Review article

Limits to Using HPA Axis Activity as an Indication of Animal Welfare

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Summary

HPA axis activity is often measured by corticosteroid release as a means to evaluate stress and well-being in animals. While the analysis of corticosteroid levels can provide useful information in some circumstances, a variety of methodological and technical problems make them difficult to accurately interpret. Furthermore, there is considerable evidence that is inconsistent with the widespread notion that high levels of stress result in a large amount of corticosteroid release, and in some cases the converse is true. This review highlights the strengths and weaknesses of the techniques used to measure corticosteroids, describes a number of studies that failed to find a positive correlation between stress and corticosteroid levels, and delineates ancillary behavioral and cognitive tests that provide insight into an animal's well-being. We conclude by emphasizing that the most holistic account of animal welfare is provided by utilizing a combination of physiological and psychological methods.

Keywords: cortisol, corticosteroids, stress, HPA axis, animal welfare

1 Introduction

Researchers in several disciplines have been interested in studying stress in animal models, ranging from biomedical research to ethology and animal conservation. Stress is a somewhat abstract concept that has been a source of much confusion and misinterpretation. Stress can be broadly defined as any stimulus that disrupts homeostasis, with the stress response referring to physiological and behavioral reactions to such a stimulus (Selye, 1950). The most salient physiological responses to stress include activation of the sympathetic nervous system (SNS) as well as the hypothalamic-pituitary-adrenal (HPA) axis. Accurate measurement of the catecholamines (i.e., epinephrine and norepinephrine) released as a result of SNS activation is difficult because these substances are only available for transient periods of time (Reeder and Kramer, 2005). Thus, evaluation of the HPA axis, most commonly by measurement of its end-product glucocorticoids, has traditionally been the primary means to make inferences about the stress response in animals.

Briefly, HPA axis activation results in corticotrophin releasing factor (CRF) being released from the hypothalamus, which in turn leads to ACTH being released from the anterior pituitary, and then finally glucocorticoids being released by the adrenal cortex (de Kloet et al., 2005, 2008; Johnson and Greenwood-Van Meerveld, 2012; Selye, 1950). The predominant glucocorticoid released from the adrenals varies from species to species, with birds, reptiles, amphibians and many rodents releasing corticosterone and humans and other mammals releasing cortisol (Romero, 2004). Of these three classes of substances, it is the adrenally released glucocorticoids that are most commonly measured. This is because CRF is immediately and transiently released, which makes it difficult to measure accurately. ACTH can be measured, but since it is a small and labile protein it degrades quickly and must be collected in tubes with specialized anticoagulant and immediately frozen (Reeder and Kramer, 2005).

There are many types of samples from which to analyze glucocorticoids, but each sample type has its own problems when trying to interpret the results. Thus, even though it is quite common, measuring glucocorticoids may not be an ideal way to assess stress. As we will outline in this review, the use of glucocorticoid hormones as an indicator of stress in animal models is problematic because HPA axis activation may not even consistently reflect what would classically be termed "stress." In addition to our discussion, good summaries of techniques and pitfalls of assaying the HPA axis can also be found in Baker et al., 2013; Cook, 2012; Johnstone et al., 2012; Novak et al., 2013; Reeder and Kramer, 2005; Sheriff et al., 2011.

Received June 16, 2014;
accepted November 18, 2014;
Epub November 24, 2014;
http://dx.doi.org/10.14573/altex.1406161

2 Difficulties assaying the HPA axis in animal models

Traditionally, glucocorticoids were measured from plasma or serum samples. However, the blood sampling procedure itself can cause enough stress to confound the results (Vachon and Moreau, 2001; Gärtner et al., 1980). There are several ways to try to circumvent this issue, which include using an indwelling catheter or drugs for sedation. Indwelling catheters can limit the number and type of animals being used and can themselves be a source of stress. Anesthetics used for sedation likewise may directly alter HPA axis activity in unpredictable ways (Bentson et al., 2003). The most promising methods of blood collection are those involving the use of either minimal restraint or positive reinforcement. For instance, allowing rats to explore a towel while collecting blood samples from the tail yielded corticosterone values that are generally considered to represent basal activity (Fluttert et al., 2000). Moreover, it is likely that training an animal to present its limb for venipuncture minimizes the stress that it experiences, although this technique is only reasonable for certain species (Coleman et al., 2008).

