PHYSIOLOGICAL INDICATORS OF ANIMAL WELFARE

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Abstract. The maintenance of ensured welfare in farm and captive animals depends on appropriate assessment of their welfare state in order to avoid inappropriate management and to maintain good housing conditions. The use of physiological indicators is encouraged, because they are quantitative and not subjective approaches. However, the assessment of ensured welfare using physiological tools is difficult, because there are no clear physiological profiles linked to this state. When we use physiological indicators of stress and pain to assess impaired welfare the interpretation of the results must be cautious and must apply a multicriteria assessment. This review summarizes the premises, applications and complexity of the use of physiological indicators in the assessment of animal welfare.

Key words: distress, nociception, pain, stress.

Resumo. Indicadores fisiológicos de bem-estar animal. Para assegurar o bem-estar em animais mantidos em cativeiro e fazendas é necessário a avaliação apropriada do bem-estar destes animais, de modo a evitar manejo inapropriado e manter boas condições de alojamento. O uso de indicadores fisiológicos é encorajado, uma vez que estes são métodos quantitativos e não subjetivos. Entretanto, a avaliação de bem-estar assegurado usando ferramentas fisiológicas é difícil, uma vez que não há perfis fisiológicos claros ligados a esse estado. Quando utilizamos indicadores fisiológicos de estresse e dor, é preciso ter cautela na interpretação dos resultados bem como aplicar uma avaliação multicritério. Essa revisão sintetiza as premissas, as aplicações e a complexidade do uso de indicadores fisiológicos na avaliação do bem-estar animal.

Palavras-chave: distresse; dor; estresse; nocicepção.

HUMAN VIEW ON ANIMAL WELFARE

After interviewing scientists, veterinarians and animal technicians from 28 institutions in the United Kingdom, HAWKINS (2002) showed that 97% of the respondents recognized that procedures used in animal care can cause discomfort, pain, suffering or distress and want to detect and alleviate it; however, the subjectivity of the criteria used to assess pain and suffering impairs the effectiveness of assessment and alleviation (HAWKINS, 2002). In this way, more objective and quantitative ways of measuring welfare should be achieved (BROOM, 2008). Physiological indicators can be a valuable tool for reducing subjectivity in the assessment of welfare. However, they are still far from being a definitive way to assess animal welfare; the data collected through these parameters also need interpretation and do not consist of a strict answer on the quality of the animal's welfare based only on the numeric value of the parameter. There are physiological signs that, undoubtedly, indicate impaired welfare, such as the presence of disease, reduced growth or tissue damage, for example. But in general one physiological parameter alone cannot be used and the variability between species, individuals and context of occurrence demands wider investigation and interpretation. In this review, we will discuss the premises, uses and limitations of physiological indicators in welfare assessment.

Animal Welfare: concept and assessment

Welfare is a complex concept that refers to the state of an animal in relation to its environment, and is considered an individual characteristic that can vary from poor (or impaired) to very good (or ensured) on a continuum (Broom, 1991) and is based on the presumption that animals can suffer and feel pain and discomfort. The term "welfare" refers to how the animal is going through life, and, although used interchangeably (Volpato *et al*., 2009), is different from the term well-being, which concerns the immediate, punctual condition (Broom, 2008). Animal welfare concern gained prominence in the scientific scenario in the last decades, after the increase in the market of domestic livestock, and the emergence of humanitarian organizations against animal suffering (for a review see Volpato *et al*., 2009).

The concept of 'Five Freedoms' was the first to detail the dimensions of animal welfare while incorporating subjective experiences, health status and behavior, and has been used as the basic philosophy of the Farm Animal Welfare Council (FAWC) of the United Kingdom, an advisor on the welfare of farmed animals. First published in 1979, based on Brambell Report from 1965, the 'Five Freedoms' (and 'Five Provisions', which represented practical advice on how each Freedom could be achieved) identify the elements that determine the animal's own perception of welfare and the provisions necessary to promote that state, addressing both physical fitness and mental suffering. According to these principles, animals should have: "*(1) Freedom from thirst, hunger and malnutrition – by ready access to fresh water and a diet to maintain full health and vigor; (2) Freedom from discomfort – by being provided a suitable environment including shelter and a comfortable resting area; (3) Freedom from pain, injury and disease – by prevention or rapid diagnosis and treatment; (4) Freedom from express normal behavior – by being provided sufficient space, proper facilities and company of the animal´s own kind; and (5) Freedom from fear and distress – by ensuring conditions which avoid mental suffering"* (Farm Animal Welfare Council, 1993 apud Webster, 2001, p.233). According to Webster (2001), these principles can be used as an Ethical Matrix, being a practical check-list to use to assess the strengths and weaknesses of a husbandry system. This paradigm is recognized worldwide and, despite the criticism, is the cornerstone of UK Government and industry policy about animal welfare (FAWC, 2009).

Limitations on the use of the 'Five Freedoms' paradigm has emerged from several prominent scientists in welfare research. First concern is about the use of the word 'Freedom' on the paradigm and the impossibility of eliminating completely the considered negative experiences such as thirst, hunger, discomfort or pain, and other states identified subsequently including breathlessness, nausea, dizziness, debility, weakness and sickness. According to Mellor (2016a), these states can only be temporarily neutralized. Each of these negative states is an important factor motivating animals to behave in a way to obtain the resources necessary to stay alive, avoid physical harm or facilitate recovery responses. Other point is that the 'freedoms' define welfare at a moment in time and do not reflect the causes and consequences of long-term chronic challenges (Webster, 2016). It is also important to consider that the paradigm did not differentiate the physical/functional elements of animal welfare (malnutrition, exposure, disease and injury) from their affective consequences (thirst, hunger, discomfort, pain, fear and distress), and do not consider whether 'freedom' from the negative states guarantees a positive experience. In fact, FAWC itself raises this question, pointing out the focus of the paradigm on impaired welfare and suffering without suggest the seek for ensured welfare. Furthermore, this paradigm does not consider an essential feature of welfare, the motivational states of the animal to show particular behaviors and the problems caused by the frustration of those needs (Broom, 2011). Despite these bottlenecks, the "Five Freedoms" are based on fundamental ethical principles in animal care, whose aim are not achieve on overall picture of mental states and welfare status, but to be signposts to right actions in what concerns animals (Webster, 2001).

