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REVIEW

What are you actually measuring? A review of techniques that integrate the stress response on distinct time-scales

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Abstract

1. The field of stress physiology has rapidly expanded, particularly in those fields interested in identifying chronic stress in wild animals. Despite this expansion, stress remains difficult to assess and understand, due in large part to the temporal complexities of common stress measurement techniques.
2. While the stress response happens on a short time-scale, chronic stress results over longer time-scales. Therefore, the temporal dynamics of techniques used to assess 'stress' need to be fully understood in order to be applied correctly.
3. In this review, we provide information on 37 physiological and behavioural metrics that are commonly used to measure stress, especially in wild free-living vertebrates, with a particular focus on which time-scale they integrate stress.
4. We organize these metrics into seven broad categories based on which physiological system they are most closely associated with (glucocorticoids, sympathetic/parasympathetic nervous system, immune, metabolic, cellular/molecular, tissue development and behaviour).
5. We conclude by summarizing which kind of biological questions and variation each technique is most suitable for.
6. This review will enable researchers to understand the temporal dynamics of stress measurement techniques for better design of future studies.

KEYWORDS

behaviour, cellular, corticosterone, immune, metabolic, stress, timing

1 | INTRODUCTION

Since its formal conceptualization by Hans Selye and Walter Cannon (Cannon, 1934; Selye, 1936), stress has become a fascination of the biomedical, psychological and wildlife conservation research communities. Despite the expansion in studies focused on stress, several key points of confusion remain. Perhaps most significant is that it is unknown how the acute, short-term, beneficial stress response transitions to, and is affected by, more prolonged stressors (e.g. chronic stress). This question becomes ever more important when assessing stress in wild animals or populations for conservation purposes.

One key difficulty is that many stress-related studies conflate the effects of short- and long-term stress. This is most clearly seen in the case of the glucocorticoids (cortisol or corticosterone depending on the species; Cort). For example, while Cort is crucial for responding to acute stressors, high levels of the hormones do not necessarily indicate a chronically stressed individual (reviewed in Dickens & Romero, 2013). Despite these findings, many studies fail to consider the temporal intricacies that underlie changes in Cort.

The purpose of this review is to catalogue techniques that have been used to measure stress in vertebrates (Table 1), providing information on how each has been used to assess stress. This is done with a specific emphasis on the temporal sensitivity of each technique. In Figure 1, we provide a representation that compares how long each parameter takes to change in response to a stressor and what duration of stressor each technique is sensitive to. There are

TABLE 1 Techniques for assessing the stress response and stress, categorized by the system each parameter is involved in

Glucocorticoids	Sympathetic/ Parasympathetic nervous system	Immune	Metabolic	Molecular/ cellular	Tissue development	Behaviour
Blood	Catecholamines	H(N):L ratio	Energy mobilization	Antioxidants	Tooth development	Perch hopping
Excretions	Heart rate	Inflammation	Metabolic rate	Heat shock proteins	Fluctuating asymmetry	Flight initiation distance
Saliva	Heart rate variability	Bacterial killing	Body temperature	DNA damage/ repair	Fault bars (ptilochronology)	Neophobia
Faeces	Breath rate	Leukocyte coping		Telomere length		Stereotypy
Urine						Vigilance
Whale blow						
Aquaria water						
Egg deposition						
Keratinized tissues						
Hair/feathers						
Whale baleen						
Whale earwax						
Nails/claws						
Scutes						
Scales						