In addition, a single sample does not accurately reflect HPA activity within an individual – instead it only provides a momentary glimpse, elucidating the necessity for repeated blood draws. But, in addition to potentially causing more stress, multiple blood draws will limit the number of samples taken from each animal, depending on the ratio of their blood volume to the volume needed for the analysis.

Also, although several studies have reported maximal or peak concentrations of cortisol in their studies, some research suggests that the duration of release or total amount, not the peak concentrations of cortisol, are most indicative of HPA axis activity. For instance, although subordinate baboons reportedly had lower cortisol spikes than dominants in response to a stressor, they had higher basal cortisol levels, which likely resulted in a down regulation of receptors and attenuated response to glucocorticoid release (Romero, 2004; Sapolsky, 1990).

The choice of whether to analyze free or total cortisol is another source of error. Free, unbound cortisol is thought to be the biologically active form. Stress appears to also alter corticosteroid-binding globulin (CBG) levels, which are transport proteins that bind free cortisol and thus prevent it from being biologically active. This can lead to problems if only total cortisol is measured. For example, some studies have reported that a change in the capacity or amount of CBG, but not total cortisol levels per se, have resulted from stressful conditions (Alexander and Irvine, 1998).

Interestingly, CBG levels can either increase or decrease in response to acute or chronic stress (Alexander and Irvine, 1998; Boonstra, 2005; Davenport et al., 2008; Delehanty and Boonstra, 2009; Qian et al., 2011). Thus, the interpretation of changes in cortisol levels in response to stress would benefit from including both free and total forms of the hormone as well as reporting changes in CBG concentrations.

Another issue with making inferences using blood glucocorticoid levels is that they naturally wax and wane throughout the day and night (Menargues et al., 2012). This circadian activity, which has generally reflected higher levels in the morning and lower levels in the evening in diurnal, terrestrial mammals and the opposite pattern in nocturnal mammals, must be controlled for, or else it can result in a significant source of variability.

Due primarily to the issues in interpreting serum or plasma glucocorticoid levels highlighted above, a variety of methods that are either less invasive and/or represent a mean hormonal level over some period of time have increased in popularity.

One example of such a method is to measure cortisol in the saliva instead of the blood. Salivary cortisol increases in a proportional rate to that in the blood across a number of different species (Cook, 2012). It also has the advantage of being a bit easier to collect than blood. Many researchers have developed collection devices that can allow animals to chew on them and subsequently voluntarily provide saliva samples (Higham et al., 2010). Nevertheless, there are many limitations to analyzing salivary cortisol. It is subject to many of the same problems that arise from blood samples. Salivary cortisol is released in different amounts as a function of the circadian rhythm. Therefore the timing of collection must be controlled for, and this can be especially problematic if the study relies on voluntary samples being donated. Salivary cortisol levels rise very quickly in response to a stressor, and thus only represent a single point on a continuum like blood cortisol. This requires the collection of multiple samples. Also, concentrations of cortisol are contingent upon salivary excretion rate, and at least one study reported an insufficient volume of saliva being collected from a portion of their test population (Novak et al., 2013; Granger et al., 2007). Finally, despite the general consensus that collecting saliva is a relatively non-invasive procedure, collecting it still requires some restraint and likely some stress in a majority of animals.

The analysis of glucocorticoids and their metabolites in feces is a method that has increased in popularity more than any other method in the recent past (Cook, 2012). It ostensibly has advantages over methods such as blood or saliva based techniques in that its collection is entirely noninvasive, does not cause an appreciable amount of stress, and is not affected by pulsatile and circadian rhythmic secretion, though even this final point may not be valid in all species (Sousa and Ziegler, 1998). Accurate interpretation of fecal assays is confounded by several factors as well. It is important to ensure that the fecal sample is not mixed with urine, as there is a much higher concentration of glucocorticoids in urine than in feces (Mostl and Palme, 2002). Diet type or availability can affect fecal glucocorticoid levels or GI transit time, respectively. A diet higher in fiber, for instance, could increase transit time and thus inflate fecal concentrations of glucocorticoids (von der Ohe et al., 2004). Different species also have various GI transit times and excrete different metabolites in their feces. Thus, it is recommended to use high-pressure liquid chromatography in order to identify and isolate the appropriate compounds from the feces (Keay et al., 2006). Also important is that all glucocorticoids in feces have been modified to some extent. Some have been metabolized and reabsorbed from the liver, and others by intestinal

bacteria (Sheriff et al., 2011). Thus, differential metabolic and reabsorption rates have the potential to affect levels.