In attempt to address the conceptual problems of the 'Five Freedoms' paradigm and develop a reliable tool to assess welfare some models of welfare assessment have been proposed, and the main points are summarized on table 1. The three models presented here are rating programs that incorporates grading scales to evaluate welfare compromise and enhancement. The 'Five Domains Model', developed by Mellor & Reid (1994) (and updated by Mellor & Beausolil, 2015), was designed to assess negative welfare impacts in a systematic and comprehensive way. The model incorporates four functional/physical domains (nutrition, environment, health and behavior) and a fifth domain, the 'mental state', was structured to first evaluate the physical/functional disruptions and the restrictions on behavioral expression, and then to determine the negative mental affects that theses imbalances would generate (Mellor, 2016a). The aim of this model is to facilitate the animal welfare assessment in a systematic, structured, comprehensive and coherent manner, facilitating the identification of internal and external circumstances that promote negative and/or positive mental experiences that have welfare significance (Mellor, 2017). Other model, the European Welfare Qual-

Table 1. Welfare assessment models.

MODEL	CHARACTERISTICS	DESCRIPTION
Five Domain Model[1]	Four physical/functional domains and one affective experience domain, divided in negative and positive experiences	**Physical/Functional:** **Nutrition** - Restricted water and food, and poor food quality (negative); enough water and food, and balanced and varied diet (positive) **Environment** – Uncomfortable or unpleasant physical features of environment (negative); physical environment comfortable of pleasant (positive) **Health** – Disease, injury and/or functional impairment (negative); healthy, fit and/or uninjured (positive) **Behavior** - Behavioral expression restrict (negative); able to express rewarding behaviors (positive) **Affective Experience:** **Mental state** – Thirst, hunger, malnutrition malaise, chilling/overheating hearing discomfort, breathlessness, pain, debility, weakness, nausea, sickness, dizziness, anger, frustration, boredom, helplessness, loneliness, depression, anxiety, fearfulness, panic, exhaustion (negative); drinking, taste and chewing pleasures, satiety, physical comforts, vigor of good, health and fitness, reward, goal-directed, engagement, calmness, affectionate sociability, maternally reward, excited playfulness sexually gratified (positive)
European Welfare Quality (WQ®)[2]	Four principles and twelve criteria	**Principles:** **Good feeding** - absence of prolonged hunger and thirst **Good housing** - Comfort around resting; thermal comfort, easy movement **Good health** - Absence of injuries, disease and pain **Appropriate behavior** - Expression of social behaviors and others, good-human relationship, positive emotional state

Table 1. Continuation.

		Components:
5-Step® Animal Welfare Program'[3]	Three components	**Health & Productivity** – raising animals so that they're healthy and productive with good quality feed and water, shelter, and free from disease, illness and injury (but treating any animals that get sick). **Natural Living** – raising animals in environments that allow them to express their natural behaviors effectively – both indoors and outdoors **Emotional Well Being** – raising animals in environments that provide them the ability to be inquisitive, happy and playful and minimize boredom, frustration, fear, stress and pain, as much as possible.

[1]Mellor & Reid, 1994; [2]Veissier *et al*., 2011; [3]Global Animal Partnership, 2008

ity assessment system for farm livestock (WQ®) was created by European Commission and aims the development of monitoring systems on-farm and of strategies to improve animal welfare on-farm, with focus on physical/functional elements of welfare, presenting itself as four welfare principles (good feeding, good housing, good health and appropriate behavior) defined by twelve criteria (Veissier *et al*., 2011). However, different from the 'Five Domain Model', in the WQ® system the affective states promoted by the physical/functional disruptions are included on the criteria list, but not separately categorized as a welfare principle. Already the '5-Step® Animal Welfare Program', created by Global Animal Partnership, define animal welfare by 3 overlapping components that consider the physical/functional and the mental states of farm animals, the health and productivity (quality off feed, water, shelter and the freedom from disease, illness and injury), natural living (provision of environments that allow animals to express natural behaviors effectively) and emotional well being (provision of environments that can provide them the ability to be inquisitive, happy and playful, minimizing boredom, frustration, fear, stress and pain). Based on these components, the 5-Step® program developed rating labels to certify the meat and other farm products according to animal welfare in the productive chain (Global Animal Partnership, 2008). Despite the inherent difficulties of the score assignment and the possibility of offsetting good against bad scores in different categories, ranking systems could provide reliable tools for welfare assessment.

Although these models presented the current knowledge in animal welfare and provides practical tools to welfare assessment, they lack the simplicity of the 'Five Freedoms' paradigm, and could be not easily assimilated by law people and general public. In this way, Mellor (2016b) updated the 'Five provisions' originally

Table 2. Welfare paradigms: Freedoms, Provisions and Animal Welfare Aims.

FREEDOMS[1]	PROVISIONS[1]	ANIMAL WELFARE AIMS[2]
Freedom from thirst, hunger and malnutrition	Provide ready access to fresh water and a diet to maintain full health and vigor	Minimize thirst and hunger and enable eating to be a pleasurable experience
Freedom from discomfort	Provide shade/shelter or suitable housing, good air quality and comfortable resting areas	Minimize discomfort and exposure and promote thermal, physical and other comforts.
Freedom from pain, injury and disease	Prevent or rapidly diagnose and treat disease and injury, and foster good muscle tone, posture and cardiorespiratory function	Minimize breathlessness, nausea, pain and other aversive experiences and promote the pleasures of robustness, vigor, strength and well coordinated physical activity.
Freedom from express normal behavior	Provide sufficient space, proper facilities, congenial company and appropriately varied conditions.	Minimize threats and unpleasant restrictions on behavior and promote engagement in rewarding activities.
Freedom from fear and distress	Provide safe, congenial and species-appropriate opportunities to have pleasurable experiences.	Promote various forms of comfort, pleasure, interest, confidence and a sense of control

[1] Farm Animal Welfare Council, 1993 apud Webster, 2001, p.233; [2] Mellor (2016B).

Table 3. Quality of Life (QoL) scale.

