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Review

Exploration of the hypothalamic–pituitary–adrenal function as a tool to evaluate animal welfare

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Abstract

Measuring HPA axis activity is the standard approach to the study of stress and welfare in farm animals. Although the reference technique is the use of blood plasma to measure glucocorticoid hormones (cortisol or corticosterone), several alternative methods such as the measurement of corticosteroids in saliva, urine or faeces have been developed to overcome the stress induced by blood sampling itself. In chronic stress situations, as is frequently the case in studies about farm animal welfare, hormonal secretions are usually unchanged but dynamic testing allows the demonstration of functional changes at several levels of the system, including the sensitization of the adrenal cortex to ACTH and the resistance of the axis to feedback inhibition by corticosteroids (dexamethasone suppression test). Beyond these procedural aspects, the main pitfall in the use of HPA axis activity is in the interpretation of experimental data. The large variability of the system has to be taken into consideration, since corticosteroid hormone secretion is usually pulsatile, follows diurnal and seasonal rhythms, is influenced by feed intake and environmental factors such as temperature and humidity, age and physiological state, just to cite the main sources of variation. The corresponding changes reflect the important role of glucocorticoid hormones in a number of basic physiological processes such as energy metabolism and central nervous system functioning. Furthermore, large differences have been found across species, breeds and individuals, which reflect the contribution of genetic factors and environmental influences, especially during development, in HPA axis functioning. Usually, these results will be integrated with data from behavioral observation, production and pathology records in a comprehensive approach of farm animal welfare.

Keywords: Stress; Animal welfare; HPA axis; Glucocorticoid hormones; ACTH; Dexamethasone suppression test; Cattle; Pig; Fur animals; Mink; Fox; Poultry; Fish

Contents

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1. Introduction

Beyond a large diversity of concepts, welfare refers principally to the subjective psychological state of the individual, as related to its internal and external environment [1,2]. Since we are not yet at the stage of being able to read directly animals' feelings and emotions, we try to infer those from measurable indices that we know or suppose to be related to them. Most of these measures – including behaviour, biology, production traits and pathology – derive from the study of emotions/stress/adaptation psychophysiology and physiopathology [3]. Stress is a general term used to describe environmental factors sollicitating adaptation mechanisms and the response to these challenges. The autonomic nervous system (ANS) [4] and the hypothalamic–pituitary–adrenocortical (HPA) axis [5] have the front of the stage in stress studies [6]. However, the interpretation of the activation of these systems is far from being straightforward.

First, these neuroendocrine systems are primarily involved in metabolic homeostasis and particularly in the regulation of energy fluxes [7]. In a teleological perspective, the reason why these systems are activated by stressors is that they are able to produce energetic metabolites either from energy storage tissues (the ANS mobilizes fat from adipose tissues and glycogen from liver) or by transformation of proteins into energetic metabolites (neoglucogenesis enhanced by glucocorticoid hormones). This energy supply is used by the defense mechanisms to cope with the stressor. Consequently, any change in HPA axis or ANS

functional parameter is not necessarily the response to a stressful stimulus, i.e. in the psychological sense, but can also reflect their involvement in homeostatic metabolic processes. The best example is the increase of cortisol levels induced by meals that are not usually considered as stressors. Furthermore, metabolic adaptive changes do not necessarily require activation of these systems, but can sometimes shut them down, depending upon the specific demands of the situation. For instance the response of the ANS to early weaning in pigs is an inhibition that can be seen in the reduction of catecholamine levels in urine [8]. This is an energy saving mechanism adapted to the deficit resulting from weaning-induced starvation. These metabolic influences on neuroendocrine systems must be taken into consideration when interpreting experimental data.

Second, the duration of the stimulus plays a pivotal conceptual role in the "general adaptation syndrome" as described by Selye, with the three successive phases, alarm, resistance and exhaustion [9]. The immediate biological responses to acute challenges (such as parturition, castration, weaning, mixing of animals from different social groups, restraint, transportation, slaughter) have been studied extensively and, like most stressors, activate biological stress systems in a more or less standardized manner (alarm phase). This common pattern of response is at the origin of the stress concept that was defined by Selye as the "non-specific response of the body to any demand made upon it" [10]. Note, however, that this non-specificity is mostly the result of the uniqueness of the response of the HPA axis, i.e. an increase in circulating cortisol levels that is exquisitely sensitive to "any demand", whatever its nature and intensity. However, if the stimulus is maintained for some time, circulating levels of corticosteroid hormones return to baseline even if the sustained activation of the HPA axis can be detected by different approaches like dynamic testing. Since many factors challenging animal welfare are long lasting – this is the case for most influences from the physical and social environment of the animals – more attention should be given to the exploration of chronic readjustments of adaptation mechanisms (allostasis) [11,12].

Third, there exists a huge variability – across species, breeds, and individuals – in the basic functioning of adaptation mechanisms and in their responses to environmental challenges. This variability has a multiple origin, genetic, developmental, and experiential. Although it may not be of primary importance in longitudinal studies with the same animals studied in basal conditions and then exposed to stressors, it has to be taken into consideration in field studies in which the history of the animal is not readily available. Therefore, a detailed knowledge of the biological adaptation mechanisms is a prerequisite to interpret the data obtained in studies focusing on stress and animal welfare. This review focuses on HPA axis functioning in the most important farm animal species.

2. The hypothalamic–pituitary–adrenal (HPA) axis

2.1. General organization

The HPA axis has the classical architecture of the major neuroendocrine systems. The main active hormone of the axis is cortisol in cattle, sheep, pig, mink, fox and fish, and corticosterone in birds and laboratory rodents. These are cholesterol-derived steroids synthesized in the fascicular zone of the adrenal cortex under the control of the pituitary hormone ACTH (adrenocorticotropic hormone). ACTH is synthesized by specialized cells of the anterior pituitary gland (corticotrophs) and its release is triggered by the coordinated action of two neuropeptides, the corticotropin-releasing hormone (CRH) and vasopressin (AVP), that are synthesized in specialized neurons of the paraventricular nucleus of the hypothalamus (PVN) and released in the capillary bed of the median eminence from where they reach the pituitary directly via the hypothalamic–pituitary portal circulation. The PVN receives numerous inputs from other hypothalamic nuclei (these inputs carry metabolic and nycthemeral signals), from the brain stem (in relation with neural inputs from the periphery), from the subfornical organ (that monitors blood plasma composition) and from the limbic system (that generates signals related to the emotional state). This multiplicity of signals converging to the PVN explains the sensitivity of the HPA axis to a wide range of stimuli from both internal and external origin. Furthermore, cortisol exerts a negative feedback on the axis by acting on the pituitary corticotrophs, the PVN and higher levels in the central nervous system. This feedback action of cortisol participates in the return of the HPA axis activity to basal levels after stimulation [13].

2.2. Physiology of the HPA axis

Due to its lipophilic nature, most circulating cortisol (approx. 90%) is bound to proteins, principally albumin and corticosteroid-binding globulin (CBG), a specialized glycoprotein that binds cortisol and/or corticosterone with high affinity, and regulates its bioavailability [14,15]. The free fraction can easily cross biologic membranes including the blood-brain barrier and cellular membranes. Cortisol interacts with intracellular receptors – mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) – that, upon activation by their ligand, translocate to the cell nucleus to activate or inhibit gene expression (transcription factors) [16,17]. In the periphery, aldosterone is the primary hormone activating the mineralocorticoid receptor. Aldosterone is released by the adrenal cortex under the influence of the renin–angiotensin system, but also ACTH. In the kidney, salivary glands and colon, all tissues that are responsive to aldosterone and involved in water and electrolytes metabolism, the mineralocorticoid receptor is protected from cortisol action by the 11β-hydroxysteroid dehydrogenase (11βHSD), an enzyme that metabolizes cortisol into its inactive derivative cortisone [18].

Detailed information on the metabolic effects of cortisol, which are numerous and complex, can be found in [19]. Altogether, cortisol has catabolic activity – proteolytic and lipolytic – in peripheral tissues and anabolic activity in liver, including gluconeogenesis and protein synthesis [20]. Since cortisol also reduces the entrance of glucose into cells, it increases blood glucose and insulin secretion (also due to the action of cortisol on ANS function in the hypothalamus). Furthermore, cortisol increases food intake by an action on the

brain so that the increase of energy availability is a coordinated process via peripheral and central mechanisms [21]. The combination of increased cortisol and insulin leads to the storage of energy as fat in the adipose tissue, if not used in the stress response, for behavioral adjustments for instance. The net effect is an increase of fat depots at the expense of tissue proteins [22].

