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Author for correspondence:
Gianfranco Gabai,
Email: gianfranco.gabai@unipd.it

Biomarkers of fitness and welfare in dairy animals: healthy living

André M. de Almeida[1], Maya Zachut[2], Lorenzo E. Hernández-Castellano[3], Marcela Šperanda[4], Gianfranco Gabai[5] and Ali Mobasheri[6,7,8]

[1]LEAF – Linking Landscape, Environment, Agriculture And Food, Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal; [2]Department of Ruminant Science, Institute of Animal Sciences Agricultural Research Organization/Volcani Center, Rishon Lezion 7505101, Israel; [3]Department of Animal Science, AU-Foulum, Aarhus University, 8830 Tjele, Denmark; [4]J. J. Strossmayer University of Osijek, Faculty of Agrobiotechnical Sciences, Vladimira Preloga 1 P.P. 117, 31000 Osijek, Croatia; [5]Department of Comparative Biomedicine and Food Science, University of Padova, via dell'Università, 16 – Agripolis 35020 Legnaro (PD), Italy; [6]Faculty of Medicine, University of Oulu, Aapistie 5 A, FIN-90230 Oulu, Finland; [7]State Research Institute Centre for Innovative Medicine, 08661 Vilnius, Lithuania and [8]Centre for Sport, Exercise and Osteoarthritis vs. Arthritis, Queen's Medical Centre, Nottingham, NG7 2UH, UK

Abstract

Increased animal productivity has reduced animal fitness, resulting in increased susceptibility to infectious and metabolic diseases, locomotion problems and subfertility. Future animal breeding strategies should focus on balancing high production levels with health status monitoring and improved welfare. Additionally, understanding how animals interact with their internal and external environment is essential for improving health, fitness, and welfare. In this context, the continuous validation of existing biomarkers and the discovery and field implementation of new biomarkers will enable us to understand the specific physiological process and regulatory mechanisms used by the organism to adapt to different environmental conditions. Thus, biomarkers may be used to monitor welfare and improve management and breeding strategies. In this article, we describe major achievements in the establishment of biomarkers in dairy cows and small ruminants. This review mainly focuses on the physiological biomarkers used to monitor animal responses to, and recovery from, environmental perturbations. We highlight future avenues for research in this field and present a timely positioning document to the scientific community.

The dramatic increase in productivity achieved in the second half of the 20th century has resulted in concomitant reduction in animal fitness, which increases susceptibility to infectious diseases, metabolic diseases, locomotion problems and subfertility (Koolhaas and Van Reenen, 2016; Friggens *et al*., 2017; Colditz, 2018; Gabai *et al*., 2018). 'Fitness' can be defined as the ability to transfer the genes that an individual, a genotype, a population or a species carries, although this definition is the subject of extensive discussion and debate (Barker, 2009). Two main features contribute to fitness: reproductive success and survival rate. In the context of farm animals, the term fitness has been often used as a synonym of 'robustness', which refers to traits such as fertility, disease resistance, health and longevity (Koolhaas and Van Reenen, 2016). According to Friggens *et al*. (2017) 'robustness' can be defined as the ability to continue activities that favour animal fitness despite environmental constraints.

Within the same population, behavioral and physiological responses to identical conditions may differ among individual animals, possibly leading to differences in health and reproductive success outcomes. Modern farm animals exhibit individual variation in the way they respond to environmental challenges and can express differential behavioral and physiological response patterns (Koolhaas and Van Reenen, 2016; Colditz, 2018). Animal brains generate expectations derived from previous experiences, rather than operating merely as a stimulus-response network, and this view is applicable to both external and internal environment. Therefore, the farm environment can be very complex, and an animal needs to be able to predict and control the cognitive, affective, physical and immunological components of the environment in order to survive, thrive and reproduce (Colditz, 2018). It is worth noting that the affective and mental state has an important implication for animal fitness (or robustness) and welfare (Koolhaas and Van Reenen, 2016; Colditz, 2018). An inability to predict and control this complex environment may result in ethological and physiological changes generating a stress response (Del Giudice *et al*., 2018).