CATEGORY	DESCRIPTION
A good life	The balance of salient positive and negative experiences is strongly positive. Achieved by full compliance with best practice advice well above the minimum requirements of codes of practice or welfare
A life worth living	The balance of salient positive and negative experiences is favorable, but less so. Achieved by full compliance with the minimum requirements of code of practice or welfare that include elements which promote some positive experience.
Point of balance	The neutral point where salient positive and negative experiences are equally balanced.
A life worth avoiding	The balance of salient positive and negative experiences is unfavorable, but can be remedied rapidly by veterinary treatment or a change in husbandry practices.

Table 3. Continuation.

A life not worth living	The balance of salient positive and negative experiences is strongly negative and cannot be remedied rapidly so that euthanasia is the only humane alternative

*Adapted from Green & Mellor (2011).

aligned with the "Five Freedoms", incorporating aspects of four welfare principles of the European Welfare Quality assessment system (WQ®) and the Five Domain Model for animal welfare assessment, and proposed a new paradigm that assign a particular 'Animal Welfare Aims' to each provision directing the welfare management towards activities that can minimize negative states and promote positive experiences. The aims emphasize the subjective experience of the animal and intend to motivate the responsible for animal care concern about the minimization of negative subjective experiences and the promotion of positive ones (Mellor, 2016b). This new proposed paradigm avoids the complications of the term 'freedom', accommodates de scientific understanding of welfare and provides a guidance for welfare management, and is proposed by the authors as an substitute for 'Five Freedoms' paradigm.

Independent of the paradigm chosen, the welfare management goes beyond the simply identification of welfare compromise and problems resolution, but should concern about the welfare enhancement, providing opportunities for animals to behave in ways they find rewarding (Mellor, 2016a), and the balance between positive and negative experiences that animals can have can be used as index of the quality of its life. Based on the notion of "a life worth living", "life not worth living" and "a good life" that consider animal's quality of life and its needs and wants, introduced by FAWC in 2009, Green & Mellor (2011) developed a "Quality of life scale" (QoL) as a comprehensive way do evaluate the balance between positive and negative experiences lived by an animal in the seek of a 'a good life'. The QoL idea focuses on the mental state of the animal that is a result of conditions from the four physical domains described in 'Five Domain Model' (Green & Mellor, 2011).

Besides the complexity of the welfare concept, it is essential consider that, even today, there are controversies about what can be considered welfare in non-human animals. Because welfare is a subjective experience, animals should have a minimum of sentience to be of welfare concern. Therefore, these animals should have brains or neural structures that are able to support conscious perceptions and behavioral responses to sensory inputs (Mellor, 2016B). According to Volpato *et al*. (2007), to evaluate the sentience of an animal it is important consider three approaches: brain machinery, sensibility to noxious stimulus and pain, and behavioral changes in response to noxious stimulation. In this sense, all the vertebrate subphylum can be considered of welfare concern, considering that evidence of sentience is present from fish to mammals. Fish, the most basal vertebrates, already present nociceptores (Sneddon *et al*., 2003a), neural pathways (Chandroo *et al*., 2004), behavioral and physiological responses to noxious stimulation (Sneddon *et al*., 2003b; Wolkers *et al*., 2013) and brain structures involved in the modulation of nociceptive output (Wolkers *et al*., 2015, 2017), suggesting that they have the necessary to sustain sentience; and groups of more derivatives vertebrates also possess these structures with an increasing degree of sophistication. Although there are evidences of nociception on other phylum, e.g. snails (*Megalobulimus sanctipauli*) (Romero *et al*., 1994), they are not sufficient to support the existence of sentience in other groups than vertebrates.

The complexity of the welfare concept provides evidence of the necessity of new research seeking knowledge about the parameters that can be used for welfare assessment, and how to provide conditions to permit the development of positive states to promote ensured welfare, especially for captive and farm animals.

The assessment of welfare

We need suitable ways to assess the state of animals in order to avoid mistreatment and to give animals under our care at least “a life worth living”. Measures of welfare, however, have to rely on indicators of ensured or impaired welfare, as the measurement of subjective experience *per se* is not possible. According to Broom (2000), the measures of welfare include: physiological indicators of pleasure; behavioral indicators of pleasure; the extent to which strongly preferred behaviors can be shown; the variety of normal behaviors shown or suppressed; the extent to which normal physiological processes and anatomical development are possible; the extent of behavioral aversion shown; physiological attempts to cope; immunosuppression; disease prevalence; behavioral attempts to cope; behavior pathology; brain changes; body damage prevalence; reduced ability to grow or breed; and reduced life expectancy. This range of assessments goes from ensured welfare indicators to impaired welfare indicators. As personal judgment can cause considerable variation in the perception of what is and what is not ensured/impaired welfare, we should preferably use quantitative, established methods to measure welfare (Broom, 2008). Several of the indicators listed by Broom (2000) are physiological; those indicators can be important tools to animal welfare assessment. They are quantitative methods, and our knowledge about the interrelationship of these parameters allows us to make a more integrative interpretation of the physiological state of the animal when a wider set of measures is used. However, variations between species and even individuals can make the interpretation of the data difficult; there is no “reference range” for these parameters we can use to assign a numeric response to the significance for welfare. The more we investigate and validate the physiological parameters together with behavioral responses and choice studies, the more we will know about the relationship between physiological responses and welfare states.

The most widely used physiological indicators of welfare are related to stress response, distress, disease and pain, which are often impaired welfare indicators, and there are several considerations to make regarding these indicators. First, the absence of these signs does not mean the animal has ensured welfare. In addition, the presence or increase of some of these parameters does not necessarily mean the animal currently has impaired welfare. Unfortunately, the search for ensured welfare physiological indicators, which could be used as counterpoints to impaired welfare indicators, has not been fruitful so far. In the subsequent sections we will discuss the absence of a physiological pattern linked to ensured welfare, the principles behind the physiological indicators of impaired welfare and the premises to interpreting these physiological parameters in the welfare approach.

Ensured welfare: are there physiological indicators?