The activity of the HPA axis is highly variable. First, the secretion of cortisol is pulsatile, with a periodicity of about 90 min. Although this feature is well documented in several species like humans [23], cattle [24] or sheep [25], it has not been detected in pigs. The second source of variability is the diurnal cycle that is genetically determined and synchronized by light. In diurnal species (including most farmed animals), a peak is observed in the morning and a trough during the evening and at night. This is indeed an important factor to be taken into consideration in experimental and clinical studies, since the difference between morning and evening levels of cortisol in biological fluids is very large. The occurrence of circadian rhythms is generally acknowledged in pigs, horses, sheep, cattle and chickens, among other species. In general, the peak in hormone levels occurs towards the end of the dark period in diurnal animals, whereas in nocturnal species there is a peak towards the end of the light period [207,27,107,28]. Feeding, however, may alter this rhythm. Surprisingly enough, although the meal-induced release of cortisol has been described in humans many years ago [26], it has not been specifically investigated in most farm animal species, but it can be found in experimental data (see for instance [27–29] for pigs). The HPA axis is also influenced by environmental factors such as temperature and humidity [30].

2.3. Individual differences in HPA axis functioning

Individual variation in HPA axis activity is well documented, but the reproducibility of the tests for HPA function discloses stable individual characteristics [31–35]. Genetic factors have been highlighted in humans by twin and family studies [36–38]. Large variations have been described between inbred strains of mice [39–43] and rats [44–50], and between farm animal breeds [51–53]. Furthermore, bird strains could be selected for divergent adrenal responses to ACTH [54], immobilisation [55], cold [56] or social stress [57]. Fish strains could be selected for divergent responses to confinement stress (rainbow trout [58] and Atlantic salmon [59]).

Several mechanisms are responsible for genetic variation in HPA axis activity. In a pioneering series of studies in pigs, Hennessy and collaborators have shown that the adrenal response to ACTH is an individual characteristic, which is inheritable and reproducible across successive testing [35,60,61]. Genetic factors also influence the bioavailability of corticosteroid hormones. In pigs, polymorphisms of the CBG gene influence circulating cortisol levels [62–64]. Finally, large differences may be found in the efficiency of corticosteroid receptors (see for instance [65,66]), but little is known in farm animals [67].

Individual variations can also arise from environmental influences, either during pregnancy and early post-natal life or as a result of experiences in later life. Early influences have been extensively studied in laboratory animals and offer new approaches to control for emotional reactivity in adults, including neuroendocrine responses [68]. Sustained changes of HPA axis responsiveness were found in pigs after prenatal restraint stress [69], repeated exposures to noise [70,71] or social isolation during early infancy [72,73] or neonatal handling [74]. However, more studies are necessary to validate these findings in other farm animal species.

3. Evaluation of HPA axis function as related to stress and welfare

3.1. Acute stress response

3.1.1. Description of the response

The HPA axis is activated during exposure to aversive situations. For instance, the blood level of cortisol is increased in pigs, cattle and sheep when they are subjected to a painful procedure like castration, but also when they are separated from their usual peers, mixed with unknown animals, restrained in a crush, transported, frustrated from not obtaining an expected reward or from not performing a behaviour, etc. [75–78]. Other measures of HPA axis activation can be used that rely upon the biological effects of glucocorticoids, e.g. increase of plasma glucose levels or the changes in circulating leucocyte number and formula (e.g. [79–81]). However, stress-induced hyperglycemia has multiple mechanisms and involves mainly a synergy between glucagon, catecholamines and glucocorticoid hormones and a reduction of insulin secretion [82].

Due to the organization of the HPA axis, the release of corticosteroids is a slow process. It is only after a few minutes from the beginning of a stressful event that corticosteroid levels are increased in blood. The response is then prolonged for about an hour after the termination of the eliciting event [79,83]. Hence, the HPA response to an aversive situation is generally assessed by measuring corticosteroid levels in blood at least 10 min after the animal has been first exposed to it. The amplitude of the response depends on the species, probably in relation to basal levels: cattle have very low basal levels (often less than 15 nmol/L) and cortisol levels in response to a stressor can increase up to 60–200 nmol/L [75,84] whereas in pigs levels of cortisol are ten times as high as those of cattle for baseline and more than twice as high in response to a stressor [85,86].

There is little evidence that corticosteroid release varies with the severity of the situation. For instance, cows which are separated from their peers and then restrained in a crush seem to have lower cortisol level than cows which are only restrained, and no difference are observed between cows which are transported or not in addition to being separated and restrained [85]. Similarly, the mere exposure of a pig to a novel environment is sufficient to increase blood cortisol to its highest possible levels [86]. The ACTH response might be more sensitive to the severity of the situation than corticosteroids. Dose-response studies show that the increase of plasma ACTH levels is much more graded with stimulus intensity, as

mimicked experimentally by injections of CRH at increasing doses ([87], in humans; [60], in pigs; [88], in calves). This reflects both the extreme sensitivity of the adrenal cortex to detect and amplify the ACTH signal, and the rapid saturation of the adrenal response with increasing ACTH concentrations. Therefore, measuring ACTH and cortisol in the same plasma samples allows a better coverage of the whole range of response intensities that should help in the evaluation of stimulus strength.

3.1.2. Psychological vs biological factors

When Selye introduced the concept of stress and highlighted the activation of the HPA axis, it was thought that the response was produced whatever the nature of the aversive situation [10]. The importance of psychological factors, rather that the physical characteristics of the situation, has been put forward by Mason [89] who observed that monkeys had an increase in corticosteroid metabolites in urine when they were not given food and that this response disappeared when they were given non nutritive pellets of the same appearance and flavour as their normal food. Hence it was not the situation *per se* (fasting) but the way the animal perceived it that produced the stress response. This has been subsequently confirmed by observing that the HPA activation in response to an aversive event depends on the control the animal can have on this event [90].

This exquisite sensitivity of the HPA axis to the emotional value of the environmental stimuli justifies the use of ACTH and adrenal hormones levels in the monitoring of animal welfare. However, the HPA axis is primarily involved in the regulation of energy fluxes in the body, and is therefore sensitive to various environmental stimuli challenging the energy balance of the body, such as nycthemeral influences, food intake and temperature regulation, as reviewed earlier. Therefore neuroendocrine data should always be interpreted in this context as there is no simple correlation between plasma cortisol levels and perceived stress.

3.2. Chronic stress response

3.2.1. Change in hormone levels

Even if the triggering stimulus is maintained, plasma cortisol levels usually decline after the acute response. For instance, pigs having received unpredictable and inescapable electric shocks during a month have blood levels of ACTH and cortisol similar to those of control animals not receiving any shock. However, they still react behaviourally to the situation (agitation followed by inhibition), suggesting that they are continuously stressed [91]. Similarly, calves subjected to social and spatial restriction have basal levels of cortisol similar to those of non-restricted calves, despite their high motivation to have contacts with other animals or to move around [92,93]. Therefore, plasma or salivary cortisol levels are not very informative to detect chronic stress situations. Although they can be slightly elevated over basal levels, these changes are difficult to demonstrate without catheterization and/or multiple sampling, as compared to spontaneous variations or the effects of blood sampling itself [94]. Furthermore, the effect of stress is not constant over the day, and increased cortisol levels have been seen mostly at night, when they are usually low [95–97]. Measurement of cortisol levels in urine – a more integrative measure of cortisol secretion – may be more sensitive to detect these small changes since rapid variations are buffered over time, but this hypothesis has still to be documented.

3.2.2. Modification of the responsiveness of the HPA to further stressors

Repeated exposures to stressful events can increase the subsequent responses of the HPA axis to aversive events. For instance, in rats, the release of ACTH in response to restraint is increased after repeated exposure to the same stressor (restraint) or to another one (cold) [98,99]. As a consequence, the release of corticosterone is also enhanced compared to that of control animals not previously exposed to stressors. According to Bhatnagar and Dallman [99], the facilitation of the ACTH response to subsequent stressors results from an increased activity of brain regions that regulate the HPA axis activity (e.g. thalamus). However, in other experiments, a decrease in the sensitivity of the HPA axis to further stressors has been reported [96].

The modification of the HPA axis response to subsequent stressors can be partly explained by modification of the behavioural reactivity of animals. There is some evidence that animals chronically stressed do not react to their environment as normal ones. Cases of increased behavioural reactivity and, on the opposite, of apathy have been reported respectively in calves subjected to social instability and sows tethered in individual stalls compared to animals reared in stable groups [100,101]. According to Boissy et al. [100], these opposite results are due to the degree of control the animal can have on its environment.

