

Chapter 11

Stress Ecology in Snakes

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Abstract

Understanding stress ecology in snakes is important to anticipate individual and population-level consequences of different types of stressors and plan for conservation efforts. Additionally, an understanding of stress ecology in snakes broadens the basic understanding of stress responses across vertebrates. We review a variety of metrics available to assess stress and provide context and resources where these metrics have been applied in snakes. We then explore how these metrics change as snakes respond to different types of stressors. Throughout, we highlight gaps in understanding, and summarize important future research directions to establish a more comprehensive understanding of snake stress ecology.

Introduction

Snakes are often associated with stress, as reinforced by common tropes throughout popular media. When Indiana Jones falls into a snake pit, or when hundreds of snakes are suddenly

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released into the cabin of an airplane, we are meant to identify the stress responses induced in humans with the sudden appearance of snakes. However, let us consider these events from the snakes' perspectives - crammed into small spaces with other snake species, including natural predators, facing sudden encounters with large agonistic primates. These events would likely be stressful to the snakes.

While stress is a difficult concept to define, a widely accepted definition of a stressor is any perturbation that disrupts or disturbs an animal's homeostatic balance (Romero and Wingfield, 2015). A stress response, therefore, comprises the physiological and behavioral changes that occur as a result of a stressor and are aimed at ameliorating the stress. In snakes and other vertebrates, these changes are initiated by the activation of two different systems that work in concert to mediate a coordinated response to the stressor: the sympathetic nervous system, which releases catecholamines from the adrenal medulla, and the hypothalamus-pituitary-adrenal (HPA) axis, which results in release of glucocorticoid hormones from the adrenal cortex (Figure 1).

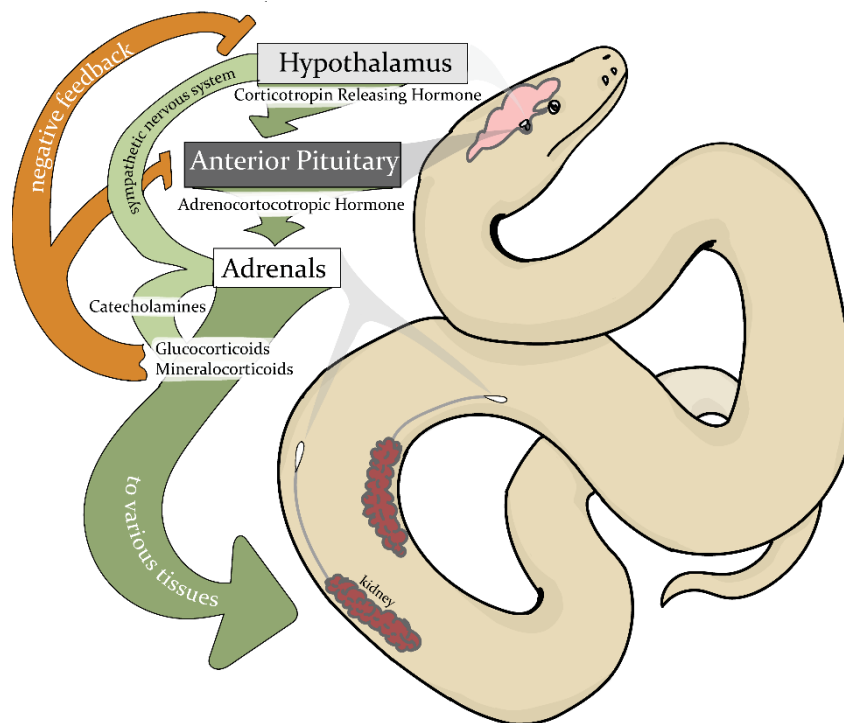


Figure 1. Conceptual diagram of the hormonal stress response and associated anatomy in snakes, focusing on the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system. When a snake perceives a stressor, the hypothalamus responds by secreting corticotropin releasing hormone and through neuronal pathways that stimulate the adrenal glands to secrete catecholamines. The anterior pituitary responds to corticotropin releasing hormone by secreting adrenocorticotrophic hormone. When received by the adrenal glands, glucocorticoids and mineralocorticoids are secreted to various tissues in the body and bind to glucocorticoid and mineralocorticoid receptors to enact downstream effects. Negative feedback occurs when high concentrations of glucocorticoids bind to receptors on the pituitary and hypothalamus; subsequently secretion of upstream hormones stimulating glucocorticoid synthesis is decreased.

Together, increases in circulating catecholamines and glucocorticoids facilitate changes in response to a stressful stimulus, such as an increase in heart rate and pulmonary respiration, modifications of energy metabolism, mobilization of immune cells to the periphery, and modulation of brain centers to heighten awareness and inhibit behaviors not critical to immediate survival such as reproduction in favor of fight-or-flight type behaviors (reviewed in Romero and Wingfield, 2015). In combination, the physiological and behavioral responses are intended to increase the likelihood of survival.

Accordingly, a canonical model of stress emerged, influenced by biomedical research on humans and laboratory rodents (e.g., Romero and Wingfield, 2015). Transient increases in catecholamines and glucocorticoids in response to noxious stimuli were categorized as acute stress and viewed as part of an adaptive response that permits an animal to cope with and survive the homeostatic challenge. Prolonged increases in catecholamines and especially glucocorticoids were interpreted as a chronic, maladaptive response to a perturbation that reflects the animal's poor health and inability to cope with stress. While the simplicity of such a model is certainly alluring, we now know that the specific effects of stress and glucocorticoids on physiology and behavior are complex, exhibit a great deal of variation, and can be difficult to interpret. This is true even for mammals upon which the canonical model of stress was built. For example, Harris (2020) identified 131 named hypotheses or models attempting to explain variation in the role of stress in health and tradeoffs (Harris, 2020). Of those, only one hypothesis considered data from reptiles.

Metrics for Assessing Stress in Snakes

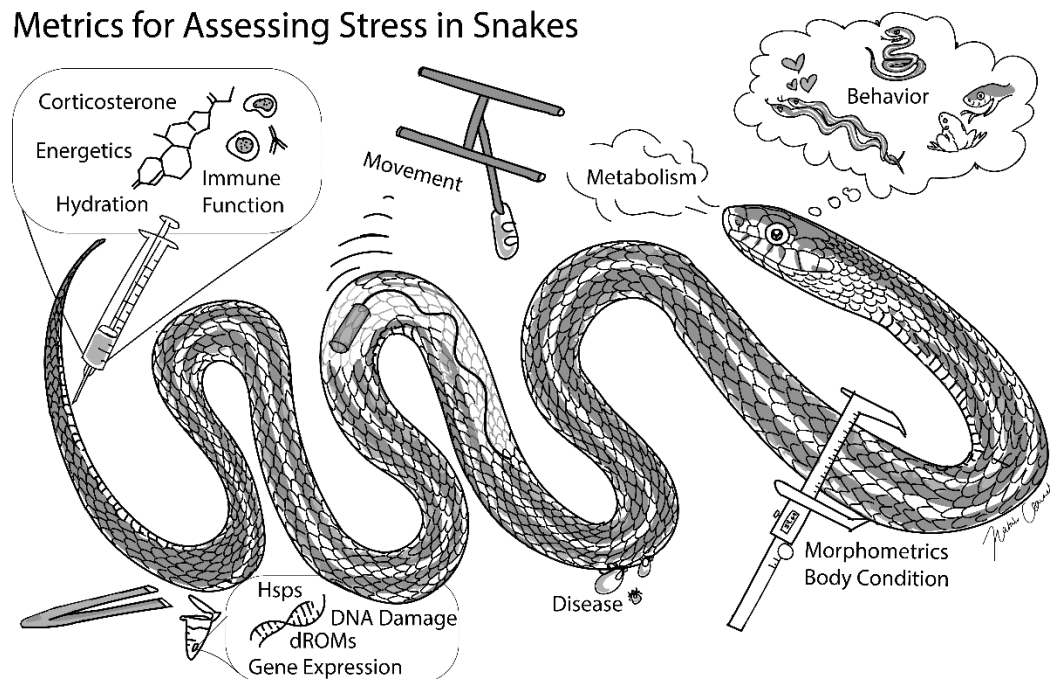


Figure 2. Conceptual diagram of a selection of the various metrics available for assessing stress in snakes. For example, from a blood sample one can obtain corticosterone concentrations, information on immune function, hydration status, and energetics. From tissue, one can extract DNA, measure heat shock proteins (Hsps), and derivatives of reactive oxygen metabolites (dROMs). With radiotelemetry, information on snake movement and activity patterns is available. One can also assess disease state, body condition, metabolic rate, and behavior with various assays.

An increase in the number of field-based studies on non-model organisms has enhanced our understanding of stress biology, but tests of current hypotheses are still limited by a lack of taxonomic diversity. The goal of this chapter is to provide a review and summary of what is currently known about stress responses in snakes. Relative to other taxonomic groups, snakes are historically understudied in many different areas of physiology and behavior (Bonnet et al., 2002; Stahlschmidt, 2011). However, snakes provide excellent opportunities for testing hypotheses about variation in stress responses because, despite being phylogenetically diverse, even closely related species can differ in life-history traits, inhabit very different environments, and exhibit unique adaptations and/or responses to stimuli. We first review the tools and metrics used for assessing stress in snakes. To facilitate an analysis that can be readily integrated with the plethora of current models in other taxa, we begin with an emphasis on what is known about the actions of glucocorticoids in snakes, followed by an exploration of other metrics available to assess stress in snakes (Figure 2). In the second section, we review data on how different types of stressors impact snake biology (Figure 3). We hope that this chapter both provides an integrative assessment of stress responses in snakes and highlights areas where additional research is needed to strengthen current models and develop new hypotheses.

Snake Stressors

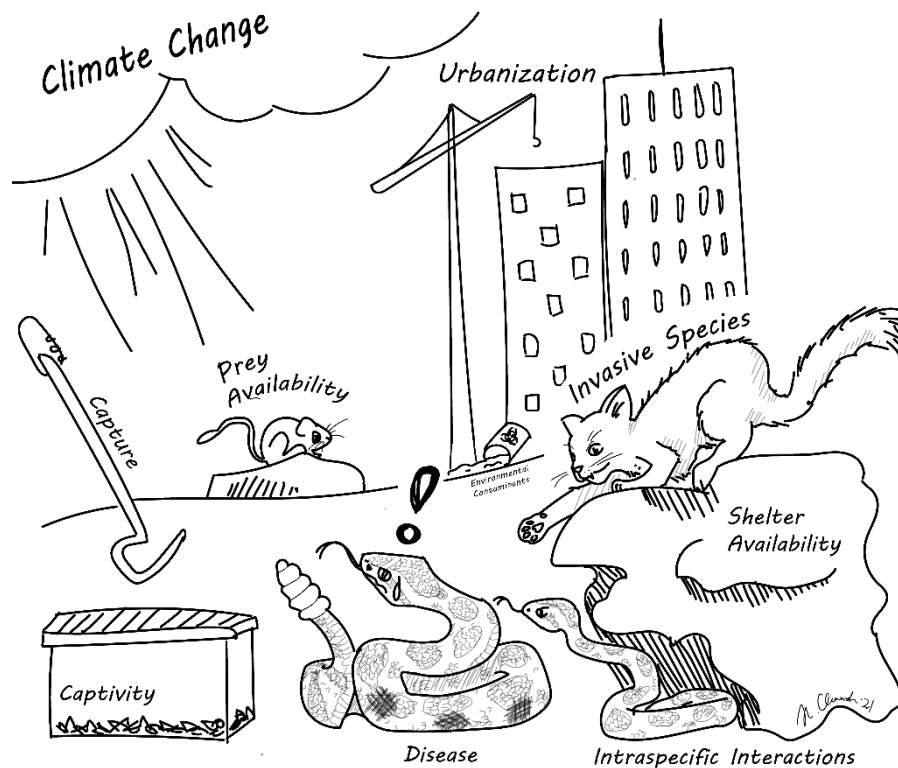


Figure 3. Conceptual figure illustrating the multitude of different stressors snakes face, including capture and captivity, climate change, urbanization, environmental contaminants, prey availability, disease, intraspecific interactions, shelter availability, and invasive species. It is important to recognize that snakes may face multiple stressors in concert.

Tools and Metrics for Assessing Stress in Snakes

Glucocorticoids: Corticosterone (CORT)

Glucocorticoids are steroid hormones that are a downstream product of activation of the hypothalamic-pituitary-adrenal/interrenal axis (Figure 1). These hormones bind to high-affinity mineralocorticoid and lower-affinity glucocorticoid receptors in tissues throughout the body to enact downstream effects (Sapolsky et al., 2000). Glucocorticoids are present in a variety of tissues in snakes, most commonly plasma, although glucocorticoids and glucocorticoid metabolites have also been measured in fecal and keratinized tissues from snakes (e.g., Berkvens et al., 2013; Halliday et al., 2015). Sampling blood plasma for CORT allows researchers to assess both baseline and stress-induced levels of circulating CORT from the same animal. The hormones can be measured using commercially available kits and measured via enzyme immunoassay, as well as via radioimmunoassay. The two methods can produce comparable results when performed using plasma samples (Claunch et al., 2021a).

Elevated Glucocorticoids Do Not Necessarily Mean Snakes Are Stressed

Glucocorticoid hormones can produce both rapid and longer-term changes in a variety of processes during a stress response (Sapolsky et al., 2000; Romero and Wingfield, 2015). Collectively, these changes in physiology and behavior facilitate an animal's ability to cope with and survive a stressor. The difficulties of interpreting what elevated glucocorticoids mean to an animal's overall health and survival were recently addressed by Romero and Beattie (2022), in which the authors provide a foundation for understanding the complexity of stress by first addressing a series of myths about glucocorticoid function. Each myth highlights a concept about glucocorticoids that is often assumed as a fundamental truth at the outset regardless of the species, season, or life-history stage.

The first two myths of glucocorticoid function addressed by Romero and Beattie (2022) are that high, acutely elevated glucocorticoids indicate that an animal is in distress and, conversely, that low glucocorticoid concentrations indicate that an animal is healthy and doing fine. This may be considered an extension of the CORT-Fitness hypothesis for which the data are equivocal (Bonier et al., 2009). As in other animals, snakes do not adhere to these assumptions about glucocorticoid function. For example, while northern populations of red-sided gartersnakes, *Thamnophis sirtalis parietalis*, in Manitoba, Canada respond to capture stress with a significant increase in plasma glucocorticoid concentrations (Moore et al., 2000), capture stress did not significantly alter male courtship behavior. Because an animal that is "in distress" would not be expected to engage in mate searching and energetically demanding courtship behavior, it cannot be assumed that red-sided gartersnakes are being negatively impacted by acutely elevated glucocorticoids. Rather, the data indicate that the hormonal stress response is dissociated from a behavioral stress response in this population of snakes (but see also Lutterschmidt and Mason, 2005, as there is annual variation in this relationship).

A second example is that changes in the sensitivity of the HPA axis and plasma glucocorticoid concentrations have been associated with female reproductive state in several snakes, including red-sided gartersnakes (Whittier et al., 1987; Dayger et al., 2018), western

diamond-backed rattlesnakes (*Crotalus atrox*; Taylor et al., 2004), timber rattlesnakes (*Crotalus horridus*; Lutterschmidt et al., 2009), western terrestrial gartersnakes (*Thamnophis elegans*; Robert et al., 2009, also see Sparkman et al., 2014), Children's pythons (*Antaresia childreni*; Stahlschmidt et al., 2013), and pygmy rattlesnakes (*Sistrurus miliarius*; Lind et al., 2020a). Should we assume, for example, that higher stress-induced glucocorticoids in gravid females indicate that these snakes are in a state of distress and poor health? It is more plausible that pregnancy and gestation are simply challenges to homeostasis, and an increase in the activity of the HPA axis during such challenges in fact indicates that an animal is coping well. These results fit better with the CORT-Adaptation hypothesis and the general idea that the stress response is adaptive (Bonier et al., 2009). Related to this conclusion, Florida cottonmouths, *Agkistrodon conanti*, on an island with limited food availability and in poor body condition showed dampened increases in glucocorticoids to capture relative to snakes on an island with continuous food availability and good body condition (Sandfoss et al., 2020). Thus, the available data on snakes support the conclusion that acutely elevated glucocorticoids should not be equated with distress and vice versa.