According to Broom (1998), the feelings, good or bad, that are present in the welfare spectrum are biological mechanisms; as with any other biological mechanisms, pain, fear, pleasure and others are adaptive and evolved through natural selection. However, as emphasized by Volpato *et al*. (2009), we will probably not find physiological pathways or patterns strictly related to ensured welfare and well-being states as we can see in very impaired welfare. These authors rationalize that when an animal is disturbed the consequent response, the stress response pattern, provides the animals the energy necessary to cope with the disturbance and it is reasonable that a standardized response evolved because this response is adaptive and enhances the chance of surviving. The well-being state, however, does not impair the survival or reproduction of animals, so it is hard to expect that natural selection would shape a pattern for this physiological state. In other words, when homeostasis is challenged, the allostatic state promotes physiological modifications that allow the body to cope with the challenge and to return to homeostasis; if everything is fine and homeostasis is not threatened, there is no need for a physiological pattern to arise to cope with it, as there is nothing to cope with.

Ensured welfare as a whole probably does not have a specific pattern, but there are measures that evaluate specific pathways involved in ensured welfare. For example, the sensation of reward has been linked to the neurotransmitter dopamine in the *nucleus accumbens* (Wise, 1980). Of course, the “pleasure” or reward sensation is just part of welfare, and is not present all the time so its measure does not define welfare. Even more, the measure of dopamine in the *nucleus accumbens* is a very delicate technique that can only be made post-mortem or using microdialysis *in vivo* in absolute laboratorial conditions. Although not applicable to captive or farm animals, the measure of dopamine levels using microdialysis techniques in the nucleus accumbens after the animal makes a choice in a preference test, combining approaches to study the physiological effects of a given choice (Cook *et al*., 2000), can help us to understand ensured welfare in its complexity. But for the inference of the welfare of captive or farm animals in a practical way, physiological indicators of ensured welfare seem to still be far away from day-to-day application.

Detecting impaired welfare with physiological indicators – The stress response

In 1936 Hanz Selye described a "general adaptation syndrome" where a typical physiological response, with non-specific symptoms, is triggered after nocuous disturbances. This day we know that the response is not non-specific to all disturbances, with different biological responses being elicited by different stimulus (Moberg, 2000); also, nowadays we use the term stress to describe a threat to homeostasis that causes a response. There are some ambiguities in the use of the term stress, however; it can be seen describing the threat (also called stressor), or it can be used as a synonymous of the physiological response elicit by the stressor (the stress response), or even can be used to name a negative state (McEwen & Wingfield, 2003). In this review we will use the terms stress to refer to a threat to homeostasis, stressor for the disturbance and stress response for the physiological responses elicited by stressors. The physiological parameters linked to the classical stress response are largely used in the assessment of impaired welfare.

The stress *per se* is a part of life, and we humans even use the trigger of the biological stress response as a psychological reward when skiing, climbing mountains, going on roller coasters and so on (Moberg, 2000). The stress response is an adaptation that permits the animal to cope with a disturbance and aims to ensure the survival of the individual (Hill *et al*., 2008). In a first moment, the setup of the stress response is not deleterious; rather, it is adaptive. In fact, the importance of this physiological response is evidenced by the high structural identity or similarity of its components across vertebrates, indicating that these physiological pathways have an evolutionary history of at least 400 million years with few modifications throughout this time (Wendelaar Bonga, 1997). The stress response, however, begins to be a threat to welfare when the animal fails to cope with the situation (Broom, 2008). We call the first physiological line of defense in the response to a stressor, being the activation of the autonomous nervous system, the release of catecholamines by the adrenal and the activation of the hypothalamic-hypophysis-adrenal (HHA) axis, the primary response to stress. The secondary response consists of the biological alterations promoted by the primary response, which provide the body the ability to cope with the stressor: an increase in heart and respiratory rates, blood pressure, sources of energy (glucose, amino acids and lipids), and the inhibition of systems not essential for the current alarm situation, such as reproduction, growth and also modulation of the immune system in order to avoid an exaggerated, and costly, immune response (Wendelaar

BONGA, 1997). When this stress response is positive or just physiological stress to adapt to the environment in the limits of normality, we can denominate it as eustress, or positive stress (SELYE, 1974). This means that if the disturbance lasts a short period (acute stress), this response is extremely adaptive and the welfare of the animal is not impaired. However, in long-term and chronic stress, the persistence of biological responses becomes maladaptive and threatens the welfare of the animals. The setup of this pathological state is called the tertiary response to stress, and it is when the initial adaptive response starts to become distress (WENDELAAR BONGA, 1997). A situation of distress means an effort that requires an amount of energy to adapt to the situation that becomes detrimental for other biological functions such as growth and reproduction (SELYE, 1974). Chronic stress, meaning the prolonged presence of one stressor or the accumulation of several consecutives stressors, results in a biological cost that invariably means a state of distress and impaired welfare (MOBERG, 2000). More rarely, even acute stress can become distress. When acute stress disrupts critical biological events, directly or by diverting the energy necessary for them to occur, it can be distress; one example is the interruption of biological events when timing is crucial, such as ovulation, that can be impaired by an acute stressor and prevent the animal from reproducing at that opportunity (MOBERG, 2000). Therefore, stress does not necessarily mean impaired welfare, but distress does (MOBERG, 2000), because some functions as immune, reproduction and growth start to fail in the distress state. According to SELYE (1936), the third phase of the "general adaptation syndrome" (stress response in the current denomination) is very similar in symptoms to the first phase. In fact, prolonged stress and its detrimental effects are no more than the persistence of the initial – and very adaptive – stress response.

To better understand the effects of the stress response in welfare and its use as an impaired welfare indicator, we first need to understand the functioning of this response and its effects in animal physiology in more detail.

The stress response in vertebrates

The stress response occurs when the central nervous system perceives a disturbance capable of threating homeostasis, leading to the triggering of general biological defense responses that are of behavioral, autonomic nervous, neuroendocrine or immune in nature (MOBERG, 2000). We will not address the behavioral response in this review, as our aim is to focus on physiological indicators. The first physiological line of defense to facing stress is the autonomic neural system. The autonomous sympathetic nervous system is usually activated after a disturbance, leading to the modulation of the cardiovascular, gastrointestinal and endocrine systems, with the increase of heart and respiratory rates and blood pressure; changes in the blood distribution to tissues; an increase in energy available in the form of increased glycaemia through glycogen degradation; a reduction in gastrointestinal activity and the release of catecholamines, adrenaline and noradrenaline, from the adrenal gland, which have similar effects on the sympathetic autonomous system (Hill *et al*., 2008). The responses of the autonomic system and circulating catecholamines appear very quickly after the animal perceives the disturbance, but have a relatively short duration; so, these systems do not have a long-term duration and do not significantly impact the animal's welfare in the long run (Moberg, 2000). Moreover, the measure of the autonomic nervous system response and even circulating catecholamines levels are only possible in a well-controlled laboratorial environment with the animal restrained, which limits the use of these parameters in welfare research.