3.2.3. Modification of the functioning of the HPA axis

Despite no change in basal levels of ACTH or cortisol under chronic stress, several indices show that the activity of the system has changed. Each level of the axis (hypothalamus, anterior pituitary, adrenal cortex) is subjected to opposite influences, trophic via their respective stimulating inputs (such as CRH to the pituitary or ACTH to the adrenal cortex) and inhibitory via corticosteroid hormones (feedback). Several changes induced by chronic activation of the HPA axis are well documented in laboratory animals: weight loss (the result of the catabolic effect of cortisol and catecholamines), shrinkage of thymus (a tissue rich in glucocorticoid receptors and a sensitive indicator of chronic cortisol action), proliferation of the corticotrope cells in the anterior pituitary (a trophic effect of CRH), inhibition of ACTH synthesis (by cortisol) and reduction of the feedback effect of GR agonists on ACTH release, increase of the size of the adrenal glands and of the response of the adrenals to ACTH (a trophic effect of ACTH) [102,103]. This resetting of the HPA axis at a different level of activity, that Selye [9] described as the stage of resistance, is also known as allostasis [11,12]. Specific protocols are necessary to detect these changes. They include stimulation tests (by CRH, CRH/vasopressin, ACTH, insulin-induced hypoglycaemia) that demonstrate the sensitization of the pituitary and/or the adrenal

cortex, and the inhibition test by dexamethasone, a synthetic steroid with glucocorticoid activity that demonstrates the reduced efficiency of the negative feedback by corticosteroids.

3.2.4. Stimulation tests

The stimulation of the HPA axis is usually performed by injecting IV synthetic ACTH (Synacthen ND, Novartis-Pharma, at the dose of 0.5–1 IU/kg $BW^{0.75}$ in young animals and 1–2 IU/kg $BW^{0.75}$ in adults [92,104,105]) or more rarely CRH (of the same species and administered at the dose of 0.03–1 μg/kg [28,60,88,96,106–108]). Blood is sampled at several intervals after the injection to determine ACTH and/or corticosteroid levels. The integrated responses are calculated as areas under the curves of corticosteroids or ACTH after the injection. The response to both challenges is rapid, with a peak of cortisol about 15 min after CRH and 1 h after ACTH, depending on the species studied and the compound injected. Around the peak, hormonal levels vary rapidly. For this reason we do not recommend to take blood samples at that time but either before or after the expected peak. Finally, to test the sensitivity of the adrenals using a CRH challenge, the ratio between the integrated response of cortisol and that of ACTH must be calculated. The response to CRH or ACTH can also be monitored with urine samples [28,109].

After injection of ACTH, some authors found an increased cortisol response in animals reared in poor conditions or subjected to repeated stressors (e.g. calves submitted to prolonged spatial and social restriction [92] or to repeated regrouping [105], growing pigs with restricted space [110], tethered sows [111], etc.). By contrast, other authors found a decreased response in cattle subjected to tethering or to crowding [104,112]. The age of the animal and the delay between the beginning of the stressful situation and the ACTH challenge might influence the activity of the adrenals (see for instance discussion in [113]). Also, as for behaviour, the control the animal can have of the situation may play a role since a behavioural hyperreactivity and an increase cortisol response to ACTH are both observed in calves submitted to repeated mixing [100,105] while apathy and a lower cortisol response to ACTH are observed in tethered animals [101,104].

3.2.5. The dexamethasone suppression test

The dexamethasone suppression test has been developed in humans to detect HPA axis changes in melancholic patients [114]. Indeed, administration of dexamethasone – a synthetic glucocorticoid with a strong feedback action on pituitary ACTH release – reduces the morning peak of plasma cortisol, but more so in healthy persons than in depressed patients. The pharmacological blockade of HPA axis is generally performed by injecting i.m. 15–20 μg/kg live weight of dexamethasone at night (e.g. Dectancyl ND, Roussel) and taking blood samples in the morning of the injection day and the next morning (humans [114], pigs [110], cattle [105]). The response to dexamethasone can also be monitored with urine samples [28].

The response to dexamethasone of animals subjected to chronic stress seems similar to that of depressive humans. For instance, in pigs, blood cortisol levels are not decreased in animals that have been submitted to a prolonged reduction in their space allowance [110]. To our knowledge, such as escape from the blockade by dexamethasone has never been observed in cattle, probably due to low basal levels of cortisol.

4. Practical issues: sampling of biological fluids and faeces

Glucocorticoid hormones can be measured in several biological samples, including plasma, saliva, urine and faeces [115]. Current methods for the assay of glucocorticoids in biological samples are radioimmunoassay (RIA), enzyme-linked immunoabsorbent assay (ELISA), and high-pressure liquid chromatography with UV detection [116]. In antibody-based assays it is important to consider their possible specificity towards cortisol and corticosterone respectively, depending on the species of interest. The assay of ACTH is based on the use of one (radioimmunoassay) or two (radioimmunometric assay) specific antibodies and has to be validated in each species (e.g. [88,117]).

4.1. Plasma

Plasma is the most widely used sample to assay glucocorticoids in animal welfare studies. A small fraction only (about 10%) of the total blood cortisol and corticosterone is in the free form, i.e. susceptible to ultrafiltration, whereas the rest is bound to CBG or to albumin. At high concentrations, such as during ACTH stimulation or stress, free plasma cortisol increases to 20–30% [118]. Most assays measure total corticosteroid levels. As the free rather than the protein-bound fraction is biologically active, methods have been developed to measure free cortisol or corticosterone [118,119]. However, whether free cortisol is a better functional measure of HPA axis activity than total cortisol is debatable. For example, in goats, an excellent correlation is observed between total plasma cortisol and the active free cortisol [120], and in general most domestic animals have little corticosteroid-binding activity compared to humans [14,15].

One of the main problems in assaying plasma levels of glucocorticoids is their large variation. As mentioned before, ultradian, diurnal, and eventually seasonal rhythms have been described in several species (see Section 2.2). All these factors have to be taken into consideration in experimental protocols. Glucocorticoid levels are also sensitive to many environmental factors. They increased simply by catching and handling the animal. This artefact can be avoided by taking the blood sample before the adrenal cortex has been activated, within 2–3 min of catching the animal. A second possibility is to habituate the animals to handling beforehand. Also, samples can be taken by means of an in-dwelling catheter. One obvious advantage of blood sampling, when done in good conditions, is that it gives access not only to glucocorticoid and ACTH levels but also to information on biological endpoints of HPA axis activation (changes in glucose levels or white blood cell count and formula), and other stress-sensitive biological systems such as the ANS (assay of catecholamines for instance).

Plasma samples, particularly with a high concentration of cortisol, shall not be stored at room temperature for prolonged storage. Also, samples that are allowed to defrost long before

being processed may have a lower cortisol concentration than at the time of collection. For ACTH assay, blood should be sampled with EDTA as an anticoagulant since heparin interferes with the assay, and samples should be kept in ice-cold water before centrifugation. Plasma samples should not be thawed and frozen several times.

4.2. Urine

Urine is the main elimination route of glucocorticoids and offers at least two practical advantages: it can be collected noninvasively, and excretion products accumulate over several hours. Therefore, urinary corticosteroids and/or their metabolites provide an integrated measure of their production over a period of time, thereby adjusting for the fluctuations in plasma levels. In pigs, urine can be collected continuously with inflatable vesical catheters [28], whereas in sheep, urine can be collected by continuous aspiration from a urinal strapped to each animal [115]. Urinary glucocorticoid hormones and their metabolites can be measured using a solid-phase extraction procedure followed by HPLC with UV detection as described by Hay and Mormède [121] or radioimmunoassay. Hormone levels are expressed as hormone/creatinine ratios in order to account for differences in urine production as creatinine is excreted at a relatively steady rate.

Urinary cortisol displays a close linear correlation with unbound plasma cortisol [122] and urinary cortisol has been used to monitor HPA activity in several farm species, including pigs [28,123] and sheep [115]. Other parameters can be measured in urine, including catecholamines and their metabolites [121], which allows a more comprehensive investigation of the neuroendocrine adaptive processes as illustrated by the work done on early weaning in pigs [8].

4.3. Saliva

Glucocorticoids have been measured in saliva in a variety of species, including pigs [124–127], cattle [128,129], sheep [118], goats [120]. The main advantages of measuring glucocorticoids in saliva are the relative non-invasiveness of the method, less stressful than blood collection, and the fact that in saliva glucocorticoids exist only in the “free” form, since saliva is devoid of binding proteins [130]. The limiting factor is the sensitivity and specificity of assays [131,132].

In sheep, saliva can be obtained by aspiration from the area of the mouth opposite to the third or fourth cheek tooth, that is, close to the opening of the parotid duct. The aspiration device consisted of a PVC tube connected to a plastic vial for collection and to a vacuum source. The animal is allowed to chew on the tube and an experienced operator can collect a satisfactory sample in 30 s [118]. A similar method has been used in calves [133]. In sheep, salivary and free serum cortisol levels are closely correlated. From observations after ACTH administration, it was concluded that the concentration of cortisol in sheep saliva is 10% that of plasma with no delay between the rise of cortisol in plasma and that in saliva (Andanson, unpublished data). When free cortisol levels are low, the correlation between saliva and plasma levels is not observed probably due to difficulties in measuring low levels of cortisol. Also, significant increases in salivary cortisol have been observed in sheep and calves submitted to rough transport, to confinement in metabolism cages or castration [133–135]. In goats, saliva can be obtained without blood contamination from adult animals by aspiration with a plastic suction tube. This is not possible in young kids, probably because of a low rate of saliva production and to damage caused by the suction tube, which results in bleeding and contaminations of the saliva sample with blood [120].