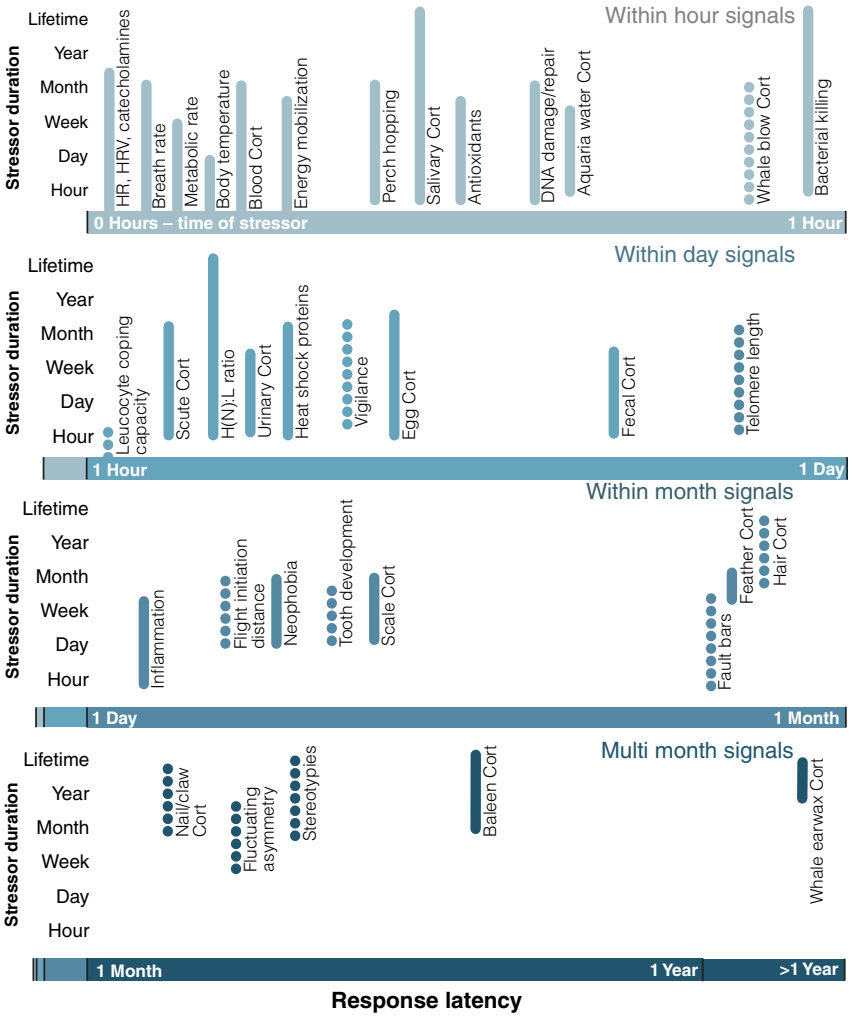

FIGURE 1 Representation of how and on what time-scale each parameter/technique integrates stress. Each metric is represented by a vertical line and is placed on the '*x*-axis' by its response latency (how long it takes to detect a signal following a stressor). The length of each line ('*y*-axis') indicates the length of stressors which can be used to measure the parameter (e.g. stressor duration). For example, blood Cort changes within minutes of a vertebrate interacting with a stressor and has been shown to be sensitive to stressors lasting minutes to months. Therefore, it is placed in the top panel (Within hour signals) and extends to 'Month' on the '*y*-axis.' In contrast, feather Cort integrates hormones over weeks and can only be used to detect longer (weeks-to-month) stressors. It therefore falls in the Within month signal section and is much shorter than blood Cort. Note that these estimates are primarily based on experimental data; dotted lines are used when theoretical extrapolations were considered due to the lack of experimental information

at least three ways to think about the timing of stress: how long the stimulus is present (e.g. exposure time); how long a stressor must be present to detect a signal in the metric (e.g. response latency); and how long after an acute event can you detect a change in the metric (e.g. response duration). In Figure 1, we catalogue each technique by response latency ('*x*-axis') and stressor duration ('*y*-axis'—either exposure time or response duration). Throughout discussion of each technique, we clarify whether references discuss exposure time or response duration. Note that this review is not meant to be species-specific, however, some techniques are limited to certain taxa. Because of the vast body of literature presented here, we have restricted techniques to those used in vertebrates. See Supplementary Material for details on the literature search to catalogue the 37 stress techniques discussed in this review. Table 1 breaks these 37 techniques into seven distinct categories based upon the physiological system and behaviour that the technique was primarily sampling. What follows are descriptions of these techniques and the time-scale of stressor exposure and duration for which they are most appropriate (see Supplementary Table for example case studies for each technique). Note that many of these techniques have limitations. We have included information on these caveats when appropriate, but emphasize that this review should be used as a starting place for those interested in these techniques. A more thorough investigation of the technical intricacies of each technique should be considered before implementation into a study.

2 | GLUCOCORTICOIDS

Cort is released into the blood from the adrenal or interrenal gland within minutes of exposure to a stressor (reviewed in Romero & Wingfield, 2016). The general role of Cort is to upregulate essential and downregulate nonessential survival systems (Vera, Zenuto, & Antenucci, 2017) by influencing transcription at glucocorticoid response elements (reviewed in Sheriff, Dantzer, Delehanty, Palme, & Boonstra, 2011). In recent decades, techniques have expanded to measure Cort in a number of biological matrices that can be used to infer circulating hormones over a variety of time-scales.

2.1 | Blood

The most common measurement of Cort is in the plasma component of blood samples. Upon acute stimulation, Cort becomes upregulated within 2–5 min, depending on the species (Romero & Reed, 2005). Thus, samples taken within 1–3 min of disturbance tend to reflect near-baseline Cort levels. Multiple blood samples can be taken over the course of minutes to hours to compare how quickly and strongly Cort is elevated (reviewed in Romero & Wingfield, 2016).

The termination of the stress response is equally important as its initiation. This is accomplished through negative feedback by which Cort shuts off the hormonal cascade. Recent studies have shown that assessing negative feedback efficacy may help deduce how an animal is coping with a stressor (Taff, Zimmer, & Vitousek, 2018). This is typically done by artificially stimulating feedback using a synthetic glucocorticoid (e.g. dexamethasone), which reduces Cort over the course of 1–2 hr (e.g. Lattin, Bauer, de Bruijn, & Romero, 2012).

Although understudied and not yet thoroughly validated, blood-sucking bugs have been used to remotely capture blood samples (Riechert, Chastel, & Becker, 2012; Voigt et al., 2004). The technique consists of allowing the bug to take a blood meal from the study animal, which is then extracted and analysed for Cort. It is assumed that the blood meal is representative of circulating blood in the animal of interest.

Regardless of how they are collected, blood samples represent a snapshot of circulating Cort at the time of sampling. Blood Cort levels change with life-history stage (e.g. Lattin et al., 2012; Romero, 2002) and during repeated stress (e.g. Cyr, Earle, Tam, & Romero, 2007). Furthermore, although blood Cort changes in response to short-term stimuli (e.g. capture and restraint), baseline and stress-induced levels and negative feedback strength are altered by longer term stressors (e.g. weather, Krause et al., 2016; captivity, Lattin et al., 2012; Table S1). This means that, if sampling is done properly, blood Cort can be sensitive to stressors lasting minutes-to-months (Figure 1; Table S1).