The most widely used parameter to assess the effect of a disturbance in the animal is the neuroendocrine response, especially regarding the HHA axis (HHI in fish, which do not have an adrenal gland but, instead, interrenal cells in the kidney), with the increase of glucocorticoids as cortisol and corticosterone. When the disturbance is interpreted as a threat, the hypothalamus secretes the corticotropin-releasing hormone (CRH; also referred to as corticotropin-releasing factor, CRF), which reaches the hypophysis (also referred to as pituitary) gland through the hypothalamic-hypophyseal portal vessels (Mormède *et al*., 2007). The paraventricular nucleus (PVN) of the hypothalamus, which synthetizes and releases CRH, receives numerous inputs from different areas that bring information about the environment, inner state and physiological state, and this explains the trigger of the HHA axis by stimuli of both external and internal origin (Mormède *et al*., 2007). In the hypophysis, CRH stimulates the release of the corticotrophin hormone (or adrenocorticotropic hormone, ACTH) in the blood circulation. The ACTH stimulates the synthesis and release of glucocorticoids by the adrenal gland; the increase in the corticoids level takes some minutes, but can be long-lasting if the disturbance continues (Hill *et al*., 2008). The hormones of the HHA axis have a broad effect on the body, modulating the immune system, metabolism, reproduction and behavior (Hill *et al*., 2008). Other hormones regulated by the hypothalamic-hypophiseal axis are also sensitive to stress, such as prolactin, somatotropin, thyroid-stimulating hormone and the gonadotropins, which are modulated by the stress itself or by the glucocorticoids increase (Moberg, 2000). The glucocorticoids regulate the energetic metabolism, stimulating the catabolism of muscle protein and fats, and promoting gluconeogenesis in the liver using amino acids as precursors and stimulating the use of lipids as an alternative source of energy in several tissues (Levy *et al*., 2006). Glucocorticoids also modulate the immune system. In the first steps of the stress response, catecholamines and glucocorticoids in low levels stimulate the immune response, but with the increase in glucocorticoids levels an anti-inflammatory effect of these hormones takes place (Hill *et al*., 2008). The occurrence of diseases in animals suffering from stress are mostly attributed to the immune suppression caused by the stress response (part of the tertiary response), modulated especially by the HHA axis; however, the central nervous system seems to have a direct role in the regulation of the immune system during the response to a stressor (Moberg, 2000).

The use of impaired welfare indicators

Several studies have used stress/distress-related parameters as indicators of welfare. Some indicators more clearly indicate impaired welfare, while others need to be compared with more parameters to give a clearer picture of the animal's state. If an animal is sick its welfare is impaired, regardless of whether diseases are part of its life. Injuries (see further section on pain) and abnormal growth are also clearer indicators of impaired welfare (Broom, 2008). Regarding other parameters linked to stress physiology, particularly linked to primary and secondary stress response, we need to base our evaluation on several parameters and multicriteria approaches, as there is not a single parameter capable of showing unequivocally the welfare state of the animal (Pascual-Alonso *et al*., 2013). Moreover, in several cases, the installation of the disease occurs due a previous state of impaired welfare or stress, and this subclinical state is more difficult to assess with certainty.

Most studies rely on the hypothalamic-hypophysis-adrenal axis, notably the glucocorticoids (cortisol and corticosterone), to assess impaired welfare. Glucocorticoids levels can be measured in the blood (Nicol *et al*., 2009), saliva (Monreal-Pawlowsky *et al*., 2017), or in the surrounding water for fish (Sundh *et al*., 2010); the rate cortisol/creatinine can be measured in the urine (Titulaer *et al*., 2013; Part *et al*., 2014); or glucocorticoids metabolites can be measured in feces (Möstl *et al*., 2002) and bird droppings (Alm *et al*., 2016). Other hormones, however, such as prolactin and growth hormone, appear to also be sensitive to stress, and hormones such as thyroid-stimulating hormone, luteinizing hormone and follicle-stimulating hormone are directly or indirectly modulated by stress, and can also be measured (Moberg, 2000), as well as neurotransmitters such as serotonin or its metabolites (Part *et al*., 2014). The sympathetic–adrenal–medullary system can also have its activity measured, such as by using catecholamines metabolites, and vanillylmandelic acid; in dogs, Part *et al*. (2014) found that all kennelled individuals showed urinary vanillylmandelic acid: creatinine ratios above baseline levels. Elevated vanillylmandelic acid: creatinine ratios indicates that the the sympathetic–adrenal–medullary system was activated, which could suggest both arousal or an alarm response in the animals; so this result should be carefully evaluated, as an arousal state is not related to impaired welfare.

Secondary responses to stress can also be measured to assess welfare. Blood glucose is a widely used parameter (Aguayo-Ulloa *et al*., 2014). Low blood glucose appears to be associated with positive choice of an environment (being in an environment of preference) in laying hens (Nicol *et al*., 2009); as stress can increase the glucose levels, this can be an indicative of absence of stress response in a preferred environment. Body temperature can be modulated by sympathetic/adrenal responses, and can increase in mammals for a prolonged time after a stressor, while normal activities such as exercise increase the temperature but with rapid recovery after the completion of the exercise (Broom, 2000). In dogs, the drop in the body surface temperature appears to reflect increased positive arousal (Part *et al*., 2014), while in laying hens lower body temperature was associated with positive choice of environment (Nicol *et al*., 2009). In addition, the eye area temperature can be used as a stress parameter in reactivity tests (in which the reaction of the animal to manipulation is measured), to infer the response of the animal to a disturbance like restraining for example (Pascual-Alonso *et al*., 2013; Aguayo-Ulloa *et al*., 2014). The heart rate can also be measured; bradycardia (decreased heart rate) can occur when animals are frightened, but the most common effect of disturbing situations is tachycardia (increased heart rate), and while these effects are generally of a short duration, during prolonged stressors such as transport, tachycardia can last for hours (Broom, 2000). The breathing rate can also increase under disturbance, and can be observed visually in terrestrial animals (Broom, 2000); in fish, the opercular beat rate is a measure of breathing rate (Wendelaar Bonga, 1997), but this parameter visualization depends on the size of the fish and the visibility of the water column (Johansen *et al*., 2006).