The third myth addressed by Romero and Beattie (2022) is that sustained elevated glucocorticoid concentrations indicate that an animal is chronically stressed. Available data in snakes again refute this conclusion. For example, in seasonally breeding animals, allostatic changes (increases) in glucocorticoid concentrations are often associated with reproductive activity (e.g., Wingfield, 2005). As discussed previously, changes in the activity of the HPA axis and plasma glucocorticoid concentrations are associated with female reproductive state in several snakes (Whittier et al., 1987; Lutterschmidt et al., 2009; Robert et al., 2009; Stahlschmidt et al., 2013; Sparkman et al., 2014; Dayger et al., 2018). In northern populations of red-sided gartersnakes, both male and female snakes exhibit elevated baseline glucocorticoid concentrations during the spring breeding season (reviewed in Krohmer and Lutterschmidt, 2011; Dayger and Lutterschmidt, 2016, 2017). Furthermore, if glucocorticoid concentrations are experimentally decreased during the breeding season, male snakes show a premature activation of feeding behavior and will choose to follow prey trails over female pheromone trails (Lutterschmidt and Maine, 2014). Together, these examples indicate that in at least some snakes, a sustained elevation in baseline glucocorticoids is necessary to facilitate and support seasonal reproductive behavior. An additional observation is that baseline CORT concentrations vary consistently with pace-of-life strategies in populations of western terrestrial garter snakes (*T. elegans*; Holden et al., 2022). Therefore, similar to the conclusion of Romero and Beattie (2022), it cannot be assumed that sustained elevations in plasma glucocorticoids indicate that snakes are experiencing chronic stress and its associated negative impacts.

The fourth myth discussed by Romero and Beattie (2022) is that glucocorticoids mobilize energy to cope with and survive short-term stressors. Those authors present a convincing argument to refute this myth: 1) the time course of the HPA response to a stressor does not align with that needed for an increase in blood glucose to facilitate coping with a stressor; and 2) one of the specific actions of glucocorticoids on cells is to remove glucose transporters from cell membranes, including those of muscle and brain cells (Romero and Beattie, 2022 and references therein). Thus, the net effect of glucocorticoids is to modify (i.e., decrease) glucose utilization by cells. This can, but does not always, result in an increase in blood glucose levels, because the net effects of stress and glucocorticoids on blood glucose vary with the fed state of the animal. In fed animals, for example, glucocorticoids often do not alter blood glucose concentrations (Romero and Beattie, 2022 and references therein).

Overall, the available data in snakes support these arguments that glucocorticoids do not directly increase glucose availability. First, plasma glucose concentrations increase more rapidly in response to stress than do plasma glucocorticoids in both common gartersnakes (*Thamnophis sirtalis*; Gangloff et al., 2017) and wild dice snakes (*Natrix tessellata*; Lakušić et al., 2020), indicating that the release of epinephrine and norepinephrine during a stress response likely induce the initial changes in glucose mobilization (see Stinner and Ely, 1993 for stress-induced measures of these metabolites in black racers, *Coluber constrictor*). Furthermore, Neuman-Lee et al. (2020) demonstrated that while capture stress significantly increases plasma glucose in wandering gartersnakes, *Thamnophis elegans vagrans*, stress-induced glucose concentrations are not significantly related to plasma corticosterone nor does treatment of snakes with exogenous corticosterone significantly change plasma glucose. Secondly, changes in plasma glucose during a stress response indeed vary with the fed state of snakes. In common watersnakes, *Nerodia sipedon*, food restriction increased plasma corticosterone but produced no changes in plasma glucose (Webb et al., 2017), whereas stress-induced glucose concentrations were higher in wild dice snakes that had a partially digested meal in their stomachs (Lakušić et al., 2020). Similarly, stress-induced increases in both corticosterone and glucose were significantly higher in Florida cottonmouths with access to plentiful food resources compared to cottonmouths experiencing a naturally occurring, acute reduction in food availability (Sandfoss et al., 2020). Finally, preliminary data in red-sided gartersnakes indicate that blood glucose concentrations are not altered by capture stress during the spring mating season, when snakes exhibit seasonal anorexia (Lutterschmidt, unpublished data). While glucocorticoids may not directly influence glucose availability, glucose may serve as a metric of stress independently- we discuss this further in Energetics and Metabolism.

In summary, advancing our understanding of stress biology will require that we embrace, rather than diminish or dismiss, observed variation in responses across seasons, years, individuals, populations, and species. Indeed, it is this variation upon which selection will act. As in other animals, the effects of stress and glucocorticoids on the physiology and behavior of snakes are complex. While it can be difficult to interpret and model these effects, a critical starting point is acknowledging the common false assumptions about glucocorticoid functions and the limitations they can impart when integrating new data into the existing framework.

Glucocorticoid Actions in Snakes

Virtually all studies of stress examine how stress-induced changes in glucocorticoids are correlated with other variables (e.g., changes in sex steroids, behavior, immune function, etc.). We acknowledge that such correlations are important for understanding the actions of glucocorticoids. However, direct actions of glucocorticoids are best understood from studies that experimentally manipulate circulating glucocorticoids. Because there are so little data on glucocorticoid actions in snakes specifically, we have highlighted what we do and do not know about the actions of glucocorticoids in snakes in Table 1 to provide a more straightforward synopsis. This information will be critical for understanding and interpreting the consequences of snake CORT responses to the stressors reviewed in the next section. We defer the discussion of CORT correlated with stress responses to the next section, where we address the responses of snakes to different categories of stressors.

Table 1. Summary of exogenous glucocorticoid actions observed in snakes as determined by manipulative experiments

Expected Effects of Glucocorticoids	Data Supporting Expected Effect	Data Not Supporting Expected Effect	Notes/Caveats
Modulation of reproductive and other behaviors	Decreased courtship behavior in male red-sided gartersnakes, <i>Thamnophis sirtalis parietalis</i> (Moore and Mason 2001; Lutterschmidt et al. 2004). Decreased receptivity in female red-sided gartersnakes, <i>Thamnophis sirtalis parietalis</i> (Dayger et al. 2013).	No effect on defensive behavior in southern Pacific rattlesnakes, <i>Crotalus helleri</i> (Claunch et al. 2017a).	Effects of glucocorticoids on female receptivity depended on body condition index (Dayger et al. 2013).
Reduction in sex steroid hormones	No data available.	No effect on androgen concentrations of male red-sided gartersnakes during the spring mating season (Moore and Mason 2001; Lutterschmidt et al. 2004) No effect on testosterone concentrations of southern Pacific rattlesnakes, <i>Crotalus helleri</i> (Claunch et al. 2017a).	Northern populations of red-sided gartersnakes typically have low sex steroid hormone concentrations during the mating season relative to other times of year, a characteristic of temporally dissociated reproductive patterns (Lutterschmidt 2012).
Inhibited feeding behavior and/or digestion	Experimentally decreasing glucocorticoids via treatment with metyrapone (Lutterschmidt and Maine 2014) or a receptor antagonist (DIL, unpublished data) prematurely induces feeding behavior in red-sided gartersnakes, <i>Thamnophis sirtalis parietalis</i> .	No effect on whole-venom composition, total protein content, or enzymatic activity of snake venom metalloproteinases or phospholipase A ₂ in southern Pacific rattlesnakes, <i>Crotalus helleri</i> (Claunch et al. 2021b).	Northern populations of red-sided gartersnakes have elevated glucocorticoid concentrations during the mating season that decline around the time that snakes begin to migrate to summer feeding grounds (Lutterschmidt and Maine 2014).
Modified glucose metabolism and/or metabolic rate	Increase in glucose following dexamethasone injection in Florida cottonmouths, <i>Agkistrodon conanti</i> (Sandfoss et al. 2020)	No effect of exogenous corticosterone, ACTH, or metyrapone on plasma glucose concentrations of wandering gartersnakes, <i>Thamnophis elegans vagrans</i> , during the fall (Neuman-Lee et al. 2020).	In Sandfoss et al. 2020 dexamethasone was administered after 1 h capture stress.
Modulation of immune function	Decrease in hemagglutination ability and H:L ratio following dexamethasone injection in Florida cottonmouths, <i>Agkistrodon conanti</i> (Sandfoss et al. 2020). Increase in H:L ratio following exogenous corticosterone application in prairie rattlesnakes, <i>Crotalus viridis</i> (Guillette 2016).		Change in H:L ratio may be due to redistribution of cells and may not represent immunosuppression. In Sandfoss et al. 2020 dexamethasone was administered after 1 h capture stress.
Modulation of biological rhythms and/or melatonin	Decreased synthesis of nighttime melatonin from tryptophan precursor in male red-sided gartersnakes, <i>Thamnophis sirtalis parietalis</i> (Lutterschmidt and Mason 2010).		
Effects on development	Treatment of gravid western terrestrial gartersnakes, <i>Thamnophis elegans</i> , with transdermal corticosterone increased offspring mortality and altered offspring morphology (i.e., decreased length in female offspring). Additional effects of maternal corticosterone on escape behaviors, the rate of ROS production, and DNA damage in offspring depended on ecotype (Robert et al. 2009; Robert and Bronikowski 2010).		

Immune Metrics

Overview of Immunity and Stress in Snakes

Snakes, like all vertebrates, have a complex immune system that is highly reactive to stress (Martin, 2009). Immunity in the context of stress can be measured in many ways, which we highlight below. There are two main branches of immunity, which interact with each other to varying degrees: innate immunity and adaptive immunity. Broadly, innate immunity is responsible for immediately responding to antigens, assessing threats, isolating and removing the threat if possible, and recruiting molecular and cellular components of the adaptive system. Most antigenic threats are cleared by the innate immune response through the detection of specific predetermined antigenic patterns via genetically programmed receptors, such as Toll-like receptors (Aderem and Ulevitch, 2000) and peptidoglycan recognition proteins (Dziarski and Gupta, 2017) in addition to a wide repertoire of molecules and cells (Table 2). All vertebrates also have some form of adaptive immunity (Fearon and Locksley, 1996), which is more plastic and more specific, but slower to respond than innate immunity. Adaptive immunity is further divided into humoral and cell-mediated immunity. Humoral immunity generally refers to antibody-mediated immunity whereby cells that recognize specific pathogens (helper T cells, TH cells, CD4 T cells) activate B cells, which produce specific antibodies for the pathogen. Humoral immunity is activated primarily for extracellular pathogens, such as most bacteria. Intracellular pathogens, such as viruses, activate the Major Histocompatibility Complex (MHC) proteins to signal direct killing by another set of cells (Cytotoxic T cells, CD8 T cells).

Stress can have a profound influence on the immune response, but the changes in immune function depend on the magnitude, intensity, and duration of the stressor, as well as associated changes in circulating glucocorticoids (Dhabhar, 2009). Most of the procedures for assessing immune function require blood or tissue samples, of which the sampling can be a stressor itself. The acute rise of glucocorticoids during a stressor typically stimulates immune activity, while chronic glucocorticoid exposure is correlated with a suppression of immune function. The pattern of immune redistribution during acute stress has been classically explained by the idea that hormones associated with stress (glucocorticoids, epinephrine, and norepinephrine) modify energy metabolism for essential survival purposes and to facilitate healing from injury incurred by the stressor. Mechanistically, the redistribution of immune components during stress likely is due to direct binding of glucocorticoids to receptors on immune cells (Sapolsky et al., 2000). Also, glucocorticoids facilitate a rapid release of pro-inflammatory cytokines with a concomitant upregulation of cytokine receptors on target cells (Dhabhar, 2009). Care must be taken when sampling snakes to collect tissues rapidly so that immune parameters represent baseline values and not those stimulated by the stress of capture.

It is important to note that immune activation may not be observed in a blood sample at all timepoints. Because cells and molecular components are mobilizing to a specific location of potential infection during the inflammatory response, the blood must be drawn during the mobilization prior to the aggregation at the tissue site. For example, if a snake has a large injury, a lower immune response in the bloodstream compared to pre-injury levels could be observed if the immune components have already mobilized and are activated at the site (Dhabhar et al., 2012). Other methods, such as observing the rapidity of wound healing, are helpful in

addressing this concern as wound healing is a holistic and integrative measure of immune function (Christian et al., 2006; French et al., 2006).

Table 2. Summary of immune functions in reptiles as they relate to components best studied in mammalian systems

Mammalian Immune Component	Function	Reptilian Analogs
Neutrophils	Phagocytosis	Heterophils
Neutrophils	Oxidative burst activity	Azurophils (de Carvalho et al. 2016, 2017)
Eosinophils	Parasitic responses	Identified only in elapid snakes (Salakij et al. 2002)
Basophils	Release of histamine	Basophils (Salakij et al. 2002)
Lymphocytes	Cells of lymphatic system; B Cells can be phagocytotic or produce antibodies and T Cells can be apoptotic or intermediary cells	Lymphocytes (Rossi et al. 2009; de Carvalho et al. 2016, 2017) Phagocytotic B cells (Zimmerman et al. 2010)
Natural Killer Cells	Apoptosis	Natural Killer Cells (Rousselet et al. 2013)
Monocytes	Matures into macrophages	Monocytes (Rossi et al. 2009; Rousselet et al. 2013)
Macrophages	Phagocytosis	Macrophages (Pasmans 2001)
Toll-like Receptors (TLRs)	Pattern recognition receptors on self-cells	TLR 2, 3, 4 and 7/8 (La Flamme et al. 2010), TLR 5 (Voogdt et al. 2016) evidence of TLR-15, which is not found in mammals (Boyd et al. 2012)
Cytokines	Signaling molecules	Many cytokines are highly conserved evolutionarily (Zimmerman, Bowden, and Vogel 2014; Secombes, Wang, and Bird 2016); cytokines such as TNF- α are found in arthropods (Wiens and Glenney 2011), fish (Roca et al. 2008), amphibians (Xi et al. 2017; Gallant and Hogan 2018), mammals, and birds (Rohde et al. 2018) and match sequences from turtles (Shaffer et al. 2013). IL-6 activity has been demonstrated in unicellular organisms (Csaba, Kovács, and Falus 1995) and amphibians (Xi et al. 2017), fish (Øvergård et al. 2012), mammals, and birds (Schneider et al. 2001).
Complement	Protein cascade that ends in membrane disruption in pathogenic cell, instigation of inflammatory response, and facilitating antibody responses	Complement (Merchant et al. 2005; Baker et al. 2019; Adamovicz et al. 2020)
Antimicrobial Peptides	Destruction of pathogenic membrane	Antimicrobial peptides (van Hoek 2014)

Because a prolonged stress response is energetically expensive, immune responsivity is often suppressed during chronic stress (Wingfield and Boonstra, 2013). Mechanistically, this is thought to occur when the higher affinity mineralocorticoid receptors are saturated and glucocorticoid receptors are activated (Sapolsky et al., 2000; see also *Glucocorticoids: Corticosterone (CORT)*). Indeed, a prolonged or chronic activation of the stress response is often thought to have pathological effects. As such, disease can be considered both a metric or consequence of stress- resulting from chronic activation of the stress response- and a contributing factor to the stress response itself. We discuss disease in more detail in the Snake Stressors section and focus here on components of immune responses relevant in both healthy and diseased animals.

Quantifying Immune Function

Blood Films and Cell Ratios

A single drop of blood can be placed on a slide to create a monolayer of cells. Using a common Giemsa-Wright staining protocol, leukocytes such as monocytes, heterophils (reptilian

equivalent of neutrophils), eosinophils, basophils, and lymphocytes can be differentiated based on size, granulation, and nuclear morphology. Profiles of all leukocytes can be useful as a measure of stress (Davis et al., 2008), though the ratio of heterophils to lymphocytes is most common (Vleck et al., 2000; Goessling et al., 2015). Theoretically, individuals experiencing chronic stress should have a higher heterophil to lymphocyte (H:L) ratio (Davis et al., 2008). In snakes, at least one study has shown this expected pattern (Stahlschmidt et al., 2013). However, there are also many studies that have seen no apparent correlation between H:L ratio and CORT or stress in snakes (Sperry et al., 2009; Sparkman et al., 2014; Fabrício-Neto et al., 2019; Holden et al., 2019; Bruschi et al., 2020a; Sandfoss et al., 2020). One study in gartersnakes found that the H:L ratio was correlated with baseline CORT, but not CORT concentrations at 3 hours post-capture or 3 days after captivity, indicating that H:L ratio may not change rapidly (Gangloff et al., 2017). While an attractive tool because of its ease, it may thus be limited in its use for monitoring snake stress.