2.2 | Excretions

2.2.1 | Saliva

Cort is transferred—via diffusion—to the acini of the salivary glands. This technique is likely most appropriate to use in humans and captive animals. In humans, stimulation by adrenocorticotropic hormone (ACTH) leads to peak salivary Cort ~5–10 min following peaks in the blood (reviewed in Gröschl, 2008). Salivary Cort can also detect longer term disruptions, including repeated stressors and social disruption (e.g. Menneson et al., 2019). The response latency (about 5 min) and stressor duration (minutes-to-lifetime) measurable by salivary Cort is represented in Figure 1.

2.2.2 | Faeces

Cort is metabolized into conjugates that are excreted with the faeces (Palme, 2019). These metabolites can be recognized by common assay antibodies, however, there are several key technical concerns that are crucial for new investigators to address (reviewed in Goymann, 2012; Palme, 2019).

Faecal Cort provides an integrated measure of the amount of excreted metabolites since the previous defaecation. Typical studies that inject ACTH to stimulate blood Cort release, report an increase in faecal metabolites hours-to-days later, depending upon species. Faecal metabolites are thus representative of circulating/active Cort hours-to-days prior to excretion; however, the length of digestion can lead to variability (e.g. Morrow, Kolver, Verkerk, & Matthews, 2002). Faecal Cort is also sensitive to longer term stimuli

including repeated stressors lasting days-to-weeks, as well as to individual differences including dominance rank, sex and age (e.g. Price et al., 2019; Table S1). Consequently, the response latency of faecal Cort metabolites is on the order of hours and they are sensitive to stressors lasting hours-to-weeks (Figure 1).

2.2.3 | Urine

In many species, urine contains the majority of excreted Cort (Romero & Wingfield, 2016). In general, urinary Cort lags behind blood Cort by ~1–12 hr depending on the species (Narayan, Cockrem, & Hero, 2013), but can also reflect changes in physiology due to chronic psychological stress (Kramer, Hiemke, & Fuchs, 1999; Table S1). Consequently, like faecal Cort metabolites, the response latency of urinary measurements is on the order of hours and they can be used to detect a stressor lasting weeks (Figure 1).

2.2.4 | Whale blow

In whales, blow, or respiratory vapour, contains Cort in expelled mucus that can be captured when whales come to the surface to expel air and seawater after long dives (Burgess, Hunt, Kraus, & Rolland, 2018). One validation using captive belugas *Delphinapterus leucas* showed that blow samples assayed for progesterone and testosterone correlate to blood samples taken within 1 hr (Richard et al., 2017). If this pattern is consistent for Cort, then we can assume that blow samples reflect relatively recent stressor exposure, on the order of about an hour. Although the duration of a stressor that can be detected using this technique has not yet been tested, it could be quite long, perhaps on the order of months, considering that mucus and saliva might be roughly analogous (Figure 1).

2.2.5 | Aquaria water

Aquaria water can be used to quantify Cort released through the gills and likely excreted in faeces and urine by fish and amphibians (reviewed in Scott & Ellis, 2007). Water-borne Cort can be used to detect the effects of handling stress (e.g. Ellis, James, Stewart, & Scott, 2004), as well as of captivity and housing condition (e.g. Ellis et al., 2007; Table S1). The response latency (minutes) and stressor duration (minutes-to-weeks) are indicated in Figure 1.

2.2.6 | Egg deposition

In avian species Cort is deposited into developing eggs, both yolk (Royo, Mayo, Carlsson, & Hau, 2008) and albumen (Downing & Bryden, 2008). Egg concentrations are thought to reflect circulating maternal levels at the time of laying (Hayward & Wingfield, 2004), however, technical concerns remain (e.g. Rettenbacher, Möstl, & Groothuis, 2009) and additional validations are necessary. Egg Cort has been shown to be sensitive to short-term stressors including simulated predation (Saino, Romano, Ferrari, Martinelli, & Møller, 2005) and handling (Downing & Bryden, 2008) as well as to long-term stressors such as persistent high temperatures (Downing & Bryden, 2008), and seasonal variation (Jenni-Eiermann, Jenni, Olano Marin, & Homberger, 2020). Because it takes several hours for albumen and yolk to form, this technique provides an integrative measure reflecting the period of egg formation. Based on current data, the response latency of egg Cort appears to be several hours and it is sensitive to stressors lasting months (Table S1; Figure 1).

2.3 | Keratinized tissues

2.3.1 | Hair and feathers

Cort diffuses from the follicular capillaries into growing hair, although other external sources (e.g. sweat, local production) may influence measured levels (reviewed in Greff et al., 2019; Russell, Koren, Rieder, & Van Uum, 2012). Similarly, Cort is integrated into a growing pin feather from the surrounding blood when cells are differentiating and keratinizing (reviewed in Romero & Fairhurst, 2016). The growth periods of hair and feathers are often species-dependent, but are typically on the order of weeks-to-months. Hair tends to grow continuously and for longer periods of time (Geyfman, Plikus, Treffeisen, Andersen, & Paus, 2015), and long hair can be partitioned to get a timeline of Cort incorporation. In contrast, feathers are molted at specific times of the year, and when complete, are dead tissue, no longer connected to a blood supply; therefore, feather Cort captures the circulating hormones *only* at those specific times when feathers were actively growing. Because of these growth dynamics, response latency of feathers and hair is on the order of weeks, and are sensitive to stressors lasting from a week to a month, possibly lifetime in hair (Figure 1). Both methods present the opportunity to retrospectively ask questions about whether and how past conditions affect future physiology and fitness (Table S1).