Stress can increase the hematocrit and blood cells count through spleen contraction (Broom, 2000), so the evaluation of these parameters can be used to evaluate the presence of stress. Alterations in leukocytes ratios, such as heterophil:lymphocyte or neutrophil:lymphocyte ratios, are also used as welfare indicators, as alterations in the leukogram indicate the occurrence of not only inflammation and disease but stress and painful events as well (Broom, 2000; Aguayo-Ulloa *et al*., 2014; Cafazzo *et al*., 2014; Alm *et al*., 2016). After a stressful event, the number of neutrophils usually increases while the number of lymphocytes decreases (Cafazzo *et al*., 2014). In laying hens, lower heterophil:lymphocyte ratios were associated with positive choice (Nicol *et al*., 2009).

Creatinine kinase, which is released in the blood when there is muscle damage (after exercise or bruising, for example), and lactate dehydrogenase, which is also released after muscle or tissue damage, can be also measured as welfare indicators (Broom, 2000). The concentration of nonesterified fatty acid (NEFA) is a negative energy balance biomarker which increases when the supply of glucose is not sufficient to the animal's current energy requirements (Adewuyi *et al*., 2005) and is used as a welfare measure (Aguayo-Ulloa *et al*., 2014). The measurement of NEFA needs to be done together with blood glucose and cortisol; a pattern of high NEFA and cortisol levels, and low blood glucose, is indicative of a negative energy balance (Pascual-Alonso *et al*., 2013).

The normal metabolic processes of the cells generate reactive oxygen intermediates and byproducts (reactive oxygen species, or ROS) that threaten cellular homeostasis through damage to DNA and other cellular constituents, the disturbance of cellular metabolism and enzymatic activity (Stoliar & Lushchak, 2012). However, the antioxidant defense systems, mechanisms that neutralize the ROS, protect the cells through making the ROS in molecules less reactive and thus less damaging (Yu, 1994). When the rate of ROS and antioxidant defense is imbalanced, we call it oxidative stress (Stoliar & Lushchak, 2012). In dogs, for example, Cafazzo *et al*. (2004) found that individuals living in shelters that performed a lower frequency of displacing activities and stereotyped behavior had a higher total antioxidant capacity. Given the potentially high damaging risk due to oxidative stress, the inclusion of this measure in welfare assessment is very important for the maintenance of animal health.

It is important to emphasize that the inference of welfare needs to be done in light of several parameters and the relations between them. For impaired welfare, there are many indicators that are potentially useful, but for the most reliable assessment we need to examine a range of measures, both physiological and behavioral, together (Huntingford *et al*., 2006). In the next section, we will consider the particularities and the care needed in inferring impaired welfare from physiological indicators that can be evoked by stress.

Considerations in the use of stress response parameters as a welfare measurement

Besides being a useful tool in the assessment of welfare, we need to proceed with caution in using the physiological indicators related to the stress response. There are several particularities regarding daily fluctuations, non-distress-related increases in the parameters and the form of sampling that need to be taken into consideration.

First, the pure absence of impaired welfare indicators does not ensure ensured welfare. The assumption that a healthy animal is in an ensured welfare state does not consider that this animal can be in physical or psychological discomfort (Volpato *et al*., 2009). Moreover, animals going through chronic disturbance cannot show the classic physiological stress parameters, such as high glucocorticoids levels, while still demonstrating the behavioral aversive response (Mormède *et al*., 2007). Under chronic stress conditions, glucocorticoids levels can rise just slightly above the basal levels (Mormède *et al*., 2007) and the differentiation between the absence of stress and a chronic stress response can be more difficult to identify before the installation of other signs of prolonged allostatic load, such as reproductive failure, immune suppression and inhibited growth. The problem with welfare assessment solely by physiological indicators is that if the impaired welfare detection is only made when these prolonged allostatic load signs are installed, the animal probably has been disturbed for a considerable time without notice.

Another problem in the measure of stress is the individual variation in the physiological response (Moberg, 2000). Characteristics such as previous experience, genetics, age, social relationships and human-animal interactions can modify and differentiate the stress response from one individual to another, from the interpretation of whether a stimulus is a threat (and whether they will have a response to it) to the particularities and magnitude of each component of the stress response (Moberg, 2000). Different stressors are perceived differently by different individuals, and the magnitude of the stress response depends on this perception (Galhardo *et al*., 2011). This process of appraisal of the stressor involves the perception of the disturbance and its evaluation based on previous experiences, followed by the evaluation of the coping mechanisms suitable for this situation (Ursin & Eriksen, 2004). In that way, the same event can be perceived and elicit a response differently by two individuals or even by the same individual in different life stages (Galhardo & Oliveira, 2009). When assessing the welfare of animals outside the laboratory, it is more difficult, if not impossible, to account for the previous experiences, current social status and genetic background to appropriately interpret the stress measures of each individual. However, for welfare it is not the absolute value of the parameter measurement that is important, but the threat to the biological function that the activation of each system represents (Moberg, 2000).