Table 3. Summary of immune assays and requirements for use in snakes

Assay/Technique	Pathogen/Challenge	Plasma State	Immune Component Tested	Amount Needed	Protocols Applied to Snakes
Flow Cytometry	NA	Fresh (unfrozen)	Phagocytosis; NK Cells; Peripheral blood cells	Variable	de Carvalho et al. 2006, 2016
Microscopy	NA	Fresh	Phagocytosis; NK Cells; Peripheral blood cells	Variable	de Carvalho et al. 2017
Microscopy	NA	Whole blood smear	Monocytes; Heterophils; Granulocytes	Single drop of whole blood	Stahlschmidt et al. 2013; Sparkman et al. 2014
Microbial Killing Assay	<i>Escherichia coli</i> (Gram – bacteria) ATCC: 8739	Fresh	Complement; Phagocytosis; Natural antibodies; Anti-microbial peptides	20 µl	
Microbial Killing Assay		Frozen	Complement; Nabs; Anti-microbial peptides	20 µl	Neuman-Lee et al. 2015a; Fabrício-Neto et al. 2019; Claunch et al. 2021a;
Hemolytic/Agglutination Assay	Sheep red blood cells (SRBC)	Frozen* + Heat Treatment	Natural antibodies	40 µl	
Hemolytic/Agglutination Assay		Frozen*	Natural antibodies; Complement	40 µl	Stahlschmidt et al. 2017; Bruschi et al. 2020a
Oxidative Stress	Reactive oxygen species and protective anti-oxidant barrier	Frozen	Phagosome burst and mitochondrial respiration	40 µl	Neuman-Lee, Van Wettere, and French 2019
Specific Antibody Production	SRBC injection	Frozen, after inoculation	SRBC-specific antibodies	20 µl	

* Hemolysis may only be apparent in fresh plasma.

Amount needed refers to the average volume of plasma necessary for one replicate of an assay based on the authors' experience. Actual amounts will vary in practice and should be optimized for each species, population, incubation temperature, and approach. Please refer to the text for more detail on variations of methodological approaches for each technique.

Antimicrobial Capacity or Bacterial Killing Assay

The antimicrobial capacity assay, often called the bacterial killing assay (BKA), is one of the most commonly used techniques in immune function research with snakes. Briefly, a known

amount of microbe (typically *Escherichia coli*, but other microbes can be used to determine activation of different immune components) is combined with plasma and compared to a positive control with no plasma added. The amount of immune activity is determined by calculating the percentage of killed bacterial colonies in the samples compared to the control (French and Neuman-Lee, 2012). This method can be performed with live blood samples, which can account for cellular and molecular activity, or frozen samples, which represent only molecular activity (Demas et al., 2011). Further, this assay is suitable for use in agar plates or 96-well plates, both of which have been validated for snakes (Sparkman and Palacios, 2009; Neuman-Lee et al., 2015a).

Some snake studies have demonstrated negative correlations between glucocorticoids and bactericidal ability (Holden et al., 2019; Claunch et al., 2021a). Other studies have shown that acute stress increases bactericidal ability, including stress associated with handling (Neuman-Lee et al., 2015a; Spence et al., 2020) and exposure to toxins (Neuman-Lee et al., 2016). Yet some studies indicate that bactericidal ability is not always correlated with stress or glucocorticoids (Neuman-Lee et al., 2015b, 2017; Fabrício-Neto et al., 2019), so the utility of this assay may be context- and species-specific.

Lysis and Agglutination Assay

This assay is conducted as a single assay, but components are analyzed separately to determine the role of natural antibodies, antimicrobial peptides, and complement in a 96-well plate (Matson et al., 2005). This assay uses red blood cells (typically derived from sheep) as an antigen. Molecular components will either lyse or agglutinate cells, both of which are optically measurable. Lysis is measured using an absorbance plate reader and agglutination is scored visually (Matson et al., 2005). Both natural antibodies and complement-mediated lysis are measured in this assay. Because complement is extremely heat-labile, it is possible to assess the relative contribution of complement by conducting and comparing two assays with the same individual—one with heat-killed plasma and one with fully functional plasma. It is important to note that while it is possible to conduct these assays with frozen plasma, necessary volumes to observe hemagglutination or lysis may be greater than in fresh plasma, and lysis may be inhibited in frozen plasma (Neuman-Lee *unpubl. data*).

There are some studies in snakes that have demonstrated a negative relationship between stress and lysis/agglutination titers (Luoma et al., 2016; Holden et al., 2019; Bruschi et al., 2020a), while others have shown no correlation between stress and natural antibodies and complement (Stahlschmidt et al., 2017). In Stahlschmidt et al. (2013), levels of CORT were reduced and the lysis titer increased post-reproduction in Children's pythons, *Antaresia childreni*, while agglutination titers remained unchanged.

Oxidative Stress

During the mitochondrial activity of respiration, reactive oxygen species (ROS) are formed (McCord, 2000; Schafer and Buettner, 2001; Turrens, 2003). Traditionally, ROS were thought to be only detrimental, but strong evidence shows that ROS can also be used to combat infections through oxidative bursts in phagosomes from macrophages in addition to possessing

other immune-mediating properties (Sena and Chandel, 2012). Measuring the antioxidant barrier- the suite of antioxidant defenses- is also critical because if ROS production outpaces the barrier's production, oxidative stress results and can cause damage to self-cells (McCord, 2000; Schafer and Buettner, 2001; Costantini and Møller, 2009). The amount of oxidative stress is calculated by converting both metrics to standard units and then dividing ROS by anti-oxidative barrier (e.g., total non-enzymatic antioxidant capacity), which can also be measured using commercially available kits (Neuman-Lee et al., 2019). While oxidative stress is a component of immunity, it is also a byproduct of other cellular functions, and is discussed in more detail in the next section: "Molecular Markers".

Inflammation

Inflammation is associated with many diseases and can be induced by a range of stressors— from infection to injury (Chovatiya and Medzhitov, 2014; Liu et al., 2017). Although inflammation is typically examined at the tissue level, through biopsy, including in snakes (e.g., Smith et al., 1988; Jakob and Wesemeier, 1995; Pereira and Viner, 2008; Mayer et al., 2015; Neuman-Lee et al., 2019), several molecular biomarkers can serve as less invasive proxies for inflammation. Transaminases (a.k.a., aminotransferases) are enzymes facilitating the synthesis of amino acids, and elevated plasma levels of aspartate transaminase (AST) and alanine transaminase (ALT) indicate inflammation in the liver and other organs in humans (Ioannou et al., 2006; Rahmani et al., 2019). Both AST and ALT have been measured in snakes (Seniów, 1976; Ramsay and Dotson, 1995; Johnson and Benson, 1996; Gillett et al., 2015), but the clinical and ecological significance of these enzymes in snake physiology is not fully understood. The tumor necrosis family (TNF) comprises cytokines that stimulate the recruitment of inflammatory cells and production of reactive oxygen species (reviewed in Chu, 2013). Lizards express TNF (reviewed in Gliński and Buczek, 1999), but TNF's role in snake stress physiology is unknown. Currently, researchers have an extremely limited understanding of the value of inflammation-related molecular biomarkers in snake stress.

Wound Healing

Wound healing is a holistic and integrative measure of functional immunity that is highly sensitive to stress (Christian et al., 2006). Inflammation, cytokines, immune cells, and growth factors interact to heal cutaneous wounds (Werner and Grose, 2003; Barrientos et al., 2008). Reptilian skin has a very thin dermis, which results in horizontal healing, versus the combined horizontal and vertical healing of mammalian dermal tissue repair (Maderson and Roth, 1972; Smith and Barker, 1988). This means that measuring the stages of wound healing (inflammation, recruitment and proliferation, granulation, and remodeling) is straightforward. Using a constant wound size produced by a biopsy punch, photographs are used to measure the rate of healing as the circumference of the wound decreases (French et al., 2006; Neuman-Lee and French, 2014).

To date, few studies have examined wound healing in snakes (Smith et al., 1988; Maas et al., 2010; Hopkins et al., 2013) and only one study has explicitly examined wound healing efficacy in response to stress. Wound healing was hindered by chronic stress and food

restriction in terrestrial gartersnakes (*Thamnophis elegans*, Neuman-Lee et al., 2015a). There are, however, multiple studies in lizards that have demonstrated chronic stress suppression of wound healing (French et al., 2007, 2010), indicating this pattern may occur in other squamates. Of note, one lizard species prioritized wound healing over bacterial killing ability while acute glucocorticoid concentrations were elevated (Neuman-Lee and French, 2014).

Microbiome

Strong evidence shows that a diverse microbiome and presence of certain microbes can indicate a more robust immune system (Lee and Mazmanian, 2010; Boehm, 2012). While there is much left to understand about the intricacies of immune/microbiome dynamics, it can unquestionably be considered an aspect of immune and physiological function (Kohl and Carey, 2016; Evans et al., 2017; Garcia-Reyero, 2018). Snake microbial communities are poorly studied, although a handful of studies have characterized microbiomes in the gut and skin across snake taxa (Colston et al., 2015; Tang et al., 2019; Dallas et al., 2021). Most snake studies have examined microbial diversity and changes in response to disease, especially ophidiomycosis (Snake Fungal Disease; Allender et al., 2018; Hill et al., 2018; Walker et al., 2019; Smith et al., 2021).

Molecular Markers

As in other animals, stress physiology in snakes is complex and involves a suite of molecular responses to stressors that mitigate damage and maintain homeostasis. Thus, researchers use a range of molecular biomarkers to assess stress in snakes. In general, stress biomarkers fall into one of two categories. First, some biomolecules indicate direct damage to critical macromolecules (e.g., fragmented DNA or products of oxidized lipid). Second, other biomolecules are up-regulated in response to damage (e.g., molecular chaperones designed to refold damaged proteins). Below, we briefly summarize the use of molecular biomarkers of stress in snakes, discuss their limitations and caveats, and suggest future directions.

Reactive Oxygen and Nitrogen Species

Reactive oxygen and nitrogen species are endogenously produced by normal metabolic processes and are important for physiological signaling (reviewed in Sies and Jones, 2020). However, many stressors induce the over-production of reactive species or free radicals, which can oxidatively damage important biological macromolecules. The measurement of reactive species in vivo can be difficult because they are typically highly unstable with biological half-lives ranging from microseconds to milliseconds (reviewed in Griendling et al., 2016). Also, the simple presence of reactive species does not ensure molecular damage because reactive species can be quenched by antioxidants before they damage DNA or other biomolecules—that is, oxidative stress reflects the imbalance between pro-oxidants and antioxidants (Hörak and Cohen, 2010; Metcalfe and Alonso-Alvarez, 2010). Therefore, physiological ecologists tend to

measure the down-stream molecular products of oxidative damage (see below) rather than pro-oxidant radicals themselves.

Lipid Peroxidation

Lipid peroxidation is the process of oxidative damage to lipids, often by free radicals, and is a useful measure of stress because lipid peroxidation leads to cell damage and cell death. Lipid peroxidation can be assessed as the amount of malondialdehyde (MDA) in blood plasma or other tissues, and MDA has been measured in the Children's python, *Antaresia childreni*, grass snake *Natrix natrix*, and dice snake, *Natrix tessellata* (Stahlschmidt et al., 2013; Gavrić et al., 2019). However, the lipid content and storage conditions of tissues can influence the amount of MDA detected by commercial assays (reviewed in Tsikas, 2017). Carbonyl groups (aldehydes or ketones) are added to protein side chains when they are oxidized (Dalle-Donne et al., 2003), and protein carbonyl assays have been used as biomarkers of oxidative damage to proteins in the asp viper (*Vipera aspis*; Stier et al., 2017). Yet, there are also caveats to measuring protein carbonyls (Rogowska-Wrzęsinska et al., 2014; Augustyniak et al., 2015)—for example, MDA can interfere with the formation and detection of protein carbonyls (Estévez et al., 2019). Derivatives of reactive oxygen metabolites (d-ROMs) serve as proxies for organic hydroperoxides, which are formed through both lipid and protein oxidation (reviewed in Costantini, 2016), and the d-ROMs test has been used to assess oxidative damage in the common gartersnake, *Thamnophis sirtalis* and corn snake, *Pantherophis guttatus* (Stahlschmidt et al., 2017; Neuman-Lee et al., 2019). Commercial spectrophotometric kits for measuring MDA, protein carbonyls, and d-ROMs are readily available. The comet assay (or single cell gel electrophoresis) is an excellent method to measure baseline and inducible DNA damage, as well as DNA damage repair, that has been used in a number of colubrid snakes: checkered garter snakes (*Thamnophis marcianus*), African house snakes (*Lamprophis fuliginosus*), diadem snakes (*Spalerosophis diadema*), king snakes (*Lampropeltis getula*), trinket snakes (*Coelognathus helena*), and corn snakes (*Pantherophis guttatus*) (Bronikowski, 2008). The comet assay requires intact cells (i.e., rather than plasma that can be stored for months prior to assaying lipid or protein oxidation), which may hamper its practical applications for some snake research.

Heat Shock Proteins

Heat shock proteins (Hsps) comprise a large family of molecular chaperones that provide protection from thermal challenges, as well as other potential stressors (e.g., hypoxia and cytotoxins), for all organisms (reviewed in Miller and Fort, 2018). Some Hsps are inducible (i.e., upregulated in response to stressors) while others are only constitutively expressed. Therefore, inducible Hsps are ideal candidates to assess an animal's stress response. Work in the common gartersnake indicates that plasma CORT, liver Hsp70, and two isoforms of liver Hsp40 are all strongly upregulated in response to a heat challenge (Schwartz and Bronikowski, 2013). Yet, the use of Hsps to assess stress in other snakes is unknown and begs further exploration. Because Hsps are largely found intracellularly, intact cells (rather than blood

plasma) are typically assayed for Hsps, and this may limit the utility of measuring Hsps for some snake researchers.

Telomeres

Telomeres are DNA-protein complexes at the ends of chromosomes that shorten with cell division (Blackburn, 1991). Thus, telomere length (TL) is typically reduced in older individuals, and telomere shortening can be accelerated by stress (Kotrschal et al., 2007; Haussmann and Marchetto, 2010; Reichert and Stier, 2017). Telomere length has been measured in common gartersnake, *Thamnophis sirtalis*, the red-sided gartersnake, *T. s. parietalis*, Siamese cobra, *Naja kaouthia*, water python, *Liasis fuscus*, and two species of ratsnakes (*Elaphe climacophora* and *E. quadrivirgata*), typically by first using a DNA extraction kit followed by real-time quantitative PCR (Bronikowski, 2008; Ujvari and Madsen, 2009; Rollings et al., 2017; Singchat et al., 2019; Cunningham et al., 2021). This work indicates a tenuous link between TL and chronological age—for example, telomeres can lengthen during early life, and females tend to have longer telomeres than males (Ujvari and Madsen, 2009; Rollings et al., 2017; Singchat et al., 2019). Further, there is equivocal evidence supporting the link between TL and stress in snakes. Body condition positively correlates with telomere length in red-sided gartersnakes (Rollings et al., 2017), but radiation exposure does not reduce telomere length in ratsnakes (Cunningham et al., 2021). These mixed results are influenced by our limited understanding of the dynamics by which snakes express telomerase, an enzyme that can reduce telomere shortening (Blackburn, 1991) and is activated by estrogen in mammals (Kyo et al., 1999).

Mitochondrial Function

Mitochondria are essential for most of animal metabolism, and mitochondrial dysfunction can occur in response to many stressors (reviewed in Picard et al., 2018; Machiela et al., 2020). Mitochondrial homeostasis is assessed by evaluating several molecular markers, including baseline and maximal respiration, ATP reactive oxygen species production, and proton leak across its inner membrane (reviewed in Little et al., 2020). The physiology of liver mitochondria has been evaluated in several species of colubrids: checkered garter snakes (*Thamnophis marcianus*), African house snakes (*Lamprophis fuliginosus*), diadem snakes (*Spalerosophis diadema*), king snakes (*Lampropeltis getula*), trinket snakes (*Coelognathus helena*), and corn snakes (*Pantherophis guttatus*; Robert et al., 2007) and western terrestrial garter snakes (*T. elegans*; Robert and Bronikowski, 2010) in the context of aging (sensu ‘pace of life’ theory or ‘free radical theory of aging’). However, mitochondrial function has not been analyzed by snake researchers to explicitly investigate stress physiology (e.g., the effects of elevated temperature or ACTH administration on mitochondrial function).

Gene Expression

RNA-sequencing (RNA-seq) is a next-generation approach that quantifies the relative expression of every gene in a sample of tissue, even in species without fully annotated genomes (Grabherr et al., 2011). Because many genes respond to stressors (Kassahn et al., 2009), RNA-

seq is a robust method for detecting stress state in animals. Snake researchers have used RNA-seq to understand the evolution of venom composition (e.g., reviewed in Calvete and Lomonte, 2015), the ‘pace of life’ theory of aging (*T. elegans*: Schwartz et al., 2015), and the physiology of feeding and digestion (Burmese python, *Python molurus*: Wall et al., 2011; Andrew et al., 2015). However, this powerful tool has not been used to understand snakes’ responses to acute or chronic stress and can be limited to assumptions of homology of annotated genomes and the species at hand. Because RNA-seq generates so much data, *post hoc* correlations with any treatment are possible; thus, it is necessary to identify candidate genes and hypotheses *a priori*.