2.3.2 | Whale baleen and earwax

Both baleen and earwax samples capture a longer term retrospective profile of Cort exposure in whales. Baleen 'plates' grow continuously from the upper gum towards the bottom gum (e.g. Hunt, Lysiak, Moore, & Rolland, 2016) and are structurally similar to hair. Cort is integrated into baleen as it grows. By subsampling plates, baleen can provide a temporal resolution on the order of months and seasons (Hunt et al., 2016, 2018) and provide a record of up to 10 years depending on the species (Hunt et al., 2018). Similarly, ear wax (cerumen) accumulates in the ear canal from birth, forming a continuously growing earplug that also contains Cort (Trumble, Robinson, Berman-Kowalewski, Potter, & Usenko, 2013). Ear wax depositions can be distinguished at approximately yearly intervals, making ear wax appropriate for longer, lifetime studies (Trumble et al., 2013).

2.3.3 | Nails and claws

Cort is also integrated in nails and claws as they grow. Recent studies have used this method to assess hormone levels in wild animals, particularly reptiles (Baxter-Gilbert, Riley, Mastromonaco, Litzgus, & Lesbarrères, 2014; Matas, Keren-Rotem, & Koren, 2016; Table S1). Additional validation studies are needed to tease apart the temporal dynamics of Cort integration into nails and claws, but it is likely that samples represent circulating levels over the course of weeks-to-months, depending on the species (Figure 1).

2.3.4 | Scutes and scales

Scutes are keratinized structures that form turtle shells and the tails of crocodilian species. Cort has been found in tail scutes from American alligators *Alligator mississippiensis* and has been shown to increase following a 2-hr handling stress, as well as due to long-term environmental contamination (Finger et al., 2019; Hamilton, Finger, Elsey, Mastromonaco, & Tuberville, 2018; Table S1). It appears, therefore, that scute Cort can change within 2 hr of a stimulus and can also be sensitive to long-term exposure (weeks-to-months; Figure 1), but exploration of this technique is just beginning.

The elasmoid scales of teleost fishes can also be used to quantify Cort. These scales are calcified external structures that grow as the fish grows, and when they are removed, they regrow within days (Metz, de Vrieze, Lock, Schulten, & Flik, 2012). Cort is detectable in these scales and is sensitive to seasonal variation, habitat quality, and repeated stressors (Aerts et al., 2015; Carbajal et al., 2019; Laberge, Yin-liao, & Bernier, 2019; Table S1). In sum, the growth rate of scales suggests a response latency of days with scale Cort sensitive to stressors lasting days-to-months (Figure 1).

3 | SYMPATHETIC/PARASYMPATHETIC NERVOUS SYSTEM

When an animal encounters a stressor, catecholamines are released in addition to Cort (reviewed in Romero & Wingfield, 2016). Due to the speed of this activation, it is often logistically challenging to capture baseline catecholamine levels, though some studies have successfully done so (e.g. Capitanio & Cole, 2015; Hoopes, Landry Jr., & Stabenau, 2000). In order the measure this response, proxy metrics are often relied upon.

3.1 | Heart rate and heart rate variability

During the stress response, epinephrine binds to β-receptors, causing nearly immediate elevations in heart rate (reviewed in Romero & Wingfield, 2016). This increase happens within seconds and heart rate gradually returns to baseline levels typically between 5 and 10 min depending on the particular disturbance (Fischer & Romero, 2016; Fischer, Wright-Lichter, & Romero, 2018). Heart rate is also controlled by the parasympathetic nervous system, which regulates the beat-to-beat interval variation known as heart rate variability (HRV; reviewed in Kim, Cheon, Bai, Lee, & Koo, 2018). As an animal encounters a stressor, the heart not only beats faster, but more consistently; thus, HRV decreases. While heart rate and HRV rapidly change during acute stress (e.g. within seconds or less after stressor onset; Figure 1), they both can be affected by chronic stress, including captivity and repeated stressors (Cyr, Dickens, & Romero, 2009; Fischer et al., 2018); thus, these techniques could be used to assess longer term stimuli on the order of months, including seasonal changes (Portugal, White, Green, & Butler, 2018; Table S1, Figure 1).

3.2 | Breath rate

Breath rate can change in response to acute stress and is thought to be a proxy for assessing parasympathetic nervous system activity. Breath rates have been assessed in a number of avian species and can quickly change within minutes in response to handling (e.g. Carere & Van Oers, 2004). Furthermore, differences in breath rate may underlie overarching environmental effects (e.g. urbanization; Abolins-Abols, Hope, & Ketterson, 2016). Taken together, these results suggest that breath rates (a) change rapidly (seconds-to-minutes) in response to stimuli and (b) can reflect underlying differences in age or external environment (e.g. stressor durations lasting months; Figure 1; Table S1). However, little validation work to fully establish this technique has been done to date.

4 | IMMUNE

The effects of stress on the immune system of wild animals have been extensively studied (reviewed in Chrousos, 2009). These immune effects are extraordinarily varied and complex, and often regulated by Cort. Note that much of the stress-immune research uses artificial infections or exogenous exposure to Cort to initiate or mimic a stress response, which might not be an accurate reflection of a natural response (MacDougall-Shackleton, Bonier, Romero, & Moore, 2019). Though there is substantial evidence indicating that the stress and immune responses are closely tied, additional studies are needed to tease apart the exact mechanisms, particularly in wild animals.