Cortisol and its metabolites can also be examined from the urine. This technique has the advantage of being less invasive than blood or saliva sampling and because a portion remains conjugated, only represents free cortisol. The cortisol levels in urine reflect those produced anywhere from a few hours to an entire day prior to collection. Thus, even if the collection technique was associated with a transient period of stress, this is less likely to confound the results than for blood or saliva. Like with feces, it is important to prevent contamination of urine with other excreta. This can be achieved with placement in specialized metabolic cages or via training animals to urinate on command (Tiefenbacher et al., 2004; Setchell et al., 1977). Also, if an entire urine sample is not collected, or if some is spilled, the concentrations of cortisol may not be accurate (Sheriff et al., 2011). Cortisol levels must be corrected for urine output as well, as the concentrations will vary with dilution. This is often circumvented by using a urinary cortisol:creatinine ratio, which allows comparison of the amount of cortisol after being controlled for the amount of creatinine in the urine (Cook, 2012). Creatinine is neither secreted nor reabsorbed in most species, so it is a reasonable indication of urine output. Unfortunately, some strains of mice have been demonstrated to actively secrete creatinine in their renal tubules, suggesting that the use of creatinine to correct for urinary excretion rate in these animals may not be valid (Eisner et al., 2010). Moreover, urinary cortisol remains affected by circadian fluctuations and pulsatile release as discussed above.

Extracting cortisol deposited in hair has the advantage of conveying cortisol levels within an organism over several months. Thus, this has the advantage of making it possible to track prolonged and/or chronic changes in HPA activity, which is of primary concern to those wishing to examine health or well-being. Indeed, several studies report a correlation between environmental or, in some cases, perceived stress and changes in hair cortisol levels, even when they failed to reveal changes in cortisol from other samples such as feces (Dettenborn et al., 2010; Malcolm et al., 2013; Stalder et al., 2014). In species where rate of hair growth is not known, hair can be shaved in order to estimate cortisol deposition over time (Davenport et al., 2008). Furthermore, cortisol levels derived from hair are not subject to variability due to the collection procedure or circadian rhythm as they are with the majority of the other methods discussed (Stalder and Kirschbaum, 2012). Despite being a promising new technique, there are several important drawbacks when interpreting the results of cortisol levels collected from hair. First, it has been revealed that human hair follicles produce cortisol in response to CRH administration *in vitro* (Ito et al., 2005). These peripheral HPA axes likely respond to external stimuli such as pain or temperature extremes independently of the central HPA system. Interestingly, even hair follicles that are in close proximity but have been subjected to different external stimuli will produce different amounts of cortisol (Sharpley et al., 2010). Additionally, the location and even color of hair follicles may alter the rate of cortisol deposition regardless of different environmental conditions (Bennett and Hayssen, 2010; Macbeth et al., 2010; Sauve et al., 2007). However, it should be noted that hair cortisol levels have also been reported to demonstrate less within-subject variability, even when sampled from different body locations, than cortisol from either feces or saliva (Bryan et al., 2013; Carlitz et al., 2014). Moreover, we have not ascertained from where the cortisol found in hair is derived. For instance, the cortisol found in hair could potentially arise from blood cortisol, cortisol produced by the hair follicle, or even via local glandular secretions that then infiltrate the follicle (Cook, 2012). Chemically altering hair with dye (a commonly used method of identifying animals) and washing hair, even with just water, can also alter cortisol levels (Novak et al., 2013).