In pigs saliva samples can be obtained by allowing the animals to chew on cotton buds until thoroughly moistened (about 1 min). The animals can be accustomed to this procedure beforehand. The cotton buds are then stored in test tubes and centrifuged before analysis (see for example [124–126,136–139]).

4.4. Milk

Cortisol can also be detected in excreted milk ([140]). Milk samples are very convenient in dairy animals (cows, goats, and ewes). There seems to be no accumulation in milk so that milk concentrations are highly correlated to plasma concentrations. Concentration in milk amounts between 4 and 10% that of plasma (Andanson, unpublished data).

4.5. Faeces

In most farm animal species, cortisol is mainly excreted in urine, but excretion via faeces also occurs [141,142], and collection of faeces is non-invasive. In particular, the measurement of fecal 11,17-dioxoandrostanes (11,17-DOA), a group of cortisol metabolites, has been proven useful to evaluate adrenocortical activity in a variety of species. The cortisol metabolites (11,17-DOA) can be determined using an enzyme immunosorbent assay (EIA) after methanol extraction [134,143]. At least in sheep, measuring 11,17-DOA with an EIA designed for 11-oxoetiocholanolone appears to be a valuable method. Other EIAs used for the determination of cortisol or corticosterone do not appear to be adequate as they do not show significant cross-reactions with the faecal cortisol metabolites. A careful validation for each species and sex is mandatory [144]. An important point to consider is that the concentration of 11,17-DOA increases in faeces at room temperature. In cows, for example, an increase of 136% was seen within a time interval of 1 h. Therefore, the time interval between defecation and freezing is critical, and faecal samples should be frozen immediately and stored at −20 °C until analysis. Even after freezing some of the 11,17-DOA producing enzymes are active, whereas enzyme activity is lost after heating the sample to 95 °C [142].

Also, although cortisol levels in faeces have been found to show diurnal fluctuations, it is likely that variations due to secretory patterns are attenuated in faeces [135]. Cortisol metabolites in cattle faeces increase after transport and after ACTH administration [145,146]. Likewise, the metabolites increase in mink faeces after ACTH injections [147] and

decreased for mink housed in enriched cages (concurrent with a reduction in abnormal behaviour) in comparison with a control group housed in a barren environment (Hansen et al., in prep).

Nevertheless, measuring cortisol metabolites in faeces is not without problems, mainly because there are important differences between species in i) the proportion of cortisol metabolites excreted in the faeces (28% in sheep, 41% in ponies and only 7% in pigs), ii) the interval between the release of cortisol and its excretion via faeces (longer in pigs than in ruminants and ponies), and iii) the main metabolites excreted in faeces (in ruminants, but not in pigs and horses, 11,17-DOA are the main excretory products). As a result, assays to measure cortisol metabolites in faeces have to be adapted to each species and at present they are not satisfactory in some of them, such as pigs [141,143,148].

5. Species-specific features

5.1. HPA axis function in ruminants with emphasis on cattle

5.1.1. Specific features of HPA function in cattle

Cortisol is the glucocorticoid most studied in cattle as it supersedes corticosterone in concentrations. Several ratios of cortisol to corticosterone have been reported: 1.5 in Guernsey, 4.0 in Holsteins and 2.8 in Jerseys [149]. In Holstein cows, Gwazdauskas et al. [150] reported cortisol to corticosterone ratios that ranged between 0.9–15.9 under saline treatment and 1.6–35.9 under ACTH treatment. Under baseline conditions (2.3 nM free cortisol), roughly 80% of cortisol is bound by CBG, 10% by albumin and 10% is free. The number of CBG binding sites in cows may be around 110 nM and at high concentrations cortisol will increasingly bind to albumin (598.5 μM), which more or less prevents free fractions from exceeding 55% [14]. It has been reported that the cortisol-binding proteins in cow plasma may interfere with cortisol assays for unextracted plasma [151]. Heat inactivation (30 min at 80 °C) of the proteins or ethanol extraction eliminates most of the problems [92].

Dunlap et al. [152] studied clearance of exogenous cortisol (20 mg) in beef cows. The half-life fluctuated around 30 min. They concluded that the clearance of cortisol was not affected by lactational status or by postpartum interval, but that the volume of distribution of cortisol in the body decreased from 14 to 28 days postpartum.

Instead of blood, other body fluids may be used to measure cortisol in cattle such as milk. With baseline cortisol levels of 10–12 ng/mL in plasma, levels in milk amount 0.7–1.4 ng/mL. Levels in whole and skimmed milk were similar, suggesting that glucocorticoids are not associated with milk fat. Contrary to estrogens and progesterone, glucocorticoid levels seem higher in plasma than in milk [153]. Measurements of cortisol metabolites in the faeces of cows may be used to detect transport and novelty stress, but not the possible disturbances caused by diagnostic procedures [142,154].

5.1.2. Baselines and rhythmicity

Mean baseline cortisol values are typically lower than 10 ng/mL, and in recent studies often under 5 ng/mL, but single point measurements may range from near zero up to around 20 ng/mL [24,149,150,155]. In bulls, 24 h profiles indicate mean levels of 2.04 [24] to 4.2 ng/mL [156]. Also in bulls, Ladewig and Smidt [104] reported a mean of 11.2 episodic peaks (mean amplitude of 4.9 ng/mL) per 24 h, which compares to the findings in cows [156]. Mean ACTH baseline levels of 10–15 pg/mL have been reported in calves (sampling every 20 min between 08:00–14:00) and 5–10 pg/mL in the same animals one year later [157]. Levels seemed somewhat lower (<10 pg/mL) before the CRH tests in studies by Vessier et al. [106]. ACTH measurements in lactating cows have been reported to range from 1–6 pg/mL between 08:00–14:00 [158].

Hudson et al. [159] found no diurnal variation for cortisol levels in non-lactating cows. Others found early morning peaks, but no evening troughs [160], or troughs between 18:00 and 02:00, with no early morning peaks [161]. Recent studies in Brown Swiss bulls indicate low cortisol levels (episodic burst<3.5 ng/mL) between 17:00–01:00 and high levels (episodic burst>8 ng/mL) with the onset of daylight [24]. Thun et al. [24] suggested that infrequent sampling, the physiological state and non-specific measuring methods contributed to the lack of diurnal variations in earlier studies. However, another controlled and detailed study with bulls [104] did not confirm these results. Lefcourt et al. [156] took 15-min blood samples sequentially over 48 h from six dairy cows. Cortisol levels had a weak circadian rhythm (a mean minimum of 3.1 ng/mL at 18:00, and maximum of 4.5 ng/mL at 05:30), but a strong ultradian rhythm. Peak to trough amplitudes of 1 to 17 ng/mL, with oscillation periods around 120 min, varied within and between animals. Collectively, these findings suggest that circadian rhythms in the cortisol levels of cattle may exist, but that these are weak. Management practices may cause patterns in cortisol secretion that resemble circadian rhythms. For example, Blum et al. [162] measured cortisol baselines between 2 and 24 ng/mL with major peaks around milking (especially in the morning). Average milking-induced rises in cortisol usually vary between 10 and 12 ng/mL [163–165]. This is in line with early findings with regards to cortisol levels and milking [166] or suckling [167].

5.1.3. Responses to pharmacological treatments

In adult cows, doses of 200 IU ACTH per animal or 1.98 IU/$kg^{0.75}$, which amounts to 209 IU for 500 kg animals, have been used most often, but other doses have been applied as well. Alam et al. [168] tested ACTH doses of 0.01 to 2.0 mg in multiparous non-lactating cows. Mean cortisol peak values reached 8.4 to 36 ng/mL. Levels returned to baseline between 2 and 8 h after injection. In non-lactating dairy cows Verkerk et al. [169] tested ACTH doses of 0.0125 to 0.4 mg. Mean cortisol peak values reached from 62.1 to 73 ng/mL. The maximum peak levels were reached after a dose of only 0.025 mg, but integrated responses increased with higher doses. Levels returned to baseline between 1.5 and 4 h after administration of ACTH. Similar results were obtained by Lay et al. in pregnant Brahman heifers [170]. Vessier and Le Neindre [83] administered ACTH in 10 mo-old heifers via the im and iv route. They concluded that im administration induced higher, but more variable cortisol responses.

ACTH-induced cortisol responses may be reduced during the first days to weeks post partum [171,172], with advancing

age [173], at high ambient temperatures [174] or in cows that suckle calves [171]. Male sex hormones suppress ACTH-induced cortisol responses as steers have higher responses than bulls [175], with steers resembling non-lactating cows [169]. Calves had lower ACTH-induced cortisol responses than heifers (the same animals 1 year later) [157]. In a longitudinal study in heifer calves, ACTH- and CRH-induced cortisol responses were significantly lower at 3 than at 13 and 26 weeks of age, whereas CRH-induced plasma ACTH responses were not affected by age [108].