4.1 | Heterophil/neutrophil: Lymphocyte ratio

One of the most popular stress immunological techniques is comparing the ratio of white blood cell types, specifically heterophils/neutrophils (depending on the species) to lymphocytes (reviewed in Davis, Maney, & Maerz, 2008). Encountering a stressor causes an increase in circulating heterophils/neutrophils and a corresponding decrease in circulating lymphocytes (historic studies reviewed in

Ottaway & Husband, 1994). In general, Cort alters the ratio of heterophils/neutrophils to lymphocytes 1–4 hr (Figure 1) following exposure to a stressor (e.g. Dhabhar, Miller, McEwen, & Spencer, 1995); however, this time can vary depending on species and type of stimulus. Finally, leukocyte counts can be affected by a variety of environmental factors, such as food unpredictability (Pusch, Bentz, Becker, & Navara, 2018) and habitat quality (Selman, Qualls, & Owen, 2013); thus changes in this metric may reflect longer term, and possibly even life-long, underlying stressors (Table S1; Figure 1).

4.2 | Inflammation

Two specific stressors—injury and infection—can trigger inflammatory responses (Martin, 2009). Most studies assess the inflammation response by injecting an antigen into the pinna (mammals) or wing web (birds) and tracking swelling. This leads to detectable elevations in inflammation after 1–2 days (Dhabhar & McEwen, 1999). Short-term stress tends to enhance whereas long-term stress tends to inhibit inflammation in response to antigen injection for up to several weeks (Cort administration, Dhabhar & McEwen, 1999; Captivity, Martin, Kidd, Liebl, & Coon, 2011). Current data suggest that the comparison between acute and chronic inflammation responses allows for the assessment of stressor duration on the order of weeks (Figure 1; Table S1). Note, however, that substantial controversy surrounds the assessment and interpretation of inflammatory responses. A robust inflammatory response may be strong and beneficial; however, it may also be a harmful overreaction. Additionally, there can be subjectivity when measuring swelling. Sorting out these complexities is key to the usefulness of inflammation in the assessment of the stress response.

4.3 | Bacterial killing capacity

The bacterial killing assay tests how effectively the innate immune system combats a pathogen—typically *Escherischia coli*—presented in vitro (Tieleman, Williams, Ricklefs, & Klasing, 2005). Restraint stress results in a decreased killing capacity within 30–90 min (e.g. Millet, Bennett, Lee, Hau, & Klasing, 2007), suggesting a response latency of about 1 hr (Figure 1). Bacterial killing capacity also can be affected by longer term stimuli, including habitat quality (Hopkins & DuRant, 2011), seasonal variation (Zimmerman, Paitz, Vogel, & Bowden, 2010), captivity (Love, Lovern, & DuRant, 2017) and repeated stressors (Gormally, Wright-Lichter, Reed, & Romero, 2018; Table S1). Consequently, bacterial killing capacity can reflect stressor durations on the order of at least months, possibly even lifetime (Figure 1).

4.4 | Leucocyte coping capacity

When leukocytes are activated, they produce an oxidative or respiratory burst, during which reactive oxygen species (ROS) are formed (Robinson, 2009). This production of ROS is considered to be adaptive, and thus failure to elicit it is perceived as negative. This oxidative burst can be monitored before and after a stressor and leukocyte coping capacity (LCC) can be calculated. LCC generally declines relatively quickly (min) after the cessation of short (10 min–2 hr) stimuli (e.g. McLaren et al., 2003). While LCC tends to decline, there does appear to be some variation in responses depending on stimuli (reviewed in Huber et al., 2019). Additional studies are needed to assess whether and how LCC is sensitive to longer disturbances (Figure 1; Table S1).

5 | METABOLIC

5.1 | Energy mobilization

A canonical role of catecholamines and Cort is to activate and mobilize energy stores, namely glucose via glycogenolysis, gluconeogenesis, and the reduction of peripheral usage (reviewed in Romero & Wingfield, 2016). The net result of these mechanisms is a rapid (minutes-hours) increase in blood glucose levels. Other energy sources can be monitored during the acute stress response. Blood ketone bodies (β-hydroxybutyrate) and fatty acids are affected by the lipolysis pathway, while uric acid is linked to the breakdown of proteins. These byproducts have also been shown to rapidly (<20 min) change in response to acute stress (Delehanty & Boonstra, 2009; Viblanc et al., 2018; Table S1). Persistent stressors related to food availability (e.g. starvation) also leads to reliance on distinct energy stores; therefore measuring energy mobilization can help assess longer term stimuli on the order of weeks (Figure 1; reviewed in Romero & Wingfield, 2016).

5.2 | Metabolic rate

There is also an associated change in metabolic rate in animals coping with acute stress and/or due to Cort exposure. Importantly, there are stark differences between the metabolic effects of stress on endo and ectotherms. Exogenous Cort elevates oxygen consumption in ectotherms for up to 11 days (e.g. DuRant, Romero, Talent, & Hopkins, 2008), whereas it tends to decrease oxygen consumption in endotherms (e.g. Buttemer, Astheimer, & Wingfield, 1991). Acute stress can substantially increase daily energy expenditure, however, this effect may depend upon stimulus type and life-history stage (Cyr, Wikelski, & Romero, 2008). Our knowledge to date suggests that metabolic rate changes nearly immediately with stressor onset, and can reflect stressor duration for at least approximately a week (Figure 1; Table S1), but there is substantial variation depending upon species, stimulus type and life-history variation that complicates interpretations.

5.3 | Body temperature

Stress can increase body temperature, known as hyperthermia (Oka, Oka, & Hori, 2001). These increases can occur in as little as

10 s (Bouwknecht, Olivier, & Paylor, 2007; Jerem, Jenni-Eiermann, McKeegan, McCafferty, & Nager, 2019) and are tuned to stressor intensity (Herborn et al., 2015). Body temperature also appears to be sensitive to longer term factors including personality and time of day (Carere & Van Oers, 2004). Finally, eye temperature has been shown to be correlated to baseline circulating glucocorticoids, suggesting it may be a useful biomarker for underlying physiology (Jerem et al., 2018, 2019). Body temperature has promise as a noninvasive indicator of physiological responses to both short (detection within minutes, if not seconds, of stressor onset) and mid-term stressors (stressor duration up to several days; Figure 1; Table S1), although more validation work is needed.