To measure welfare, we need to know the biology of the species and, more importantly, the tools species uses to cope with disturbances, to infer if the animal is being successful or if it is failing to cope with the challenges (Broom, 2008). Also, the stimulus can be not a real threat; what is important is that it is perceived as a threat by the animal, and the stress response will be triggered (Moberg, 2000). So, we need to try to infer impaired welfare in the actual animal response, and not solely in the assumption that a situation will be stressful for some individuals. Of course, there are situations so disturbing that they would bring impaired welfare to any animal, such as being injured or ill. However, there are conditions that usually would be harmful, such as being submitted to hunger until it becomes malnourished; however, for some species, fasting in certain periods of the year, usually during winter, is natural, and the animals will stop eating by themselves. In this case, anorexia is not a sign a sickness and neither would interrupting the supply of food during this period configure mistreatment; so we should be careful in evaluating each case in light of the biology of the species. Similarly, there are situations in which the physiological indicators commonly used to assess the stress response are increased, and this absolutely does not mean that the animal is experiencing a bad stimulation. The stress response releases hormones that modulate metabolism, preparing the animal to be able to cope with the situation in several cases, including exercise – which is not stressful (McEwen & Wingfield, 2003). For example, stallions show a similar increase in glucocorticoids levels either after a stressor (restraint) or after exercise and mating (Colborn *et al*., 1991) and the two latter activities do not bring discomfort to the animal. Therefore, pleasure activities that also demand an increase in the metabolic rates can result in the release of these indicators. Moreover, the levels of several parameters naturally fluctuate throughout the day. Glycaemia increases after a meal (Randall *et al*., 1997); cortisol levels usually increase in the hours immediately before the beginning of the activity of the species, reaching one or two peaks for the day (Reece, 2004); and there are seasonal fluctuations of glucocorticoids release, with peaks of the secretion of these hormones in periods of high energetic demand (Hill *et al*., 2008). Further, natural activities of extreme importance for fitness, such as migration, result in physiological demands that show the same pattern as the stress response. In fish, migration leads to increases of glucose and lactate levels as well as parameters used as impaired welfare indicators (Crossin *et al*., 2009), and besides being a severe challenge, migration behavior is a part of life for fish and essential to reproduction. In order to use these parameters to more accurately infer impaired stress, we need to know the biology of the species, knowing if there are seasonal changes in the activity and food intake, and the pattern of daily fluctuations of the physiological parameters that are species-specific. With these information in hands, we are able to distinguish between a stress related anorexia and a seasonal natural decrease in the food intake; a marked stress response and a natural physiological preparation for migration; and so on. Also, if we know the natural fluctuation of physiological parameters we can better schedule the samples collection to avoid natural daily peaks that could be erroneous interpreted as impaired welfare. For example, the evaluation of glycaemia should not be done after a meal, when the glucose levels probably are high due to digestion, neither the glucocorticoid levels should be done right before the beginning of activity (early morning for diurnal and end of the afternoon for nocturnal animals), when a peak of these hormones is natural. The more we know about the biology of the species under our care, the more capable of better interpret these physiological parameters we will be; so studies about the circadian and circannual rhythms are an important tool in the use of physiological parameters as welfare indicators.

Another issue to be taken into account is that the assessment of physiological stress parameters can be stressful itself and this can confound the results (Cook *et al*., 2000). For example, to measure glucocorticoids levels outside the laboratory environment (where the animal can be cannulated) using blood samples, we probably will need to capture, restrain, and then draw the blood through a puncture, and all these handlings can be stressful. The blood withdrawal procedures must take less than ~3 min in order to not show an increase in cortisol levels due to the procedures (Fox *et al*, 1997; Mikics *et al*., 2004); for catecholamines inference, the animal must be cannulated, as the release of adrenaline and noradrenaline takes only seconds (Reece, 2004). Currently, there are ways to measure stress indicators other than through the blood, such as the measure of cortisol in the surrounding water for fish (Ellis *et al*., 2004; Sundh *et al*., 2010) or in feces for mammals (Möstl *et al*., 2002), fish (Huntingford *et al*., 2006) and droppings or egg yolk of birds (Alm *et al*., 2016). We need to take in account that these kinds of assessments show a more long-term state of the animal than the measure in the blood: for example, in birds, the metabolites of corticosterone rise in the droppings only hours after the hormonal plasma increase (Rettenbacher *et al*., 2004) and it takes 1 to 11 days for this response be detectable in the egg yolk (Rettenbacher *et al*., 2005). In the case fish, the increase in plasmatic cortisol levels is not always followed by an increase in the surrounding water cortisol: Sundh *et al*. (2010) found an increase in plasmatic cortisol levels in Atlantic salmon *Salmo salar* submitted to low dissolved oxygen in tanks, but the measure of cortisol in the water did not showed this increase. Of course, the accuracy of the measure of glucocorticoids has to be evaluate, as the hormone concentration is smaller in the surrounding water; with the increase of the minimal level of detection of the methods, we can be able of better use the surrounding water to infer the inner state of the fish. Moreover, frequently, measures of animal waste (surrounding water, feces) cannot differentiate between the individuals of animals that live in a group, being only suitable for the evaluation of distress and impaired welfare in general. If we need to evaluate the individual state of the animals, we need to be able of track the animal and the sample (to which animal belongs the egg, the feces, etc.); this is easier with bigger animals, as cattle, or animals keep in individualized spaces, but can be quite difficult with animals kept in large groups as broiler chickens of fish in a tank. The analysis of glucocorticoid levels in the group as a hole in the surrounding water, egg yolk and feces is interesting when we need to evaluate if an event or manipulation was stressful for the animals, taking in account the time gap between the event and the response. For a punctual evaluation, when the immediate effect of a stressor is the target, the analysis of the circulating levels of hormones in the blood is the better alternative. One alternative to blood sampling is to measure the glucocorticoids in the saliva; besides being ten or more times lower than the plasma glucocorticoids levels, the rate of diffusion through plasma to saliva is high and an increase in plasmatic hormone levels is followed by a correspondent increase in salivary hormone levels (Broom, 2000). However, if the contact with humans is stressful, even the collection of saliva can result in increase in the primary and secondary parameters due to manipulation. One alternative is to acclimatize the animals to the presence and manipulation by humans, minimizing the stress of the samples collection. In all types of sampling, the better the interaction between the animals and the human, less stressful will be the sampling and more reliable will be the results.

Nociception, pain and welfare

Although impaired animal welfare it is not always related to suffering, the presence of pain will always promote a decrease in welfare and the presence of pain is one of the most important aspects to determining animal welfare. In this way, assessing the presence and intensity of pain is crucial for pain prevention and alleviation.

Pain is a somatic sensation with a protective function, which alerts the organism about a real or imminent injury (Basbaum & Jessel, 2000; Julius & Basbaum, 2001). The International Association for the Study of Pain (IASP, 1979) describes pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage", evidencing the subjective and emotional attributes of pain sensation. While the IASP concept is applied for human pain, Mollony (1997) described animal pain as "an aversive sensory and emotional experience representing an awareness by the animal of damage or threat to integrity of its tissues; it changes the animal's physiology and behavior to reduce or avoid damage, to reduce the likelihood of recurrence and to promote recovery; non-functional pain occurs when the intensity or duration of the experience is not appropriate for the damage sustained and when physiological and behavioral responses are unsuccessful in alleviating it".