To conclude, researchers can quantify a dizzying array of stress-related molecules, each of which entails advantages, disadvantages, and caveats. For example, RNA-seq offers considerable advantages over older, more conventional methods of characterizing gene expression (e.g., quantitative PCR, which measures fewer gene products), but it can be expensive. Likewise, the comet assay attractively measures both DNA damage and repair, but it requires intact, living cells. Further, it is important to validate that a given biomarker truly represents stress (i.e., is biologically relevant) in one’s study system. If not, a statistically significant result (e.g., an immune challenge significantly increases ‘biomarker x’ by 10%) may not be biologically significant and, therefore, be misleading (*sensu* Martinez-Abraín, 2008). Also, it is often difficult to assign causality versus correlation in observational studies. While an increase in a molecular marker might occur in response to a stressor, it is often unclear if the marker is indicative of a detrimental effect or an adaptive response, unless we know more about the role of that marker. Therefore, we recommend integrating multiple molecular methods (if possible) and also combining them with non-molecular metrics (see above and below) when assessing stress in snakes. Thus, when choosing a molecular biomarker for stress, a number of practical concerns should be taken into consideration, including the type and amount of tissue, duration of tissue storage, standardization and validation of technique, and financial limitations.

Energetics, Metabolism, and Hydration

Glucocorticoids have for decades been thought of as hormones directly involved in the mobilization of stored energy required to effectively cope with stressors (Sapolsky et al., 2000; Romero, 2002). This classic role of glucocorticoids has been invoked to explain patterns observed between plasma GC levels and a variety of stressors and seasonal life-history events in free-living snakes (e.g., Taylor et al., 2005; Cease et al., 2007; Lind et al., 2010; Neuman-Lee et al., 2015a). Yet, no study has experimentally examined the effect of glucocorticoids on metabolic rate in snakes, and only recently have studies begun to critically examine the long-assumed relationship between glucocorticoids and glucose mobilization (Gangloff et al., 2017; Neuman-Lee et al., 2020; Lakušić et al., 2020; Sandfoss et al., 2020). The effect of glucocorticoids on metabolic rate and the mobilization of stored energy, as well as the contexts that impact the energetic consequences of stress-coping strategies, are ripe areas for future research. Below, we summarize our current knowledge of the impact of stress on host energetics and highlight key areas where targeted studies may vastly improve our current state of knowledge.

Changes in Glucose Metabolism and Availability

As indicated in the section “Glucocorticoids: Corticosterone”, recent research has called into question the direct role of glucocorticoids in the short-term mobilization of glucose to cope with acute stressors (see Romero and Beattie, 2022). Acute glycemic responses are mediated by catecholamines released by the sympathetic nervous system and are an important component of the acute stress response (Romero and Wingfield, 2015). However, most work aimed at characterizing the stress response in snakes and other reptiles has focused on the glucocorticoid response (Moore and Jessop, 2003). Inexpensive and easy to use glucometers developed for the measurement of glucose in human whole blood have recently been validated for use in snakes (Kelley et al., 2021), and a small group of recent studies has used these technologies to examine the impact of acute capture stress on glucose mobilization in snakes. Results thus far indicate a consistent elevation of glucose in response to capture stress in several species (Gangloff et al., 2017; Neuman-Lee et al., 2020; Lakušić et al., 2020, Sandfoss et al., 2020; Kelley et al., 2021). Additionally, baseline CORT is positively correlated with baseline glucose in free-living pygmy rattlesnakes, *Sistrurus miliarius* (Lind et al., 2020a), wandering gartersnakes, *Thamnophis elegans* (Neuman-Lee et al., 2020), common gartersnakes, *Thamnophis sirtalis* (Gangloff et al., 2017), and dice snakes, *Natrix tessellata* (Lakušić et al., 2020).

Relationships between stress-induced CORT and glucose have been less consistent, with no significant relationship observed in wandering gartersnakes (Neuman-Lee et al., 2020) and a positive relationship observed in both dice snakes (Lakušić et al., 2020) and Florida cottonmouths, *Agkistrodon conanti* (Sandfoss et al., 2020). Taken together, these results support that glucose metabolism is modified during the acute stress response in snakes, and that glucose may provide a useful metric for characterizing the short-term fight or flight response. However, baseline glucose levels may vary due to responses unrelated to stress (e.g., feeding responses) and may not be responsive to certain types of stressors (e.g., Webb et al., 2017). Because stress responses vary seasonally in most animals, understanding if stress-induced changes in plasma glucose also vary seasonally would help in assessing the relationship between stress and glucose metabolism. For example, Neuman-Lee et al. (2020) found that season did not significantly affect the relationship between stress-induced CORT and glucose. Additionally, phylogeographic variation must be considered. For example, baseline and stress-induced glucose and CORT differed according to pace-of-life ecotypes of western terrestrial gartersnakes (*T. elegans*) in non-drought years (Holden et al., 2022), though the influence of temperature on these responses did not vary between them (Gangloff et al., 2016). However, the time course of stress-induced changes in plasma glucocorticoids and glucose differed significantly between populations of common gartersnakes (Gangloff et al., 2017). Further research to elucidate the potential effects of season, life history stage, and phylogenetic relationships on the acute glycemic response is needed to clarify the contexts in which glucose may serve as an informative stress metric.

Metabolic Rate

The mobilization and reallocation of energy is a fundamental component of the stress response (Sapolsky et al., 2000; Romero and Wingfield, 2015). Yet, few studies have directly quantified the metabolic cost of stress coping responses in snakes and other reptiles (but see DuRant et

al., 2008; Preest and Cree, 2008). While glucocorticoids are negatively related to body condition in diverse snake taxa (reviewed in Lind et al., 2018), studies addressing the relationship between glucocorticoids and energetics have been largely descriptive and indirect, leaving directions of causality unresolved. A recent study in eastern cottonmouths, *Agkistrodon piscivorus*, reported a weak positive correlation between metabolic rate and both baseline and stress-induced CORT (Richard, 2020). Exogenous CORT increases metabolic rate in amphibians (Wack et al., 2012) and lizards (DuRant et al., 2008; Preest and Cree, 2008), but experimental studies linking glucocorticoids and metabolism in snakes are not currently available. Studies have established significant metabolic increments associated with reproduction (e.g., Dupoué and Lourdais, 2014) and disease (e.g., Agugliaro et al., 2020) in snakes, and these may be interpreted broadly as metabolic coping responses. That being said, our current understanding of the metabolic costs of stress coping responses in snakes is lacking in general. In particular, the neuroendocrine regulatory mechanisms that adjust metabolically costly coping responses across contexts deserve direct attention.

Measuring metabolism as a metric to quantify energy mobilization during the vertebrate stress response makes intuitive sense; however, methods to assess metabolic stress coping responses often involve placing animals into stressful situations (e.g., respirometry chambers) that may disrupt natural HPA function. Quantifying the metabolic consequences of the stress response will require novel and creative approaches, and snakes exhibit a range of traits and behaviors that make them good models for field-based energetic studies (Beaupre and Duvall, 1998). In particular, the extreme low energy life-history and infrequent feeding strategies of many ambush-foraging taxa allows for examination of energetics while controlling for, or even manipulating, energetic status (e.g., Taylor et al., 2005). Additionally, many snakes can be manipulated and monitored in the field via radio telemetry, thus avoiding the impacts of captivity on HPA function and allowing natural movements and thermoregulatory behaviors. Snakes, as a model system, present a great opportunity for future research aimed at directly quantifying the energy mobilized to cope with diverse stressors under natural conditions.

Hydration

Maintaining homeostatic hydration is another challenge that many snake species face, especially sea snakes and desert species for which fresh water is often not available. Despite adaptations for coping without water for long time periods, the increasing frequency of drought may exacerbate hydration stress in snakes (see the section: “Climate Change”). Metrics for measuring hydration in snakes generally rely on collecting a blood sample to measure variables associated with water content of the snake’s body. These include hematocrit, plasma osmolality, and total body water. Hematocrit is the percent by volume of red blood cells in the plasma, and so hematocrit is expected to be elevated in dehydrated individuals due to a decrease in plasma volume. However, this metric is not ideal for measuring hydration per se because the amount of red blood cells in circulation can be independently impacted by numerous other variables (e.g., Webb et al., 2017). Plasma osmolality, measured using an osmometer, directly quantifies the solute concentration in plasma samples and is therefore a better measure of hydration.

Although poorly studied, snakes likely vary in the extent to which they defend homeostatic plasma osmolality at the expense of protein metabolism to liberate water. In other words, some

dehydrated snakes may maintain homeostatic plasma osmolality while they break down muscle tissue to obtain water (Brusch et al., 2018), potentially masking dehydration related stress if only osmolality is considered. Measuring total body water is the most accurate way to assess hydration in snakes but is also the most challenging. Snakes may be euthanized, weighed, then desiccated and re-weighed (Lillywhite et al., 1988); however, this method obviously is not helpful in studies of individual snakes over time or of sensitive species. Alternatively, total blood volume can be measured by dilution of red blood cells labeled with ^{51}Cr , and extracellular fluid volume by injection of thiocyanate (Lillywhite et al., 1988). Another way to measure total body water is with stable isotopes (e.g., Christian et al., 2007). After equilibration following injection with known quantities of isotopes, researchers then draw a blood sample and quantify the relative amounts of labeled and unlabeled water in the sample, which can be used to calculate the total amount of water in the body. Notably, if doubly labeled water (i.e., with hydrogen and oxygen stable isotopes) is used, then this technique can also be used to measure field metabolic rate in snakes (e.g., Beaupre, 1996; Peterson et al., 1999). In this case, the snake is released after the baseline blood draw, then recaptured weeks or months later for another blood draw. The change from baseline to resample in hydrogen isotopes represents total hydrogen lost in water during the time period between measurements, and the change in oxygen isotopes represents total oxygen lost in water and in carbon dioxide during that time period, and so the difference between the two can be calculated to show how much carbon dioxide was produced by the snake during that time period.

In addition to metrics of hydration, researchers interested in hydration stress may wish to quantify variables associated with thirst and/or regulation of water loss. Though poorly studied in snakes, thirst threshold can be quantified by offering fresh water to snakes. The easiest way to do this is to capture and carefully weigh snakes, offer them water, then reweigh them to measure how much water was ingested (Lillywhite et al., 2012). Sea snake drinking behavior can also be studied by observing them drinking fresh water floating on the surface of seawater (Lillywhite et al., 2015). For some wild terrestrial snakes, a good strategy to non-invasively study drinking is to simulate rainfall by spraying snakes with water from a pump-sprayer. This works well in species like rattlesnakes, which will coil and flatten their bodies to drink the water that collects on their skin (Phadnis et al., 2019).

Calculating water loss over long periods of time can be done via the doubly labeled water method described above. In addition, measuring the water content of fecal and urate samples by drying and weighing fresh samples can provide information on how much water the snake is losing via excretion. Snakes also lose water via ventilation and evaporation across their skin and cloaca. Total evaporative water loss is typically measured using flow-through hygrometry, where air is scrubbed of water before entering a respiratory chamber housing the snake, then water content of the air is measured as it leaves the chamber (Guillon et al., 2013). Cutaneous evaporative water loss has traditionally been measured by quantifying water movement across shed skins or skin of dead snakes (Dunson and Freda, 1985; Agugliaro and Reinert, 2005), and more recently using hand-held evaporimeters (Lourdais et al., 2017). Researchers can also measure the critical limit of dehydration by evaluating snakes for behavioral lethargy, loss of righting reflex, and changes in skin tone as water is withheld over time (see the Chapter “Drinking behavior and water balance in insular cottonmouth snakes”). Water loss and retention is partly regulated through fluctuation of hormones such as arginine vasotocin (AVT) and mineralocorticoids such as aldosterone (Mancera et al., 1990). Measurement of these hormones in association with hydration state may be informative in evaluating hydration stress.

It is also important to note that CORT has binding affinity for mineralocorticoid receptors (Sapolsky et al., 2000) and may directly influence water balance, so disentangling the role of CORT in responding to hydric and other stressors may be difficult (see Climate Change for more details).

Morphometrics

Morphology can be affected by stress, often in the form of variation in metrics of body condition or the expression of symmetrical physical traits. This variation can be developmental and long-lasting or much shorter in duration.

Condition Indices

An animal's body condition (or relative body mass) is typically assumed to reflect its energetic state, and body condition has long been linked to a range of important traits—from immunocompetence to survival (Evans, 1969; Navarro et al., 2003). Stored lipids are the primary energy reserve of animals (Bell and Coleman, 1980; Arrese and Soulages, 2010), and a number of body condition indices (BCIs) have been used to non-destructively estimate an animal's standardized fat content (e.g., mass of fat in animal after correcting for body size; Peig and Green, 2009, 2010; Labocha et al., 2014; Warner et al., 2016). BCIs are especially useful when considering nutritional stress. The unique elongate shape and lack of limbs in snakes means that variation in mass among individuals of similar length is due primarily to gut contents, embryos or eggs in females, muscle mass, or fat mass.

Taking care to account for gut contents and embryos allows comparison of individuals based on BCIs that therefore may reflect relative health, energetic status, and nutritional stress of individuals in a population. For example, a recent paper showed that numerous snake species in a tropical community experienced declines in body condition and abundance following a major die-off in their amphibian prey (Zipkin et al. 2020), providing data to confirm the expected result of nutritional stress following collapse of prey. Various BCIs are used as variables in analyses, ranging from ratio indices (e.g., mass divided by SVL) to residual indices (e.g., residuals from regression of log SVL and log mass) to specialized indices like the scaled mass index (SMI; Peig and Green, 2009, 2010); see Falk et al. (2017) for an extensive list of specific BCIs used in snake studies. Which BCI to use has been debated by some researchers (Weatherhead and Brown, 1996; Green, 2001; Hayes and Shonkwiler, 2001; Schulte-Hostedde et al., 2001; Peig and Green, 2010; Labocha et al., 2014; Barnett et al., 2015; Wilder et al., 2016; Falk et al., 2017), while others have questioned the necessity of selecting the best BCI (Labocha and Hayes, 2012; Cox and Calsbeek, 2015; Wilder et al., 2016).

Ideally, a BCI should be useful for all ages and sexes and insensitive to changes in body size, such that it removes the effects of ontogenetic growth or sexual size dimorphism (Peig and Green, 2010). Falk et al. (2017) showed that ratio-based BCIs may introduce more bias into analyses due to their strong association with body size than residual BCIs. This likely accounts for the more frequent use of residual-based and SMI indices in the literature on snakes. No matter which BCI is used, they are most useful when data sets are large (Peig and Green,

2010; Cox and Calsbeek, 2015) and when they contain data from individuals across the species' range of body sizes.

Fluctuating Asymmetry

Developmental differences that lead to morphological asymmetry in typically symmetrical physical traits without a clear directional bias are termed fluctuating asymmetry. In reptiles, fluctuating asymmetry (FA) may be a product of maternal or environmental stresses that occurred during pregnancy, egg formation, or incubation stages during development (reviewed in Laia et al., 2015). FA has been recorded in snakes, but is mostly limited to the number and shape of specific facial scales. Dice snakes (*Natrix tessellata*) from more recently established populations and from smaller habitats show asymmetry in several facial scales (Herczeg et al., 2005). Extreme temperatures during incubation were associated with FA in scales in western terrestrial gartersnakes (*Thamnophis elegans*; Arnold and Peterson, 2002) and grass snakes (*Natrix natrix*; Lowenborg et al., 2011).

Some studies found FA among individuals, but FA was not related to potential stressors. For example, Caspian whipsnakes (*Hierophis caspius*) and dice snakes (*Natrix tessellata*) did not show patterns of FA with disturbed habitat (Bellaagh et al., 2010). Other morphological traits, such as skeletal and hemipene asymmetry remain largely unexplored in snakes (but see Shine et al., 2000a). This may be in part due to high rates of measurement error associated with caliper measurements and measurements taken on photographs (Goodenough et al., 2012; Niemeier et al., 2019). High rates of measurement error are a common criticism of FA studies and may limit the utility of FA as a stress metric in living snakes. This has placed micro-3D-computed tomography-based measurements for geometric morphometrics of preserved specimens as the gold standard for FA studies (Niemeier et al., 2019). Another criticism is the potential for confirmation bias when the "stress" grouping of specimens is known a priori (Kozlov and Zvereva, 2015). However, there is still utility in FA studies of effects on stress on snake development, either through double-blind experimental methods or correlative investigations of environmental stressor gradients among museum specimens.