6 | MOLECULAR/CELLULAR

6.1 | Antioxidants

When an animal responds to an acute stimulus there is often associated oxidative stress due to the overproduction of ROS during cellular respiration (Bayir, 2005). In general, handling/restraint stress or exogenous Cort affects measures of the antioxidant system quickly, suggesting a response latency of 30 min–1 hr (Cohen, Klasing, & Ricklefs, 2007; Lin, Decuypere, & Buyse, 2004b; Figure 1; Table S1); however, the direction of these changes is not always consistent, likely because of the variability of methods. Additionally, measures of oxidative physiology vary seasonally and between sexes (Cohen, McGraw, & Robinson, 2009; Pap et al., 2018). Finally, repeated and persistent stressors lasting days-to-weeks change uric acid, an antioxidant in birds (e.g. Gormally, Fuller, McVey, & Romero, 2019; Gormally et al., 2018; Lin, Decuypere, & Buyse, 2004a). Note that a number of reviews have addressed the complexities involved in assessing the antioxidant systems (e.g. Costantini, 2011; Hõrak & Cohen, 2010) and it is important to be well-versed in these intricacies before interpreting results. In conclusion, it appears that antioxidants can detect the onset of a stressor within 30–60 min, and can detect stressor durations of several weeks (Figure 1).

6.2 | Heat shock proteins

Heat shock proteins (Hsp) play crucial roles in maintaining proper protein folding and protecting cells against stress (Li & Srivastava, 2003) and are also associated with Cort receptor signal transduction (Mayer & Bukau, 2005). Therefore, changes in Hsp expression may have the capacity to link cellular- with organismal-level stress. Hsp70 and Hsp90 significantly decrease following acute (30 min–2 hr) stressors, lasting up to several hours (e.g. Finger, Hoffman, & Wada, 2018). Longer stressors lasting weeks can also increase or decrease Hsp expression, depending on the stimulus (Li et al., 2011; Pusch et al., 2018). It thus appears that appropriate time frames for Hsp measurements are hours for response latency and up to a month for duration (Figure 1; Table S1).

6.3 | DNA damage and repair

A number of studies have assessed connections between stress-related hormones and DNA damage and repair pathways. In vitro studies found that 10-min applications of physiologically relevant doses of Cort, epinephrine and norepinephrine led to significant increases in DNA damage (Flaherty et al., 2017; Flint, Baum, Chambers, & Jenkins, 2007). This finding has been replicated in vivo in house sparrows (Gormally et al., 2020), king penguins *Aptenodytes patagonicus* (Stier et al., 2019) and gilthead seabream *Sparus aurata* (Malandrakis et al., 2016), but not in mice (Flint et al., 2005). It is currently unclear by what mechanisms these changes are occurring and whether this accumulation of damage is harmful. DNA damage can also accumulate in animals exposed to more persistent stress, including introduction to captivity (Gormally, Fuller, et al., 2019), chronic epinephrine exposure (Hara et al., 2011), repeated stressors (Gormally, Estrada, Yin, & Romero, 2019) and radiation exposure (Bonisoli-Alquati et al., 2010). Our current knowledge suggests DNA damage changes rapidly (within minutes) following a stressor, but can also be affected by stressors lasting months (Figure 1; Table S1).

6.4 | Telomere length

Telomeres are the protective caps at the ends of chromosomes which are crucial for genome stability. As cells divide, telomeres progressively shorten. Additionally, these areas of the genome are particularly susceptible to oxidative damage (Richter & Zglinicki, 2007). Thus assessing telomere dynamics (change in length over time) can be connected with both ageing and lifetime stressor exposure (Aubert & Lansdorp, 2009). Telomeres are traditionally measured when assessing long-term (days-to-months) stressors, which result in significantly shortened telomeres (Zollinger, Heidinger, & Brumm, 2018). A recent model has proposed that short periods of stress (hours-to-weeks) can also shorten telomeres and that this change can be adaptive (Casagrande & Hau, 2019). It is not yet clear if the degree of telomere shortening can be tuned to stressor intensity or duration, but can certainly be reflective of years of stressor exposure (Figure 1; Table S1).

7 | TISSUE DEVELOPMENT

7.1 | Tooth development

A number of studies in humans and non-human primates have examined the development of teeth and found that external stressors can disrupt the pattern of dentine and enamel deposition (e.g. Austin et al., 2016; Schwartz, Reid, Dean, & Zihlman, 2006). This technique assesses changes in the growth lines, the chemical (e.g. deposition of barium relative to calcium) composition of teeth, as well as the deposition of proteins, including Hsps (Austin et al., 2016). While this technique is limited to species with teeth, it provides the opportunity

to detect signatures of longer term stressors including illnesses and injuries, thus stressors lasting days-to-months (Figure 1; Table S1). Because this technique relies on the deposition of enamel and dentine, it is likely that stressors must last days-to-weeks in order to be detectable (Figure 1).