5.1.4. Stress-induced changes in HPA axis activity and reactivity

5.1.4.1. Acute stress. This section merely illustrates the range of stress responses in cattle and is not an overview of the large amount of experiments on the subject. Alam and Dobson [176] studied cortisol responses to 5 commonly practised veterinary procedures. Palpation per rectum of the reproductive tract, intramuscular injection, single venepuncture, repeated venepuncture and jugular vein catheterization increased plasma cortisol concentrations from 2 ng/mL to mean values between 6 and 14 ng/mL at around 20 min after the manipulations. Return to baseline occurred approximately 80 min later. Separating cows from their herd induced mean cortisol peak values of 15 ng/mL [177]. Cortisol levels are elevated around calving, and on the day of calving mean concentrations of 14 ng/mL [178] and 17 ng/mL in first lactation cows may occur [172]. Intra-mammary administration of endotoxin induced maximum cortisol concentrations around 23 ng/mL after 3 to 5.5 h [179]. In response to the introduction into a novel arena, cows show mean cortisol peak values between 23 and 35 ng/mL at 10 to 25 min after the beginning of the test [83]. Note that the individual differences in such cortisol responses are substantial [179]. Average plasma cortisol responses (elevations of post-test levels from corresponding pre-test baseline levels) in heifer calves exposed to 10-min novel arena tests were 6, 11 and 12 ng/mL at 3, 13 and 26 weeks of age [108]. Mean maximum cortisol concentrations after freeze-branding in young [180,181] and adult cows [84] ranged between 30 and 35 ng/mL, with mean responses as high as 55 ng/ml in Angus calves [182]. In dairy cows, Hopster and van der Werf [177] measured mean cortisol concentrations of 55 ng/mL immediately after 1 h of transport by truck.

5.1.4.2. Chronic stress. Fisher et al. [183] associated restricted housing conditions of heifers with reduced baseline cortisol levels. Calves that were socially isolated for 4 weeks, after having been raised in groups, had lower baseline cortisol levels (prior to administration of exogenous ACTH) than group-housed controls [184]. Relatively high levels of oral stereotypies shown by dairy calves housed in tether stalls were associated with relatively low baseline levels of plasma ACTH [157]. The authors' interpretation was that calves with higher stereotypy levels may have a higher resistance to tethering. Crowding-stress for 2–7 days, but not for 1 day or 9 days, increased ACTH-induced cortisol responses [185,186], whereas longer-lasting crowding stress suppressed adrenocortical responses [183,187]. Subjecting bulls to restrictive housing conditions, i.e. tethering on a slatted floor for 5 weeks when the animals were accustomed to group housing on deep litter, reduced high dosage ACTH-induced cortisol responses [104]. Likewise, in calves subjected to social isolation for 4 weeks, the integrated ACTH-induced cortisol response was lower than in group-housed calves [184]. Notably, ACTH-induced cortisol responsiveness was depressed in red deer stags after exposure to chronic social stress [188]. These findings suggest that under stressful conditions adrenal responsiveness in cattle increases on the short term, but decreases on the long term. This result, however, is not found consistently.

Some authors have reported that cattle adrenal responsiveness was minimally affected by potentially aversive treatments. Munksgaard and Simonsen [189] found minimal increases in ACTH-induced cortisol responses in lactating cows that were transferred from tie stalls to pens (4 animals in 3.6×3 m). Lactating cows that were deprived of lying (14 h per 24 h), crowded or isolated showed no changes in ACTH-induced cortisol responses [190,191]. In more recent studies, no differences in HPA axis reactivity to ACTH (or CRH) were found between individually housed and group-housed calves [106], or between visually isolated and non-isolated calves [192]. Other authors, using different types of stressors or more permanent housing conditions, have reported increased HPA axis reactivity [92,193]. It seems that adaptations to environmental challenges at the level of the pituitary–adrenocortical system are flexible and dynamic over time. They may involve both sensitization and desensitization, for example, due to reversible changes in the density and sensitivity of ACTH and cortisol receptors. Thus, depending on factors such as the nature of the stressor and the timing after the onset of the stressful condition, the reactivity of the pituitary–adrenal system may be increased, unchanged or depressed.

5.1.5. Implications for the assessment of welfare in cattle

Cortisol responses to short lasting management procedures increase in magnitude with different treatments that we assume to be increasingly aversive. For example, the cortisol responses to routine veterinary procedures are much lower to the response to freeze-branding (cf. paragraph 5.1.4). This implies that HPA axis activity in cattle may help to evaluate in what way short lasting farming procedures and treatments are perceived by the animals. For instance, the fact that cortisol levels in cows are similarily increased by transvaginal follicular puncture and only handling, is seen as evidence that this procedure for collecting ova for biotechnological practices does not compromise the welfare of the donor cows. This interpretation has been confirmed by measurements of milk production and blood cell counts, which were not influenced by follicular puncture [194].

For longer lasting stressors the situation seems more complex. There are indications that in cattle, enduring stress reduces HPA axis activity and reactivity, but findings to support the opposite exist and the available data are far from conclusive. It seems fair to say that our knowledge of HPA axis reactivity in cattle is currently insufficient to use response patterns to exogenous ACTH (or CRH) as a reliable indicator of animal

welfare status. Significant differences in cortisol (or ACTH) responses between treatments that putatively differ in the amount of stress imposed have been documented, but these effects are not consistent across the studies that use similar treatments. Also, different treatments that are all assumed to be stressful are known to produce profoundly different effects. In addition to the environmental factors, the dynamics in adaptive processes may strongly determine the HPA axis responsiveness of individual cattle at a given time.

5.2. HPA axis function in pigs

5.2.1. Measuring HPA axis activity in pigs

The most direct and reliable approach to determine HPA axis responses (cortisol, ACTH, CRH) is the collection of blood. However, in some breeds of pigs this may be difficult, as prominent superficial veins at the ears or the tail which can easily be punctured are sometimes not well-developed. It is also possible to access the *vena cava anterior* or the jugular vein by direct venipuncture, especially in younger animals [195,196]. This procedure requires some experience to be performed in short time in order to avoid too much stress for the animal. In cases where blood samples have to be taken frequently and over longer periods of time indwelling jugular catheters are commonly used. The method requires surgery and, hence, recovery time has to be considered (usually at least 1 week), but non-surgical techniques have been also described [197]. For smaller piglets up to 6 kg a minimally invasive technique for catheterization, applicable for 2–5 days, has been described by Carroll et al. [198] using the commercial Cook Single Lumen Central Venous Catheters kit. As with jugular venipuncture some experience is necessary to locate the guide needle without visual control. In free ranging catheterized pigs, care has to be taken that the extension tube will not be damaged. Therefore, catheterization is mostly used in single-housed animals. If, however, catheters are required for measurements of HPA axis in social interactions, the exit of the tube can be protected by neck straps where the end is covered in an integrated bag [199]. In this case the experimenter has to approach the animal which therefore must be familiar with the procedure. In addition, particular care has to be taken that the wound where the catheter exits the body (usually at the neck–shoulder region) is well protected against infections.

In order to overcome the drawbacks of punctures and catheters, various non-invasive methods for measuring cortisol in pigs have been used. Saliva is usually collected by allowing the pig to chew on cotton buds for some 30 s. A method to obtain integrative salivary samples over hours has been described by Schönreiter et al. [127]. The authors used Oral Diffusion Sunk (ODS) columns fitted inside the mouth by elastic bands around the animals' upper jaws. The method was reported to work well during transport but less if the mouth dried out due to specific circumstances, such as frequent vocalizations. When ODS columns have been placed in the mouth, the pigs must not be fed in order to avoid contamination of the column.

Cortisol can also be measured in spontaneously voided urine [53,116]. The diurnal cycle found in urine cortisol secretion is similar to that in plasma samples. However, after CRH challenge, maximum levels were measured in urine with a delay of about 1 h as compared to plasma cortisol [28]. Urinary cortisol is therefore well suited as a non-invasive measure of acute and chronic stress [8,109,200,201]. Cortisol metabolite extraction from faeces of pigs and horses has also been described [141]. In this study, using a radioimmunoassay for 11-oxoaetiocholanolone, pigs gave poorer positive results compared to horses.

5.2.2. Specific features of HPA axis in pigs

Different pig breeds may differ in basal HPA axis activity and responses to stress. For instance, Meishan pigs display higher basal and post-stress cortisol levels than Large White pigs [52]. The hypercortisolism of Meishan pigs is probably related to an increased responsiveness of the adrenal cortex to ACTH [202]. Other differences were found at the level of CBG [63] and corticosteroid receptors [67].