Behavior

There are many aspects of snake behavior that can be assessed as potential metrics of stress in observational, experimental, and comparative contexts. Snake behavior is well-studied (Gans and Tinkle, 1978; Gans and Crews, 1992) and as such, there are established ethograms for scoring snake behaviors that may be adapted for use in other snake species (e.g., Arnold and Bennett, 1984; Burger, 1998; Moore et al., 2000; Lutterschmidt et al., 2004). Ford (1995) provides a useful overview of experimental designs for studies of snake behavior. Below, we outline examples of behaviors that may be relevant to consider in studies of snake stress ecology.

Exploratory Behavior

Exploratory behavior of snakes may be useful in assessing how snakes respond to novel environments, which may potentially be stressful. Exploratory behavior, sometimes termed boldness behavior, is usually assessed in a standardized artificial environment or when presented with a stimulus. Behaviors include tongue flicking, freeze time (time before action), and interaction time with a stimulus (Chiszar et al., 1976, 1979). Refugia are important for thermoregulation and sources of shelter from predators for snakes. When shelter is available, exploratory behavior can be placed in context of shelter use, such as shelter exploration or residence time versus time spent locomoting in an open area (Stahlschmidt et al., 2016). Shelter use may be influenced by immune status in snakes as some snakes spend more time outside of a shelter attempting to bask when undergoing an immune challenge (e.g., Lorch et al., 2015; Todd et al., 2016).

Activity Area

Relative activity or movement following a stimulus may also be informative for field studies of stress in snakes. For example, snakes translocated to a new area tend to move more (Nowak et al., 2002; Sullivan et al., 2015; Wolfe et al., 2018a; but see Holding et al., 2014a), and snakes with reduced food availability (in areas managed specifically for prey suppression) also increase movements (Christy et al., 2017), while snakes in disturbed areas with high human use may move less (e.g., Lomas et al., 2019). Studies on movement generally require a protocol involving manual tracking or automated recording of location at regular intervals (Macartney et al., 1988; Ward et al., 2013; Smith et al., 2018; Whitney et al., 2021). Movement can then be assessed by comparing distances travelled at different scales (per week, per day, per movement), or sizes of activity areas (Macartney et al., 1988; Row and Blouin-Demers, 2006; Greenberg and McClintock, 2008). Analytical methods are important to consider when evaluating snake movement data (e.g., Macartney et al., 1988; Row and Blouin-Demers, 2006; Greenberg and McClintock, 2008).

Reproductive Behavior

When snakes are resistant to the disruptive effects of stress on reproductive behavior, as is the case in northern populations of red-sided gartersnakes (*Thamnophis sirtalis parietalis*), reproductive behaviors (or the absence of these behaviors) can serve as a useful measure for assessing the effects of stress in both sexes (Moore et al., 2000; Moore and Mason, 2001; Lutterschmidt et al., 2004; Dayger et al., 2013). Some metrics to consider include tongue-flicking, chin-rubbing, copulation (or latency to copulate), bridging, twisting, and combat.

Defensive Behavior

Perhaps the most obvious candidates for assessing stress are defensive behaviors in snakes. These behaviors can be assessed both in field and laboratory studies. When faced with a

potential predator or harassment, snakes will often attempt to escape rather than defend (Greene, 1988; Burger, 1998). As such, some studies will employ separate ethograms for fleeing/protective and defensive behavior (Burger, 1998). Fleeing behaviors can be categorized according to relative speed and shelter-seeking, for example freezing versus slowly crawling to hide in vegetation versus rapidly escaping under a cover object or into a tunnel (Burger, 1998). Sometimes snakes will exhibit head-hiding behaviors as a protective response (Claunch et al., 2017a).

Snakes can exhibit many defensive behaviors, sometimes simultaneously. Coiling to face the threat, rattling the tail, hissing, striking, musking, and defecating are observed in multiple species (Greene, 1988) and have been used in the context of assessing snake stress responses (e.g., Herr et al., 2017; Claunch et al., 2017a; Brashears et al., 2020). Regurgitation can occur when snakes are harassed (Greene, 1988) and may serve as a behavioral metric of extreme stress in laboratory-based studies where snakes are regularly fed (Bonnet et al., 2013), although this behavior would not likely be useful in field-based contexts where prey consumption rates are unknown. Depending on the snake species in question, there are several other antipredator display behaviors to consider incorporating into an ethogram: hooding, puffing, and head-flattening behavior (Greene, 1978), tail displays (Greene, 1978), thanatosis or death-feigning (Gehlbach, 1970), venom spitting (Westhoff et al., 2005), and cloacal eversion/popping (Young et al., 1999). Beyond the context of predator defense, snakes also exhibit similar behaviors (mouth gaping, body eversion, cloacal eversion, and/or tetany) to toxin exposure or pain (e.g., Mohammadi et al., 2017).

Whether to incorporate behavior and which types of behavioral responses to integrate into a study in snake stress will depend on careful consideration of the species, methodology, and specific research questions in mind.

Snake Stressors

There are a multitude of biotic and abiotic factors that may induce stress in snakes. Here, we review evidence for a number of potential snake stressors, based on their ability to disrupt homeostasis as outlined above. This section also serves to point out knowledge gaps related to the classification or magnitude of certain stressors and their consequences to snake ecology.

Capture

Capture stress and the resulting change in physiological metrics, primarily glucocorticoid hormones, have long been a standard method of evaluating the stress response in vertebrates. When applied to free-living animals, this technique can give baseline estimates (if the sample is taken rapidly after capture), peak or stress-induced estimates, as well as estimates of recovery from capture stress, all of which can be used to assess potential variation in the reactivity and sensitivity of the HPA axis. Ideally, capture and restraint stress protocols are designed and hypothesized to mimic, at least to some extent, the effects of a predatory attack on animals.

It is well-established across multiple taxonomic groups that responses to stressful stimuli are context-dependent, and this holds true for capture stress. For example, male rough-skinned

newts (*Taricha granulosa*; Rose et al., 1995; Coddington and Moore, 2003) subjected to capture stress exhibit a significant increase in plasma corticosterone and an inhibition of reproductive clasping behavior, and newts treated with exogenous corticosterone similarly exhibit reduced clasping behavior. However, if exogenous corticosterone is applied after newts have already engaged in clasping a female (or after treatment with arginine vasotocin), corticosterone fails to elicit an inhibition of reproductive behavior (Coddington and Moore, 2003).

Stress responses to capture can vary dramatically for many reasons, including, but not limited to, differences in the sensitivity of the HPA axis to stress, the rate of synthesis and/or secretion of HPA axis hormones at all levels, the expression of glucocorticoid and mineralocorticoid receptors in the brain that contribute to negative feedback regulation of the stress response, and even the perception of a potential stressor relative to other stimuli (Romero and Wingfield, 2015). In addition, the physiological and behavioral state of an animal, which is regulated and often characterized by elevated levels of other hormones, can also influence how an animal responds to capture stress. For example, female reproductive cycles are characterized not only by changes in plasma estradiol and progesterone concentrations but also by changes in the sensitivity and activity of the HPA axis in response to capture stress (Dayger and Lutterschmidt, 2016). Responses to stress can also vary with time of day, season, social experience, energetic state, activity patterns (e.g., migration), resource availability, environment, and phylogeny. For experimental convenience and repeatability, a standardized stressor was therefore developed: capture and restraint of an animal. In most studies, this entails capturing an animal and placing it in a relatively small, confined space, such as an opaque cloth bag or bucket for a given period. The primary feature of a capture stress protocol is that it must induce a stress response in the animal of interest, and therefore the natural history of each species should be considered when developing capture stress methods. For example, based on the hypothesis that sit-and-wait predators may not find small spaces “stressful,” Brashears et al. (2020) restrained pythons in cloth bags within buckets that were then placed on a plate shaker (set to 800 rpm for 1 h) to induce a significant stress response in different species of pythons.

The utility of capture stress techniques in understanding stress biology stems from the ability to standardize the stressor experimentally and then apply this standardized stimulus across different conditions and contexts. In this way, we can use capture and restraint stress protocols to understand when stress responses vary. In addition, capture stress is a tool with which further questions regarding “why” can be delineated. For example, if a difference in baseline or stress-induced corticosterone concentrations is observed in response to capture stress, a subsequent experiment using an ACTH challenge can help pinpoint if the observed variation results from differences in the sensitivity of the adrenal glands to ACTH. Variation may be due to differences in ACTH receptor expression on cells of the zona fasciculata layer of the adrenal cortex, which synthesize glucocorticoids (Figure 1).

A thorough review of the variation in responses to capture stress is beyond the scope and goals of the current chapter, particularly because variation in stress responses must first be studied thoroughly within a given population, species, or system before patterns can emerge that more broadly explain variation in stress biology across organisms and taxonomic groups. As such, the need for additional research on all aspects of stress biology, both within the species discussed in this chapter as well as other snake species, is clear and pressing. We provide a few examples here of how the application of standardized stress protocols across time,

environments, and species has provided clues as to the function of stress and its importance to an animal's ecological niche.

Studies employing capture and restraint stress have been used extensively in northern populations of red-sided gartersnakes, *Thamnophis sirtalis parietalis*, to understand if and how the HPA axis regulates seasonal changes in physiology and behavior. Moore et al. (2000) first demonstrated that the predicted behavioral response to capture stress (i.e., decreased reproductive behavior) is uncoupled from the hormonal response to capture stress (increased corticosterone and decreased testosterone) in male snakes during the spring mating season. Similar to other northern-latitude animal populations with limited breeding opportunities, these results help explain the observed phenomenon of reduced sensitivity to potential stressors (including the presence of researchers) during the mating season. Subsequent studies using an identical capture stress protocol have determined that hormonal responses to capture stress vary by season (Moore et al., 2001; Lutterschmidt and Mason, 2005), age (Moore et al., 2000), body condition (Dayger et al., 2013), reproductive and/or migratory state (Cease et al., 2007; Dayger and Lutterschmidt, 2017; Dayger et al. 2018), sex (Dayger and Lutterschmidt, 2017), and population (Moore et al., 2001; Lutterschmidt and Mason, 2005). For example, male red-sided gartersnakes do not exhibit blunted stress responses during the summer (Moore et al., 2001), and female snakes retain both hormonal and behavioral responses to capture stress during the mating season (Dayger et al., 2013; Dayger and Lutterschmidt, 2017). Treatment of snakes with an ACTH challenge demonstrated that male snakes are significantly less sensitive to ACTH during the spring compared to females in all seasons and males during the fall pre-dormancy period (Dayger and Lutterschmidt, 2016). These results suggest that a seasonal change in the responsiveness of the adrenal glands is important for maximizing male reproductive opportunities in this population. Similarly, responses of female snakes to capture stress during the spring mating season were greater in snakes in negative body condition (Dayger et al., 2013), although these differences do not appear to result from variation in the amount of glucocorticoid receptor protein expressed in the brain during the spring (Dayger et al., 2018). Together, these studies using capture stress provide a framework for understanding how modulation of the HPA axis is associated with and influences seasonal physiology and behavior within an ecological context.

In turn, comparing responses to capture stress across multiple populations and species helps establish patterns of HPA axis modulation and function. For example, one pattern that emerges from studies of capture stress in female snakes is that the sensitivity and/or activity of the HPA axis varies with reproductive state in Children's pythons (*Antaresia childreni*; Stahlschmidt et al., 2013), timber rattlesnakes (*Crotalus horridus*; Lutterschmidt et al., 2009), western terrestrial gartersnakes (*Thamnophis elegans*; Robert et al., 2009; also see Sparkman et al., 2014), asp viper (*Vipera aspis*; Lorient et al., 2016), and red-sided gartersnakes (*Thamnophis sirtalis parietalis*; Dayger et al., 2018). In addition, studies using standardized capture stress protocols underscore variation in HPA axis responsiveness and possibly function among different snake species, populations, and life-history ecotypes (Moore et al., 2001; Lutterschmidt and Mason, 2005; Palacios et al., 2012; Schwartz and Bronikowski, 2013; Brashears et al., 2020; Spence et al. 2020; Holden et al., 2022).

While capture stress protocols are useful for understanding when and why stress responses vary, they may or may not accurately model the responses of animals to real or perceived stressors, including food shortage, dehydration, severe weather, aggressive encounters, infection, parasitism, and/or predation, which we discuss below. Comparing the response of an

animal to a “real” stressor to that of a standardized capture stress protocol will be critical in understanding how well capture stress approximates the effects of stressful stimuli on physiology and behavior. In the following sections, we review the available literature examining the effects of different types of stressors on the physiology and behavior of snakes.

Captivity

The relationship between captivity (i.e., being captured and then housed for at least 1 d post-capture) and stress in wild animals, including snakes, is complex (Van Waeyenberge et al., 2018). On one hand, captivity can eliminate much of the unpredictability of a wild environment. Captivity offers a respite from predators, parasites, and weather. Captive animals usually receive regular feedings and constant access to water sources. By meeting all basic needs, captive environments may be considered less stressful than wild ones. On the other hand, some animals fail to thrive in captivity (Pasmans et al., 2008), despite best attempts to re-create a suitable environment (e.g., HACC, 2004). Even in the best care, an animal is taken from the wild and introduced to an enclosure which limits long-distance movements with limited refugia choices, a new diet, and regular human disturbance. Many assume captivity to be stressful and a disease risk for reptiles (Warwick, 1990; Jacobson, 1993; Pasmans et al., 2008). The initial exposure to captivity is considered stressful by many researchers as well, given the acclimatization protocols employed prior to beginning laboratory experiments (e.g., Pintor et al., 2016). Acclimatization protocols are also required by many animal care and use committees, but it is important to note these guidelines are developed primarily from literature on mammals (Obernier and Baldwin, 2006). Despite its implication as potentially stressful, studies explicitly assessing captivity as a potential stressor are rare (Fischer and Romero, 2019), especially in snakes.

Captivity can induce behavioral and physiological changes. For example, reptiles kept in cages with transparent doors or windows may spend the majority of their time attempting to escape through them, which may cause injury (Warwick, 1990). Despite access to a thermal gradient, some captive reptiles exhibit preferences for low temperatures, which may be followed by inactivity, anorexia, and disease (Warwick, 1990). Snakes kept in captivity may be more likely to exhibit defensive behaviors (Warwick, 1990), potentially due to the consistent inability to escape within an enclosure. On the other hand, snakes may also begin to associate humans with food and exhibit food seeking behavior during cleanings. These potential changes should be considered when designing studies, especially those that investigate thermoregulatory preference, prey preference, or defensive behaviors.

For some colubrid snakes, captivity may also prove costly to fitness-related traits, such as reproduction. For example, in brown treesnakes, *Boiga irregularis*, captivity led to cessation of reproductive activity compared with wild individuals (Aldridge and Arackal, 2005). Recent captivity (10-30 days post-capture) led to decreased spermatogenesis and epithelial height of the sexual segment of the kidney and testis in brown treesnakes, although spermatogenesis was recovered after 60 days (Siegel et al., 2009). Warwick (1990) notes brown treesnakes among reptiles notoriously “unadaptable to unnatural environments”. Captivity may also influence other aspects of physiology. Baseline CORT and H:L ratios increased in western terrestrial gartersnakes, *Thamnophis elegans*, held captive for 6 days (Robert et al., 2009) and for over 3 months (Sparkman et al., 2014). Similarly, northern watersnakes *Nerodia sipedon*, held captive

for up to 8 days showed increases in both baseline and 1 h post-handling CORT (Sykes and Klukowski, 2009). One population of common gartersnakes, *Thamnophis sirtalis*, in Iowa, US exhibited increased circulating CORT relative to baseline from 15 minutes through 3 days in captivity, while another population with higher at-capture baseline CORT in California, US only showed increased levels relative to capture at 45 minutes, which then returned to within-baseline levels (Gangloff et al., 2017). The ultimate effects of such changes in CORT require further study- but see above section on “Glucocorticoids”.