7.2 | Fluctuating asymmetry

Bilateral organisms develop symmetry through a series of carefully orchestrated developmental and molecular mechanisms. Disruption during growth, including from stress, can lead to asymmetrical structural development (e.g. differently sized limbs; Lens & Van Dongen, 2000), termed fluctuating asymmetry. A number of reviews have detailed the advantages and drawbacks of this method, namely that fluctuating asymmetry does not consistently indicate developmental stress (Bjorksten, Fowler, & Pomiankowski, 2000; Knierim et al., 2007). If fluctuating asymmetry *is* linked with long-term, developmental stressors, it has the capacity to be a useful retrospective tool for assessing developmental stressors lasting days-to-months, depending on the species (Figure 1; Table S1).

7.3 | Fault bars (ptilochronology)

Ptilochronology refers to the growth of feathers over a 24-hr period during molt, which can result in differential protein deposition and the formation of visible bars. Fault bars can form when birds experience nutritional stress that forces a temporary reduced investment in feather growth; thus, examining growth bar width can provide a retrospective analysis of resource conditions during molt (reviewed in Grubb, 2006). There is also evidence that suggests that fault bars can be representative of much shorter stressors (e.g. handling; reviewed in Jovani & Rohwer, 2017). Fault bars can also be caused by repeated stressors (Strochlic & Romero, 2008). However, it has been suggested that different feather follicles, individuals and species may have a different propensity for fault bars and different severities of stressors will influence these factors in specific ways (see Figure 1 in Jovani & Rohwer, 2017). From the knowledge that we currently have, it appears that fault bars can form in response to relatively mild and short-term stressors (e.g. handling), and can increase in severity with stronger or longer term stressors (e.g. malnutrition; Figure 1; Table S1), but more validation work is needed.

8 | BEHAVIOUR

8.1 | Perch hopping

Perch hopping—often termed 'activity' in birds—has also frequently been studied to compare underlying physiological differences with expressed behaviour. Direct Cort and corticotropin releasing hormone administration elevate activity within minutes to hours (Breuner, Greenberg, & Wingfield, 1998; Maney & Wingfield, 1998). One interpretation of these results is that increased perch hopping during acute stress in captivity is an expression of escape behaviour (redirection away from reproduction towards survival) that would be displayed in the wild. Note, however, that interpretations of acute changes in perch hopping are not consistent. Longer term stressors—including social defeat and repeated stressors—can decrease activity as well (Carere & Van Oers, 2004; Gormally et al., 2018). Therefore, it appears that elements of the stress response have the capacity to affect activity on a short time-scale, but also that longer term stimuli can affect overall behaviour. Consequently, perch hopping appears to have a response latency of seconds-to-minutes, and is sensitive to stressors lasting at least 3 weeks (Figure 1; Table S1).

8.2 | Flight initiation distance

Flight initiation distance—or the distance at which an animal flees a simulated predator approach—has become a popular metric of wariness in wild animals. This method is more appropriate when assessing longer term external stressors, including predation pressure (Berger, Wikelski, Romero, Kalko, & Rödl, 2007), urbanization (Abolins-Abols et al., 2016; Berger et al., 2007) and food availability (Beale & Monaghan, 2004). Therefore, flight initiation distance is sensitive to stressors lasting days-to-months (Figure 1; Table S1).

8.3 | Neophobia

The balance between the desire to access novel resources with the potential for predation underlies the neophobic response—the fear of the new (reviewed in Greenberg & Mettke-Hofmann, 2001). This is traditionally assessed by presenting a novel object along with food that attracts the animal. Cort administration alters neophobic responses in both short- and long-term contexts (Bebus, Small, Jones, Elderbrock, & Schoech, 2016; biomedical literature reviewed in Korte, 2001). Other long-term stimuli can also affect neophobia such as repeated stressors (Gormally et al., 2018) and habitat alteration and urbanization (Grunst, Grunst, Pinxten, & Eens, 2019). Consequently, neophobia appears to have a response latency of days-to-weeks, and stressor duration of months-to-years (Figure 1; Table S1).

8.4 | Stereotypy

Stereotypic behaviour is defined as a compulsive, repetitive behaviour that has no discernable goal (reviewed in Mason, 1991) and is often associated with a decrease in welfare (Broom, 1991). Examples of these behaviours include pacing in zoo animals, cribbing in horses and feather plucking in birds. Though there is the assumption that

TABLE 2 Questions and variations each stress technique/parameter can explain.[a] ✓ = a technique that has been shown to appropriately examine a type of response/variation; -- = a technique that cannot address this kind of question. A blank cell indicates a technique that requires further exploration

Category	Technique	Acute emergency strategies[b] (acute changes in response to unpredictable events)	Long-term health indicators[c] (in response to chronic unpredictable events)	Individual differences (between sex, rank, condition, etc.)	Phenotypic flexibility (in response to predictable circadian and circannual rhythms)	Environmental differences (differences across environments)
Glucocorticoids	Blood	✓	✓	✓	✓	✓
	Saliva	✓	✓	✓	✓	✓
	Faeces	✓	✓	✓	✓	✓
	Urine	✓	✓	✓	✓	✓
	Whale blow	✓		✓	✓	
	Aquaria water	✓		✓		
	Egg deposition	✓	✓		✓	✓
	Hair	--	✓	✓		✓
	Feathers	--	✓			✓
	Whale baleen	--	✓	✓	✓ (seasonal only)	✓
	Whale earwax	--	✓		✓ (circannual only)	✓
	Nails/claws	--	✓	✓	✓	✓
	Scutes		✓			
	Scales	--	✓		✓	
Sympathetic/parasympathetic nervous system	Catecholamines	✓		✓		
	Heart rate	✓	✓	✓	✓	✓
	Heart rate variability	✓	✓	✓	✓	✓
	Breath rate	✓				✓
Immune	H(N):L ratio	✓	✓	✓		✓
	Inflammation	✓	✓			
	Bacterial killing	✓	✓	✓	✓	✓
	Leukocyte coping	✓				
Metabolic	Energy mobilization	✓	✓		✓	
	Metabolic rate	✓	✓		✓	✓
	Body temperature	✓		✓		✓
Molecular/damage	Antioxidants	✓	✓	✓	✓	
	Heat shock proteins	✓	✓			
	DNA damage/repair	✓	✓			
	Telomere length		✓		--	
Tissue development	Tooth development	--	✓		--	
	Fluctuating asymmetry	--	✓		--	
	Fault bars (ptilochronology)		✓		--	
Behaviour	Perch hopping	✓	✓			
	Flight initiation distance	--	✓	✓	✓	✓
	Neophobia	--	✓		✓	✓
	Stereotypy	--	✓		--	✓
	Vigilance	✓				✓