To counteract some of the difficulties of assaying the HPA axis as outlined above, other less direct methods have also been utilized to measure its output in the context of stress. Blunting of HPA axis responsiveness to negative feedback or stimulation has been found with multiple human behavioral disorders including posttraumatic stress disorder (PTSD) (Yehuda et al., 1991, 1995; Yehuda, 2009), major depressive disorder (Marques et al., 2009) and generalized anxiety disorder (Stansbury and Gunnar, 1994). This is presumed to be an adaptive response to the high circulating glucocorticoids of chronic stress. However, the relationship between HPA function and stress per se is unclear, with examinations of stressful situations showing increased reactivity in some contexts and blunted response in others (Miller et al., 2007). Where this relationship has been examined in animal models, the results are similarly inconsistent. In nonhuman primates with high frequencies of self-injurious behavior (SIB), it has been demonstrated that suppression with a synthetic steroid and stimulation with ACTH are both blunted in comparison to those with low frequency SIB (Tiefenbacher et al., 2004). The adrenal glands taken from mice exposed to chronic stress likewise show a reduced response to stimulation with ACTH, but *in vivo* those same animals actually experience an increased release of corticosterone in response to an acute stressor (Uschold-Schmidt et al., 2012). As more work is done, HPA function may become a better proxy for measuring stress, but the inconsistency of results thus far makes interpretation difficult.

The known effects of corticosteroids on other biological systems have also been exploited in the attempt to measure stress in animals. The link between high circulating glucocorticoids and lymphocyte suppression has been a topic of intense study with respect to the consequences of stress, and this link has also been utilized as a proxy measure of stress. In a study of the effects of sedatives on stress parameters in macaques and baboons, for instance, counts of circulating lymphocytes were used as an indicator of acute stress, and suggested that animals experienced transient decreases following the injection procedure regardless of what was being injected (Bentson et al., 2003). In contrast, a recent study failed to find changes in the neutrophil-lymphocyte ratio in rats after acute stress (Swan and Hickman, 2014). Instead, they reported an increase in corticosterone levels after acute stress and found that following

chronic stressors, corticosterone levels were not increased but the neutrophil-lymphocyte ratio was increased. Although more work needs to be conducted in order to elucidate the extant literature, neutrophil-lymphocyte ratios do seem to have advantageous qualities when compared to measuring glucocorticoids alone, which includes the fact that less sophisticated equipment is needed for analysis and that the ratio does not vary as much as a function of time of day and sex. Moreover, the neutrophil-lymphocyte ratio may be of more use for indicating chronic stress, since it takes hours for elevations in glucocorticoids to result in alterations to white blood cell populations (Davis et al., 2008).

In our own work examining the effects of mouse enrichment on stress parameters, we used the ratio of immature to mature thymocytes as a measure of chronic HPA output, since these immature cells are known to be more sensitive to corticosteroid induced apoptosis (Webster et al., 2002). In addition to demonstrating that pre-weaning enrichment conditions determined the reaction of adult mice to environmental enrichment, our results also showed concordance between measures of urinary corticosterone and immature:mature thymocyte ratios with regard to which conditions were most stressful (Hutchinson et al., 2012). The effects of stress on the reproductive cycle also show potential as an indirect measure of HPA axis activation. Monkeys categorized by the ease with which their estrous cycle is disrupted by external stressors demonstrated differences in HPA axis sensitivity to those same stressors, though not higher baseline cortisol levels (Herod et al., 2011). Though the use of these and other systems to reflect stress holds some promise, the primary difficulty with these methods is that each of these systems is subject to a wide array of inputs, of which HPA axis activation is only one. Any other pathologic disruption, such as a transient infection in the case of the immune system, could thus be misinterpreted as reflecting corticosteroid release if the experimenter is not careful to consider all variables.

3 Problems relating glucocorticoid output to stress in animal models

Aside from the many variables discussed above that make it challenging to interpret such data is the problem that even if accurate, an increased amount of glucocorticoids does not categorically imply stress. HPA and SNS activation can be elicited by appetitive and affectively neutral stimuli as well as aversive stimuli. For instance, cortisol levels have been found to increase during sexual behavior, periods of increased exercise, while hunting prey, and when expecting either a punishment or reward (Dawkins, 2006). Although both alpha males and low ranking baboons have high levels of glucocorticoids, only low ranking males suffer from delayed wound healing (Archie et al., 2012). The authors mention that one possible explanation is that the nature of the stress is different, potentially being due to acute periods of physical activity in the alpha males but from chronic social stress in the low ranking animals. Human studies have even found it challenging to discern anger, excitement and fear when limited to physiological data (Oatley and Jenkins, 1996). Furthermore, different types of aversive stimuli have different effects on glucocorticoid levels (Dickerson and Kemeny, 2004). Thus, an increase in autonomic and HPA activity may simply reflect physiological arousal that does not necessarily denote undesirable experience. Interestingly though, glucocorticoid levels do not appear to rise in a manner that relates to the intensity of the stressful experience; not only is the HPA limited to assessing the arousal component of a negative experience, it also only provides limited information with respect to the magnitude of the component (Rushen, 1986).