In newborn piglets a stress non-responsive phase of the HPA axis, as found in rats during the first weeks of life, is absent [195,203]. The circadian cycle occurs at an age of about 7 days, and in female pigs somewhat earlier than in males (6 and 10 days, respectively [204]). The mesor of basal salivary cortisol at an age of 41 d is about 1.3 ng/mL with an amplitude of ±0.5 ng/mL [205] and decreases with age to 0.5 ng/mL ± 0.2 ng/mL at 24 weeks of age [27]. Accordingly, the amount of free cortisol is highest immediately after birth and decreases with age. As the amount of CBG bound cortisol is virtually constant after day 1 of age [206] this indicates a gradual decline of HPA axis activity in the postnatal phase. Forced weaning of the piglets causes a transient increase of ACTH and cortisol, which seems to be largely independent of weaning age. Increased levels, probably resulting from the emotional stress of mother–infant separation and new environment, have been found at very early weaning (day 6) and at day 28 [8,196].

No ultradian rhythm has been described in pigs. The acrophase of the nycthemeral cycle starts shortly after midnight and the maximum is reached in the morning to decline until the afternoon trough is reached [27,28,107,207].

5.2.3. Responses to pharmacological treatments and stress

The maximum cortisol response is achieved with a high dose (typically 200 IU/animal) of synthetic ACTH resulting in a peak of plasma cortisol reaching 150 ng/mL on average [208], with large differences across breeds [107,209]. Other frequently used HPA challenges are novel environment exposure or nose snaring (psychological stressors) for typically 5–15 min and lipopolysaccharide injection (typically 100 μg/kg BW) [196,210–213].

There are comparatively few reports dealing with the effects of chronic stress on the HPA axis in pigs. Pregnant gilts and male fattening pigs housed in pens with little space allowance resulted in lower basal free cortisol concentrations in the gilts and unchanged levels in the fattening pigs compared to controls [110,214,215]. The cortisol response to ACTH was, however, increased in the crowded fattening pigs. A chronically poor environment resulted in more vigorous social interactions with

elevated basal cortisol levels and a prolonged glucocorticoid reaction to an acute stressor in piglets, as compared to an enriched environment [216]. Similarly, pregnant sows tethered in stalls separated by bars have higher corticosteroid concentrations than not tethered sows, whether in groups or in stalls [217].

A recent study on effects of chronic intermittent noise stress (daily or three times a week 2 h, 90 dB) in post-weaning male Landrace pigs revealed complex results. The daily stressed animals displayed more rapid dynamics of cortisol increase after an ACTH challenge [70]. The immediate ACTH and cortisol increase to the daily repeated noise stressor was higher in the first week of stress, but returned to control levels thereafter. By contrast, animals stressed only three times a week displayed increased ACTH levels to the stressor and steadily increasing cortisol concentrations, while basal levels remained unchanged, compared to controls in both chronic stress regimens [71]. Further, the daily stressed animals had an increased size of the adrenal cortex but a reduced cell number on day 29 at the end of the experiment.

Taken together, chronic stress in pigs appears to be quite ineffective with respect to basal ACTH and cortisol levels. It may change, however, the responses to various additional acute stressors or to challenges and morphological features of the adrenals.

5.2.4. Implications for the assessment of welfare in pigs

The HPA axis in pigs is exquisitely sensitive to environmental stimuli, both acute and chronic. It is therefore a precious index to monitor the psychobiological status of the animal. But how is HPA activity, and cortisol particularly, correlated to emotional stress? Short stress and the resulting transient response of the HPA axis during anticipation and successful coping may not really affect welfare but rather may be a suitable way to overcome boredom [218]. Also, in chronically sustained or repeated stress, the HPA activity is not only a function of the stressor but may depend also on breed-specific genetic traits, dominance status and individual experience. Individuality in the stress response of pigs must not be underestimated and becomes clearly evident when the important role of the highly modifiable neuronal networks in the amygdala and hippocampus are considered, which are crucially involved in both the mediation of psychological stress and the control of the HPA axis [13].

5.3. Farmed fur animals: mink and foxes

A range of different mammals is farmed for production of fur (e.g. mink, fox, sable, raccoon dog, chinchilla, and rabbit). In this review we focus on mink and foxes, being the economically most important species in fur production, with approximately 38.7 million mink and 3–4 million fox pelts produced annually world-wide [219]. Farmed mink are descendants of the American mink, *Mustela vison*. The two species of farmed foxes are the silver fox, bred from *Vulpes vulpes*, and the blue fox, which is bred from the arctic fox *Alopex lagopus*. Several features in farmed fur animals contrast with the other species of farm animals. For instance, mink and foxes are opportunistic carnivores with one fixed yearly cycle of reproduction and a different social structure compared to most other farmed mammals. As discussed below some of these features may affect the measures of HPA axis function.

5.3.1. Specific features of HPA axis activity in farmed fur animals

The majority of blood cortisol reports in mink are on females, with the mean concentration ranging from 25 to 95 nmol/L in plasma. These values cover a range of sampling procedures (nail clipping [220,221], vein puncture [147] and catheterization [222]) across seasons (February–December) in different breeds. Nearly all reports consider rightfully the duration from capture to sampling as important and blood samples taken within 2–2.5 min are generally regarded to reflect basal levels. Moreover, it is important to consider the order of testing/housing, since it is well-known that the capture and sampling of one animal may affect animals housed nearby, leading to increased cortisol concentrations. Other methods, such as non-invasive urine sampling, and recently also faeces sampling for mink have been used to evaluate the HPA axis function, but a more thorough validation of these techniques is still awaited [144].

The ACTH induced response, with peak cortisol concentrations of 330–375 nmol/L 30–45 min after injection [223], is comparable to the profile of response towards acute stressors such as capture (200–250 nmol/L; peak 15 min) [147], and immobilisation for 30 min in a trap (314–432 nmol/L) [220]. In blue fox, mean basal concentrations of cortisol range from 49–83 nmol/l in serum with a mean response of 340–389 nmol/L peaking 2–4 h after ACTH injection (October–December) [224,225]. In silver foxes, the basal concentrations of cortisol appear more variable (27–221 nmol/L in plasma [226–228], 86–136 nmol/L in serum [224]), with peak levels of 393–728 nmol/l in serum 2 h after ACTH injection [224,229]. Several studies report responses to acute stressors (e.g. an open field test) in silver foxes, with mean peak response values ranging from 190–326 nmol/L in plasma after 10–30 min [226–228]) and 154–173 nmol/L in serum [224], all sampled in the period September–November.

HPA axis indicators other than blood cortisol have been used to evaluate effects related to welfare. These include weight/size/morphology of adrenals (mink [230], blue fox [224,225,229,231–235], silver fox [224,236–239]), weight of pituitary (blue fox [240]), ACTH in serum or plasma (mink [147], blue fox [231,233,234]), cortisol binding protein/transcortin (mink [241], silver fox [242]), urine cortisol (mink [77,242–246], blue fox [224,235,247], silver fox [224,238]), and cortisol/corticosteroid metabolites in faeces (mink [248,249]).

5.3.2. Seasonality and sex differences

Farmed mink and foxes are seasonal breeders, with the photoperiod as the main regulator of reproduction, fur moulting and hormonal rhythms. In mink, seasonality in the secretion of glucocorticoids has been observed in both females [250] and males [251]. These studies found peak values of serum cortisol

early in the year (January to primo February, before the mating time) and again ultimo April/primo May (around the birth period), regardless of sex. Furthermore, Weiss et al. [250] found a large peak in females in September, whereas males had a low value at that particular time of year, but two additional peaks in June and November [251]. These early data are based on relatively few animals (6–18 per sex); moreover it may be difficult to compare values across studies, and thus further investigations of the variation in the HPA function in both sexes during the yearly cycle are desirable. Hansen and Damgaard also noted a pronounced seasonal variation in mink plasma cortisol between sexes [220], and reported higher basal concentrations in female than in male mink during September–November [252]. Seasonal changes in activity of the pituitary–adrenal system are described for silver foxes as well [237]. Besides the influence of natural photoperiods on the hormonal levels, the normal farm routine with changing feeding strategies during the reproductive cycle may contribute to the variation. Mink, for instance, are typically fed in amounts close to *ad libitum* in the growing season (autumn), followed by a period of restrictive feeding of breeding animals during winter, before excessive feeding of females until ovulation. This feeding routine may contribute to the seasonal fluctuations in HPA function measures, since food restriction increases the overall level of activity and stereotypies, which may affect the baseline cortisol levels [253]. The increase of HPA axis activity during food restriction may also be directly related to the effect of cortisol on metabolism. When deprived of food for 24 h the minks' urinary cortisol may increase by 50% above baseline [77].