Because it is a subjective experience, the major difficulty of the assessment of pain perception in animals is the definition of parameters of indicators of pain, since verbalization and self-reporting are not possible. Behavioral signs, such as the absence of normal behavior, can be considered one of the most important signs of pain in an animal. However, because of its species- and injury-specific nature, behavior may not be accurate as a pain indicator. In this way, physiological parameters as indicators of distress can be complementary to behavioral observations in animal pain assessment (Anil *et al*., 2002), since pain experiences are accompanied by physiological changes that can indicate its presence and intensity, providing indirect evidence of the mental state of an animal.

Physiological changes related to pain can be caused by two interrelated mechanisms: stress response, which promotes activation of the HHA/HHI axis and sympathetic systems, and tissue damage, which activates the immune system and releases inflammatory mediators, which also promotes, indirectly, stress responses (Prunier *et al*., 2013). Therefore, according to Prunier *et al*. (2013), the hormones produced by the adrenal and sympathetic axes, their metabolites and physiological alterations, and inflammatory mediators can be considered physiological indicators of pain.

Painful stimuli are, undoubtedly, an important stressor for animals that promotes a strong activation of the sympathetic system and HHA axis. The use of laboratory models for pain studies demonstrated that physiological stress indicators, such as an increase in glucocorticoid levels, are deeply influenced by both acute and chronic pain, including the neuropathic kind, and this response can last from minutes to hours after the painful experience (Friedman *et al*., 1967; Taylor *et al*., 1998; Benedetti *et al*., 2012). Common handling procedures applied to farm animals that are considered potentially painful also promote stress responses that can be evaluated by hormonal measures. For example, an increase in glucocorticoid and noradrenaline levels is related to castration and tail docking procedures in lambs (Kent *et al*., 1993; Mollony & Kent, 1997, Mellor *et al*., 2002), and branding with a hot iron and freeze, cautery disbudding and amputation dehorning in cattle (Schwartzkopf-Genswein *et al*., 1997; Stafford & Mellor, 2011). Although in addition to being painful these procedures are also stressful per se, there is evidence that the HHA axis activation, in some contexts, such as in castration and tail docking procedures in lambs, is directly related to the pain experience, since the application of anesthetic can inhibit the glucocorticoid response (Thornton & Waterman-Pearson, 1999). Surgical procedures applied to veterinary care considered as painful, e.g. exploratory celiotomy for colic in horses, can also promote HHA axis and sympathetic system activation with a glucocorticoid increase associated with an increase in heart rate (Prichett *et al*., 2003). Sympathetic system activation, as evidenced by an increase in the respiratory rate was also demonstrated in fish submitted to potentially painful stimuli (Newby *et al.*, 2007; Reilly *et al*., 2008; Alves *et al*., 2013), with this response being inhibited by morphine treatment (Sneddon, 2003b).

All kind of procedures that cause tissue damage are considered potentially painful for animals and the evaluation of tissue damage indicators associated with stress response physiological indicators, can help in pain assessment. Studies demonstrate that procedures such as tail docking (Simonsen *et al*., 1991; French & Morgan, 1992) and beak amputation (Breward & Gentle, 1985), which are common procedures in farm animals, can produce neuromas that are formed in peripheral nerves when injured axons form sprouts in damage sites. These peripheral neuromas can be detected by palpation and result in unbearable neuropathic pain and functional impairment. Considering its painful nature, the presence of neuroma in an animal can be an indicator of pain.

Limitations of pain assessment

Although there are several biological signs that can be related to nociception and pain experience, there is not one that can give definitive evidence of pain. Because of the complexity of the pain concept, the measurable processes (physiological, pathological and chemical) are only underling events leading to the perception of this subjective feeling (Anil *et al*., 2002). Therefore, the physiological and biochemical alterations listed above that are promoted by painful stimuli should be interpreted carefully, since they are related to stressful situations that can also be promoted by non-painful stimuli, including normal behaviors, such as eating, exercise, reproduction, besides being influenced by individual characteristics such as age, species, gender, previous experiences, etc. In this way, to avoid mistakes in pain assessment a holistic evaluation, involving physiological and behavioral parameters, and a careful analysis of these parameters are essential. Furthermore, considering that the attempt to avoid suffering is the key point of welfare management, especially in captive and farm animals, it is ethically reasonable to consider that if an animal is submitted to a procedure that causes pain in a human, it can be assumed that it can cause pain in animals also (Anil *et al*., 2002) and should be avoided. However, since several currently-applied procedures in farm animals are potentially painful, it is important to develop strategies to minimize this suffering. In this perspective, the French National Institute for Agricultural Research chose an expert committee to review the issue of pain in farm animals. In this review, the authors developed a '3S' approach accounting for 'Suppress, Substitute and Soothe' by analogy with the '3Rs' approach of 'Reduction, Refinement and Replacement' applied in the context of animal experimentation. The 3S approach indicates that every effort should be made to 'Suppress' the procedures or environments that are a source of pain; when the suppression is not possible, to 'Substitute' such procedures by others causing no or less pain and distress, and to 'Soothe' pain when it cannot be avoided (Le Neindre *et al*., 2009 apud Guatteo *et al*., 2012). According to Guatteo *et al*. (2012), several painful procedures can be suppressed (e.g. tail docking in dairy cows), or performed just when necessary (e.g. cutting piglets' teeth). Furthermore, choosing methods that promote less pain responses is preferable than more painful methods (e.g. the use of cauterization for disbudding and/or dehorning cattle rather than the use of chemical substances or amputation with a scoop). When a painful procedure is unavoidable, the alleviation of pain should be considered by pharmacological treatment using analgesic and anti-inflammatory drugs.

Conclusions

The use of physiological parameters as indicators of distress and pain is a suitable form of measurement; however, the assessment of welfare has to be done using a multicriteria approach, with several parameters of a physiological and behavioral nature being analyzed concomitantly, and we need to take into account the particularities of the physiological parameters in the interpretation of the results.

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