In addition to there being problems interpreting elevated glucocorticoid levels insofar as how they relate to wellbeing, there are myriad reports of putatively stressful conditions failing to report elevations. Different experiments have yielded conflicting reports concerning the change in glucocorticoid levels in laying hens as a function of being housed in battery cages, with some showing an increase, some showing a decrease and some showing no changes (Rushen, 1991). Spotted salamanders living in deforested habitats had lower basal corticosterone levels than those living in unaltered conditions, which might suggest that the disrupted habitat was actually less stressful. However, those salamanders also had higher corticosterone response results (Homan et al., 2003). Interpreting this result is complicated because of the many factors that could explain the results, including facilitation (hypersensitivity to stressful stimuli due to acclimation to another stressful stimulus), acclimation (reduced HPA activity after repeated exposure to a stressful stimulus), or hypocortisolism from chronic stress (Romero, 2004). Similarly, a series of porcine and bovine studies failed to reveal changes in glucocorticoids in several adverse housing conditions (Rushen, 1991). However, they were able to detect changes in other indicators of adversity/stress, such as decreased weight gain, immune function and luteinizing hormone concentrations. Cats exposed to various unpredictable stressors for five days demonstrated an increase in gastrointestinal symptoms and a decreased N:L ratio, but did not show any differences in serum cortisol levels (Stella et al., 2013). In a study of capuchin stereotypy, elevated levels of fecal glucocorticoids were found to be correlated with levels of head twirls but not with pacing (Pomerantz et al., 2012). The authors concluded that one potential explanation is that, since head twirling but not pacing was also associated with an increased propensity to make pessimistic judgments, that pacing may not be indicative of animal wellbeing. However, this conclusion is surprising when it is considered that increased activity levels can elevate glucocorticoids and that pacing behavior is more likely to manifest under undesirable conditions.

Not all types of stressful stimuli are equally effective in eliciting a cortisol response either. A meta-analysis of 208 experiments examining the relationship between cortisol levels and laboratory-induced stressors in humans found that only stimuli that were perceived as uncontrollable or those that involved the potential for judgment/criticism by others reliably raised cortisol levels (Dickerson and Kemeny, 2004). Importantly,

subjects did not report that they experienced a higher level of distress when exposed to the aforementioned conditions than they did after exposure to other types of stimuli. Thus, because the conditions that reliably raised cortisol levels were not reported to be more distressful than other types of conditions by the subjects, differential distress levels do not explain the higher cortisol levels in these experiments. This is true even if the experimental conditions including both features that reliably raise cortisol (uncontrollability and social evaluation) were compared to all other conditions which lack these features. In addition, no relationship was found between reported distress level and cortisol levels. Several studies included in the meta-analysis confirmed that a negative affective state was successfully induced but failed to find a corresponding increase in cortisol levels.

Macaques raised exclusively with peers, a condition leading to high rates of abnormal behavior and anxiety, and those with self-injurious behavior showed decreased glucocorticoid levels with respect to controls (Novak et al., 2013). It was posited that perhaps the HPA axis is altered during development in the face of stressful conditions and that the typical responses and activity are consequently depressed. Consistent with this notion is research with rats that demonstrated an attenuated response to moderate stressors as adults if they were subjected to handling stress as neonates (Caldji et al., 2001). However, handling stress did not affect stress responses to intense stressors, and severe stressors such as prolonged maternal separation resulted in an enhanced glucocorticoid response to all future stressors.

