisol, DHEA, DHEA-S and their ratios and address the allostatic load during some critical phases of growth, as post-natal and post-

weaning. Similarly, in bulls this approach was used to evaluate their stress under semen collection routine, whereas in sows the stress of parturition and lactation has been evaluated. Furthermore, one of the main advantages of hair is that an integrated measure of hormone concentrations over longer periods of time is provided. This allows a retrospective assessment of the stress, that can be useful to modify farming techniques and daily procedures. These evaluations are even more significant when associated to automated and real time monitoring systems, such as those obtainable with PLF technologies.

In this project of thesis, a method has been proposed to estimate the amount of feed intake through a 3D depth camera and a proper algorithm to measure the volume of the feed. The high feasibility of the method was demonstrated by errors lower than 2%. Therefore, although it was targeted to dairy calves, this technique can be adopted to almost all the species to monitor feed intake, supplying valuable information about the nutritional management of the farm. Further studies are needed to improve the technology and record this information in real time and several times a day. The technology based on 3D depth camera has also been used to automatically estimate the live weight of grazing sheep in extensive conditions. In this case too, the concordance correlations coefficients showed an overall agreement between predicted and observed live weight, with concordance correlation coefficient ranging from 0.692 and 0.967 and a correction bias factor always higher than 0.90.

In conclusion, the integrative approach among biomarkers for welfare evaluation and PLF technologies is a promising strategy to improve welfare assessment of livestock, since it provides a twofold input: gaining insight into the adaptive efforts of the animal by the mean of biomarkers of allostatic load and resilience and developing targeted technological tools that can be integrated in the facilities of the farm. Further studies are needed to integrate the results and elucidate if this approach can be used in the different modern livestock productive systems, taking into account a systematic comparative investigation on the biology of the steroids and considering species-specific behaviours.

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