The endocrine, immune and central nervous systems are responsible for coordinating appropriate behavioral and physiological responses to environmental challenges (Koolhaas and Van Reenen, 2016; Colditz, 2018; Gabai *et al*., 2018). Recent data obtained from laboratory animals and human studies highlights the presence of bidirectional interactions between the nervous system, metabolic organs and the immune system. Peripheral nerves and specialized structures within the nervous system can detect immune stimuli, which allows the brain to orchestrate complex behavioral and physiological responses (Soto-Tinoco *et al.*, 2016). Inflammatory processes affect social behavior, leading to changes that may help the individual to adapt to the social environment during times of sickness. Conversely, adverse social behavior affects aspects of immunity and inflammatory state, predisposing the body to situations in which injury and infection will be more likely (Eisenberger *et al.*, 2017).

The concept of an integrated whole organism regulatory network needs to be characterized in dairy animals. In addition, a better understanding is needed of how environmental conditions affect genetic and phenotypic links between temperament, immune function, metabolic performance, affective state, resilience and welfare. Future animal breeding strategies should focus on balancing high production levels with optimal health status and welfare. In this context, the discovery, validation and contextual implementation of biomarkers will contribute to our understanding of physiological processes and regulatory mechanisms used by animals to adapt to new conditions. Biomarkers may also be used to monitor health status and welfare and to establish new management and breeding strategies.

In this article, we review the definition of biomarker and summarize the major achievements in the establishment of health and fitness biomarkers in dairy cows and small ruminants. This review mainly focuses on physiological biomarkers used to monitor animal responses to environmental disturbances. It also highlights future avenues for research in this field, allowing us to present a timely and impactful positioning document to the scientific community.

Scientific definition of biomarker

Biomarkers, also called molecular markers, biochemical markers or signature molecules, are biological molecules used to understand a physiological process or diagnose an abnormal process or a disease. In healthcare, biomarkers are used to diagnose diseases, assess their progression and monitor responses to new treatments, combination treatments and repositioned drugs. In 2001, the Biomarkers Definitions Working Group defined a biomarker as ‘a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention’ (Biomarkers Definitions Working Group, 2001). Different scientific disciplines have refined the original definition provided by the Biomarkers Definitions Working Group. In this paper we present a biomarker definition that is relevant to the major stakeholders in dairy production science.

Emergence of new analytical technologies to improve dairy production

Over the last years, research in the dairy production science field has progressed immensely. Indeed, research in animal and dairy science has moved from a traditional cause-effect perspective, to a broader and more comprehensive view on several fundamental fields such as physiology, health, productivity and welfare, among others. The use of bioinformatics and high-throughput technologies, namely ‘omics’ techniques such as proteomics and metabolomics, has allowed researchers to perform different experiments in the dairy science field in a more consistent and comprehensive manner. Proteomics can be defined as the analytical science that studies proteomes and their functions. The proteome refers to the proteins that exist in a given cell, tissue, organ, organism, population or the proteins that are produced and secreted by cells to form the extracellular matrix and soluble components of biological fluids. Metabolomics is the analytical science that studies the metabolites present within an organism, cell, or tissue. Metabonomics is a subset of metabolomics and is defined as the quantitative measurement of the multiparametric metabolic responses to pathophysiological stimuli or genetic modification, with particular emphasis on the elucidation of differences in population groups due to genetic modification (Ramsden, 2009). For further details on the subject, kindly refer to our previous reviews on the subject (Eckersall *et al.*, 2012; Ferreira *et al.*, 2013; Almeida *et al.*, 2015). The use of these two omics technologies has been crucial in dairy sciences and several proteins and metabolites have been associated to specific study topics. Thus, studies about tolerance to seasonal weight loss in dairy goats (Hernández-Castellano *et al.*, 2015), insulin resistance in transition dairy cows (Zachut, 2015), response to seasonal heat stress in late pregnant dairy cows (Zachut *et al.*, 2017) and amino acid composition of milk replacer on muscle metabolites (Yu *et al.*, 2018) have been published over the last few years. Moreover, as newborn survival is an important factor affecting fitness, assessment of colostrum quality and intake represents an important area of investigation in both large and small ruminants (Banchero *et al.*, 2015; Buczinski and Vanderveerd, 2016). Therefore, the establishment of biomarkers of adequate colostrum quality and intake related to newborn health status is an area that will grow in importance. Examples in this field include for instance the works of Hernández-Castellano et al. (2014*a*, 2014*b*, 2015) that used proteomics to establish markers of proper colostrum intake and consequently adequate immune status in lambs.