[a]This table is akin to table 6.4 in Romero and Wingfield (2016) and the distinctions made by Wingfield and Romero (2001).

[b]This includes studies that use short-term stressors including physical stressors (e.g. restraint) or exogenous Cort administration.

[c]This includes studies that have showed changes either in response to long-term/repeated exogenous Cort administration or other psychological/physical stress.

stereotypies are correlated with stress, few studies have found consistent relationships between their expression and other physiological markers of stress (e.g. Costa et al., 2016). *If* a mechanistic relationship between stress and stereotypies exists, it has yet to be elucidated. Because stereotypies are only seen in captive scenarios, it is difficult to design experiments that test how they develop. One study did find that transferring bank voles *Clethrionymus glareolus* to captivity did not increase the expression of behavioural stereotypies within 60 days, whereas they were prevalent in their laboratory-bred counterparts (Cooper & Nicol, 1996). This suggests that stereotypic behaviour is a result of a stressful environment lasting multiple months-to-years (Figure 1; Table S1). Note, however, that some suggest that the presentation of stereotypic behaviour may actually help animals better cope with acute stressors (Pomerantz, Paukner, & Terkel, 2012).

8.5 | Vigilance

One key to successful foraging is being vigilant to potential predators. The proportion of time spent displaying vigilance behaviours is associated with both short- and long-term stressors (reviewed in Morgan & Tromborg, 2007); specifically, factors such as capture methods (Arzamendia, Bonacic, & Vilá, 2010), group size, and habitat characteristics can influence vigilance (Chmura, Wey, & Blumstein, 2016). Based on current literature, the response latency of vigilance appears to be a minimum of hours, but can be sensitive to stressors lasting months or maybe even years (Table S1; Figure 1).

9 | CONCLUSION

This review was motivated by the fact that the markers and effects of short- and long-term stress are too often conflated; this makes it extraordinarily difficult to interpret results across studies and stagnates continued efforts in the field. Therefore, we provide a synthesis of the techniques commonly used to assess stress in vertebrates, with a focus on which time-scale they can be used. To summarize, we suggest which types of questions and variations can best be explained by each technique (Table 2). Each of the 37 techniques is categorized by which types of questions it has already been shown to reflect (✓), which they cannot explain (--), or which need additional exploration (blank cell). The first category—acute emergency strategies—encompasses techniques that reflect rapid (minutes-to-day) changes in response to unpredictable events (e.g. handling, predation attempts). The second category—long-term health indicators—include the techniques that capture the effects of 'chronic' stress lasting days-to-months (e.g. repeated stressors, captivity). The third category—individual differences—include variation between factors such as sex, rank, condition or age. The fourth category—phenotypic flexibility—refers to changes that occur in response to predictable stimuli, including circadian or circannual rhythms (Piersma & Drent, 2003). Finally, the fifth category—environmental differences—provide opportunities for comparisons across different environments (e.g. urbanization, climate).

These categories are loosely based on those established by Wingfield and Romero (2001), and repeated in table 6.4 of Romero and Wingfield (2016) and capture the major types of questions that many stress physiological studies focus on. They help synthesize the information that was provided in each section and cover everything from the short-term responses to acute stressors to the long-term differences that can exist between species and environments. We hope that this highlights the fundamental differences between these kinds of questions and as a result, between the various definitions of and approaches to 'stress'. These results emphasize the importance of clarifying what temporal level of stress is of interest. Similarly, it is crucial to take multimodal approaches in studies of stress in wild animals. By focusing on single endpoints, studies can often miss how stress—at any scale—might be impacting other systems.

It should be obvious from this review, and from Figure 1 and Table 2 more specifically, that despite the development of dozens of techniques, assessing stress in vertebrates is fraught with difficulties. Even more apparent is how much remains to be studied. We hope that the information provided in this review helps readers (a) understand how different techniques integrate the effects of stress on different time-scales and (b) effectively design their own studies to accurately capture stress in their systems. Finally, we want to emphasize that we do not feel that the temporal complexities of stress should discourage others from studying it. Assessing stress physiology in wild animals and populations can add great value to studies, but it is crucial that results are interpreted with a deep understanding of what type of information each technique provides so that we can understand how the stress response can impact animals on a variety of time-scales.

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AUTHORS' CONTRIBUTIONS

B.M.G.G. and L.M.R. collaborated on the conception of the ideas; B.M.G.G. led the writing of the manuscript; L.M.R. contributed critically to the drafts and both B.M.G.G. and L.M.R. gave final approval of or the publication.

DATA AVAILABILITY STATEMENT

There is no underlying data included in this review that requires archiving.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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