In other species, there is evidence for generally neutral or positive effects of captivity. When exposed to different disturbance regimes, captive southern Pacific rattlesnakes, *Crotalus helleri*, did not differ in baseline CORT concentrations, although they responded to disturbance with rattling (Claunch et al., 2017b). Burmese pythons, *Python bivittatus*, exposed to differing lengths of time in captivity up to 109 days only showed an initial increase in baseline CORT which subsided over time, and did not show differences in functional immunity, H:L ratios, or 1 h post-restraint CORT relative to field levels or initial captivity (Claunch et al., 2022). Lettoof et al. (2021) demonstrated increased body condition and changes in plasma biochemistry associated with consistent feeding in western tiger snakes, *Notechis scutatus occidentalis*, after 6 months of captivity. Namely they reported that globulin, albumin, and total protein increased with captivity, likely due to consistent diet and access to water. Lower blood glucose was also observed in captive western tiger snakes, providing evidence of either increased protein in diet or reduction of stress, although it is important to note that snakes were held up to six hours prior to the field reference measurement (Lettoof et al., 2021), and blood glucose can increase in as little as 15 minutes in some snakes and remain elevated for hours (Gangloff et al., 2017). Regardless, these studies demonstrate that some captive snakes may be representative of field-sampled animals or may serve as a baseline for developing health assessments.

It is important to note there may be a type of survivorship bias highlighting minimal effects of captivity on well-studied species. Namely, the current well-studied snake species or snake ‘model systems’ (*Thamnophis* sp., *Crotalus* sp., *Python bivittatus*) may persist in popularity due to the relative ease of maintenance in captive laboratory environments. As with many traits, response to captivity may be species-specific (Fischer and Romero, 2019). Thus, captivity may exist as a stressor on a case-by-case basis, and purported lack of effects in one species should not be used to justify ignoring potential effects in other species when designing experiments. Additionally, as we have belabored, CORT levels do not equate to stress. For example, brown treesnakes, which cease reproductive activity during 10-30 days of captivity (Siegel et al., 2009) exhibit baseline CORT indistinguishable from wild CORT levels after 3 days in a wire mesh modified minnow trap (Mathies et al., 2001). Because reproductive activity is often halted in favor of other physiological processes during a stress response (reviewed in Wingfield and Sapolsky, 2003), it is best to conclude that brown treesnakes perceive captivity as a stressor for at least 30 days, although CORT shows a shorter acclimatization time. Finally, an understanding of physiological effects of captivity is limited for most animals- not just snakes. A recent literature review revealed that only about half of tested species adjusted to captivity after any length of time (reviewed in Fischer and Romero, 2019). Overall, there are so few studies explicitly investigating captivity in snakes that drawing conclusions about captivity’s role as a stressor in snakes is difficult and remains an area for future study.

Climate Change

Global climate change represents an existential threat to much of life on Earth, as human activities have warmed the climate at a rate that is unprecedented in at least the last 2000 years (IPCC, 2021). Higher average temperatures increase the water and nutritional needs of animals, lead to shrinking body size, and disrupt the phenology, behavior, and interspecific interactions of animals (Traill et al., 2010; reviewed in Angilletta, 2009; Sheridan and Bickford, 2011). Gradual warming continues to drive other climatic changes, including an increase in the frequency and intensity of extreme weather events, such as heat waves, heavy precipitation, and droughts, depending on global region (IPCC, 2021). Some thermal hazards are greater in human-developed areas (e.g., the urban heat island effect), which are undergoing rapid expansion worldwide (Oke, 1973; Grimm et al., 2008; Youngsteadt et al., 2015). In sum, environmental change exhibits tremendous spatial and temporal variation. Therefore, understanding how the physiology of snakes will continue to respond to changing environments requires characterizing the effects of multiple facets of climatic variation on animals' physiological safety margins across ecosystems. Below, we focus on our understanding of two interconnected types of stress associated with climate change—heat and hydric stress—experienced by snakes.

Heat Stress

Snakes that cannot achieve their preferred body temperature exhibit increased CORT and decreased immunocompetence (Dupoué et al., 2013; Fabrício-Neto et al., 2019), but thermoregulation requires careful decision-making that weighs many important needs, such as reproduction, foraging, self-maintenance, and predation avoidance (Burns et al., 1996; Gregory et al., 1999; Shine et al., 2000b; Blouin-Demers and Weatherhead, 2001; Todd et al., 2016). Snakes heavily rely on thermal heterogeneity in their environments to behaviorally thermoregulate (e.g., by shifting their daily surface activity patterns and choice of microhabitat) (Blouin-Demers, 2002; Deutsch et al., 2008; Kearney et al., 2009; Aubret and Shine, 2010). As the thermal quality of their environments degrade with climate change, snakes may endure heat stress that exhibits variation in time and predictability—from stress experienced regularly during certain times-of-day or season over the course of a snake's life to less predictable heat waves that span only a few days at a time. Thus, researchers have taken several experimental approaches to understand climate change-induced heat stress in snakes.

Acute heat stress has diverse effects on snakes. Within 2-3 h of heat exposure, western terrestrial gartersnakes, *Thamnophis elegans*, increase their levels of plasma CORT and expression of genes encoding heat shock proteins in their liver, but they also reduce the expression of the gene encoding superoxide dismutase, a key antioxidant (Schwartz and Bronikowski, 2013; Gangloff et al., 2016). The reduced ability to deliver sufficient oxygen to tissues at elevated temperatures may be an underlying mechanism for heat sensitivity (Pörtner, 2002, 2010), but an 8-h ramping exposure to heat in ball pythons (*Python regius*) does not affect oxygen transport (Fobian et al., 2014). A simulated heat wave reduces body condition in corn snakes, *Pantherophis guttatus*, but it also reduces oxidative damage and does not affect antioxidant capacity or overall performance of several innate immune metrics (Stahlschmidt et

al., 2017). Therefore, snakes from both tropical and temperate regions appear physiologically adapted to acute heat stress events.

Studies examining the longer-term effects of elevated temperatures on snake physiology tell a similar tale—snake physiology is robust to chronic warming. Prolonged exposure (14 months) to warm microhabitat options (i.e., to simulate gradual climate warming) enhances growth and body size in juvenile tiger snakes, *Notechis scutatus*, after controlling for food intake, likely due to behavioral plasticity in thermoregulatory tactics (Aubret and Shine, 2010). A similar experimental design in juveniles indicates that corn snakes also exhibit developmental plasticity (i.e., irreversible phenotypic plasticity) in several behavioral traits (Stahlschmidt et al., 2016). Warmer microclimate options promote growth and energy savings (i.e., reduced rates of O₂ consumption at fixed temperatures) in juvenile corn snakes only when food intake is high—that is, the benefits of warming may be food-dependent (Stahlschmidt et al., 2015). Snakes may also exhibit beneficial reversible plasticity because six months of warm acclimation in adult grass snakes, *Natrix natrix*, reduces oxidative damage (Bury et al., 2018). The potential benefits of warming for snakes may scale up to the population level due to the positive effects of higher temperatures on aspects of performance and embryonic development (e.g., Aubret and Shine, 2010; reviewed in Noble et al., 2018).

Hydric Stress

Snakes and other squamate reptiles are common inhabitants of arid and mesic areas, with numerous adaptations to survive prolonged periods without water including skin with high resistance to water loss, production of highly concentrated urine, and tolerance of dehydration and hypernatremia (Brischoux and Kornilev, 2014). The hormone arginine vasotocin is released in snakes in response to dehydration (Mancera et al., 1990) and may impact their cutaneous water loss, urine water content, and drinking behavior. Despite many snake species being tolerant of short-term dehydration, numerous studies demonstrate negative impacts of drought on snake activity, body condition, and reproduction (Willson et al., 2006; Sperry and Weatherhead, 2008; Pomara et al., 2013; Vogrinc et al., 2018). Drought conditions also appeared to induce convergence on similar glucose and HPA-axis reactivity phenotypes in populations of western terrestrial gartersnakes (*T. elegans*) which display distinct phenotypic differences in non-drought years (Holden et al., 2022). Some snake species may be more active when humidity is higher (Daltry et al., 1998), and most snakes seek refuge underground or in other refugia during dry portions of the year as well as extended drought. Furthermore, even desert-adapted and marine snakes cannot rely on preformed water from food and instead must drink liquid fresh water to rehydrate (Lillywhite et al., 2015; Murphy and DeNardo, 2019).

With droughts expected to increase in frequency and magnitude due to climate change in the coming years, we can expect to witness concomitant influences on snake populations, especially in deserts and near cities that may confer an urban heat island effect. The tight interplay between water balance and thermoregulation is also an important consideration when examining how drought and rising temperatures may impact snakes. For example, dehydrated tiger snakes choose lower body temperatures in a gradient than hydrated snakes (Ladyman and Bradshaw, 2003), a common strategy among terrestrial reptiles that likely helps to conserve further water loss (Crowley, 1987; Sannolo and Carretero, 2019). Clearly, global climate change may increase hydration stress and simultaneously limit the ability of snakes and other

ectotherms to select thermally suitable microhabitats, a double whammy that could have severe impacts on snakes in numerous ways.

The most obvious way that hydration stress could deleteriously impact snakes is via its effect on reproduction. Water as a currency for reproduction in snakes has not been studied nearly as much as energy, but a number of key studies provide several important results as well as hypotheses for future study. During one study conducted during a severe drought, wild female rattlesnakes did not produce litters unless they were given supplemental water (Capehart et al., 2016), suggesting that viviparous females may resorb developing embryos when dehydrated. Pregnancy involves increases in blood volume, which may be related to increased cutaneous evaporative water loss observed in pregnant asp viper (*Vipera aspis*, Lourdais et al., 2017). When dehydrated, these pregnant female vipers prioritize water transfer to their embryos at the expense of their own water balance (Dupoué et al., 2014a), and growth is enhanced in offspring of dehydrated mothers, possibly via the elevated CORT levels she displays due to dehydration (Dupoué et al., 2016). Indeed, pregnancy and dehydration both lead to increased baseline and/or stress-induced CORT levels in Children's pythons, *Antaresia childreni*, as well (Dupoué et al., 2014b; Bruschi et al., 2020b), suggesting that CORT may mediate numerous impacts of dehydration on pregnant females and their offspring. In gravid European adders (*Vipera berus*), 15 days of water deprivation not only increased maternal corticosterone, dehydration, and loss of muscle mass, but also increased embryonic mortality (Dezetter et al., 2021). Thus, even short-lived periods of hydric stress could negatively impact both current and future reproductive efforts.

Dehydration during pregnancy may result in additional dramatic impacts on offspring, including apparent overriding of heterogametic sex determination resulting in male-biased sex ratios in litters of dehydrated females (Dupoué et al., 2019). The authors of this study, who also demonstrated the same result in viviparous lizards, suggest a hypothesis for this phenomenon. Because these species are consistently exposed to differences in habitat water availability that lead to a female-biased adult sex ratio, production of more males when mothers are dehydrated could be adaptive (Dupoué et al., 2019). While this hypothesis needs to be tested, it highlights the important concept that hydration “stress” could be adaptive and should not be viewed merely as a detrimental outcome. In this same vein, research on snakes has consistently demonstrated that dehydrated individuals have enhanced immune function compared to hydrated individuals (Brusch and DeNardo, 2017; Bruschi et al., 2017, 2020a). While the ultimate reasons for this counterintuitive effect are unknown, one potential proximate mechanism is upregulation of an inflammatory cascade that may be activated by osmotic stimuli (Brusch et al., 2017). An emerging theme in this chapter is that stress is not always a negative, maladaptive phenomenon but rather a means by which snakes and other organisms attempt to maintain homeostasis in a changing environment. Future research opportunities are ample for studying how (and why) hydration stress leads to such counterintuitive phenomena as altered offspring sex ratio and improved immune function; Additionally, research opportunities abound on yet-unstudied effects of snake hydration stress on fitness, survival, and more.

Field-based experiments, modelling, and meta-analyses provide key insights into how snake populations will fare with ongoing climate change. For example, a recent study comparing coastal and inland populations of the Pacific rattlesnake, *Crotalus oreganus*, indicates that accurate thermoregulation may buffer temperature shifts associated with future climate change (Crowell et al., 2021). Warming is expected to improve the thermal conditions

or prey availability for populations of rat snakes, *Pantherophis obsoletus*, spanning a latitudinal range (Weatherhead et al., 2012), the high-altitude Taiwan pit viper (*Trimeresurus gracilis*; Huang et al., 2013), and the western whipsnake (*Hierophis viridiflavus*; Capula et al., 2016). Modelling approaches further indicate increased environmental suitability under climate change scenarios for invasive California kingsnakes, *Lampropeltis getula californiae*, in Canary Islands (Piquet et al., 2021), and an increased range size for the Caspian whip snake (*Dolichophis caspius*; Sahlean et al., 2014). However, climate change is expected to dramatically reduce the range sizes of approx. 100 snake species in a Brazilian Atlantic Forest hotspot that is poorly protected (Lourenço-de-Moraes et al., 2019), and similar declines are predicted for the endangered broad-headed snakes (*Hoplocephalus bungaroides*, Penman et al., 2010) and some populations of the horseshoe whip snake (*Hemorrhoids hippocrepis*; Bombi et al., 2011). Further, meta-analysis results indicate that climate change is expected to have generally negative outcomes for squamate reptiles (Diele-Viegas and Rocha, 2018). In sum, although experimental work suggests that snake physiology is resilient to certain features of climate change (e.g., heat exposure), modelling approaches indicate that some snake species or populations may be critically vulnerable to climate change in its entirety.

Urbanization

Urbanization, or the encroachment of human activity into areas that were historically remote, may present several stressors to snakes. First, urbanization increases encounters between snakes and humans. Many snake species view humans as a threat, and will flee when approached (e.g., Gibbons and Dorcas, 2002; Glaudas et al., 2005), although human presence for 30 minutes did not impact CORT levels in eastern cottonmouths (*Agkistrodon piscivorus*, Bailey et al., 2009). Snakes may be more likely to be hunted or targeted as humans move to more remote areas. Hunted populations of mamushi, *Gloydius blomhoffii*, in Japan show morphological, physiological, and behavioral differences compared with non-hunted populations, where hunted populations are smaller, show increased reproductive effort, and higher propensity to flee from humans (Sasaki et al., 2008). The disturbing practices of rattlesnake roundups are certain to induce population and individual-level stress responses, especially when considering the majority of hunters for these events target the same dens yearly (Adams et al., 1994).

Conflicts can arise when snakes, particularly venomous species, are discovered in residential or recreational areas. Areas with newer housing are associated with increased calls to a professional snake removal company which uses translocation as a mitigation method (Cornelis et al., 2021). Translocation can have varying effects on snakes. Translocation influences activity levels, and longer distance translocation is associated with increased mortality in some snake species (reviewed in Cornelis et al., 2021). Physiological effects of translocation are not well understood. CORT and testosterone increase in southern Pacific rattlesnakes, *Crotalus helleri*, following long-distance (Heiken et al., 2016) but not short-distance translocation (Holding et al., 2014b). Short-distance translocation also does not appear to influence thermal ecology in southern Pacific rattlesnakes (Holding et al., 2014a).

Urbanization also affects habitat type and fragmentation. In a habitat fragment, fecal parasites were more prevalent in snakes found closer to the urban forest edge (Davis et al., 2012). Urbanization has been shown to influence body size in several species of snakes. In

large-bodied species, snakes display smaller sizes in urban areas relative to rural areas (e.g., dugites, *Pseudonaja affinis*, Wolfe et al., 2018b; African pythons, *Python sebae*, Luiselli et al., 2001), despite the increased availability of rodents as potential prey. In contrast, several species of snakes were found to be larger in smaller patches of natural area (Kjoss and Litvaitis, 2001), although there were fewer snakes and less snake diversity in those smaller patches. Brown treesnakes, *Boiga irregularis*, reach greater body sizes in urban areas in their invaded range, but this can be attributed to their depletion of native prey in natural areas (Savidge, 1988).

There are relatively few studies that assess the physiological impacts of urbanization on snakes. A recent meta-analysis found no relationship between baseline reptile CORT levels and different indices of urbanization (Injaian et al., 2020). This is not particularly surprising, given the context-dependent nature of CORT, and the fact that reptiles with very different life history traits (squamates, turtles, and crocodylians) were combined for the analysis. Copperheads captured on roads showed dampened 30-minute post-restraint CORT compared with those on the forest interior, and increased road traffic was associated with lower baseline and post-restraint CORT (Owen et al., 2014). Higher H:L ratios were observed in northwestern gartersnakes, *Thamnophis ordinoides*, at a highly disturbed urban site as determined by increased human and predator presence (Bell, 2010). Exposure to pesticides and toxicants found in urban environments also induces a CORT response in common gartersnakes (*Thamnophis sirtalis*) exposed to Indoxacarb (Neuman-Lee et al., 2016) and leads to decreased baseline CORT and increased bacterial killing ability in western terrestrial gartersnakes (*Thamnophis elegans*) exposed to PBDE-47 (Neuman-Lee et al., 2015b). Overall, there is a lack of understanding how urbanization influences different snake species (French et al., 2018), despite the pressing need to understand these responses to develop and assess conservation efforts.