The application of omics technologies in dairy science and their association with the establishment of biomarkers is of particular interest from commercial and welfare perspectives. However, omics-based research requires specific analytical instrumentation, specialized reagents and technical knowledge which is uncommon for researchers that traditionally work in this area (Almeida *et al.*, 2015; Boschetti *et al.*, 2019). These facts may explain the difficulties of omics-based studies in farm animals compared to human medicine and preclinical animal models (e.g. rats and mice), where omics approaches are very commonly employed and the relevant databases are well-established. This situation is even more complex in studies performed in sheep and goats, as the reduced size of databases makes the identification and quantification of proteins and metabolites very challenging (Soares *et al.*, 2012; Almeida *et al.*, 2015). As previously described, the number of entries in publicly available databases for farm animals is extremely low compared to humans and animal models. Furthermore, homology searches may present some difficulties as there are no ruminant model species. Therefore, new research needs to be done to establish more complete and more extensive databases for farm animals.

Despite all these technical challenges and the practical difficulties associated with biomarker identification and verification, establishing biomarkers for applied ‘field and farm’ applications in animal and dairy sciences is extremely relevant to our community. This requires efficient integration of researchers, veterinarians, extension professionals, legislators, dairy farmers and industry professionals.

Stress biomarkers

Stress occurs when a physiological control system detects a state of real or presumptive threat to the animal's homeostasis or a failure to control a fitness-critical variable internal or external to the organism (Ralph and Tilbrook, 2016; Del Giudice *et al*., 2018). Unambiguously defining stress is problematic because many factors can act as stressors, and the stress response is multifaceted and multidimensional and possesses an individual component (Romero *et al*., 2015). Animals respond to stress by activating a wide array of behavioral and physiological responses, collectively referred to as the 'stress response'. In animal husbandry, stress is often associated with poor health or hampered welfare. The definition of a stressful condition has mostly relied on glucocorticoids measurement. However, glucocorticoids only represent one stress-response system, the hypothalamus-pituitary-adrenocortical (HPA) axis. Therefore, it seems that this type of response needs to be combined with biomarkers of other physiological systems in order to provide a holistic characterization of the stress response.

Monitoring the HPA axis using different biological matrices

The HPA axis is responsible for initiation of stress responses in all vertebrates, and corticosteroids are widely recognized as stress biomarkers (Mormède *et al*., 2007; Cook, 2012). However, laboratories running cortisol assays find that results are often contradictory. In cattle, several 'social' and 'physical' stressors can influence the animal's physiological systems. How the individual perceives a specific stressor affects both the intensity and duration of the stress (Mormède *et al*., 2007; Bova *et al*., 2014).

Stressors activate the HPA axis through different pathways and several neuronal circuits from different brain districts (hippocampus, amygdala and the medial prefrontal cortex) converge in the hypothalamus, which secretes the corticotrophin releasing hormone (CRH), the primary hypothalamic factor that trigger HPA axis activation. CRH stimulates corticotrophin (ACTH) release by the pituitary gland that triggers the release of glucocorticoids from the adrenal cortex. Arginine vasopressin (AVP) is another factor that can potentiate CRH effects. The glucocorticoid feedback inhibition plays a prominent role in regulating the magnitude and duration of glucocorticoid release and their physiological and behavioral effects (Smith and Vale, 2006; Mormède *et al*., 2007; Jankord and Herman, 2008; McEwen *et al*., 2015). Comprehensive studies about HPA axis regulation in dairy animals are still lacking, and most available information is based on studies performed in laboratory animals, or in the relationship between stress and reproduction in farm animals (Ghuman *et al*., 2010; Ralph *et al*., 2016).

Considering the complexity of the HPA axis regulation and the variability in the stress response, the suitability of cortisol as a stress biomarker is controversial (Mormède *et al*., 2007; Otovic and Hutchinson, 2014; Ralph and Tilbrook, 2016). Cortisol concentrations increase rapidly in blood when animals are subjected to a specific stressor. As cortisol is commonly measured in plasma or serum samples, immobilization of the animal for blood collection may already trigger the stress response. Moreover, glucocorticoids participate in metabolic regulation independent of stressors, and their release is pulsatile and follows a circadian pattern (Lefcourt *et al*., 1993; Marinelli *et al*., 2007; Ogino *et al*., 2014; Otovic and Hutchinson, 2014). All these factors are significant sources of variability and act as confounding factors. Consequently, it is unlikely that blood cortisol, particularly measured once daily, can be used for detecting cows under stressful conditions. As glucocorticoids can be measured in several biological matrices, less invasive procedures may contribute to avoid stressful conditions at sampling (Mormède *et al*., 2007; Cook, 2012).