A number of conditions in humans also result in a so-called hypocortisolism, including PTSD, fibromyalgia, chronic fatigue syndrome, and IBS. It has been posited that hypocortisolism may be an adaptive response to an earlier period of HPA hyperactivity that is characterized by increased sensitivity to stress, fatigue, and pain (Fries et al., 2005). This could potentially explain the low cortisol in the monkeys with SIB or those who were peer-reared, as both of those ailments likely result from deprivation early in life. However, perhaps even more confusing is that monkeys with SIB did not differ from controls in their response to a dexamethasone challenge test. In contrast, rodent models of hypocortisolism revealed that chronically stressed animals had heightened dexamethasone challenge tests and thus proposed that enhanced sensitivity to glucocorticoids was the mechanism resulting in lower cortisol levels. Although there are certainly more potential mechanisms to explain low cortisol levels, enhanced sensitivity to glucocorticoids has been a relatively robust finding in humans with hypocortisolism, regardless of etiology (Fries et al., 2005). To make it even more difficult to relate the monkey findings to those in human and rodent models, studies in different species of monkeys revealed either no differences or higher levels of glucocorticoids being associated with abnormal behavior and SIB (Novak et al., 2013).

4 Alternative and adjunctive methods of measuring stress in animals

Due to the problems outlined above, it is clear that analyzing glucocorticoid levels paints, at best, an incomplete picture of an animal's wellbeing. For example, environmental enrichment plans are legally required for nonhuman primates by the Animal Welfare Act in the US (AWA, 1990). They have been shown to increase species typical behavior and can help prevent or mitigate the frequency of abnormal behaviors under some circumstances (Novak et al., 2013; Lutz and Novak, 2005). Yet they have also been found to have little effect on HPA axis activation. In this case, a practice that is commonly accepted as beneficial to welfare and indeed can be observed to positively impact behavior would be dismissed as ineffective if evaluated solely by the modality of cortisol activation.

It is thus important to evaluate alternative approaches to assessing animal welfare, including the use of multiple modalities, in order to capture a fuller picture and help explain contradictory findings. Some of the more common ancillary methods used to investigate stress and well-being include observational studies, behavioral testing, and, more recently, cognitive tasks that indirectly examine stress and well-being by measuring the effects it might have on attention or judgment and decision making.

There are several indices of animal stress and well-being that can be collected by non-invasive, strictly observational techniques. Some of the more traditionally "clinical" measures include body condition scoring, appetite, urination and defecation, abnormal behavior such as self-mutilation or some forms of stereotypy, and cleanliness (Siegford, 2013). Work examining facial expressions has more recently provided insight into distress in animals ranging from apes to rodents in some situations, with ostensibly similar facial reactions apparent in many of these species (Berridge and Robinson, 2003; Defensor et al., 2012). Recent studies examining changes in facial anatomy, including eyelid aperture, whisker and ear position in mice, have been found to have good inter-observer reliability and provide insight into pain and distress in mice (Langford et al., 2010). Similarly, farm animals that have had their tails docked or been castrated without adequate pain management exhibit alterations in gait or body posture (Molony and Kent, 1997; Sprecher et al., 1997), and changes in ear position have been correlated with emotional state in sheep (Reefmann et al., 2009). While specific to hunger, the frequency and intensity of food vocalizations is another honest signal of impaired wellbeing in many species (Weary and Fraser, 1995).

In addition to passive observation of an animal's appearance and behavior, several behavioral tests have been designed to study stress and well-being. For example, rodent nesting behavior has recently been demonstrated to provide insight into pain and distress, with mice undergoing surgery without post-operative analgesia measurably slower to integrate new material into their nests than controls (Rock et al., 2014). Observing stimuli or situations that animals seek out versus those they avoid is another way to assess distressful conditions that