5.3.3. Factors modulating HPA axis response

Records of mink and fox farming only go back to 1866 and 1895, respectively [219]. It has therefore been debated whether these animals are domesticated to the same extent as are other farm animals (e.g. [254]). However, the tendency to maintain some flight distance and physiological stress responses when exposed to humans persists even in animal populations domesticated for a long time (e.g. [255]). The majority (ca. 75%) of mink on commercial farms react with curiosity and exploration, rather than with fear, when confronted with a human [256]. Kruska [257] estimated that approximately 200 generations of domestication are necessary to change the brain size of a wild mink strain into that of a highly domesticated form, such as the change reported for cats and dogs. The hippocampus is reduced in modern farm mink; it is unknown whether the decrease in brain size supports functional or behavioral changes along with the ongoing domestication process and equally unknown are the precise consequences for the HPA function. Selection for tame behavior has influenced the HPA function in silver foxes as well as in mink. In these species, long-term selection for tame behavior towards humans has led to genetic lines with a lower response of the HPA-axis (e.g. secretion of cortisol) to human exposure and sampling [147,237,258–260]. Thus a certain genetic variation exists within the species in their sensitivity towards humans and handling. Further, many different color types and variants of mink and foxes exist on farms [261]. At least anecdotal evidence suggests that the black types are more reactive than the brown color types of mink. Only a few studies have investigated differences between color types; one study found that the color breed 'Pastel' had a higher mean plasma cortisol level, was more active, and showed more stereotypic behavior than the color breed 'Pearl' [221]. Thus, the diversity of color types on farms makes it further difficult to compare levels of HPA function. Care should therefore be taken when comparing animals of different genotypes and across studies in an attempt to evaluate welfare consequences of e.g. different management or housing systems.

5.3.4. Implications for the assessment of welfare in farmed fur animals

Since both behavioral and physiological measurements may be helpful in assessing welfare, discordance between (and within) the behavioral and physiological parameters should make one careful when drawing final conclusions [262]. Furthermore, when using the HPA function to evaluate the welfare of fur animals one should particularly consider the influence of their fixed annual cycle, whether induced by management routines or natural conditions.

Handling and capture of the individual may be stressful for mink and foxes, e.g. eliciting secretion of ACTH and cortisol. Additionally, the capture of one animal in a cage may disturb other not yet sampled animals in the same house [263]. Sampling procedures should therefore be considered carefully when sampling. Different methods of capturing the two species of foxes have been compared; silver foxes became more stressed when caught with a pair of tongs instead of by hand only, whereas the opposite was true for blue foxes. However, silver foxes are a greater risk for the caretaker when caught by hand, while the two procedures are equally safe when handling blue foxes [264]. Non-invasive methods, such as passive collection of urine for determination of cortisol to creatinine ratio and measuring metabolites in faeces, may be particularly useful when investigating fearful or hard-to-handle animals, in large-scale studies or when a time-integrated response is needed. The diurnal variation and effect of handling become more evident when blood sampling is used. Repeated blood sampling by insertion of permanent catheters is possible [265,266], but this method has not been commonly used; perhaps also since relatively large group sizes (e.g. 20–100 individual per treatment group) often are investigated. The future value of HPA function as a tool to evaluating welfare would benefit from the development of better sampling methods, combined with a better understanding of the influence of biological factors, as well as focus on the relationship between HPA function and long-term stress.

5.4. HPA axis function in birds

5.4.1. Specific features of HPA axis function in birds

Although the knowledge of HPA axis functioning is far less important in birds than in mammals, it appears that its general organization and its main biological roles are comparable; however, some known specific features are worth to be mentioned.

Frankel [267] first published a review of the neurohormonal regulation of the avian adrenal gland. It seems likely that the

pituitary release of ACTH is controlled in a manner similar to that found in mammals. Results from earlier studies suggested that the avian hypothalamus contains a CRF-like molecule controlling ACTH release [268,269] The nucleotide sequence and the deduced amino acid composition of CRF have been recently published in chicken [270] and quail [271]. Predicted amino acid sequence of the mature peptide reveals a complete homology between bird, human, pig and rat peptide, while it differs from bovine and ovine. Consequently, it further validates the physiological meaning of earlier functional and neuroanatomical studies [271–279]. Two classes of CRF receptors have been described in mammals but we do not know if they are present in birds. The chicken genome contains a sequence presenting more than 87% homology with mammal CRF-1 receptor genes. The protein product of this sequence is structurally similar to that of mammalian CRF-1 receptors, while exhibiting ligand-binding properties similar to those of the CRF-2 receptors [280]. Furthermore, administration of a mammalian CRF-1 receptor antagonist potentiates the increase in corticosterone induced by a physical constraint in quail [281]. Therefore, the existence of both types of receptor in birds is still putative and their biological activities may differ from those described in mammals. Furthermore, other peptides, such as arginine vasotocin (AVT) or mesotocin, were also reported to be active in the release of ACTH [272,277,282–284].

ACTH is secreted from the pituitary corticotrope cells upon excision by specific endoproteases from a multifunctional precursor protein, the proopiomelanocortin or POMC, which primary structure has been established in chickens [285]. In birds, partial ACTH amino acid sequences were reported for turkey [286], chickens [287,288], ostrich [289,290] and quail [277]. Nucleotide sequence and full amino acid composition have been determined in chickens [285,291]. ACTH is a 39 amino acid peptide with a common 1–24 N-terminal sequence in both mammals and birds, which is sufficient to elicit its biological activity.

The secretion of ACTH into the general circulation results in the biosynthesis and release of corticosteroids within a few minutes in the adrenal gland. Avian adrenal glands are paired organs located anterior to the kidneys and posterior to the lungs. In sexually mature birds, the left adrenal gland is often embedded within the ovarian stalk [292]. As in all vertebrates, adrenals consist of two histologically and functionally distinct cell types, i.e. steroid-producing cells and catecholamine-producing cells. However, the avian adrenal gland does not have a distinctly arranged cortex and medulla although a functional zonation of the steroidogenic tissues has been described for the duck adrenal gland, suggesting some analogy to that of the mammalian adrenal cortex in this species [293]. Corticosterone is the main corticosteroid hormone [294,295].

Despite a direct relationship between ACTH and corticosterone release, adrenal steroidogenesis is also under direct and indirect negative feedback effects and may be partially under extra-hypophyseal controls. Indeed, the injection of dexamethasone, a potent glucocorticoid analogue, has been shown to suppress corticosterone secretion in hens [296,297]. On the other hand, hypophysectomy does not result in a decrease in plasma levels of corticosterone in turkey [298,299] or only in a partial one in quails [269], and is not associated with adrenal insufficiency in cockerels [300,301]. These results clearly differ from those observed in mammals. A circadian rhythm of plasma concentrations of corticosterone has been documented for various species of domestic birds such as the quail [302], turkey [303], pigeon [304], domestic duck [305] and chicken hen [295,306]. Recent investigations did not provide any indication of the existence of a circadian rhythm in quail [307]. Moreover, results from these different studies are even somehow controversial as, for example in immature chickens, the acrophase is reported to occur during the middle of the light period [308], toward the end of the light period [309], at the onset of darkness [295] or a few hours after darkness [310]. These discrepancies may result from the influence of various environmental factors such as season and day length [302]. In addition, changes in this rhythm appear prior to the onset of sexual maturity, i.e. between 15 to 17 weeks of age in the duck [305] and the domestic hen [311]. In hens, time of ovulation and oviposition [306,312] has a further impact on this rhythm. Long photoperiods have also been shown to be associated with higher basal corticosterone levels and higher corticosterone adrenal response capacity to 1–24 ACTH [307]. The effect of ageing and acquisition of sexual maturity are somehow controversial and often confounded with the effect of photoperiod length. It has been recently shown in quail that an increase in basal B levels is associated with the acquisition of sexual maturity while it did not affect HPA axis responsiveness or adrenal corticosterone response capacity [307]. On the other hand, age affected HPA axis responsiveness and adrenal corticosterone response capacity in one strain but not in another.

5.4.2. Reactivity of HPA axis in birds

Although it varies depending on species, genotype and age, birds have a relatively low sensitivity to social stress in term of adrenal responses [277]. On the other hand, various physical stressors will generally elicit adrenal responses, with a large variability between species in terms of responses to comparable physical stressors and to ACTH. Thus maximal corticosterone levels in response to a pharmacological challenge with 1–24 ACTH (Immediate Synacthen, 5–10 μg/kg B.W.) varies from 20 to 350 ng/mL plasma. It is of interest to note that in birds, the response has a very short delay, with a peak between 2 to 20 min depending upon the intensity of the physical stress or pharmacological challenge (using CRF, AVT or ACTH). Larger doses of ACTH only marginally increase peak levels but increase the duration of the corticosterone response [277]. Different time courses have been reported in broilers in response to two different forms of 1–24 ACTH (immediate and delayed Synacthen) [313].

Chronic stress or repeated acute stressors induce long-term changes in the regulation of the HPA axis resulting first in a hypersensitivity, then to a progressive decrease in adrenal capacity. Thus, chronic stress can be investigated in using ACTH challenges with low and high dose [314] to address the HPA sensitivity and maximal reactivity, respectively [315–318].