Salivary cortisol reflects the free hormone fraction measured in plasma (Negrao *et al*., 2004). In cattle, there is a time lag of approximately 10 min to reach a salivary peak in cortisol compared to plasma (Hernandez *et al*., 2014). However, this finding is still controversial (Schwinn *et al*., 2016). A positive correlation between plasma and salivary cortisol is found during an ACTH challenge, but basal cortisol concentrations in saliva are poorly correlated to cortisol in blood during different feeding and drinking procedures (Schwinn *et al*., 2016). It is possible that only acute and intense stress or pain can affect salivary cortisol, while mild or chronic stress and circadian variation can only be observed in plasma. These observations should be considered when salivary cortisol is used for monitoring HPA axis.

Besides saliva, circulating cortisol can accumulate in matrices such as milk, urine, feces and hair over different time intervals. Therefore, cortisol measured in those matrices gives an average representation of the HPA activity during those periods. Milk may be considered as a preferred medium in dairy cows to point out short-term stimulation of the HPA axis (Cook, 2012) and this can be measured in real-time, especially in intensive dairy farms that employ robotic milking. Milk cortisol represents the average plasma cortisol concentrations between two consecutive milkings. Social challenges such as group relocation can be detected within herds by measuring milk cortisol (Pošćić *et al*., 2017), suggesting that milk cortisol measurement can detect short- and mid-term HPA axis activation. However, several factors (e.g. herd, breed, somatic cell count) not directly related to the stress exposure can affect milk cortisol concentrations (Sgorlon *et al*., 2015). In the case of intense stressor exposure (ATCH or transportation), cortisol concentrations in milk reflect those in plasma, but are dependent upon the duration of the stimulus and the consequent elevation in blood cortisol, suggesting that cortisol can freely cross the milk-blood barrier following a concentration gradient. Consequently, if an animal is exposed to a mild or moderate stressor and has sufficient time to recover before milking, milk cortisol concentrations may not be able to reflect that specific perturbation (Verkerk *et al*., 1996).

Urine cortisol represents the average free hormone filtered by the kidney between two consecutive emptyings of the bladder, providing that the total volume of urine produced in that period can be measured (Cook, 2012). Feces is a potentially useful biological matrix to measure glucocorticoids. Glucocorticoids are continuously secreted into the intestine by bile secretions. Some of these glucocorticoids are further metabolized by the intestinal microbiota into diverse glucocorticoid metabolites (GM) such as 11-oxoaetiocholanolone, this being the most abundant metabolite in ruminants (Mostl *et al*., 2002). Fecal GM may be useful biomarkers for long-term adrenocortical activity if the non-adrenal sources of variation can be controlled or the animals are used as their own controls. Fecal GM concentrations can be affected by several processes including conjugation in the liver, intestinal transit time, bacterial metabolism, type of food and procedures of sample collection and preservation (Cook, 2012). The measurement of fecal glucocorticoids requires a solvent extraction which can be only performed in well-equipped laboratories. Therefore, it is difficult to apply this method for monitoring large herds at the farm level.

Circulating cortisol can be incorporated in growing hair. Therefore, cortisol concentrations in hair are representative of cumulative cortisol exposure over a period of several weeks or months and can be successfully measured in humans (Stalder and Kirschbaum, 2012). Promising examples of hair cortisol measurements in cattle have been obtained in experimental settings. Hair cortisol concentrations were higher in ACTH-treated heifers on weeks 2 and 3 but not on week 6 after challenge and were affected by hair color and age (del Rosario González-de-la-Vara *et al*., 2011). However, Tallo-Parra *et al.* (2018) did not find any correlation between hair cortisol concentrations and age. Hair location and the sampling method (clipping *vs.* plucking) affects cortisol concentrations. Indeed, cortisol concentrations are higher in hair sampled from the tail than from the head or shoulders. Similarly, cortisol concentrations are higher in samples collected by clipping (Moya *et al*., 2013) than those collected by plucking. These authors suggested that clipping hair from the tail seems the most suitable method for measuring cortisol concentrations in hair. Braun *et al.* (2017) and Tallo-Parra *et al.* (2018) were not able to detect any increase in hair cortisol concentrations following cows' illness, while others found a significant rise in response to illness in cows (Comin *et al*., 2013; Burnett *et al*., 2015). The lack of standardization of the methodology is a critical issue that can explain the differences observed among studies and limits the exploitation in the field of this methodology for the assessment of stress and welfare. Rapidly growing hair can take-up cortisol from the circulation more efficiently than hair obtained from a previously unshorn area. Additionally, growing hair samples can provide a better retrospective picture of the HPA axis activation related to a mild stressor such as approaching to parturition (Braun *et al*., 2017). Hair cortisol concentrations show a circannual variation with the higher concentrations observed in early summer and in cold-temperate regions (Uetake *et al*., 2018).