animals may experience. These preference test paradigms allow us to "ask" an animal what conditions they prefer. Experiments analyzing cortisol and beta endorphins failed to find a difference between sheep being sheared or those subjected to electro-immobilization, but when given the choice the sheep reliably elected shearing (Jephcott et al., 1987; Rushen and Congdon, 1986). When given the choice, studies have shown that rats will elect to gain access into a cage with a conspecific rather than an empty one (Patterson-Kane et al., 2002). Several studies have used preference testing to inquire about preferred food in relation to enrichment items (Dawkins, 2006; Siegford, 2013). One historical and astonishing study revealed that rhesus monkeys would refrain from accessing food if doing so meant that a conspecific would receive a shock (Masserman et al., 1964). Standardized behavioral tests such as open field tests or plus mazes have been validated as measures of anxiety and can offer insight into welfare in some, but not all, cases and species (Janczak et al., 2002). For instance, the elevated plus maze does not seem to be a reliable indication of anxiety in pigs (Janczak et al., 2002), and there are certainly myriad alternative interpretations that explain an animal's inclination to avoid open areas that do not involve anxiety or fear (see Ennaceur, 2014 for a review). Another interesting behavioral method involves recording startle responses from animals that have been subjected to different conditions, as this reflex is primed to respond faster or in a more intense manner after an experience leading to fear or anxiety (Lang et al., 2000). This technique has been used to study stress for quite some time but is also beginning to be used to assess animal welfare (Mineur and Crusio, 2009).

More recently developed techniques are based on the premise that an individual's affective state influences cognitive functioning. One such method is centered on the valence component of emotion (i.e., whether an emotion is positive or negative), which is arguably more relevant to well-being than the arousal (i.e., the intensity of an emotion) measured by most physiological assays. Specifically, this paradigm uses performance in judgment and decision making tasks to make inferences about underlying mental states. It has been substantiated that human beings with negatively-valenced emotions like anxiety interpret ambiguous stimuli more cynically than humans with positive affective states like joy (Mathews and Macleod, 1994). Extrapolating from the human data, Harding et al. (2004) trained rats to press a lever in response to a tone of a particular frequency (i.e., 4 kHz) to receive a reward and to refrain from pressing the lever in response to a tone of a different frequency (i.e., 2 kHz) in order to avoid punishment. They found that when a tone was played with a frequency close to or equal to 4 kHz, rats housed in unstable conditions were slower to press the lever than rats housed in stable conditions, which is consistent with a decreased anticipation of reward. This "pessimistic" judgment bias has since been demonstrated in a few other species (including humans) and situations (Brilot et al., 2010; Daros et al., 2014; Hales et al., 2014; Mendl et al., 2009; Neave et al., 2013; Papciak et al., 2013; Scheele et al., 2013; Verbeek et al., 2014). Conversely, Swan and Hickman (2014) interpreted a longer latency to investigate an unrewarding situation as evidence for pessimistic bias and supplemented their findings with elevated neutrophil-lymphocyte ratios in the chronically stressed animals with pessimistic bias.

Clearly, more work needs to be done to parse out the relevance of different biases as they pertain to welfare. For example, a decreased proclivity to seek reward might indicate something different about an animal's emotional state than a lower motivation to investigate unrewarding situations or terminating those that could lead to punishment. Capuchins with a higher rate of head twirls (a stereotypic behavior observed in captive capuchins) were more likely to judge ambiguous stimuli in a pessimistic manner than those with lower rates of head twirls. Interestingly, no such relationship was found for pacing and pessimistic bias (Pomerantz et al., 2012). Thus, perhaps there are different underlying affective states for different abnormal behaviors; and some may be less indicative of psychological distress than others.

Based on the premise that affective states can influence the direction of attentional resources in humans, some studies have designed experiments to determine whether non-human animals also display this tendency. Following a stressful situation (i.e., a veterinary health check), rhesus monkeys avoided looking at images depicting conspecifics performing threat displays, but displayed the opposite tendency following a positive experience (i.e., receiving enrichment) (Bethell et al., 2012).

5 Conclusion

Although measuring HPA activation as a means to study stress undoubtedly has the potential to provide useful information, there are several limitations and pitfalls that must be understood. To summarize, it is challenging to obtain glucocorticoid measurements that are truly reflective of HPA activation. Furthermore, there are several circumstances where changes in glucocorticoids are not consistent with putative stress levels. This often leads to post-hoc hypotheses being devised about the relationship (or lack thereof) between stress and glucocorticoid levels. Consequently, the most holistic view of the stress that an animal experiences is obtained by examining multimodal indices of welfare and would ideally include behavioral and/or cognitive components to augment and help interpret hormonal or other physiologic assays.

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Conflict of interest

The authors declare they have no conflicts of interest.

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