5.4.3. Implications for the assessment of welfare in poultry

Activation of the HPA axis of birds in response to an acute stress has been demonstrated and is reflected by an increased

concentration of corticosterone while the effect of chronic stress, which can also be assessed by corticosterone measurement in plasma following challenge of the adrenal glands, is variable. It is also possible to measure corticosterone in the eggs or its metabolites in faeces, but the interest of these approaches is limited since they represent only cumulative changes that may result from other regulatory mechanisms. Consequently, plasma corticosterone measurement, which implies blood collection, will be the more appropriate method to assess stress in poultry. However, several studies have shown that blood sampling procedures can greatly influence corticosterone levels depending upon the species [277,319–321]. As a consequence, methodological and species differences can mask differences in reactivity and functionality of the HPA axis. Venipuncture of the wing vein is the most common technique used to sample blood in birds and have been shown to be satisfactory if samples can be collected in less than 2 min. However, blood collection by occipital sinus puncture or following decapitation has been shown to be more appropriate in waterfowls [321] and quail [277], respectively. Last but not least, as reported previously, various environmental conditions are known to up- or down-regulate corticosterone concentrations with differing effects depending upon the genotype [277]. Extreme caution should therefore be the rule before stating firm conclusions in term of stress and thus welfare assessment, after measurement of circulating corticosterone levels.

5.5. Hypothalamic–pituitary–interrenal (HPI) axis function in fish

In commercial fish industry, acute stress can occur when fish are counted, graded or harvested. Chronic stress, more potent to jeopardize animal welfare, can occur when fish are reared at too high densities, in poor quality water (low O_2, high ammonia, high suspended solids, etc.), or when fish are confined, transported, sick or exposed to social interactions between individuals.

5.5.1. Specific features of HPI axis function in fish

α-MSH (melanophore-stimulating hormone) and ACTH are the two main candidates for the control of cortisol secretion, while β-endorphin is probably a potentiating factor [322]. In salmonids, a rise of ACTH (and cortisol) is always observed in fish subjected to a stressor whatever the stressor type [323,324]. In tilapia (*Oreochromis mossambicus*), the handling-induced rise in cortisol is not associated with a rise of ACTH but when tilapia are submitted to confinement, cortisol rise is associated with a rise of ACTH [325]. This suggests that, in this species, rapid elevation of plasma cortisol levels does not rely on circulating ACTH.

The corticosteroid hormone (cortisol in teleost fish) is produced by the interrenal cells. These cells do not form a compact gland but rather are distributed around the walls of the posterior cardinal veins and its branches at the kidney head.

5.5.2. HPI axis responses to stress

Short-term stressors (handling, chase, etc.) typically elicit marked elevation of plasma cortisol concentrations. For example, in salmonid fish and white sucker (*Castostomus commersoni*), acute stress induces a rapid increase in plasma cortisol concentrations followed by a rapid decrease [326–328].

A more prolonged stress, such as 24 h confinement in sea bass (70 kg m^{-3}) resulted in sustained high plasma cortisol levels associated with an increase in the interrenal cells activity (enlarged nuclear diameters measured after 1 and 4 h of confinement) [329]. Similarly, in subordinate rainbow trout, plasma cortisol level remains high for 3 days after mixing compared to dominant animals [330].

Longer confinement periods (several weeks in salmonids) induced a rise in plasma cortisol at the beginning of the confinement and a decrease back to initial values after several days [331,332]. Furthermore, no significant differences in interrenal histology were detected after several days and therefore, cortisol concentrations were no longer suitable to evaluate this stress situation [331,333]. However, some papers describe a slightly higher level of cortisol in confined fish compared to the controls after several weeks (sea bream) [334].

The same discrepancies in the long-term response of cortisol were observed with another type of stressor, social interactions. In arctic charr (*Salvelinus aipinus*) after 6 days of social interactions, there was no more difference between dominant and subordinate fish [335]. But, in subordinated eels, plasma cortisol remained high and interrenal cells were enlarged after 10 days of social interactions [336]. High levels of cortisol have also been described in rainbow trout after 2 weeks of social interactions [337].

These results show that a single measurement of plasma cortisol concentration is not a reliable parameter to evaluate fish status in relation to chronic stress. Several studies have been carried out to evaluate if the application of an acute stressor may help to reveal a chronic stress situation. Fish subjected to chronic stress (confinement), and nevertheless with basal levels of circulating cortisol were submitted to an acute stressor. In rainbow trout [338] (Geslin and Auperin, unpublished data), in coho salmon raised in seawater [342], in brook charr [339] and in carp [340], this type of treatment induced a higher cortisol increase in confined fish than in control fish, showing that the HPI axis has been sensitized to the effect of the acute stress by exposure to the chronic stress situation. However, there are exceptions and, in tilapia [341] and coho salmon raised in freshwater [342], plasma levels of cortisol after acute stress were lower in confined fish than in control fish.

In fish, few reports are available on the use of dexamethasone to analyze the functioning of the HPI axis and no time course study has been published. In brown trout (*Salmo trutta*) the ability of dexamethasone to block the HPI axis has been demonstrated [343]. This blockade is associated with a dose-dependent decrease of cortisol concentrations and a decrease in ACTH concentration in control and stressed fish [343,344] probably because of an interaction between dexamethasone and corticosteroid receptors at the level of the brain, hypothalamus and pituitary [345,346].

In fish, elevation of plasma cortisol levels is attributed, at least in part, to ACTH stimulation of interrenal cells. Therefore, ACTH injection may be used as a tool to reveal a sensitized state of the interrenal gland due to a chronic stress situation [325]. We

demonstrated (Geslin and Auperin, unpublished data) that rainbow trout confined for 21 days (with low cortisol levels) or handled daily during 19 days are more sensitive to ACTH than control fish. However, interrenal tissue from chronically stressed brook charr and rainbow trout were less sensitive to ACTH stimulation [323,339]. In carp, interrenal response to ACTH was unaffected by 7 days of confinement although the quantity of ACTH receptor mRNA was down-regulated after such treatment [347]. These controversial results deserve more comprehensive investigations.

5.5.3. Implications for the assessment of welfare in fish

All these data show that the use of HPI as a tool to evaluate animal welfare is possible but needs a proper validation for each species and each type of stressor. Despite these limitations, the functionality of HPI axis has been used to select fish for their responsiveness to a standardized confinement stressor. Thus, lines of rainbow trout with divergent low and high cortisol response to acute confinement stress have been produced by selective breeding, and functional tests show that the interrenal gland is more reactive to ACTH in the high responsive line [58,348]. In striped bass, high responsive fish had significantly greater plasma cortisol levels than low responsive fish after bovine CRH injection [349]. These different studies illustrate the possibility of using these criteria as tools for genetic selection for stress resistance in fish.

6. Conclusion

Basal HPA axis functioning and its response to various environmental factors, both acute and chronic, has been extensively studied in the farm animal species reviewed in this paper. Although blood cortisol (or corticosterone) levels are the golden standard to study the HPA axis, many complementary investigation tools have been developed to overcome the various problems related to blood sampling such as the stress introduced by animal handling and vessel puncture or the rapid oscillations of circulating levels. The assay of hormone levels in saliva, urine, milk, faeces offers several interesting alternative approaches. A special mention must also be given to dynamic testing of HPA axis functioning with stimulation (CRH, ACTH) or inhibition (dexamethasone) protocols that may reveal changes otherwise undetectable by mere plasma levels, especially with chronic, low levels stimuli. All these tools have revealed a wide range of variation of HPA axis activity, as a result of internal factors (such as pulsatility of cortisol secretion, endogenous nycthemeral rhythm, genetic and developmental influences) as well as external factors (such as food intake, temperature and hygrometry, lighting regimen). These changes are mostly related to the pivotal role of corticosteroid hormones in metabolisms and several major physiological functions such as the cardiovascular system, immune functions and brain activity, just to cite a few. It is therefore of critical importance to take all this information into consideration when it comes to the interpretation of experimental data in the context of the evaluation of animal welfare. In acute situations, a large hormonal response is usually measured, reflecting the exquisite sensitivity of the HPA axis to a large range of stimuli that are not necessarily harmful to the animal. Complementary measures are necessary to evaluate the intensity of the response. For instance, the increase of ACTH levels in plasma is more gradual and proportional to the intensity of the stimulus. In many cases, behavioral observations have been used together with biological measures. With low-level, long-lasting stimulations, the situation is more difficult, mainly because the straightforward measure of HPA axis activity with plasma cortisol levels is much less informative as with acute stimuli and alternative experimental approaches including dynamic testing are still scarce in the literature. In that case, a multidimensional approach of animal welfare is necessary, integrating data from behavioural observations, production traits, and disease incidence. Although the study of HPA axis activity will probably remain the standard approach to the evaluation of stress and welfare in farm animals, it is necessary to keep in mind the limitations to the information this neuroendocrine system can deliver. Indeed, these limitations will be largely reduced by the use of suitable experimental approaches, as described in this paper, by the knowledge of physiological roles of corticosteroid hormones and the mastering of confusion factors in the context of the situation under study and finally, by relying upon a multidisciplinary approach of animal welfare.

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