As described by Ralph and Tilbrook (2016), assessing health status and welfare by measuring glucocorticoids is not precise enough as glucocorticoids are also released in non-stressful related situations such as pleasure, excitement and arousal. The stress response is mediated by the activation of several circuits in the brain (Reeder and Kramer, 2005; Smith and Vale, 2006; Romero et al. 2015), which can affect other physiological systems and consequently animal behavior. Therefore, it is important to understand which mechanisms activate the HPA axis, which receptors are involved in the stressor identification, and what effects glucocorticoids exert in both the brain and target tissues (Ralph and Tilbrook, 2016). Based on these facts, studying the downstream effects of glucocorticoids may contribute to better define the stress response and identify animals under stressful conditions.

Adrenal androgens

Several studies in humans and laboratory animals have evidenced the role of the adrenal androgens dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) in the stress response. Both DHEA and DHEAS are mainly synthesized in the adrenal glands and gonads, and there is evidence that they are synthesized also in the brain. The function of these hormones is still unclear, but available data suggest their antagonistic effects on glucocorticoids and a role in the stress response (Maninger *et al*., 2009). In humans, DHEA and DHEAS release can be differently affected by psychosocial stress. For instance, increased circulating DHEA and DHEAS levels were observed in response to an acute psychosocial stressor (Lennartsson *et al*., 2012). Conversely, exposure to prolonged stress did not affect circulating DHEA concentrations, while it seemed to negatively affect the capacity to produce DHEAS in response to a subsequent acute stress (Lennartsson *et al*., 2013).

Only a few published studies have explored DHEA and DHEAS release in cattle in response to stressors. In young beef bulls, 9-hours transportation induced a significant rise in blood cortisol, a decrease in DHEA and an increase in the cortisol/DHEA ratio (Buckham Sporer *et al*., 2008). Almeida *et al.* (2008) observed a significant decrease in serum DHEA in lame cows, while Fustini *et al.* (2017) observed increased plasma DHEA in cows kept in overcrowded conditions during the last month before parturition. Cows with metritis displayed higher plasma DHEA concentrations only in subjects with low white blood cells count (Gundlach *et al*., 2017).

The stressors considered in the above-mentioned papers are different in nature and duration and may affect DHEA release in different ways. For example, in cattle ACTH is a poor secretagogue for DHEA and the adrenal contribution to circulating DHEA seems to be quite modest compared to humans. In addition, placenta from cows in late pregnancy seems to be a major source of circulating DHEA (Marinelli *et al*., 2007).

Therefore, the characterization of DHEA release affected by the nature (inflammatory, physical or social) and persistency of specific stressors over time may provide new information to improve the definition of the stress phenotype in dairy animals.

Sympathetic nervous system

The sympathetic nervous system is another important player during the stress response. When the sympathetic system is activated and catecholamines (in particular adrenaline) are released, a plethora of physiological adaptations are triggered to prepare the animals for the 'fight or flight response': heart rate (HR) increases, blood flow is redistributed and metabolic fuels (glucose and fatty acids) are mobilized. However, the sympathetic nervous system has been poorly studied in farmed animals, possibly due to the complexity of measuring catecholamines. Their very short half-life (approximately 1–2 m) means that measuring catecholamines requires particular care in sample collection and storage, and complex pre-analytical steps (Peaston and Weinkove, 2004), all of which makes them unsuitable as biomarkers for monitoring stress in large groups of animals under field conditions.

Sympathetic nervous system activity can be monitored indirectly by assessing physiological variables regulated by catecholamines, such as reticulum-rumen motility, HR and superficial body temperature. The development of sensors that automatically measure physiological parameters, such as rumen and heart functions, can potentially help the early detection of undesirable health or welfare conditions (Caja *et al*., 2016). In healthy cows, for instance, the frequency of reticular contractions is significantly lower in stressed animals (Braun and Rauch, 2008). Therefore, the continuous measurement of reticulum-rumen motility by ruminal boluses can assess the autonomous nervous system (ANS) activity (Caja *et al*., 2016). Measurement of HR has also been used as a good indicator of the physiological response to acute stress (Kovács *et al*., 2014).