Invasive Species and Invasion

Snakes may experience stress caused by invasive species, or during their own introduction and establishment in new environments. Introduced species are attributed to population declines in many snake species. Although introduced mammals may supplement available prey for some snakes (e.g., Wolfe et al., 2018b), they also prey on snakes and their eggs. Feral cats attack, kill, and consume snakes (Whitaker and Shine, 2000; Yip et al., 2015). Invasive rodents are implicated in population suppression of snakes as well; following removal of invasive rodents, populations of milk snakes, *Lampropeltis triangulum*, on San Pedro Martir (Samaniego-Herrera et al., 2011) and Antiguan racers, *Alsophis antiguae*, on Great Bird Island greatly increased (Daltry, 2006). Introduced arthropods can have similar effects. For example, the red imported fire ant, an aggressive colonizing insect with a painful sting, is implicated in the declines of southern hognose snake (*Heterodon simus*; Tuberville et al., 2000) and may affect shelter use (Stahlschmidt et al., 2018), and recruitment of other snake species through predation on young and eggs (Allen et al., 2004). Pentastome parasites were introduced with invasive Burmese pythons, *Python bivittatus*, now infect native snakes (Miller et al., 2017), and have since spread to many native snakes outside of the range of the Burmese pythons (Miller et al., 2020).

Smaller snakes, such as the pygmy rattlesnake, may be particularly at risk of stress induced by this non-native parasite as it can grow to sizes encompassing the majority of the lung cavity (Farrell et al., 2019). Introduced cane toads, *Rhinella marina*, in Australia are toxic and can be

physiologically challenging or fatal to snakes when consumed (Phillips and Shine, 2006; Phillips et al., 2009). Anticipated snake population declines predicted by laboratory toxicity studies are not apparent in the field (Brown et al., 2011), which may be in part due to some populations evolving behavioral, morphological, and physiological resistance to toads (Phillips and Shine, 2004, 2006). Studies on how invasive species induce stress in snakes are often lacking beyond population-scale measurements or injury rates, and much remains to be studied. For example, lizards decrease foraging in the presence of predatory mammal scents (Webster et al., 2018), and it is possible snakes respond similarly.

During introduction to new environments, snakes face a variety of potential stressors, such as novel prey, predators, competitors, and environmental challenges. Long-term research on invasive brown treesnakes, *Boiga irregularis*, in Guam suggests body condition and levels of baseline and post-restraint CORT may be influenced by fluctuating resource availability, management pressure, and snake population change during the course of the invasion (Claunch et al., 2021a). Invasive populations may also experience differences in predatory pressure compared to the native range. This can occur via release from predators or exposure to different predator communities, as implicated by different tail breakage rates in horseshoe whip snakes, *Hemorrhoids hippocrepis* and ladder snakes, *Zamenis scalaris* between introduced and mainland populations (Febrer-Serra et al., 2021). Introduction to a new environment also facilitates exposure to novel pathogens and parasites (Holldorf et al., 2015). Invasive snakes may face thermal stress in novel conditions. Invasive Burmese pythons in Florida weathered a cold snap that caused mortality in some individuals (Jacobson et al., 2012) but may have driven adaptation to cooler physiological tolerance in offspring of survivors (Card et al., 2018). Snake invasions provide opportunities to investigate the role of novelty and ecosystem changes as stressors and the influence of stress responses in the success (or failure) of invasion.

Shelter

Shelters (or refuges or refugia) are key habitat features for animals because they provide a range of benefits. The access to and quality of shelter may play key roles in snake stress ecology. For snakes, shelters help maintain homeostasis while balancing ecological needs. Snakes use shelters to facilitate thermoregulation across time scales—for example, snakes can retreat to cool shelters during the heat of the day, use warm shelters to achieve higher body temperatures facilitating digestion, and use burrows or other hibernacula while over-wintering (Webb and Shine, 1998; Shine et al., 2001a; Whitaker and Shine, 2003; Webb et al., 2009; Gregory, 2009; Lelièvre et al., 2010; Gienger and Beck, 2011). Shelter use can also maintain snakes' water balance when ambient conditions are hot or windy, or during periods of high water demands (DeGregorio et al., 2021). Snakes have many predators, and shelter use can further serve to reduce predation risk (Webb and Whiting, 2005; Coombs, 2016). Shelters can facilitate social interactions in some snakes (e.g., common gartersnake, *Thamnophis sirtalis sirtalis*: reviewed in Skinner and Miller, 2020).

Shelter use is also important during oviposition-site selection because snakes' parchment-shelled eggs are highly vulnerable to the abiotic and biotic characteristics of the nest environment (i.e., temperature and humidity, and the presence of predators, and fungal and bacterial pathogens) (e.g., Madsen and Shine, 1999; Stahlschmidt et al., 2011). However, snakes must balance all of the benefits of shelters with foraging, mating or mate-searching, and

the exploitation of thermal resources outside of the shelter (Shine et al., 2001a; Bonnet and Brischoux, 2008; Wasko and Sasa, 2012; Todd et al., 2016). Therefore, the ability of snakes to move freely among shelters and exposed features of their environment is critical to their success.

Many snakes prefer sheltered microhabitats, and the availability of suitable shelters may influence the distribution of some snakes, including the Eastern indigo snake, *Drymarchon couperi*, and the blue-lipped sea krait, *Laticauda laticaudata*, (Bonnet et al., 2009; Hyslop et al., 2009). The location of a suitable shelter typically depends on a given snake's lifestyle (e.g., fossorial snakes prefer subterranean shelters while arboreal snakes rely on shelters in tree hollows or cavities), which can mediate inter-specific competition. Snakes readily use an array of shelters constructed by animals (e.g., tortoise or mammal burrows: Hyslop et al., 2009, 2014; Miller et al., 2012), including humans (Akani et al., 2002), which has allowed researchers to perform population surveys on snakes using artificial cover objects (e.g., Grant et al., 1992; dos Santos et al., 2008; Stahlschmidt et al., 2018). Snakes incorporate thermal, hydric, and predator-related information when choosing shelters, and laboratory studies indicate that snakes can quickly learn to efficiently locate shelters (Weldon et al., 1990; Holtzman et al., 1999; Stahlschmidt et al., 2011). Given the importance of shelters to snakes' success, it is clear that snakes exhibit a remarkable adaptive plasticity in decision-making related to finding and using shelters.

Because shelters are key resources for snakes, stress can result when these critical habitat features are limited. Indeed, the availability of shelter for captive snakes is assumed to be of paramount importance (Van Waeyenberge et al., 2018), yet very few manipulative studies have examined the relationship between shelter availability and stress physiology in snakes. Shelter deprivation in aspik vipers, *Vipera aspis*, reduces energy intake by increasing the rate of regurgitation, and it increases energy expenditure by increasing basking behavior (Bonnet et al., 2013). Further, aspik vipers without access to shelter exhibit elevated baseline CORT levels in some conditions, similar to other taxa (e.g., cattle and domestic cats) (Tucker et al., 2007; Bonnet et al., 2013; Ellis et al., 2021). In corn snakes, *Pantherophis guttatus*, shelter availability also affects thermoregulation, and immune activation can influence the tradeoff between thermoregulation and shelter use (Todd et al., 2016). Shelter-seeking is a common feature of sickness behavior in many animals (reviewed in Sullivan et al., 2016), but parasites' roles in snake shelter use requires investigation given the potentially increasing prevalence of parasitism (Harvell et al., 2002; Cumming and Van Vuuren, 2006; Brooks and Hoberg, 2007). Humans continue to exert both positive and negative effects on the availability of shelters for snakes (e.g., Webb and Shine, 2000; Akani et al., 2002; Pike et al., 2011). Thus, future work is required to better understand the dynamics of snake shelter and physiology.

Prey

Snakes are obligate carnivores and likely experience stress related to seeking and acquiring prey items through three main sources: 1) danger associated with prey-seeking behavior, 2) possibility of injury during capture or digestion, and 3) unpredictability or lack of sufficient prey. The first two of these sources are typically acute stressors, while the final source is most consistent with chronic stress.

Snakes exhibit a continuum of foraging behavior from active pursuit (e.g., coachwhip snakes, *Masticophis flagellum* and small-eyed snakes, *Rhinoplocephalus nigrescens*) to ambush foraging (e.g., sidewinder rattlesnakes, *Crotalus cerastes* and broad-headed snakes, *Hoplocephalus bungaroides*; Secor, 1995; Webb et al., 2003). While no studies have explicitly tested stress or glucocorticoid concentrations in species exhibiting the two extreme foraging strategies, there is evidence of physiological differences. Snake species that primarily use ambush foraging have lower hematocrit levels, an indicator of blood oxygen carrying capacity, than active foragers. Species that use both methods had an intermediate level of hematocrit (Lourdais et al., 2014). These findings are consistent with the studies indicating that ambush snake predators have lower metabolic rates than active foragers (Stuginski et al., 2018, Secor and Nagy, 1994), although temperature and climate can heavily influence these relationships (Dupoué et al., 2017).

Snakes routinely capture and ingest large prey items whole (Shine, 1991). Although not explicitly tested in snakes, this could elicit a stress response due to the relative immobility of the snake during ingestion and possible injury during subduing the prey. Further, even the digestion of immobile prey (killed, frozen, and thawed mice) induces oxidative damage in cornsnakes (*Pantherophis guttatus*), potentially due to associated immune activation (Butler et al., 2016; Luoma et al., 2016). There is some evidence of a stress response due to prey acquisition when examining species of snake that routinely consume toxic prey items. The tiger keelback snake, *Rhabdophis tigrinus*, a toad-eating specialist that sequesters bufotoxins, had a lower concentration of circulating corticosterone than non-toad specialists after acute cardiotoxic steroid exposure (Mohammadi et al., 2017). Adrenal gland enlargement has also been detected in toad eating species, especially in males (Mohammadi et al., 2013). Common gartersnake, *Thamnophis sirtalis*, individuals have varying resistance to the toxin tetrodotoxin, which is found in a commonly consumed prey item- the rough-skinned newt, *Taricha granulosa*. Interestingly, the resistance level in individual snakes was not associated with CORT concentrations, although females demonstrated a marked increase in CORT after any exposure to the toxin. Males and juveniles exposed to increasing doses of tetrodotoxin showed no change in CORT (Neuman-Lee et al., 2017). An anthropogenic chemical with a similar mechanism of action as tetrodotoxin did elicit a CORT response, though (Neuman-Lee et al., 2016).

Because of their ability to reduce energy investment into digestive tissue and their lower metabolic rate relative to endotherms, snakes are very resistant to the stress associated with food limitation (McCue et al., 2012). Food deprivation of six weeks had no influence on baseline or stress-induced corticosterone in western terrestrial gartersnakes, *Thamnophis elegans*, while chronic stress elicited a greater change from baseline after acute stress (Neuman-Lee et al., 2015). However, a study with another natricine snake (northern watersnake, *Nerodia sipedon*) found that food restriction of 15 days did elicit a higher baseline CORT concentration when compared to a fed group (Webb et al., 2017). Chronic food restriction may have a greater impact on the stress response than on baseline levels of CORT. When comparing two populations of Florida cottonmouth, *Agkistrodon conanti*, snakes from a food-restricted island had a dampened increase in CORT concentrations after an acute stressor, although baseline concentrations of CORT were similar (Sandfoss et al., 2020). In a longitudinal study of the invasive brown treesnake, *Boiga irregularis*, on Guam, snakes had lower body condition and higher baseline CORT when food resources were limited after the extirpation of native fauna but before the introduction of new prey items (Claunch et al., 2021b). There is still much to

learn about how snakes with different foraging strategies respond to different durations and severity of food restriction as a stressor.

Intraspecific Interactions

While a normal and natural part of life history, some intraspecific interactions may be viewed as stressors based on the challenges they pose to homeostasis. Many male snakes will engage in combat to gain mating opportunities (Carpenter, 1986; Senter et al., 2014). In eastern copperheads (*Agkistrodon contortrix*), males that lose these fights retreat, engage in submissive behavior, show suppressed sexual behavior, and display increased concentrations of plasma CORT and lactate 1-hour post-fight relative to winners and males that did not fight (Schuett et al., 1996; Schuett and Grober, 2000). Little is known about physiological effects of combat in other snake species, or long-term effects post-combat, in part because natural combat events are rarely witnessed by prepared snake physiologists.

Beyond agonistic interactions, the act of courtship may also be stressful. Female red-sided gartersnakes (*Thamnophis sirtalis parietalis*) leaving a communal densite following hibernation may be harassed by males attempting to mate for several days (Shine et al., 2000c), which can expose them to predators (Shine et al., 2001b) and lead to exhaustion. Given the choice, females select areas with less male scent (Shine et al., 2003). Copulation may also be painful in some species. Females have evolved keratinized protections to the cloaca due to the spiny architecture of hemipenes (Siegel et al., 2011) and allow longer copulations when the area is anesthetized (Friesen et al., 2014). During copulation, males are at risk of hemipene damage from female twisting and rolling (King et al., 2009). The act of mating is energetically demanding for both sexes, as evidenced by increased plasma lactate (Shine et al., 2003). While potentially controversial, it may be useful to consider copulation as a stressor in future studies of snake physiology and behavior. Finally, population density may influence intra-specific interactions in snakes and may determine whether interactions are stressful based on magnitude (Claunch et al., 2021a), but this is largely unstudied beyond the gartersnake system.

Disease

While links between stress and disease are well-established in a range of biomedical contexts (Sapolsky et al., 2000), the direction of causality linking stress and disease is not clearly understood in wildlife (Romero and Wingfield, 2015). This lack of clarity results from the fact that, in free-living organisms, infectious diseases may interact with other stressors in complex ways (Marcogliese and Pietrock, 2011; Romero and Wingfield, 2015), and the causal mechanisms driving observed interactions are rarely defined in controlled experiments. Proposed mechanisms linking stress and disease include immunosuppression via the direct action of adrenal glucocorticoids (Dhabhar, 2009) and resource allocation tradeoffs that arise between stress coping responses and immune functions (Lochmiller and Deerenberg, 2000; Sapolsky et al., 2000; Martin, 2009). As such, disease may be understood as either a stressor or as a potentially useful stress metric, and the need to distinguish between disease severity as a cause or consequence of stress sets disease apart from other stress metrics discussed in this review. Outside of a handful of specific host-pathogen systems, the mechanisms linking stress

and disease have received little direct investigation in snakes. Below we outline what has been learned from systems in which the relationship between disease and stress has been investigated in snakes and discuss specific areas where further research is sorely needed and could provide fruitful data applicable across vertebrates.

Fungi (*Ophidiomyces ophiodiicola*)

Ophidiomycosis (also known as snake fungal disease) is caused by the keratinophilic skin fungus *Ophidiomyces ophiodiicola* (Oo; Lorch et al., 2015) and has been identified as an emerging threat to North American snake populations (Lorch et al., 2016). Ophidiomycosis has been linked to high mortality rates in captivity (Allender et al., 2011) and local declines in wild snake populations (Clark et al., 2011). However, field-based studies indicate that snakes can clear the infection and mortality rates may not be as high as indicated in captive settings (Allender et al., 2011; Lind et al., 2018, McKenzie et al., 2021). Both the origin of the disease and the role of stress as a causal factor driving outbreaks and negative impacts on captive and free-living individuals are currently murky.

In pygmy rattlesnakes, *Sistrurus miliarius*, ophidiomycosis is associated with a suite of stress-associated metrics including reduced body condition (McCoy et al., 2017), increased plasma CORT (Lind et al., 2018), suppression of sex steroid hormones (Lind et al. 2019), and metabolic up-regulation (Agugliaro et al., 2020). Further implicating a role for stress in disease outbreaks, seasonal patterns of disease severity closely follow seasonal patterns in baseline CORT (Lind et al., 2018). These associations between ophidiomycosis and diverse stress metrics may indicate that the disease causes stress and associated increases in plasma CORT. However, results are observational, and the direction of causality linking stress and ophidiomycosis remains unclear.