Changes in superficial body temperature are influenced by changes in blood flow and metabolism and can be accurately measured using infrared thermography (IRT). However, superficial body temperature may be affected by the anatomical site where IRT is measured (Salles *et al.*, 2016). In cattle, the superficial eye temperature can be considered as an indicator of ANS activity as it reflects changes in the capillary blood flow of the conjunctiva, which is under ANS control (McManus *et al.*, 2016). Interestingly, the exogenous stimulation of the HPA axis alone is not associated with any modifications in eye temperature, while psychological/cognitive stressors (i.e. catheterization procedures) can increase cortisol concentrations and eye temperature (Stewart *et al.*, 2007). Adrenaline infusion can decrease eye temperature in calves, associated with increased blood cortisol concentrations, suggesting that changes in eye temperature are mediated by the sympathetic nervous system (Stewart *et al.*, 2010). In addition, IRT may be used for detecting variations in metabolism, inflammatory processes and diseases, and reproduction (McManus *et al.*, 2016). However, limitations to the use of IRT are related to environmental (temperature, humidity, sunlight exposure) and animal's coat conditions (color, dirtiness).

Neurotrophins

Neutrophins are considered putative biomarkers for chronic stress. These molecules are actively investigated in laboratory animals and humans as potential biomarkers of stress and psychiatric disorders (Bath *et al.*, 2013). Unfortunately, there is no published information in the literature about the involvement of neurotrophins in stress responses in domestic animals.

One of the most investigated neurotrophins is the brain-derived growth factor (BDNF). This molecule is a dimeric protein that participates in neuronal development and plasticity. It is also found in the brain areas regulating mood, emotion and cognition. Additionally, BDNF is implicated in the development of stress-associated pathologies in humans. As BDNF can cross the blood–brain barrier, high blood concentrations of this molecule are highly correlated with BDNF levels in cerebrospinal fluid (Fernandes *et al.*, 2014). In rodent models, several stressors (e.g. maternal separation, social defeat, social isolation, acute or chronic restraint) can affect BDNF expression throughout animal life (Bath *et al.*, 2013). In rats, increased plasma BDNF concentrations are detected in response to both acute and chronic stress (Saruta *et al.*, 2010*a*, 2010*b*). In the rat, the submandibular gland seems to be the major source of circulating BDNF during acute immobilization stress (Saruta *et al.*, 2010*a*). Conversely, circulating BDNF concentrations are poorly influenced by BDNF secreted by the submandibular glands during chronic stress, since increased circulating BDNF concentrations were observed in both sialoadenectomized and non-sialoadenectomized rats (Saruta *et al.*, 2010*b*). The involvement of neurotrophins in the stress response should be investigated in dairy animals, as these molecules are promising biomarkers that may be suitable for assessing animal welfare.

Pheromones

Pheromones are molecules released in the environment by most mammals. They can elicit behavioral and physiological responses (reproduction, maternal care, aggression and alarm) in conspecific animals. In terrestrial mammals, pheromones are mainly volatile molecules, although proteins and peptides with pheromonal or odorant binding protein properties have been detected in several species (Brennan and Keverne, 2004), including cattle (Japaridze *et al.*, 2012). Pheromones can be classified as 'releasers' if they trigger immediate short-term responses, or 'primers' if they trigger medium to long-term changes in behavior or physiology. Another class of pheromones, named 'signallers', carry information about the identity of an individual (Swaney and Keverne, 2009). Since animals can perceive the environment through 'deciphering' these chemosignals, altered pheromone sensing may contribute to poor welfare.

In cows, pheromones are released through body fluids (vaginal fluids, urine, saliva, feces and milk). A pheromonal message can be delivered either by a single compound or a mixture of compounds, whose proportion is crucial for conveying specific reproductive and social signals (Archunan *et al.*, 2014). This may pose limitations to pheromone detection. In dairy animals, pheromones are used in 'pheromone therapies' to induce favorable effects such as estrus stimulation or to appease agitated animals. Pheromones could also be used as biomarkers to detect estrus (Archunan *et al.*, 2014) and therefore increase fertility rates. In the future, estrous pheromones could be detected by electronic noses (Wiegerinck *et al.*, 2011; Cave *et al.*, 2019), providing relevant information to farmers.

A class of pheromones that may be worth investigating in farm animals are those related to the alarm status. Alarm pheromones are secreted by animals in threatening situations to inform other members of the same species of perceived danger. So far, alarm pheromones have been identified in insects and aquatic organisms (i.e. invertebrates and fish), even though behavioral responses ascribed to alarm pheromones have also been observed in ungulates (Hauser *et al.*, 2011). They are highly volatile compounds, characterized by high propensity for spread and penetration and by a short duration of action. (Hauser *et al.*, 2011). Information about alarm pheromones in ungulates is scarce, although novel findings may become available in the near future, as detection of these molecules by electronic noses can inform the farmer or the veterinarian about conditions of fear, uneasiness or pain.

Biomarkers in dairy sheep and goats

Small ruminants represent above 3–5% of the total milk produced worldwide. In Mediterranean countries, most milk produced by small ruminants is transformed in valuable and high-quality dairy products, namely cheeses. Compared to dairy cows, the small ruminant dairy field is poorly studied and the establishment of biomarkers in these species is no exception. One of the reasons is the multiplicity of small ruminant production systems around the world. In contrast, in developed countries, the dairy cattle sector is fundamentally based in one type of animal, the Holstein-Friesian dairy cow, and a production system that favors high yields per animal and, consequently, has to supply adequate environmental, nutritional and management practices to maximize animal performance. Based on the economic importance of small ruminants in the production of highly valuable and high-quality dairy products, increased research effort in strategic topics related to small ruminants are suggested. These strategies may be grouped into different major categories as summarized in Fig. 1.

One of these strategies is related to the different production systems, particularly extensive *vs.* intensive production systems. In small ruminant species, extensive production systems are the most common worldwide. These systems are characterized by the use of natural pastures with seasonal variations in the ewe and goat's live weights arising due to variation in the availability

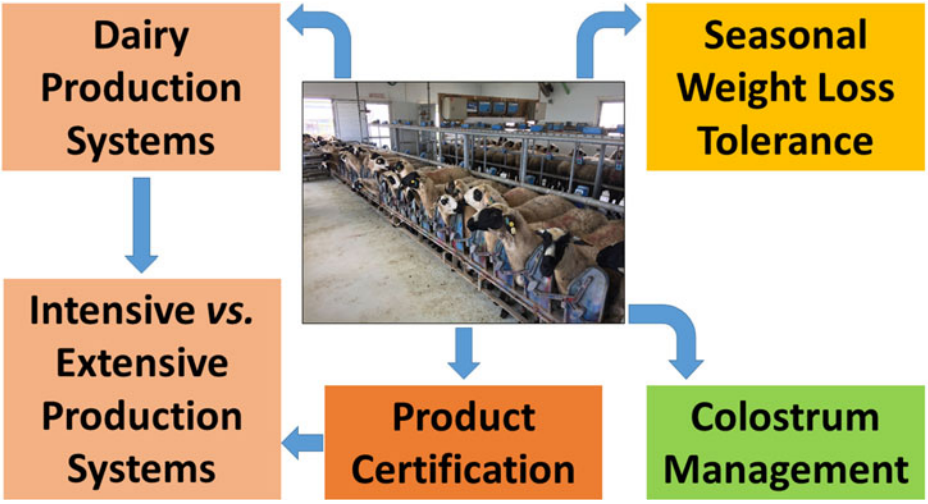

Fig. 1. Schematic representation of the major areas of research for the establishment of biomarkers in dairy small ruminants. They include the classification and characterization of dairy production systems, particularly highlighting contrasting intensive and extensive production systems; product certification; seasonal weight loss tolerance and finally colostrum management. The picture shows dairy Chios sheep in a milking parlor in an intensive milk production farm near Nicosia (Republic of Cyprus).

of pasture. On the contrary, intensive systems imply the use of high-quality forages and high amounts of concentrate. The extensive vs. intensive management is perhaps the most important aspect regarding dairy small ruminant research and the use of biomarkers. Indeed, the definition of biomarkers to differentiate both systems may have important implications in the future, mainly related to the sustainability of the production system and animal welfare. Examples of this type of research already exist in the literature, such as the studies performed in Israel on high yielding Awassi and Assaf sheep breeds (Pollott and Gootwine, 2004; Gootwine, 2011). These breeds have been selected to be reared in intensive production systems. In these studies, molecular genetics markers were used to better understand how the Awassi breed was created during domestication. Authors have also uncovered differences in its genetic structure compared to other breeds, particularly highlighting genetic differences between high and low yielding populations.