Oo is an opportunistic fungus that can persist in soil (Campbell et al., 2021), and it may be that individuals are chronically exposed to the pathogen and that other stressors predispose individuals to severe infection. In the latter scenario (i.e., increased disease severity as a consequence of stress), ophidiomycosis may be indicative of other challenges to homeostasis (e.g., habitat degradation, resource limitation, and/or shifting thermal regimes) and serve as a metric to assess accumulated stress in individuals or populations. That ophidiomycosis is either a cause or consequence of stress are testable, but not mutually exclusive, hypotheses that require experimental examination. Ophidiomycosis presents researchers with a useful model system for future exploration of the causal relationships between stress and disease in free-living snakes. Establishing directions of causality is important and can inform: (1) the future use of ophidiomycosis as a stress metric in snake populations, and (2) conservation and management approaches that effectively target the causal variables that drive negative individual and population-level outcomes.

Hemogregarine Parasites (Phylum Ampicoplexa)

The wide distribution and prevalence of hemogregarine blood parasites in snake hosts has resulted in the most taxonomically and geographically diverse group of studies investigating the relationship between stress, fitness, and endoparasite infection in snakes. Quantifying

parasite loads from stained blood smears is relatively straightforward, and researchers have sought to examine the potential efficacy of hemogregarine loads as a proxy for individual condition (e.g., Madsen et al., 2005; Brown et al., 2006) or stress experienced by populations (Xuereb et al., 2012; Sandfoss et al., 2020). While individual hemogregarine loads may correlate negatively with fitness in some contexts (Madsen et al., 2005; Ujvari and Madsen, 2006), the majority of studies indicate that hemogregarine loads do not correlate with other stress metrics (e.g., CORT; Sperry et al., 2009; H:L ratios; Xuereb et al., 2012, or body condition; Davis et al., 2012) or fitness proxies (e.g., growth rate; Brown et al., 2006; Xuereb et al., 2012). However, in a population comparison, Sandfoss et al. (2020) found that natural food resource limitation in an insular population of Florida cottonmouths, *Agkistrodon conanti*, was associated with a low body condition index, an attenuated glucocorticoid and glycemic response to acute capture stress, reduced natural antibody function, and a higher prevalence of hemogregarines. Related, multiple species of snakes in a mesic community had lower humoral immunity and higher plasma osmolality when found infected with hemogregarines (Brusch et al., 2020a). Moreover, while Xuereb et al. (2012) found no correlation between hemogregarines and other stress metrics in individual eastern foxsnakes, *Pantherophis gloydi*, significant differences in parasite loads and H:L ratios were observed between populations, and higher hemoparasite loads were associated with higher H:L ratios at the population level.

The significant association between infection severity and other stress metrics in population comparisons may indicate the potential for hemogregarine loads to serve as an indicator of the degree of stress experienced by populations. However, several caveats should be considered. First, significant population-level reports have, thus far, been the result of comparisons between two populations and should be interpreted with caution (Garland and Adolf, 1994). Parasite loads may vary among populations due to local adaptation of host resistance versus tolerance strategies (Sternberg et al., 2013), or as the result of biotic and abiotic factors, including temperature and the distribution of intermediate host vectors (Chapa-Vargas et al., 2020). Additionally, the fact that a majority of studies have found no significant relationship between individual parasite load and stress or fitness metrics (Brown et al., 2006; Sperry et al., 2009; Xuereb et al., 2012; Minter et al., 2013) calls into question the efficacy of haemoparasite loads as a metric for stress. Parasite loads may also be correlated with age or size class (Madsen et al., 2005; Minter et al., 2013), and life-history strategy may influence the relationship between haemoparasite loads and fitness (Mason et al., 2005); further complicating the use of hemogregarine parasite loads as a stress metric. Lastly, it is worth noting that a variety of hemoparasites have been identified in reptiles (Telford, 2009), and the relationship between non-hemogregarine blood parasites and stress in free living snakes is unknown and in need of further research.

Ectoparasites (Class Arachnida: Ticks, Mites, and Chiggers)

Despite extensive research on the relationship between stress and ectoparasitism in lizards (reviewed in Bower et al., 2019), comparatively little research exists on snakes. This taxonomic bias may result from geographic and phylogenetic patterns in ectoparasite prevalence (Bury et al., 2020). The few studies which have investigated the effects of ectoparasitism on snakes suggest little relationship between parasitism and either stress or fitness. In Texas ratsnakes, *Pantherophis obsoleta lindheimeri*, ectoparasitism by chiggers was unrelated to plasma CORT

(Sperry et al., 2009). Similarly, Natusch et al. (2018) found that rates of tick ectoparasitism were unrelated to a body condition index in six Australian snake species. Due to the limited research on the relationship between ectoparasitism and stress, it is currently not possible to use ectoparasitism rates as a metric for assessing individual or population-level stress. Extensive work in lizards indicates that ectoparasite loads may serve as important metrics for stress induced by anthropogenic disturbance (Amo et al., 2006, 2007; Lindsay et al., 2016; Alaasam et al., 2021), and ectoparasite loads may have unexplored utility as stress metrics in tropical snake species where rates of ectoparasitism may be higher compared to temperate populations (Bury et al., 2020).

Helminths (Cestodes, Trematodes, Nematodes, and Pentastomes)

Research on the relationship between helminth endoparasites and stress in snakes is limited. We mention them here as a potentially overlooked cause or consequence of stress in snake populations and due to emerging threats to snake populations driven by the spread of helminth parasites. The helminths are a polyphyletic catch-all grouping composed of phylogenetically diverse vermiform endoparasites (e.g., nematodes, pentastomes, cestodes and trematodes). In Australian keelback snakes, *Tropidonophis mairii*, high nematode parasite loads (Gnathostomidae) are counterintuitively associated with a higher body condition index in wild snakes, and deworming in captive keelback snakes has no impact on growth rate (Mayer et al., 2015). In a comprehensive study of two species of gartersnake (*Thamnophis sirtalis* and *T. radix*), Uhrig (2015) examined fitness metrics associated with cestode, nematode and trematode infections and found population and species-specific relationships between helminth infection and fitness proxies. Additionally, pentastome lung parasites are an increasing conservation concern for Australian (Kelehear et al., 2014) and North American snakes (Farrell et al., 2019; Miller et al., 2020). Yet, we currently lack any understanding of pentastome infection as a cause or consequence of stress in snake hosts. As metrics for stress, helminth endoparasites present a range of challenges beyond a lack of study in snakes. Paramount among these is that methods to quantify infection severity are invasive and often require sacrificing the host. In the absence of new methods to assess severity (e.g., genetic detection), helminth endoparasites may have limited utility as a stress metric, but may certainly serve as an important cause of stress that should be explored in controlled experiments.

Bacteria, Viruses, and Stress

While snakes are known to carry a variety of bacterial (e.g., *Salmonella*, Pulford et al., 2019) and viral (e.g., iridoviruses, Hyatt et al., 2002; Ariel et al., 2015; reptarenaviruses, Stenglein et al., 2017, and flaviviruses, Reisen and Wheeler, 2019) pathogens, studies have been limited to field reports of presence or prevalence in populations and clinical reports in captive individuals. Understanding bacterial and viral pathogens as a cause or consequence of stress in free-living snakes remains an unexplored frontier.

The Need for Experiments Linking Disease and Stress

Experimental infection studies in snakes are currently lacking. This is a substantial issue because it limits the ability to differentiate causes from effects of disease. The experimental impact of infection on stress metrics has primarily been explored using sterile antigens as proxies for infection (e.g., lipopolysaccharide; LPS). Immune challenge via LPS results in an elevated H:L ratio (Lind et al., 2020b), increased metabolic rate (Lind et al., 2020b), and increased CORT (Urhig, 2016), suggesting that immune activation may cause stress in snakes. However, it is likely that infection and stress interact in positive feedback cycles where disease causes stress, stress exacerbates disease, and vice versa (*sensu* Beldomenico and Begon, 2010). Carefully designed infection studies, coupled with HPA manipulation, would be useful in examining the causal mechanisms linking disease, stress, and ultimately fitness in snakes.

Using disease as a metric for stress in snake populations has promise and may provide an informative complement to other metrics discussed elsewhere in this review, especially when examining the effects of human disturbance on populations (e.g., Amo et al., 2006, 2007; Alaasam et al., 2021). However, the relatively unexplored potential for local adaptation or plasticity to impact resistance versus tolerance coping strategies and, in doing so, drive patterns of disease severity independent of stress levels may hinder clear interpretations. Experimental examination of the causal links between stressors, coping strategies, and disease dynamics in snakes would provide clarity and promote efforts to understand and predict how populations respond to emerging pathogens. Importantly, experimentally differentiating between disease as a cause or consequence of stress in snakes would have practical implications and would allow conservation biologists and managers to maximize their impact by focusing efforts on the variables that directly drive negative outcomes in individuals and populations.

Conclusion

Snakes undergo a variety of physiological and behavioral changes when experiencing stress. Researchers have employed a variety of tools and methods to assess stress in snakes. The base of knowledge of snake stress ecology is derived from correlative studies, with a large focus on descriptive comparisons of plasma CORT. However, our knowledge is continually growing as certain tools and metrics become more accessible to ecologists, and there is a promising frontier with new methods for assessing immune function and gene expression in particular. Additionally, methods for assessing metabolism, morphology, and behavior are being honed for use in snakes, improving the use of these metrics for future studies of snake stress ecology. Through the application of standardized capture and restraint assays across many studies, we understand that snakes modulate their physiological stress responses depending on a variety of environmental and physical factors. By combining the results of correlative study and experimental manipulation, we gain understanding of the different types of stressors snakes face and their context-dependent coping mechanisms.

As discussed above, the studies on stress in snakes demonstrate that ectothermic vertebrates, such as snakes, provide important tests of hypotheses developed from stress ecology studies on endotherms (mammals and birds; Harris, 2020). The continued study of snake stress ecology will be important to explore convergence and continuity in stress responses among vertebrates, as well as develop an understanding of how and why ectotherms may respond in distinct manners.

Table 4. A summary of select molecular metrics available to assess stress in snakes and their limitations

Metric	Tissue Type	Limitations	Example References
Lipid peroxidation (MDA, d-ROMs)	Plasma + others	Lipid content and storage of tissues; cross-reactivity and sensitivity of assays	Stahlschmidt et al. 2013, 2017; Gavrić et al. 2019; Neuman-Lee et al. 2019
Protein oxidation (protein carbonyl groups, d-ROMs)	Plasma	Cross-reactivity and sensitivity of assays	Stier et al. 2017
DNA damage (comet assay)	Whole intact cells, including blood	Storage of samples	Bronikowski 2008
Molecular chaperones (Hsps)	Liver (+ others)	Mostly unknown in snakes	Schwartz and Bronikowski 2013
Telomere length	Whole intact cells, including blood	Storage of samples	Bronikowski 2008; Ujvari and Madsen 2009; Rollings et al. 2017; Singchat et al. 2019; Cunningham et al. 2021
Mitochondrial function	Liver (+ others)	Storage of samples	Robert et al. 2007; Robert and Bronikowski 2010
Gene expression (RNA-seq, reverse transcription quantitative [RT-q] PCR)	Tissue with whole cells	Storage of samples; interpretation relies on assumptions of homology of genes in annotated genomes for phylogenetically disparate species	Wall et al. 2011; Andrew et al. 2015; Schwartz et al. 2015

There is still much to understand about stress ecology in snakes. As with other vertebrates, stress ecology in snakes is complex and the state of stress is best inferred from the use of multiple metrics. We encourage researchers to develop a habit of including many metrics rather than single metrics (e.g., CORT) in their investigations of snake stress ecology. By doing so, and especially by publishing ‘null results’, these efforts can help advance understanding of the utility of these metrics in assessing stress at a more rapid pace. Many of the metrics we outlined above have been used rarely, if at all, in snakes, so there is much to learn. In the same vein, we echo calls to avoid conflating CORT with stress in future studies (MacDougall-Shackleton et al., 2019; Romero and Beattie, 2022) and encourage researchers to think critically about using CORT as a default metric of stress. It is imperative to acknowledge CORT as a complex, context-dependent hormone that requires further mechanistic research in snakes before we can fully interpret the consequences of fluctuations of this hormone. We encourage continued experimental research on the context-dependency of the other metrics discussed herein (Table 5).

Next, we explored different stressors in snakes, again highlighting the gaps in knowledge on several fronts. Much of what we know about stress ecology in snakes is limited to a few North American taxa. Because stress is so context-dependent, it is imperative that we broaden the taxonomic and geographic scope of snake species assessed to achieve a comprehensive understanding of snake stress ecology. Snakes also experience multiple stressors in concert in their natural environment. Experiments targeting cause-and-effect relationships between diverse stressors, coping responses, and fitness outcomes are sorely needed. We have summarized some important future directions for snake stress ecology in Table 5. While beyond the scope of this chapter, we encourage researchers to scour the stress ecology literature in other taxa (in particular, birds and lizards), which may provide inspiration for hypothesis generation and experimental methodologies that can be applied to snake ecology beyond what we have compiled here.

Table 5. Areas of future study to advance understanding of snake stress ecology

Category	Topic	Questions	Approaches
Utility of glucocorticoids/CORT as a metric of stress	Function of Glucocorticoids	What are direct consequences of elevated or dampened glucocorticoids?	Experimental manipulation of glucocorticoids in snakes in field and laboratory
	Glucocorticoids and Fitness (Reproduction and Survival)	How do changes in glucocorticoid levels influence fitness in snakes?	Experimental manipulation of glucocorticoids in snakes in field and laboratory, following generational outcomes and performance
	Drivers of variation in glucocorticoids	What triggers acute <i>versus</i> chronic elevation in glucocorticoids in snakes?	Experimental manipulation or field comparisons of environment, diet, disease, reproductive state etc., measuring glucocorticoid expression
Utility of 'X' as a stress metric	Understanding snake responses to stress	Are there predictable patterns of change in a given metric in response to certain stressors?	Repeated studies of metric in response to standardized and naturally occurring stressors across different contexts
Among-stressor comparisons	Utility of capture assays to inform general stress responses	What utility is capture stress for understanding snake responses to 'naturally-occurring' stressors?	Comparisons of within- and among-individual responses to capture/restraint and other stressors
Consequences of stress	Metabolic consequences of stress	What is the energetic cost of coping with diverse stressors?	Field methods to assess costs in nature or modeling approaches that can incorporate natural behavioral and physiological coping responses
	Fitness consequences of stress	How do different stressors influence survival and reproduction?	Experimental manipulation of stressors in snakes in field and laboratory, following generational outcomes and performance
When is 'X' a stressor? (duration, magnitude, consequences)	Captivity	In what contexts is captivity a stressor <i>versus</i> a safe haven?	Multi-metric longitudinal study of different species of snakes accessioned into different captive environments
	Climate change	In what contexts is thermal and hydric change a stressor for different snake species?	Multi-metric longitudinal study of snakes experiencing naturally-occurring environmental change; Experimental manipulation of environment in cohorts of different snake species
	Urbanization	In what contexts is urbanization a stressor and how does this vary among snake species?	Multi-metric longitudinal study of snakes in gradient of urban/rural environments or during ongoing habitat loss/land use conversion
	Shelter availability	How do shelters ameliorate stress in snakes?	Experimental manipulation of shelters in field and laboratory
	Prey availability and feeding	When do changes in prey type or availability induce stress in snakes?	Experimental supplementation/restriction of prey availability/type; follow cohorts with naturally occurring fluctuations in prey availability/type
	Intraspecific interactions	When are interactions with conspecifics stressful?	Manipulation of individuals interacting in different contexts
Stress, immune function, and disease	Immune consequences	How is immune function altered during stress responses?	Measuring multiple immune metrics during experimentally induced stress.
	Disease status	What immune metrics are useful in predicting disease outcomes?	Cohort studies of infected animals with longitudinal sampling correlated to disease outcomes
	Disentangling stress and disease	In what contexts is disease a cause versus a consequence of stress?	Experimental approaches that can directly target cause and effect relationships between disease and stress
Broad patterns of stress in snakes	Snake stress	Are there phylogenetic or trait-based factors that influence snake responses to different stressors?	Multi-species multi-metric comparisons of snake responses to standardized stressors

It is important to understand the stress ecology of snakes for several reasons. By examining the stress response in this diverse and evolutionarily successful group, we are better able to understand the physiological and ecological mechanisms that influence stress in animals. A comprehensive understanding of stress in many snake species can also help develop better conservation initiatives. For example, the utility of stress metrics and consequences of different stressors are necessary to understand when monitoring at-risk populations, the success of repatriation programs, and to understand how snakes may act as indicators of stress in their respective environments. It is also important to understand how snakes respond to stressors such as captivity and disease to inform the consequences of their use in other research and commercial applications. Overall, there is still much to accomplish before we can truly understand the total magnitude and consequences of stress snakes experience when accosted by an archaeologist in a dark pit or when suddenly dropped into an airline cabin.

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