Extensive small ruminant dairy production systems are very dependent on local ecosystems. Rainfall pattern defines growth and nutritional value of the pasture, with a consequential impact on animal performance. Seasonal Weight Loss (SWL) tolerance is one of the most important aspects in this area. During pasture scarcity due to restricted rainfall, live weight is significantly reduced in grazing animal, affecting animal performance. The selection of breeds or certain genetic groups that are particularly well-adapted to SWL confers significant commercial advantage, so biomarkers of tolerance to SWL are of particular importance as they could provide tools to improve animal production. In dairy goat production, research has compared Majorera (tolerant to SWL) and Palmera (susceptible to SWL) dairy goat breeds from the Canary Islands (Spain). This research involved the quantification of production levels in these two breeds subjected to nutritional stress (Lérias *et al.*, 2013). This study also allowed the establishment of different molecular markers of tolerance in blood (Lerias *et al.*, 2015), some of them classic biomarkers such as creatinine, urea, non-esterified fatty acids (NEFAs), cholesterol, IGF-1 and T3. Similarly, this experiment also investigated differences between breeds on the mammary gland secretory tissue from proteomics (Cugno *et al.*, 2016; Hernández-Castellano *et al.*, 2015), metabolomics (Palma *et al.*, 2016) and fatty acid (Palma *et al.*, 2017) perspectives. The last two studies also involved analysis and the establishment of biomarkers in milk. Biomarkers of tolerance to SWL include for instance cadherin-13, collagen alpha-1, nidogen-2, clusterin and protein s100-A8 that were found to have higher expression in the SWL-tolerant breed than in the SWL-susceptible breed (Hernández-Castellano *et al.*, 2015). Furthermore, these results indicated that the susceptible breed had higher expression of apoptosis related proteins.

As mentioned above, extensive production is the most common system used while raising small ruminants. However, the use of intensive productions systems for raising small ruminants has increased over recent decades. Such intensification has led to an increased occurrence of metabolic diseases, including acidosis, foot rot or mastitis, among others. The establishment of biomarkers to prevent or detect these metabolic diseases in sheep and goat will be relevant for farmers. In addition, the use of genetic biomarkers will contribute to the establishment of more resistant or tolerant populations to these metabolic diseases within certain breeds. In order to reach these goals, it is necessary to define reference values for well-known biomarkers of stress in small ruminants. For instance, Miglio *et al.* (2018) established reference values for different acute phase proteins in Lacaune sheep. In another study performed by Banos *et al.* (2017), the authors established several genetic markers of mastitis resistance in dairy Chios sheep.

As can be observed, further studies need to be performed in small ruminants to determine biomarkers specifically suitable for sheep and goats. The growth and the specific needs of the sector, combined with novel genetics and omics-based tools, may contribute with important information in the near future.

Future prospects

Further studies will need to identify welfare biomarkers and develop accessible, inexpensive and sensitive analytical or non-invasive techniques to measure them. The development of automated monitoring technologies will provide a more precise quantification of animal responses to environmental perturbations (Caja *et al.*, 2016; Friggens *et al.*, 2017). In addition, biomarkers of robustness can be used to design effective breeding strategies to select more robust and resilient animals (Friggens *et al.*, 2017).

Stress cannot be measured with a single biomarker but requires the use of a combination of physiological and behavioral indicators. The hypothesis of non-invasively monitoring HPA axis activation at the farm level has not been tested so far, although the development of technologies for measuring cortisol in milk may not be a limitation (Daems *et al.*, 2017). Milk cortisol concentrations can change as a result of several genetic and environmental factors (Sgorlon *et al.*, 2015; Pošćić *et al.*, 2017), which require more thorough analysis and mathematical modeling before this biomarker could be exploited in the field. Finally, it should be emphasized that the information available in this field for small ruminants is limited compared to cows. Similarly, differences between the two bovine dairy species, *Bos taurus* and *Bos indicus* and in other bovine dairy breeds such as the Jersey or the Ayrshire could also be explored as most of the results available in the literature are obtained using the Holstein-Friesian breed. Therefore, more research is suggested in both small ruminants and non-mainstream bovine dairy breeds.

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